REGULATION OF THE RAT 5-AMINOLEVULINATE SYNTHASE mRNA: THE ROLE OF mRNA STABILITY.

A THESIS SUBMITTED TO THE UNIVERSITY OF ADELAIDE
FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

by

Timothy John Sadlon B. Sc. Hons. (Adelaide University)

Department of Biochemistry
University of Adelaide
Adelaide, South Australia

July 1995.

CONTENTS

CHAPTER 3: THE EFFECT OF HEME ON ALAS-1 mRNA LEVELS IN FRL 4.1 CELLS.

CHAPTER FOUR: ANALYSIS OF THE 3'UTR OF THE RAT ALAS-1 MRNA FOR DESTABILISING ACTIVITY.

CHAPTER FIVE: LOCALISATION OF THE HUMAN ALAS-1 MRNA INSTABILITY DETERMINANT TO THE CODING REGION.

THESIS SUMMARY

All animal cells need to synthesise heme for their respiratory cytochromes, however, the demand for heme can vary greatly within the same cell in response to various endogenous and exogenous signals. This, coupled with the fact that excess heme can be toxic to cells, requires that heme synthesis be tightly regulated. The mitochondrial matrix protein 5-aminolevulinic acid synthase (ALAS) is the first and rate-limiting enzyme of the heme biosynthesis pathway. There are two isozymes of ALAS in animals, an ubiquitous form (ALAS-1) and an erythroid-specific form (ALAS-2). In the liver, ALAS activity can be negatively regulated by heme (the end product of the pathway) and positively regulated by a number of exogenous chemicals which also induce the synthesis of the cytochrome P450 hemoproteins. We and others have shown that heme can repress the steady state level of ALAS-1 mRNA in a number of systems, however the point at which this repression occurs is still controversial. This thesis is concerned with the mechanism by which heme causes negative feedback inhibition on ALAS-1 mRNA.

The mechanism of heme repression of ALAS-1 mRNA was examined in the fetal rat hepatoma cell line FRL 4.1 where it was shown that heme treatment resulted in a rapid (80%) decline in the level of ALAS-1 mRNA. This repression could have been caused by a decrease in the transcription rate of the ALAS-1 gene, a decrease in the half-life of the ALAS-1 mRNA, or a combination of the two. Therefore, the effect of heme on the synthesis of the ALAS-1 mRNA was examined at these two potential control points. The transcription rate of the ALAS-1 gene, examined by *in vitro* nuclear run-on experiments, was found to be unaffected by heme. The transcriptional inhibitor actinomycin D was then used to show that the degradation rate of ALAS-1 mRNA was specifically increased, approximately 3-fold, in the presence of high heme concentrations. Further characterisation of this heme-mediated destabilisation of ALAS-1 mRNA included showing:

1.) The destabilising effect on ALAS-1 mRNA was specific for heme. Neither iron nor Tin-mesoporphyrin could elicit the same destabilisation of the ALAS-1 mRNA.

2.) Destabilisation occurred independently of translation. Protein synthesis was inhibited in FRL cells by cycloheximide prior to the addition of heme. Interestingly, treating FRL cells with cycloheximide did not inhibit the heme-mediated destabilisation of the ALAS-1 mRNA. This result suggested that translation of ALAS-1 mRNA itself

was not required for the heme-mediated destabilisation effect. Furthermore, the proteins involved in this effect appear to be relatively stable.

3.) The role of the polyA tail in the heme-mediated destabilisation effect was also examined. The decay of several other short-lived mRNAs occurs via a poly(A) tail dependent pathway. De-adenylation of the polyA tail was found not to be a prerequisite for the heme-mediated destabilisation of the ALAS-1 mRNA.

The second part of this thesis investigates the location of the *cis*-elements which target ALAS-1 mRNA for rapid degradation in the presence of high heme levels. Initially the 3'-untranslated region (UTR) of the rat ALAS-1 mRNA was tested for destabilisation activity. A hybrid gene construct containing a fragment of the rat ALAS-1 3'UTR inserted into the 3'UTR of the rabbit β-globin gene was synthesised. Stably transfected FRL cell lines were established and the effect of heme on the stability of the β-globin mRNA was assayed by RNase protection assays. However, no significant change in the half-life of the β-globin mRNA was observed. Hence, the *cis*-acting sequence(s) responsible for the heme-mediated destabilisation of the ALAS-1 mRNA was presumed to be located elsewhere.

A second series of constructs was then synthesised in which the untranslated and translated portions of the human ALAS-1 and β-globin genes were transposed. Stably transfected cell lines were again established and analysis of the half-life of the hybrid mRNA produced from these constructs indicated that the *cis*-acting element(s) responsible for the heme-mediated destabilisation effect are located in the coding region of the ALAS-1 mRNA. However, one construct containing the ALAS-1 coding region, and therefore expected to be unstable, was found to be stable. Several possibilities for this contradictory result were investigated but, as yet, the reason for this difference is still unclear.

In conclusion, heme was found to specifically decrease the half-life of ALAS-1 mRNA, leading to a rapid drop in the level of ALAS-1 mRNA following heme treatment. It is becoming increasingly apparent that regulating the turnover of cytoplasmic mRNA is an important mechanism for controlling gene expression in eukaryotic cells. The heme-mediated destabilisation of ALAS-1 mRNA provides an interesting model for studying the mechanism by which mRNAs are differentially targeted for degradation. Elucidation of the biochemical pathway of ALAS-1 mRNA degradation should not only provide insights into the mechanism by which mRNA

molecules are degraded but also into how the cell senses its metabolic requirements and alters gene expression accordingly.

DECLARATION.

This thesis contains no material that has been accepted for the award of any degree or diploma by any university. To the best of my knowledge it contains no material that has been previously published by any other person, except where due reference has been made in the text. I consent to this thesis, when deposited in the university library, being available for photocopying and loan.

Timothy Sadlon

ACKNOWLEDGMENTS

I am grateful to Professors W. H. Elliott and G. E. Rogers for permission to undertake this research in the department of Biochemistry.

I wish to thank my supervisor Dr B. K. May for his continual interest, support and unwavering enthusiasm throughout my doctorate. I would also like to thank the entire department for providing a stimulating, friendly and enthusiastic environment in which to work. In particular, the support and encouragement of "Prof" Elliott and Drs John Wallace and Julian Wells was very much appreciated.

A big thank you to all members of the CSU team, not only for cleaning up the mess generated by pigs like me; but for actually remaining so cheerful while doing it. The office staff deserve another big thank you, particularly Jan, for her good humour in the face of yet another late Bresatec order. I would also like to thank Darren, Jenny, Joe and especially Jacquie Beale for all their help in tissue culture.

Thanks to all past and present members of the BKM group for their help and friendship, in particular Tony Hanson, Helen Healy, Raman Bhasker, Jack Omdahl, Chris Hahn and Ben Davidson. A special thank you to Satish Dogra and Mike Bawden for their continuing help and friendship. A special thank you also goes to Tim Cox, Giovanna Braidotti and Kathy Surinya for their friendship and the opportunity to exchange juicy gossip. Thanks also to Chris Mathews for all his help in my continuing battle with the computer and for his help in preparing the figures for this thesis.

Thanks also to a large number of other folks for some great moments, especially Lones, Mr Michael Reed, Linda and Keith, Brett-San, Jules, Kim, Dora, Gary, Nick Kalincos, Mac and Nick Coates.

Huge thanks to Mum and Dad for their endless patience, interest, support and free board!

Lastly, but most importantly, I would like to thank Rebecca for all her understanding, help and encouragement, especially when it counted most.

During this work I was supported by a University of Adelaide Postgraduate Research Scholarship.

ABBREVIATIONS

Abbreviations are as described in The Journal of Biological Chemistry "Instructions to authors" (1989). Additional abbreviations are listed below.

AIA:	allylisopropylacetimide
ALA:	5-aminolevulinate
ALAS:	5-aminolevulinate synthase
bp:	base pair
Ci:	Curie
cpm:	counts per minute
dNTP:	deoxyribonucleotide triphosphate
DMSO:	dimethyl sulphoxide
DMEM:	Dulbeccos modified Eagles medium
DTT:	dithiothreitol
F12:	Hams F12 media
FRL 4.1:	Fetal rat hepatocyte cell line 4.1
HO-1:	heme oxygenase 1
kb:	kilobase
PB:	phenobarbital
PBS:	phosphate-buffered saline
PMSF:	phenylmethylsulphonyl fluoride
poly (A):	poly adenylic acid
RNase:	ribonuclease
Tris:	Tris(hydroxymethyl) aminomethane
TCA:	trichloroacetic acid
UTR:	untranslated region

CHAPTER ONE

GENERAL INTRODUCTION

CHAPTER 1: INTRODUCTION.

1.1: Introduction.

Heme, as iron protoporphyrin IX, plays a vital role in all animal cells, as a prosthetic group to a diverse family of hemoproteins. Crucial to the function of heme as a prosthetic group is the central atom iron, which can exist in two stable oxidation states, namely the ferrous (Fe^{2+}) or ferric (Fe^{3+}) states. Importantly, the ferrous state can be readily and reversibly oxidised to the ferric state by the transfer of a single electron. This characteristic has made heme ideal to serve as a single electron carrier in electron transport chains and as a catalyst in redox reactions involving molecular oxygen and certain other oxygen containing compounds. In addition, in the ferrous state, heme has a high affinity for oxygen, allowing it to also act as an oxygen carrier. These features of the heme molecule have led to its exploitation in a wide variety of aerobic processes in vertebrates.

Such processes include oxygen transport and storage by haemoglobin and myoglobin, mitochondrial respiration carried out by the respiratory cytochromes, the oxidative metabolism of lipophilic compounds by the microsomal and mitochondrial cytochrome P450s (CYP)[1], steroid biosynthesis by the steroidogenic CYPs, the desaturation of fatty acids by microsomal cytochrome b5, tryptophan catabolism by tryptophan pyrrolase and the destruction of hydrogen peroxide by peroxisomal catalase and peroxidase. A labile heme protein (acting as an O_2 sensor molecule) is implicated in

---

[1] A new nomenclature has recently been applied to the cytochrome P450 superfamily. In this system the italicised root symbol "CYP" represents "cytochrome P450" followed by an Arabic number denoting the family, a letter designating the individual gene within the subfamily and lastly an Arabic number representing the individual gene within the subfamily (Nelson *et al.*, 1993). This nomenclature has been adopted throughout this thesis.

the increase in the transcription of the erythropoietin gene in the kidney in response to hypoxia (Blanchard *et al.*, 1993). Recent studies have also implicated a hemoprotein in the signal transduction processes elicited by nitric oxide and carbon monoxide (Verma *et al.*, 1993). These gases activate guanyl cyclase by binding tightly to the heme moiety of the enzyme leading to a change in the concentration of the intracellular messenger molecule cyclic GMP.

In addition to its role as a prosthetic group, heme is increasingly recognised as an important regulator of a number of cellular processes including cellular metabolism and differentiation. Heme's effect on cellular metabolism can occur at multiple steps. For example, in erythroid cells heme has a global effect on translation, by modulating the activity of a specific initiation factor-2α (eIF-2α) kinase, termed the heme-regulated inhibitor (Reviewed Chen and London, 1995). Heme, acting at both the transcriptional and post-transcriptional levels, also regulates the expression of a variety of genes, including the rate-limiting enzymes involved in its own synthesise and degradation (Reviewed May *et al.*, 1995). In yeast, heme co-ordinates the transcription of nuclear genes encoding respiratory proteins with aerobic conditions by activating the yeast transcription factor HAP 1 (Pfiefer *et al.*, 1989). Heme has also been implicated in the transcription of some erythroid-specific genes, including the β-globin gene, in erythroleukemic cell lines (Solomon *et al.*, 1993 ; Fukuda *et al.*, 1994). This, transcriptional activation appears to be mediated through a NF-E2 consensus site, although the *trans*-acting factor responsible has not yet been positively identified as the erythroid-specific transcription factor NF-E2 (Ikuta and Tan, 1991 ; Miller *et al.*, 1993 ; Palma *et al.*, 1994).

Apart from its effect on cellular metabolism heme has been implicated in the differentiation of several cell types. It has been found to enhance the differentiation of neuroblastoma cells (Padmanadan *et al.*, 1989), skeletal muscle myotubes (Funanage *et al.*, 1989) and white blood cells (Novogrodsky *et al.*, 1989 ; Ruutu *et al.*, 1987). Heme may also be important for the differentiation of erythropoietic cells. Evidence for such a role in the differentiation of erythropoietic cells has been suggested by the finding that heme treatment can stimulate the differentiation of several erythroleukemic cell lines in

culture. Whether heme also plays a role in the differentiation of erythroid progenitor cells in whole animals is yet to be established, although heme addition apparently augments the frequency of primitive Burst-forming units erythroid (BFU-E) colonies formed in bone marrow cultures (Monette and Holden, 1982).

While all nucleated cells must synthesise heme for incorporation into respiratory cytochromes, clearly the cells requirement for heme will vary depending on each cell's requirements. For example, about 80% of the total heme synthesised by the body takes place in the erythropoietic tissues, while the majority of the remaining 20% occurs in the liver to supply heme to the drug-metabolising CYPs.

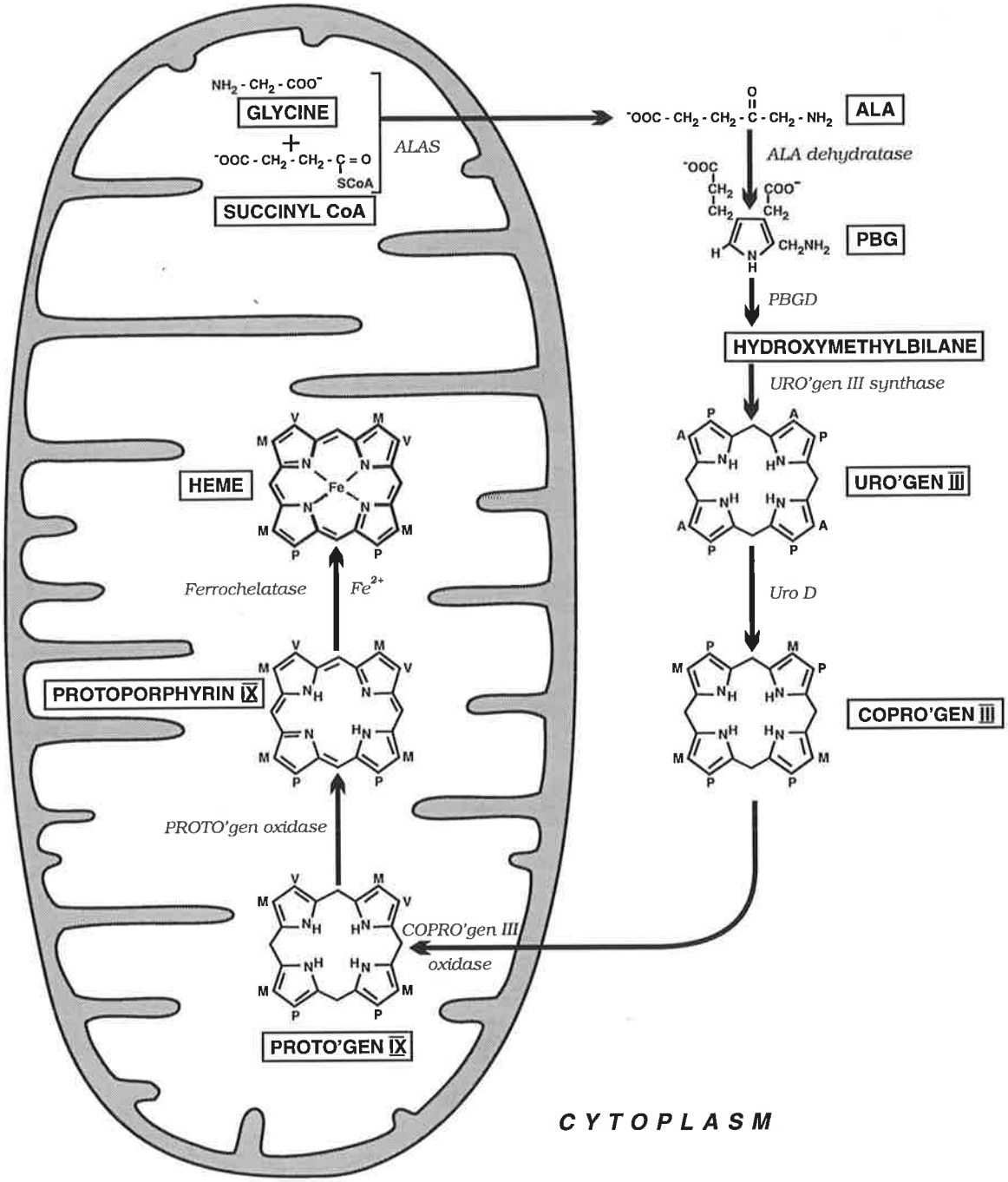
1.2: The Heme Biosynthesis Pathway.

The heme biosynthesis pathway consists of eight enzymes in animal cells (fig 1.1). The first enzyme 5-aminolevulinic acid synthase (ALAS) and the last three enzymes, coproporphyrinogen-III oxidase (Copro'gen oxidase), protoporphyrinogen-III oxidase (Proto'gen oxidase) and ferrochelatase (FC) are located in the mitochondrion, while the remaining enzymes, 5-aminolevinate dehydratase (ALAD), porphobilinogen deaminase (PBGD), uroporphyrinogen-III synthase (Uro III sythase) and uroporphyrinogen-III decarboxylase (UroD) are found in the cytoplasm. All the enzymes are encoded by nuclear genes, are synthesised in the cytoplasm with the four mitochondrial enzymes, and subsequently transported into the mitochondria.

The reason for this compartmentalisation of the enzymes between the mitochondria and the cytoplasm is unknown, but may partially reflect both substrate availability and the dual function of at least one of the heme pathway enzymes. The localisation of ALAS to the mitochondria is thought to reflect its substrate availability since succinyl-Coenzyme A (succinyl-CoA) is generated in the mitochondrial matrix. Although, succinyl-CoA is generated by several enzyme systems such as methyl-malonyl-CoA mutase, succinate thiokinase and acetacetyl-CoA:succinate transferase, the major source of succinyl-CoA for heme synthesis is from the citric acid cycle. The

Figure 1.1: **The Heme Biosynthetic Pathway in Non-Plant Eukaryotes.**

Illustrated are the intermediates of heme biosynthesis, the enzymes catalysing each of the eight steps of the pathway and the unique sucellular compartmentalisation of these enzymes. **ALA**, 5-aminolevulinic acid; **ALAS**, 5-aminolevulinic acid synthase; **ALAD**, 5-aminolevulinic acid dehydratase; **PBG**, porphobilinogen; **PBGD**, porphobilinogen deaminase; **Uro'gen III synthase**, uroporphyrinogen III synthase; **Uro'gen III**, uroporphyrinogen III; **UroD**, uroporphyrinogen III decarboxylase; **Copro'gen III**, coproporphyrinogen III; **Copro'gen oxidase**, coproporphyrinogen III oxidase; **Proto'gen IX**, protoporphyrinogen IX; **Proto'gen oxidase**, protoporphyrinogen IX oxidase; **A**, acetate; **M**, methyl; **P**, propionate and **V**, vinyl.


GLYCINE
SUCCINYL CoA
ALAS
ALA
ALA dehydratase
PBG
PBGD
HYDROXYMETHYLBILANE
URO'gen III synthase
URO'GEN III
Uro D
COPRO'GEN III
COPRO'gen III oxidase
PROTO'GEN IX
PROTO'gen oxidase
PROTOPORPHYRIN IX
Ferrochelatase
Fe2+
HEME
CYTOPLASM

reason that ALAD is found in the cytoplasm on the other hand may reflect the recently proposed second function of ALAD as a component of the proteasome complex (Guo *et al.*, 1994).

The proteasome is a large 26S protease complex responsible for the selective degradation of protein-ubiquitin conjugates and thus forms one of the major proteolytic pathways in the cell (Reviewed Ciechanover, 1994). The 26S proteasome consists of three subunits, a 20S subunit (CF-3) which contains the catalytic core, a 600kDa subunit termed CF-1 and a 240-250kDa subunit termed CF-2. All three subunits are required for the 26S proteasome complex assembly and/or activity. The CF-2 subunit is thought to be an ATP stabilised inhibitor of the proteasome (Driscoll *et al.*, 1992). Purification of the CF-2 subunit by Guo *et al.* (1994) led to the surprisingly suggestion that the CF-2 factor consisted of ALAD. This assignment of a second function to ALAD was based on the following findings; i) the first 14 amino acids at the N-terminal of CF-2 were identical to that of ALAD, ii) both CF-2 and ALAD had identical migration patterns on native and SDS-polyacrylamide gels, iii) both proteins had identical isoelectric points, iv) both proteins cross-react with specific polyclonal antibodies raised against each other, v) purified ALAD and CF-2 demonstrated both ALA dehydratase and proteasome activity and vi) recombinant ALAD also demonstrated proteasome activity, excluding the possibility that ALAD co-purified with the CF-2 factor. However, the native ALAD enzyme exists as a 280-290kDa octameric complex, consisting of eight 35-36kDa ALAD monomeric subunits (Wu *et al.*, 1974), which is substantially larger than that reported for CF-2.

In the overall reaction, as depicted in figure 1.1, the first enzyme, ALAS catalyses the formation of 5-aminolevulinic acid (ALA) from glycine and succinyl CoA. ALA then leaves the mitochondria for the cytosol, where two molecules are dimerised by the cytosolic enzyme ALA Dehydratase (ALAD) to form the pyrrole porphobilinogen (PBG). Four molecules of PBG are then tetra-polymerised by PBG deaminase (PBGD) to give the unstable intermediate 1-hydroxymethylbilane. This polymerisation is though to occur in a stepwise manner with each PBG molecule being added one at a time to the growing polymer. This linear tetrapyrrole is then re-arranged and cyclised by

uroporphyrin III synthase to produce the ring tetrapyrrole uroporphyrinogen III. In the absence of Uro'gen synthase, the highly reactive 1-hydroxymethylbilane undergoes a non-enzymatic cyclisation, without prior re-arrangement, to give the isomer uroporphyrinogen I, which has no biological function. The final cytosolic enzyme, UroD catalyses the decarboxylation of all four acetic acid side chains of uroporphyrinogen III to methyl groups yielding coproporphyrinogen III. Coproporphyrinogen III then re-enters the mitochondrion for the remaining reactions. Coproporphyrinogen III oxidase, which is found in the intramembrane space usually loosely associated with the outer surface of the inner mitochondrial membrane, subsequently catalyses the conversion of the two propionate groups at positions R2 and R4 to vinyl groups, to form protoporphyrinogen IX. This compound is then oxidised by protoporphyrinogen IX oxidase to protoporphyrin IX. Protoporphyrinogen IX oxidase is an integral inner mitochondrial membrane protein, with its active site on the intramembrane space side of the molecule (Ferreira *et al.*, 1988). Finally, ferrochelatase, which is also an inner mitochondrial membrane protein catalyses the insertion of ferrous iron into protoporphyrin IX to produce heme. The active site for ferrochelatase is believed to be located on the side of the protein that faces the mitochondrial matrix (Harbin and Dailey, 1985). Heme can then be utilised within the mitochondrion, for example as a prosthetic group for the respiratory cytochromes or mitochondrial CYPs, or transported out of the mitochondria to other cellular locations for use by various hemoproteins.

Although ALAS catalyses the formation of ALA in animal cells as well as in yeast and some species of bacteria, plants, algae and other bacteria, lack ALAS and instead synthesis ALA by an alternative pathway, termed the C5 pathway. ALA synthesise is initiated in the C5 pathway by the attachment of glutamate to a tRNA molecule. The glutamate group is subsequently reduced to form glutamate-1-semialdehyde prior to its transamination to form ALA; the subsequent steps in the synthesis of heme appear to be conserved (Schoen *et al.*, 1986 ; Avissar and Beale, 1989).

1.3: Intra-cellular and Inter-cellular Transport of Heme and its Precursors.

In plasma, the major heme-binding proteins are albumin and the 60kDa glycoprotein hemopexin. However, due to hemopexins extremely high affinity for heme, almost all free heme in the serum is bound to the latter molecule. In addition, a second heme-transport system (serum haptoglobin) exists in plasma. This protein is involved in the binding and delivery of free haemoglobin, released during intravascular hemolysis, to the liver as a haemoglobin-haptoglobin complex. Both heme-hemopexin and haemoglobin-haptoglobin complexes are predominantly taken up by the liver, in a receptor-mediated process. In the case of hemopexin, upon release of heme, hemopexin is recycled back to the circulation (Reviewed Smith, 1990a). This sequestering and delivery to the liver of heme and haemoglobin-heme by hemopexin and haptoglobin respectively is thought to have several biological roles. These include, preventing extra-cellular oxidation, particularly lipid peroxidation, by heme following tissue damage and intravascular hemolysis (Gutheridge and Smith, 1988). This protective role is extremely important given that intravascular hemolysis is thought to account for about 10% of the daily turnover of red blood cells (Garby and Noyes, 1959). Other roles include the recycling of iron (Davies *et al.*, 1979) and the sequestering of potential iron sources from invading microbes (Smith, 1990a).

Heme and its precursors, are in general highly reactive, lipophilic molecules and this poses a problem to the cell, given the separation of the pathway between the mitochondria and cytoplasm, in terms of the intracellular movement of these pyrrole compounds. The mechanism by which heme and its precursors move across the mitochondrial membranes is still unclear (Muller-Eberhard and Fraig, 1993 ; Smith, 1990). A recent study has implicated the peripheral-type benzodiazepine receptor (PBR) as a possible candidate receptor for the import of coproporphyrinogen III into the mitochondria (Taketani *et al.*, 1994), however direct evidence for the PBR receptor in the transport of the heme precursor is still lacking. An enzyme complex which spans the inner mitochondrial membrane has been suggested to form between coproporphyrinogen III oxidase and the integral membrane enzymes protoporphyrinogen IX oxidase and

ferrochelatase during the conversion of coproporphyrinogen III to heme. This complex is though to provide the means by which the precursors are simultaneously converted and transported across the membrane into the mitochondrial matrix as each substrate is channelled from one enzyme active site to the next (Ferreira *et al.*, 1988).

The mechanism by which heme is then delivered to other intracellular locations from its site of synthesis in the mitochondria is also not well defined. Several *in vitro* studies, have found that cytosolic proteins facilitate the efflux of heme from the mitochondria to the endoplasmic reticulum protein apo-cytochrome b5 (Senjo *et al.*, 1985 ; Liem *et al.*, 1990 ; Boyer and Olsen, 1991). This has led to the proposal that specific heme-binding proteins act as carriers to accomplish an organised intra-cellular distribution of heme. However, the exact identity of the carrier protein(s) remains controversial (Muller-Eberhard and Fraig, 1993). Two cytosolic proteins that bind to heme avidly, namely, the Yb_2Yb_2 isozyme of glutathione-S-transferase and the heme-binding protein (HBP), a member of the liver fatty acid binding proteins, have been implicated as possible intra-cellular heme-transporter proteins.

A role for the Yb_2Yb_2 isozyme was suggested by Senjo *et al.* (1985). These workers, purified to apparent homogeneity, a protein from rat liver cytosol, which was capable of facilitating the reconstitution of cytochrome b5, when incubated *in vitro* with isolated mitochondria and apo-cytochrome b5. Characterisation of the physical and immunological properties of the protein led to its identification as the glutathione-S-transferase isozyme Yb_2Yb_2. However, Boyer and Olsen (1991) found that purified heme-free Yb_2Yb_2 had only a very limited capacity to stimulate the transfer of heme from mitochondria to apo-cytochrome b5, compared to crude cytosolic extract. Purified Yb_2Yb_2 however did facilitate the reconstitution of cytochrome b5 when heme was added exogenously, suggesting that the role of Yb_2Yb_2 may be to increase the amount of heme available to apo-cytochromes. This raised the possibility that the protein purified by Senjo *et al.* (1985) may have been contaminated by cytosolic heme, thus the increase in cytochrome b5 holo-enzyme observed may have been due to the transfer of this heme and not due to an increased efflux of mitochondrial heme. The second potential cytosolic carrier protein HBP was observed by Leim *et al.* (1990) to increase

the efflux of radiolabelled heme from isolated intact mitochondria into the media, whereas Yb_2Yb_2 and serum albumin another heme-binding protein had little effect on the amount of heme efflux. This suggested that HBP may be involved in the export of heme from the mitochondria to the cytosol.

1.4: Structure and Properties of ALAS.

ALAS activity was first described independently in bacterial extracts (Kikuchi *et al.*, 1958) and in avian erythrocytes (Gibson *et al.*, 1958). Since then, the enzyme has been purified from a number of organisms including Rhodobacter spheroides (Warnick and Burnham, 1971), rat liver (Srivastava *et al.*, 1982), Euglena (Dzelzkalns *et al.*, 1982), chick embryo liver (Borthwick *et al.*, 1986) and yeast (Volland and Felix, 1984).

Compared with the other enzymes of the heme biosynthesis pathway, ALAS exhibits the lowest activity in both liver and erythroid tissues, the major sites for heme biosynthesis in the body (Bottomley and Muller-Eberhard, 1988), and thus is the rate-limiting step in heme synthesis. Numerous studies have clearly established ALAS and heme oxygenase, the rate limiting enzyme in heme degradation, as the major control points in the regulation of heme levels in the cell (May *et al.*, 1995).

Eukaryotic ALAS was first localised on the matrix side of the inner mitochondrial membrane by immuno-gold labelling studies (Rhode *et al.*, 1990). In animals, ALAS is encoded for by a nuclear gene. Therefore, ALAS must first be made in the cytosol as a precursor protein containing an N-terminal signal sequence that targets the protein to the mitochondria. This signal sequence is removed during the import of the precursor into the mitochondria to generate the mature protein. Further electron microscopy and cross-linking studies on purified chicken liver ALAS suggested that the enzyme existed as a homodimer with each monomer associated in opposite polarities (Pirola *et al.*, 1984).

ALAS activity is critically dependent on the cofactor pyridoxal phosphate (PLP), a derivative of vitamin B6 (pyridoxine). Studies with the bacterial ALAS (Nandi, 1978)

and purified chick embryo liver ALAS (Pirola, 1986), indicated that PLP formed a schiff base with a lysine residue of the enzyme, to which glycine binds as the first step in the synthesis of ALA. For a detailed review on the mechanism of ALA synthesis by ALAS the reader is referred to Jordan (1990).

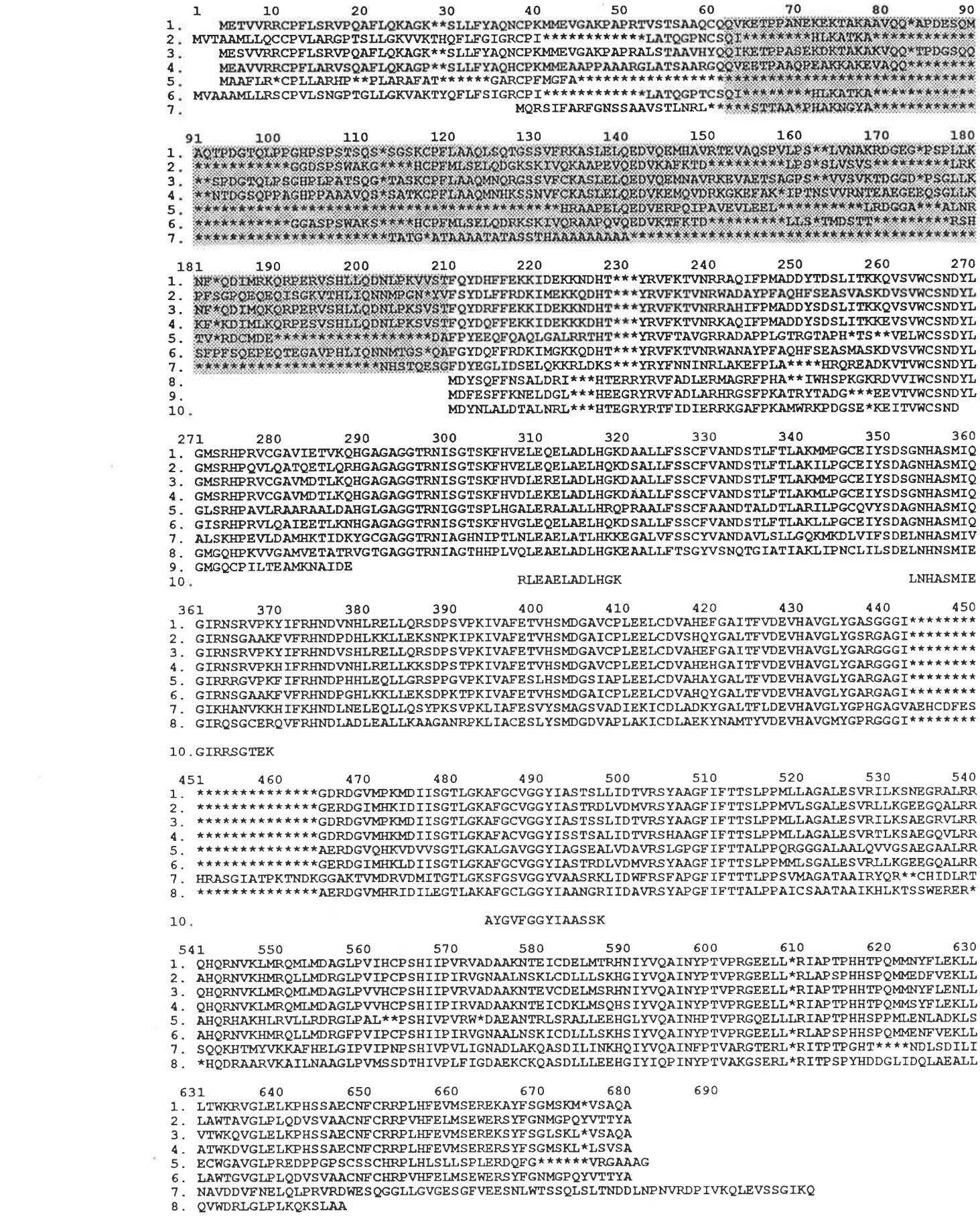
Two isozymes of ALAS exist in mammalian and avian organisms and these enzymes are the products of separate genes (May *et al.*, 1995). The expression of one isozyme (ALAS-2) is restricted to erythroid tissues, including fetal sites of erythropoiesis (Yamogida *et al.*, 1993). This isozyme is thought to be primarily responsible for the supply of the large amounts of heme required for haemoglobin assembly. The second isoform (ALAS-1) is expressed ubiquitously, to supply heme to a diverse range of ubiquitous and tissue-specific hemoproteins. This "housekeeping" form is present in erythroid tissues but its expression appears to be down-regulated during erythroid differentiation, presumably due to the high levels of heme present, indicating that this isoform contributes little to the synthesis of haemoglobin-heme (Yamogida *et al.*, 1993 ; Fujita *et al.*, 1991b).

cDNA clones representing both the ubiquitous and erythroid ALAS isozymes have been cloned from several mammalian and avian species. ALAS-1 has been cloned from chick embryo liver (Borthwick *et al.*, 1985), rat liver (Srivastava *et al.*, 1988 ; Yamamoto *et al.*, 1988) and human liver (Bawden *et al.*, 1987 ; Bishop, 1990) . The erythroid isozyme (ALAS-2) was first cloned from chickens (Riddle *et al.*, 1989) and has subsequently been cloned from mice (Schoenhaut and Curtis, 1989), humans (Cox *et al.*, 1990 ; Bishop, 1990) and rats (Munakata *et al.*, 1993). ALAS has also been cloned from the yeast Saccharomyces cerevisae (Urban-Grimal *et al.*, 1986) and the bacterial species Bradyrhizobium japonicum (McClung *et al.*, 1987) and Rhizobium melitoti (Leong *et al.*, 1985). In these single cell organisms there is apparently only one ALAS gene and enzyme.

The mammalian and avian enzymes have been proposed to be made up of three regions or domains (Elliott *et al.*, 1989). This division of the enzyme was based upon the predicted amino-acid sequence of both the prokaryotic and eukaryotic forms (fig 1.2). Region 1 corresponds to the N-terminal signal sequence of the eukaryotic precursor

Figure 1.2: **Comparison of Amino Acid Sequence of Eukaryotic and Prokaryotic ALAS Proteins.**

Comparison of Amino Acid Sequence of Eukaryotic and Prokaryotic ALAS Proteins from cDNA clones or direct protein sequencing. Region 2 found in the mature eukaryotic proteins but not in the prokaryotic enzymes is shaded. The amino acid sequences used for this comparison were obtained from: **1**) rat ALAS-1, Srivastava *et al.* (1988); **2**) human ALAS-2, Cox *et al.* (1990); **3**) human ALAS-1, Bishop, (1990); **4**) chick ALAS-1, Borthwick *et al.* (1985); **5**) chicken ALAS-2, Riddle *et al.* (1989); **6**) mouse ALAS-2, Schoenhaut and Curtis, (1989); **7**) Saccharomyces cerevisae, Urban-Grimal *et al.* (1986); **8**) Bradyrhizobium japonicum, McClung *et al.* (1987); **9**) Rhizobium melitoti, Leong *et al.* (1985) and **10**) Rhodopseudomonas sheroides, Elliot *et al.* (1989).



protein, and is required for the import of ALAS into the mitochondria. This region is removed during the transport of the enzyme into the mitochondria.

N-terminal sequencing of the mature chicken and rat ALAS-1 protein indicated that the cleavage of the precursors occurred between two glutamine residues resulting in a signal sequence of 56 residues along (Borthwick *et al.*, 1985 ; Pirola, 1986). On this basis the human ALAS-1 leader sequence is also predicted to be 56 amino acids in length, with the proteolytic cleavage site located between two glutamine residues. The length of the signal sequence for the erythroid enzymes has not been determined but based on the sequence alignment it is predicted to take place between the serine residue at position 49 and the glutamine residue at position 50 in both the human and mouse proteins. The chicken ALAS-2 signal sequence however appears to be much smaller and has been estimated by Riddle *et al.* (1989) to be approximately 18 residues in length. Analysis of the signal sequences suggests that this region most likely forms an amphipathic, helical structure which is characteristic of a mitochondrial signal sequence (Vassarotti *et al.*, 1987). Only, limited sequence homology exists between region 1 of the ALAS-1 and ALAS-2 isozymes, apart from two sequences that resemble the heme-binding site of the yeast transcription factor HAP-1 (Pfeifer *et al.*, 1989). The role of these putative heme-binding sites are discussed later in section 1.6.3.ii.

The mature vertebrate ALAS enzymes can be further divided into regions on the basis of sequence and catalytic activity (Elliott *et al.*, 1989). Located at the N-terminus of the mature protein in eukaryotes is a region (region 2) that appears to be non-essential for catalytic activity. The role of region 2 is currently unknown, but papain digestion which removed almost all of this region had little effect on either the chicken ALAS-1 (Elliott *et al.*, 1989) or rat ALAS-2 (Munakata *et al.*, 1993) enzymes kinetics *in vitro*. Whether, region 2 plays some regulatory role *in vivo* is currently being investigated.

The remaining C-terminal portion of the mature enzyme, which represents approximately 75% of the mature protein, is highly conserved between all ALAS enzymes and has been termed region 3 (Elliott *et al.*, 1989). The N-terminus of region 3 corresponds to the N-termini of the bacterial ALAS enzymes. Region 3 has been proposed to represent the catalytic core of the animal ALAS enzymes due to its strong

sequence conservation. In addition, proteolytic cleavage resulting in the loss of region 2 from the mature protein still results in a catalytically active region 3 fragment. Recently, the active site lysine which forms the schiff base with pyridoxal phosphate co-factor has been localised to this region (Ferreira *et al.*, 1993)

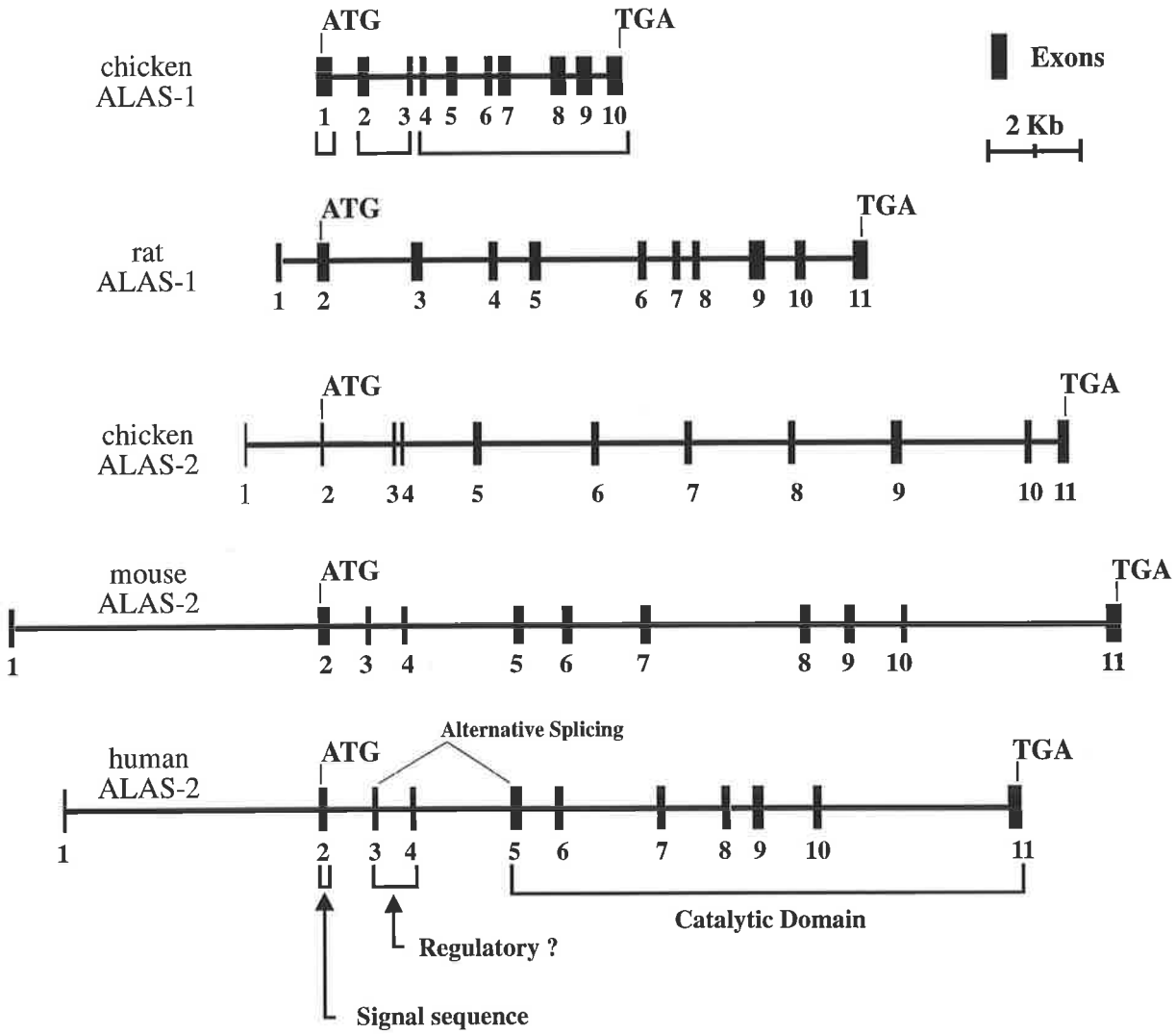
1.5: Structure of the Ubiquitous and Erythroid ALAS Genes.

The genes that encode the chicken (Maguire *et al.*, 1986), rat (Yomogida *et al.*, 1993 ; Braidotti *et al.*, 1993) and human (Healy, 1990) ALAS-1 and chicken (Lim *et al.*, 1994), rat (Munakata *et al.*, 1993), mouse (Schoenhaut and Curtis, 1989) and human (Conboy *et al.*, 1992) ALAS-2 have been isolated. The complete exon-intron boundaries for the chicken and rat ALAS-1 and chicken, mouse and human ALAS-2 have been determined (fig 1.3). The genomic organisation of the different genes appears to be conserved with all the genes made up of eleven exons, apart from the chicken ALAS-1 gene which unlike the others lacks an intron in its 5'-untranslated region. Overall, there is a good correlation between the exon-intron boundaries and the putative functional domains. Exon 1 and 2 make up the 5'-UTR, with part of exon 2 also coding for the mitochondrial signal sequence; in the chicken exon 1 and 2 appear to be fused by the loss of the 5'-UTR intron. Exons 3 and 4 code for region 2 of the mature protein (exon 2 and 3 in the chicken ALAS-1 enzyme), while exons 5 to 11 encompass region 3. The highly conserved arrangement of the exon-intron boundaries suggests that the two genes probably arose in vertebrates from a common ancestral gene. In human's the two ALAS genes are located on separate chromosomes. The human ALAS-1 gene has been localised to position p21 on the short arm of chromosome 3 (Sutherland *et al.*, 1988 ; Bishop *et al.*, 1990), while the human ALAS-2 gene has been mapped to Xp11.2 on the short arm of the X-chromosome (Cox *et al.*, 1990 ; Cotter *et al.*, 1992).

Alternative splicing of the erythroid ALAS-2 mRNA has been observed in two species but to date there has been no reported alternative splicing observed for the ubiquitous ALAS-1 mRNA. In humans, two mRNAs products were identified by PCR

Figure 1.3: **Structural Organisation of the Genes for ALAS-1 and ALAS-2.**

Structural Organisation of the Genes for ALAS-1 and ALAS-2. Exons are numbered. The ATG initiation and TGA termination codons are shown. The proposed functional roles of the exons are indicated. The alternative splice pattern for human ALAS-2 mRNA is also shown.


chicken
ALAS-1
ATG
TGA
1 2 3 4 5 6 7 8 9 10
Exons
2 Kb
rat
ALAS-1
ATG
TGA
1 2 3 4 5 6 7 8 9 10 11
chicken
ALAS-2
ATG
TGA
1 2 34 5 6 7 8 9 10 11
mouse
ALAS-2
ATG
TGA
1 2 3 4 5 6 7 8 9 10 11
human
ALAS-2
Alternative Splicing
ATG
TGA
1 2 3 4 5 6 7 8 9 10 11
Catalytic Domain
Regulatory ?
Signal sequence

amplification of the ALAS-2 mRNA from reticulocyte RNA (Conboy *et al.*, 1992). The smaller of these mRNAs lack a portion of the mRNA that encodes for part of region 2 which corresponds exactly to exon 4 of the gene. Both ALAS-2 mRNA isoforms are present in approximately equal amounts in a number of erythroid cell lineages, which represent most stages of erythroid development, including proerythroblasts, reticulocytes, first trimester fetal liver and adult bone marrow indicating that the alternative splicing of exon 4 was not restricted to any particular developmental or differentiation state (Conboy *et al.*, 1992 ; Cox, 1993). Interestingly, this alternative splicing event is not phylogenetically conserved, with no evidence for transcripts lacking exon 4 in mice or dog erythroid cells (Conboy *et al.*, 1992). However, Schoenhaut and Curtis (1989) noted the existence of a second, smaller ALAS-2 transcript in murine MEL cells. In this case the two mRNA isoforms are the result of the inclusion or exclusion of the first 45 nucleotides from the 5'-end of exon 3 in the transcript. The exclusion of these nucleotides from the mRNA appears to be dependent on the utilisation of an alternative downstream 3'-slice acceptor site (Schoenhaut and Curtis, 1989). However, neither the relative levels nor cell-type distribution of these mice isoforms has been established.

As with the removal of exon 4 in the human mRNA, this differential splicing of the mouse ALAS-2 mRNA produces mature protein isoforms that are heterogenous in their N-terminal domain (region 2). The significance of the alternative splicing of the human and mice ALAS-2 mRNAs is not known. In humans the alternative splicing produces a protein which lacks 37 amino acids in region 2, while the mouse isoform lacks 15 residues. The domain (region 2) affected is probably not involved directly in catalysis since it is absent from bacterial ALAS proteins and can be proteolytically cleaved from the eukaryotic proteins without apparently affecting enzyme activity. Indeed, both human ALAS-2 isoforms showed comparable activity when expressed in *E. coli*. Currently, we are attempting to purify both bacterially expressed human isoforms to perform a more detailed analysis of the kinetics of the different isoforms. However, it has been proposed that region 2 may play some regulatory role *in vivo* (May *et al.*, 1995), and therefore the removal of this region may have an indirect effect on

ALAS activity *in vivo* by potentially modifying such processes as monomer interaction, mitochondrial import or the localisation of ALAS to the matrix side of the inner mitochondrial membrane.

1.6: Molecular Regulation of ALAS Expression.

As mentioned previously, heme participates in a vast array of cellular processes within a eukaryotic cell as either a prosthetic group to a number of hemoproteins or as a regulatory molecule in its own right (Padmanaban *et al.*, 1989 ; May *et al.*, 1995). As heme participates in inducible and tissue-specific as well as ubiquitous processes, the metabolic requirement for heme can vary markedly in a given cell at different times or between different tissues. For example, heme synthesis must rapidly increase in hepatocytes following drug induction of the cytochrome P450 system, to meet the demand for extra heme caused by increased cytochrome P450 apoprotein synthesis. Heme synthesis must also increase in erythroid precursor cells in order to supply the vast amounts of heme needed for haemoglobin assembly during terminal differentiation of erythrocytes. Coupled with this varied demand for heme is the fact that a build up of heme in the cell is toxic due to protein and lipid oxidation (Muller-Erberhard and Fraig, 1993 ; Balla *et al.*, 1991). As a consequence of these temporal, tissue-specific and metabolic variations in the cells heme requirements, the cell has had to develop a number of different control mechanisms to co-ordinate heme-synthesis with the requirements of the cell. Several decades of research has demonstrated that most of these regulatory checks act upon the first enzyme of the biosynthetic (ALAS) and degradative (heme oxygenase) pathways making them rate-limiting. The next section summarises the current knowledge of ALAS regulation in different tissues with the focus being upon its regulation in hepatic and erythroid tissues, the major sites of heme synthesis.

1.6.1: Erythropoiesis and Heme Synthesis.

The differentiation and maturation of red blood cells from pluripotent hemopoietic progenitor cells is termed erythropoiesis. In vertebrates, the site of erythropoiesis changes with the developmental status of the animal. In humans, erythropoiesis begins in the yolk sac, shifts to the fetal liver and then progressively shifts to the bone marrow during the third and final trimester, where it remains throughout adulthood (Karlsson and Nienhuis, 1985). Erythrocytes originate from a self-renewing population of pluripotent hemopoietic stem cells, located in the bone marrow in adults. These stem cells generate progenitor cells that become irreversibly committed to differentiate into a restricted number of lineages (Metcalf, 1989). A variety of hemopoietic growth factors then guide these cells further along a defined pathway to finally produce a progenitor cell irreversibly committed to terminally differentiate into a single lineage type.

Erythropoiesis is a continuous processes in the body to counterbalance the loss of aged and damaged cells. In healthy humans, it has been estimated that approximately 1% of all circulating erythrocytes are lost in this manner daily, to be replaced by new reticulocytes. This rate of formation (~2-3x10^{11} cells/day) can be altered in response to a number of factors which cause low blood oxygen (O_2) tension including altitude, haemorrhage or infection. For a detailed review on factors influencing erythropoiesis the reader is referred to Jelkman, (1992).

The main hemopoietic growth factor involved in regulating erythropoiesis is the hormone erythropoietin (Epo). Epo is a secreted 165 amino acid glycoprotein which is predominantly produced in the kidney and to a lesser extent the liver in adults. In the foetus the liver is the major site of Epo production. The effect of Epo is mediated by a specific transmembrane receptor, which is a member of the hemopoietic/cytokine receptor family. The molecular events following Epo binding to its receptor are beginning to be understood. Firstly, binding of Epo to its receptor is thought to activate the latter by promoting receptor homodimerisation (Watowich *et al.*, 1994). This activation then leads to the phosphorylation of tyrosine residues on a number of cellular

substrates, including the intracellular domain of the receptor itself. However, unlike a number of growth factor receptors, the Epo receptor lacks a kinase domain. It is now believed that the activation of the Epo receptor induces the binding and or activation of JAK 2 kinase to the intracellular domain and it is this kinase which is responsible for the phosphorylation of the receptor (Witthuhn *et al.*, 1993). The molecular events following Epo receptor phosphorylation are not well understood but recent data indicates the intracellular signalling protein, phosphatidylinositol 3-kinase (PI 3-kinase) may be involved. PI 3-kinase has been shown to bind to the phosphorylated Epo receptor via an SH2 domain. However, the role of PI 3-kinase in Epo stimulation is still unclear, since cells containing mutant Epo receptors, which prevent PI 3-kinase binding, are only partially inhibited in their Epo induced proliferation (Miura *et al.*, 1993). This finding could indicate that the Epo receptor contains functionally redundant intracellular signalling domains as seen with the PDGF receptor (Valius and Kazlauskas, 1993). Alternatively, as only Epo induced proliferation was investigated in this study, PI 3-Kinase may be involved in mediating the Epo induced differentiation of erythroid cells.

As mentioned the main stimulus for Epo production is tissue hypoxia which can be defined as a low ratio of O_2 availability to demand (Blanchard *et al.*, 1993 ; Jelkman, 1992). These subtle changes in O_2 tension are though to be sensed via an O_2 sensor protein in the erythropoietin producing cells. This O_2 sensing protein has been proposed to be a hemoprotein (Goldberg *et al.*, 1988 ; Blanchard *et al.*, 1993). In the model proposed, reversible O_2 binding to the heme moiety of the protein causes a conformational change in the protein leading to a change in its activity. Specifically when the O_2 tension is low the protein is thought to be active and this somehow triggers increased Epo expression. Conversely, when the O_2 tension is high the protein is in an inactive conformation and does not stimulate Epo production. Although a candidate O_2 sensor protein has not yet been identified in eukaryotes, a hemoprotein which senses O_2 concentrations has been isolated and cloned from the nitrogen-fixing bacteria Rhizobium melitoti (Gilles-Gonzalez *et al.*, 1991). It is not known how the signal is transmitted from the putative O_2 sensor, but hypoxia leads to an increase in the transcription of the Epo gene (Schuster *et al.*, 1989) and an increase in the stability of

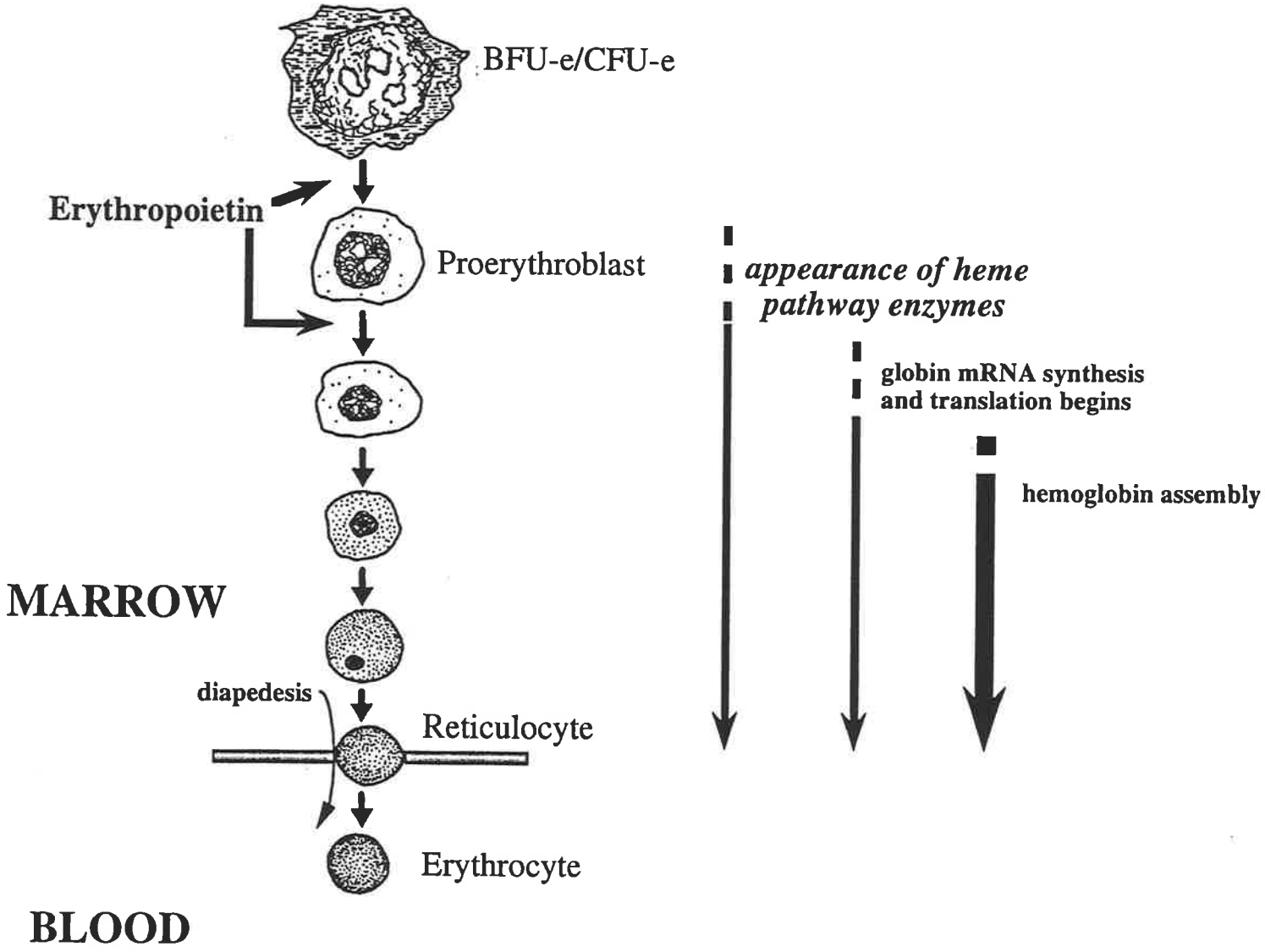
the Epo mRNA (Goldberg *et al.*, 1991). Recently, two regions have been implicated in the hypoxia mediated induction of the Epo gene. One region lies within the promoter (-118 to -65) of the gene, while the other is located 120bp downstream of the poly(A) tail site (Blanchard *et al.*, 1992) and these regions appear to act synergistically. Two proteins have been identified which bind to these regions. One termed hypoxia-inducible factor 1 (HIF-1) increases its DNA-binding and transcription-activation activities under hypoxia conditions (Wang and Semenza, 1993a). Blocking protein phosphorylation inhibits hypoxia induction of HIF-1 implicating a protein kinase in the signalling mechanism (Wang and Semenza, 1993b). The second protein which binds to these regions and appears to be important for hypoxia-mediated induction of the Epo gene is the "orphan" nuclear receptor; (HNF-4) hepatic nuclear factor 4 (Galson *et al.*, 1995).

Epo appears to be most important in the early stages of erythropoiesis where it has been shown to be vital for the viability, proliferation and differentiation of the early erythroid progenitors, (BFU-E) burst-forming unit-erythroid and (CFU-E) colony-forming unit-erythroid (Lim and Wintour, 1993 ; Jelkman, 1992). The earliest defined erythroid precursor is the BFU-E cell whose viability is reliant upon Epo, interleukin-3 (IL-3) and granulocyte-monocyte colony stimulating factor (GM-CSF). Epo appears to trigger these cells into S phase and differentiation, resulting in the formation of CFU-Es. CFU-Es are though to be the main target for Epo and contain the highest number of Epo receptors on their surface. In culture, CFU-Es have an absolute requirement for Epo to maintain their viability and prevent apotosis (Kouryand and Bondurant, 1990). Epo also initiates the proliferation and differentiation of the CFU-Es into proerythroblasts. The formation of reticulocytes from proerythroblasts then proceeds via four consecutive mitotic steps (fig 1.4), which show a progressively decreased dependence on Epo as evidenced by the down-regulation of the receptor (Landschulz *et al.*, 1989). The reticulocytes are then released into the circulation via a process termed "diapedesis" where they undergo further maturation to form mature erythrocytes.

Shortly after the Epo stimulated proliferation and differentiation of the proerythroblasts there is a noticeable induction of many of the heme pathway enzymes,

Figure 1.4: **Heme and Hemoglobin Synthesis During Erythropoiesis in Adult Bone Marrow.**

Erythropoietin(Epo) stimulates both the production of proerythroblasts from BFU-Es and CFU-Es as well as the progression from the proerythroblast stage into the four consecutive mitoses leading to the formation of reticulocytes. Immediately, following the Epo-stimulated onset of cellular proliferation there is an increase in all the enzymes of the heme pathway. The induction of heme synthesis in turn promotes the subsequent synthesis and assembly of hemoglobin.


BFU-e/CFU-e
Erythropoietin
Proerythroblast
appearance of heme
pathway enzymes
globin mRNA synthesis
and translation begins
hemoglobin assembly
MARROW
diapedesis
Reticulocyte
Erythrocyte
BLOOD

which precedes globin gene transcription. There is some evidence that the up-regulation of heme synthesis is a prerequisite for proper erythroid cell development and differentiation from *in vitro* studies of MEL cell (Grandchamp *et al.*, 1985 ; Fukuda *et al.*, 1994) and primary human bone marrow cultures (Abraham *et al.*, 1989). Therefore, it appears that induction of heme synthesis in erythroid cells in response to Epo stimulation has a dual role of supplying heme for haemoglobin assembly as well as participating in cellular signalling of the differentiation program leading to the induction of globin synthesis.

1.6.2: Regulation of Heme Pathway Enzymes in Erythroid Cells.

To date most of the studies have been carried out in murine erythroleukemic (MEL) cells which are virus-transformed erythroid precursor cells blocked at a developmental stage comparable to the CFU-E. Upon exposure to a chemical inducer such as Dimethyl Sulphoxide (DMSO), the cells will differentiate down the erythroid lineage, in a manner that exhibits many characteristics of natural erythropoietic maturation. However, Mel cells do not respond to Epo the natural inducer of erythropoiesis. An Epo responsive cell line J2E has been developed by Klinken *et al.* (1988) which has allowed Epo-induced differentiation to be studied. These cells are thought to correspond to the proerythroblast stage and upon Epo stimulation, proliferate and differentiate in a manner very similar to normal proerythroblast maturation (Busfield and Klinken, 1992 ; Klinken *et al.*, 1993). Generally, the events following Epo or chemical induction of erythroid cells are similar but there are some differences (Busfield and Klinken, 1992 ; Chern *et al.*, 1990).

Shortly, after Epo or chemical induction of erythroid differentiation, the activities of most, if not all, of the heme pathway enzymes are increased (Beaumont *et al.*, 1984 ; Abraham *et al.*, 1989 , Busfield *et al.*, 1993) and are accompanied by an increase in the mRNAs for these enzymes (Fujita *et al.*, 1991a ; Grandchamp *et al.*, 1985 ; Fukuda *et al.*, 1993). The cloning of the genes for heme pathway enzymes has

begun to provide the molecular mechanism for this induction during erythropoiesis (Reviewed May *et al.*, 1995). Whereas, there are two genes for ALAS in vertebrates, one of which is expressed exclusively in differentiating erythroid cells, all the other enzymes cloned so far have a single structural gene. The reason for two ALAS genes is not known, but it has been suggested that two genes has allowed the development of different mechanism to regulate the stability and translation of the two mRNAs.

Erythroid and ubiquitous mRNA isoforms have been detected for ALAD and PBGD, the second and third enzymes of the pathway respectively (Bishop *et al.*, 1991 ; Kaya *et al.*, 1994 ; Chretien *et al.*, 1988 ; Mignotte *et al.*, 1989). These isoforms are produced from the same gene by the use of alternative promoters and alternative splicing. The erythroid-specific promoters contains several binding sites for erythroid-specific factors such as GATA-1 (Ko and Engel, 1993 ; Merika and Orkin, 1993), EKLF (Miller and Bieker, 1993) and NF-E2 (Andrews *et al.*, 1993) and activation of this promoter is thought to be responsible for the increase in the mRNA during erythroid differentiation. While the erythroid and "housekeeping" ALAD isozymes are identical, since the alternative splicing occurs in the 5'UTR, the alternative splicing of the PBGD gene results in an addition 17 amino acids at the N-terminus of the housekeeping enzyme.

Of the remaining vertebrate heme pathway enzymes, the genes for uroporphyrinogen III decarboxylase (Romana *et al.*, 1987), coprophyrinogen oxidase (Martasek *et al.*, 1994) and ferrochelatase (Taketani *et al.*, 1992) have been cloned and their promoters examined. Coproporphyrinogen oxidase and ferrochelatase produce apparently identical transcripts in all cell types, including erythroid cells from a single gene that contains a composite promoter lacking a TATA box but containing several copies of the ubiquitous Sp1 and erythroid-specific GATA-1 binding sites. Although the expression of uroporphyrinogen III decarboxylase is increased during DMSO induced differentiation of Mel cells (Fujita *et al.*, 1991a ; Fujita *et al.*, 1991b) this is not observed in Epo stimulated J2E cells (Busfield *et al.*, 1993). So far the elements involved in the induction of uroporphyrinogen III decarboxylase in Mel cells is unknown.

1.6.3: Regulation of Erythroid ALAS.

(i) Activation during Erythropoiesis.

Studies performed in both Mel and J2E cells indicate that the expression of the erythroid-specific ALAS-2 mRNA is increased during erythroid differentiation and that this reflects an increase in gene transcription (Fujita *et al.*, 1991b and Busfield *et al.*, 1993). In contrast, the level of the ubiquitous ALAS-1 mRNA rapidly drops following DMSO induction of Mel cells (Fujita *et al.*, 1991b) but the mechanism involved in this decrease is not known. In J2E cells ALAS-1 mRNA was undetectable.

The promoters for the mouse, human and chicken ALAS-2 genes have now been isolated (Schoenhaut and Curtis, 1989 ; Cox *et al.*, 1991 ; Lim *et al.*, 1994). Interestingly, the early promoters show little conservation of potential control elements. The chicken and mouse genes surprisingly, lack a TATA-like box while in humans the TATA box resembles more closely a GATA-1 binding site. Non-canonical TATA boxes which bind GATA-1 in preference to TFIID have been identified in several other erythroid-specific genes, including the chicken β-globin (Fong and Emerson, 1992), human glycophorin B (Rahuel *et al.*, 1992) and rat R-pyruvate kinase (Max-Audit *et al.*, 1993). Conversion of this GATA-1 like sequence to a authentic TATA box substantially interfered with erythroid specific expression in these cases. This has led to the proposal that GATA-1 is involved in forming a stable pre-initiation complex on these genes which ensures appropriate erythroid-specific expression (Barton *et al.*, 1993).

The human ALAS-2 immediate promoter also contains a number of putative binding sites for both erythroid-specific and ubiquitous factors (Cox *et al.*, 1991). These include two GATA-1 elements (-100bp and -125bp), a CACCC box (-55bp), a CCAATT box (-87bp), a NF-E2 consensus (-44bp) and a thyroid hormone like (TRE) element (-15bp). Interestingly, the GATA-1 site at -100 in the human promoter is the only element conserved between the murine and human promoter sequences. Currently,

the importance of these putative binding sites for the erythroid-specific expression of the human gene is being pursued, with preliminary data indicating that each contribute to the overall expression of the promoter (Surinya, 1995). Of particular interest is the presence of a TRE-like element in the early promoter of the human gene (Cox *et al.*, 1991) given that a study in avian erythroid cells indicated that the thyroid hormone receptors v-erbA and c-erbA, in the absence of hormone repressed transcription of a subset of erythroid-specific genes including ALAS-2 (Zenke *et al.*, 1990).

The human, and presumably the mouse, ALAS-2 gene also contain an erythroid-specific enhancer in intron 8 (Surinya, 1995). This enhancer was initially identified on the basis of a conserved clustering of putative erythroid-specific transcription factor binding sites in both genes (Cox, 1993) and this region corresponds to a DNase 1 hypersensitivity site in Mel cell nuclei (Schoenhaut and Curtis, 1989). DNase 1 hypersensitive sites are often associated with transcription factor binding (Gross and Garrard, 1988).

Two regions important for the erythroid-specific expression of the chicken ALAS-2 gene have also been identified (Lim *et al.*, 1994). These are a region in the proximal promoter (-155bp to +21bp) and a distal region (-784bp to -505bp). Sequence analysis of the distal region identified a putative GATA-1 site but its role in the expression of the gene is unclear, since mutating this site did not affect the expression of the reporter gene. The chicken ALAS-2 promoter is unusual in that it resembles a type 1 "housekeeping" promoter, which lack a TATA box and contain multiple Sp1 binding sites (Lewin, 1990). Footprint analysis of the proximal promoter identified seven footprints that protected sequences that matched the consensus sequence for the ubiquitous transcription factor Sp1, with a additional footprint centred around a sequence at -130bp which shared similarity with both an Ap-1 binding site and a retinoic acid response element (RARE). The identity of the proteins binding to these sites is currently unknown since neither a 200-fold excess of authentic Sp1 oligo could affect the protected signal in a footprint competition assay, nor could AP-1 or its erythroid homologue NF-E2 bind to the -130 sequence in an *in vitro* gel mobility assay (Lim *et al.*, 1994). A possible candidate protein for the binding activity detected at the

Sp1-like elements in the chicken promoter is the erythroid-specific transcription factor EKLF, which is known to be able to bind Sp1-like elements in the promoters of other erythroid-specific genes (Merika and Orkin, 1995).

(ii) Heme Regulates ALAS-2.

Although ALAS levels are induced during erythroid differentiation, ALAS is still thought to be rate limiting in erythroid cells since the addition of ALA to cells greatly enhances the *de novo* synthesis of heme (Gardner and Cox, 1988). As in hepatic cells, ALAS activity in differentiating erythroid cells appears to be regulated by heme (Beaumont *et al.*, 1984 ; Elferink *et al.*, 1988 ; Gardner *et al.*, 1991). Succinylacetone, a *de novo* heme synthesis inhibitor, massively increases ALAS activity in DMSO induced Mel cells, which is prevented by heme addition (Elferink *et al.*, 1988 ; Dierks, 1990), but since neither succinylacetone, nor heme alter the level of ALAS-2 mRNA in differentiating Mel cells (Fujita *et al.*, 1991b ; Dierks, 1990) it has been concluded that heme regulates ALAS activity at a step later than mRNA accumulation.

One mechanism by which heme regulates ALAS activity in erythroid cells has been identified by Lathrop and Timko (1993). High heme concentrations have been known for some time to inhibit the import of the ubiquitous ALAS-1 precursor into the mitochondria (Hayashi *et al.*, 1972 ; Srivastava *et al.*, 1983) and Lathrop and Timko (1993) have reported a similar phenomenon for the erythroid ALAS-2 protein. An examination (Dierks, 1990 ; Lathrop and Timko, 1993) of the amino acid sequence of the ALAS-1 and ALAS-2 presequences revealed two sequences that resembled the heme binding sequence (HRM) required for the heme activation of the yeast transcription factor HAP-1 (Pfiefer *et al.*, 1989). *In vitro* studies by Lathrop and Timko (1993), demonstrated that these motifs where required for the heme inhibition of the import of the mouse ALAS-2 precursor and furthermore that a single motif was sufficient to confer heme inhibition to the import of a normally unregulated protein.

Although not proven, it seems probable that the similar motifs in the presequence of the ALAS-1 are also responsible for heme-inhibition.

The precise mechanism by which heme inhibits the import of the ALAS precursor into the mitochondria is unknown. Recently, heme was shown to bind directly to the consensus HRM motif from the yeast HAP 1 protein (Zhang and Guarente, 1995) and so presumably heme also binds directly to the ALAS precursors. The binding of heme to ALAS could inhibit import by several mechanisms. For example, bound heme could prevent the interaction of the precursor with a specific mitochondrial transporter protein. Alternatively, heme bound to the presequence may prevent the unfolding of the protein, a prerequisite for transport (Eilers *et al.*, 1988). In reticulocytes heme may also regulate ALAS-2 translation indirectly, by inhibiting the release of transferrin-iron from internalised vesicles (Ponka *et al.*, 1988 ; Gardner and Cox, 1988). The role of iron in regulating ALAS-2 translation is discussed in the next section.

(iii) Heme Synthesis is Coupled to Iron Availability Via ALAS.

The enormous demand for iron during erythropoiesis, for incorporation into heme, necessitated the development of a specialised mechanism to co-ordinate its uptake with its utilisation for heme production. That erythroid ALAS activity is decreased *in vitro* and *in vitro* by iron deficiency and conversely increased in over-load, suggested that this co-ordination may occur through the modulation of ALAS activity (Houston *et al.*, 1991 ; Bottomley, 1992).

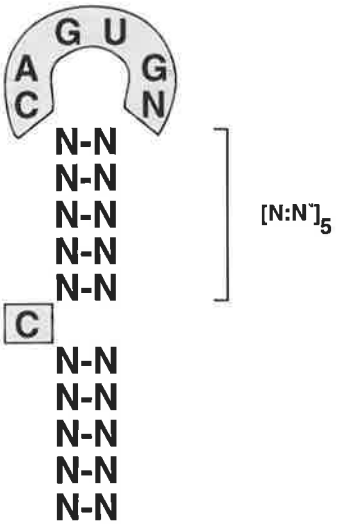
The cloning of the ALAS-2 gene has led to a molecular explanation of this co-ordination. Present in the 5'UTR of the mouse (Dierks, 1990), human (Cox *et al.*, 1991) and chicken (Lim *et al.*, 1994) ALAS-2 mRNA is a secondary structure that resembles the iron response element (IRE) used to co-ordinate the synthesis of ferritin and transferrin receptor proteins with iron availability (reviewed O'Halloran, 1993 ; Klausner *et al.*, 1993). The consensus IRE (fig 1.5) is a stable stem-loop structure which consists of a) a six-membered loop, the first five bases of which are most often CAGUG

Figure 1.5: **The Sequence and Proposed Structure of an IRE.**

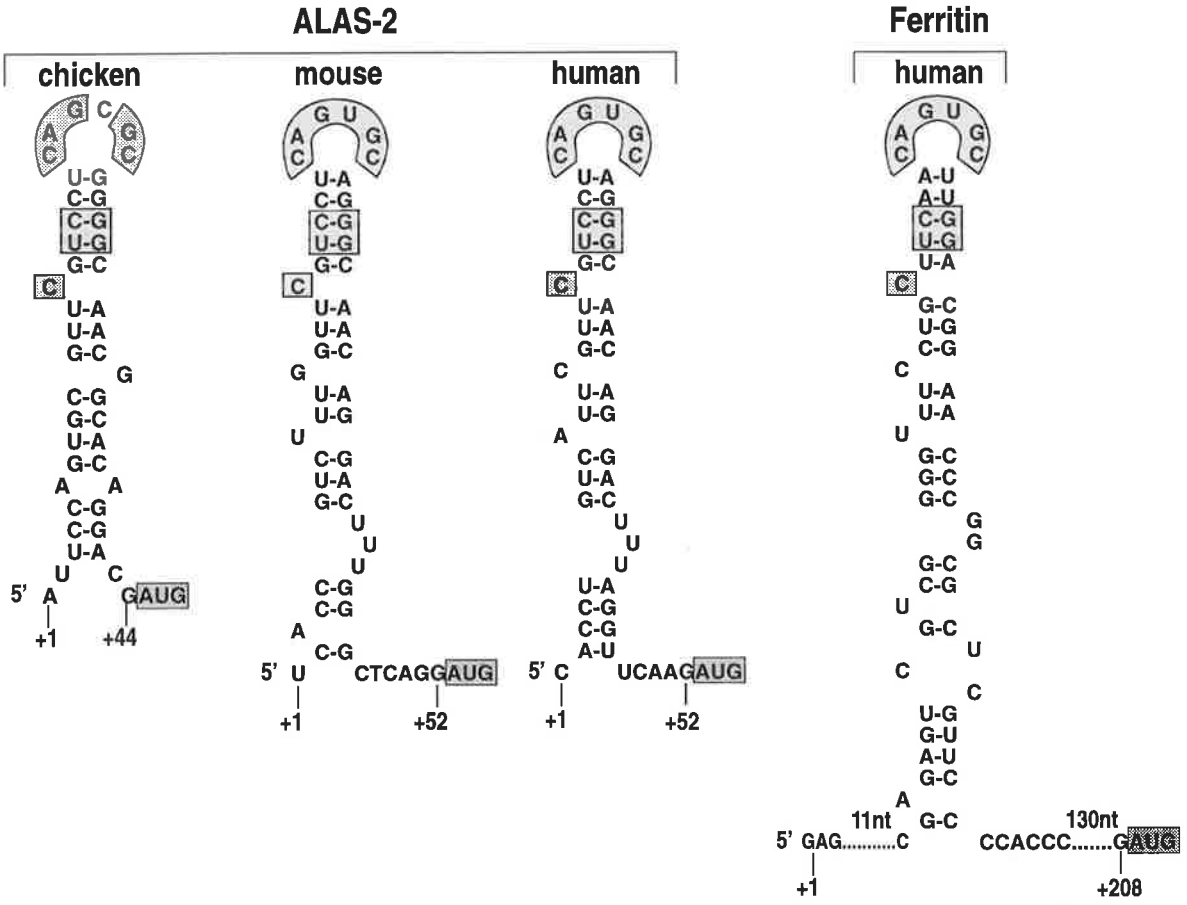
The consensus IRE motif is depicted in part A. Critical features of a functional IRE are the hexanucleotide loop of sequence 5'-CAGUGN-3', an unpaired cytosine residue five nucleotide pairs 5' to this loop and a lower stem of variable length.

Depicted in part B is a comparison of the proposed IRE structure in the 5'-UTR of the chicken, mouse and human ALAS-2 mRNAs and human ferritin mRNA. The AUG initiation codon is shaded as are the conserved bases. The first nucleotide (CAP site) of the mRNAs is at +1.

A


G U
A G
C N
N-N
N-N
N-N
N-N
N-N
C
N-N
N-N
N-N
N-N
N-N
[N:N]5

B


ALAS-2
Ferritin
chicken
mouse
human
human
5' A
+1
+44
AUG
5' U
CTCAGGAUG
+1
+52
5' C
UCAAGAUG
+1
+52
5' GAG.....C
11nt
130nt
CCACCC.....GAUG
+1
+208

and the sixth base most often a pyrimidine; b) an upper stem usually of five base pairs, in which the bases can be any complementary pair of RNA base pairs; c) a bulge that is invariably a cytosine residue and d) a lower stem of variable length and sequence. The activity of the IRE is mediated by the binding of a cytosolic protein referred to as the iron-responsive element binding protein (IRE-BP). The IRE-BP is the metal sensor and its IRE binding activity is modulated post-translationally by iron (O'Halloran, 1993 ; Klausner *et al.*, 1993 ; Beinert and Kennedy, 1993). If iron is abundant, the IRE-BP's RNA binding activity is low; when iron is scarce, the IRE-BP has a high affinity for the IRE.

Cloning of the IRE-BP revealed its homology to mitochondrial aconitase, responsible for the conversion of citrate to isocitrate. Indeed the IRE-BP has been shown to have a high aconitase but low RNA binding activity when purified from iron replete cells but minimal aconitase and high RNA binding activity when purified from iron starved cells (Haile *et al.*, 1992). It is now thought that the IRE-BP corresponds to the previously identified cytosolic aconitase, which like its mitochondrial counterpart was thought to be an iron-sulphur containing protein (Beinert and Kennedy, 1993). According to the present model, the IRE-BP exists in two forms with mutually exclusive functions; when an iron-sulphur cluster forms in the presence of high iron, the protein gains aconitase activity with subsequent loss of RNA binding activity, in low iron the iron-sulphur cluster cannot form and the protein functions as a IRE binding protein. Evidence for this model has recently been obtained with the generation of mutants unable to form an iron-sulphur cluster. These mutants are unable to be converted to an active aconitase form and are constitutively active as RNA binding proteins in tranfected cells, independent of the cells iron status (Philpott *et al.*, 1993 ; Hirling *et al.*, 1994). Presumably, the formation of the iron-sulphur group provoke a conformational change in the protein which alters the RNA binding site or buries it within the protein. It is also unclear whether the IRE-BP protein binds iron reversibly *in vivo* since the removal of iron and hence its conversion from its aconitase to RNA binding form, requires strong reducing conditions *in vitro* (Beinert and Kennedy, 1993).

A possible role for cytoplasmic aconitase activity in high iron conditions has been proposed (O'Halloran, 1993). In the cytoplasm iron is thought to be complexed with citrate so that an equilibrium exits between iron-citrate and iron-isocitrate. Therefore, when the IRE-BP gains aconitase activity the isocitrate concentration increases at the expense of the citrate pool and this facilitates the loading of ferritin with iron since iron-isocitrate is thought to be more labile.

The position of the IRE appears to be important for defining its function. A single IRE is located in the 5'UTR of ferritin mRNA and in this position it regulates the translation of ferritin mRNA. In the case of transferrin receptor mRNA, five IRE's are located in the 3'UTR of the mRNA and in this position they regulate the turnover of the mRNA in response to changes in the iron status of the cell. When the IRE-BP binds to the ferritin IRE, under low iron conditions, the initiation of translation of the mRNA is inhibited and the synthesis of ferritin is prevented, whereas the binding of the IRE-BP to the transferrin receptor mRNA increases the stability of the mRNA and hence increases the synthesis of transferrin receptor. The binding of the IRE-BP to the transferrin receptor IRE's is thought to indirectly lead to the stabilisation of the mRNA by blocking the access of a site specific endo-ribonuclease to its target (Binder *et al.*, 1994).

On the basis of its position in the 5'UTR of the ALAS-2 mRNA it was proposed that the translation of the ALAS-2 mRNA was also controlled by iron availability (Dierks, 1990 ; May *et al.*, 1990). Since then the IRE-BP has been shown to bind to the human ALAS-2 IRE in RNA gel mobility assays (Cox *et al.*, 1991) and to control the translation of a reporter gene *in vitro* (Bhasker *et al.*, 1993 ; Dandekar *et al.*, 1991) and in Mel cells (Melefors *et al.*, 1993). Recently, the IRE-BP/IRE complex was shown to inhibit translation by sterically preventing the association of the 43S translation pre-initiation complex with the CAP structure of the ferritin and ALAS-2 mRNAs (Grey and Hentze, 1994) and this would seeming explain the importance of the position of the IRE with respect to the CAP site for efficient inhibition of translation (Bhasker *et al.*, 1993).

1.6.4: Molecular Regulation of ALAS-1.

(i) Expression in Different Tissues.

A cDNA for ALAS-1 was first isolated from drug-induced chick embryo livers (Borthwick *et al.*, 1985) and subsequently cDNAs were isolated for rat (Srivastava *et al.*, 1988 ; Yamamoto *et al.*, 1988) and human (Bawden *et al.*, 1987 ; Bishop, 1990) ALAS-1. In Northern blots, a single mRNA species of approximately 2.3kb was detected by the ALAS-1 cDNA probe in all rat and chicken tissues examined, including adrenal gland, brain, heart, intestine, kidney, liver, lung, spleen and testis (Srivastava *et al.*, 1988 ; Srivastava *et al.*, 1989a ; Drew and Ades, 1989a ; Srivastava *et al.*, 1992). Furthermore, primer extension and RNase mapping experiments indicated that this mRNA species was identical in all tissues tested (Srivastava *et al.*, 1988 ; Elferink *et al.*, 1987). This indicated that the ALAS-1 protein functioned as a "Housekeeping" enzyme to supply heme for respiratory cytochromes and other hemoproteins. The level of ALAS-1 mRNA varied several fold between different tissues in untreated animals and this probably reflects the different metabolic requirements for heme in each tissue.

The developmental profile of ALAS-1 mRNA has been examined in rat liver (Srivastava *et al.*, 1988 ; Yomogida *et al.*, 1993). During fetal development, when the liver is the major site of hemopoiesis, the level of ALAS-1 mRNA is very low up to day 20 where it increases rapidly to peak around birth, at day 22, the level of ALAS-1 mRNA then decreased to about half this level before increasing again in the adult liver. These fluctuations in mRNA parallel that of the hepatic cytochrome P450 CYP2B1 mRNA and presumably the increase at day 20 reflects the shift of erythropoiesis from the liver to the bone marrow and the development of liver function. On the other hand the erythroid-specific ALAS-2 mRNA was detectable in the fetal liver from day 15 and continued to increase up to about day 20, from whence it steadily declined to be undetectable after birth. This pattern of ALAS-2 expression mimicked that of α -globin mRNA and thus this data supports the proposal that ALAS-1 performs a "housekeeping"

role, while ALAS-2 is chiefly responsible for supplying heme for haemoglobin synthesis.

(ii) Regulation of ALAS-1

Most of our current understanding on the control of ALAS-1 has been developed from studies in mammalian and avian liver cells, but it is assumed that many of these principles are valid in other tissues. Firstly, in liver, and probably most other tissues, the mechanisms that control the rate of heme synthesis are designed to accommodate transient fluctuations in the rate of apo-hemoprotein synthesis. Secondly, heme regulates its own rate of synthesis by controlling the production of ALAS the first enzyme of the pathway. These principles were first demonstrated by Granick (1966). Granick showed that the administration of certain substances such as barbiturates specifically induced the activity of ALAS in the liver of experimental animals, and that heme could prevent this induction. As these compounds are substrates for the heme containing cytochrome P450 mono-oxygenase and specifically induced the synthesis of a subset of the P450 enzymes associated with their metabolism, it was proposed that the induction of ALAS was to met the increased demand for heme. Furthermore, since heme inhibited the induction of ALAS activity without blocking the drug-mediated induction of cytochrome P450 synthesis, it was concluded that heme regulated its own rate of synthesis by controlling the production of ALAS. The regulation of ALAS by drugs and heme will be discussed in detail in the next section.

1.6.5: Regulation of Heme Biosynthesis in Hepatic Tissues.

About, 15% of the heme synthesised in the body is produced in the liver parenchymal cells and of this *de novo* heme, at least half is utilised by the CYP microsomal mono-oxygenase system (Kappas *et al.*, 1989). Therefore in liver the rate of heme synthesis is intimately associated with the synthesis of CYP enzymes.

The superfamily of microsomal CYP proteins, together with the NADPH-dependent P450 reductase, catalyse the mono-oxygenation of a broad spectrum of lipophilic compounds such as cholesterol, steroids, fatty acids and foreign substances. In general, the microsomal CYPs found in the liver are involved in metabolising foreign substances, converting these hydrophobic substances to more hydrophilic derivatives that can be more easily eliminated from the body. CYPs catalyse the insertion of a single oxygen atom derived from molecular oxygen into a substrate, then depending on the nature of the unstable intermediate produced, a variety of different reactions can occur including hydroxylation, deamination and dealkylation. In many cases a functional group such as a hydroxyl group is formed, to which a battery of transferase enzymes can add various hydrophilic moieties including, glucuronic acid, glutathione, glucose, sulphate and cysteine (Jakoby and Ziegler, 1990). The net result of the addition of these groups is an increase in the hydrophilicity of the compound which facilitates its excretion from the body in bile or urine

However, not all CYP proteins are associated with catabolic processes, with some CYPs catalysing steps in the synthesis of steroid hormones. Many of these steroidogenic CYPs are located in the adrenal gland but other member's exist in the liver, kidney and gonads (Gonzalez, 1989 ; Porter and Coon, 1991 ; Darwish and Deluca, 1993). Unlike the catabolic CYPs, which usually possess a broad and overlapping substrate specificity, the steroidogenic CYPs are selective in their substrate specificity.

To date, over 200 CYP cDNAs have been isolated and these have been separated into 36 gene families on the basis of amino acid homology, 12 of which exist in mammals (Nelson *et al.*, 1993). The majority of the vertebrate CYPs are expressed in a tissue-specific manner and can be roughly divided into two broad groups; in one group are CYPs that are constitutively expressed while the other group consists of CYPs that show inducible expression. This latter group is of considerable interest as they metabolise a variety of phamacologically important drugs as well as a number of environmental toxins. The inducible CYPs include enzymes whose expression is increased by their substrates, which can be exogenous compounds such as drugs and

environmental pollutants or endogenous substances such as steroid hormones. The expression of other CYPs particularly those involved in steroid biosynthesis can be induced by certain hormones such as adrenocorticotropic hormone (ATCH) and glucocorticoids.

Although ALAS plays no direct role in drug metabolism, ALAS activity and mRNA levels, are strongly induced in the liver of animals, by a wide range of foreign chemicals (Granick, 1966), which interestingly, also induce CYP enzymes (May *et al.*, 1986 ; Srivastava *et al.*, 1989b). This induction of ALAS-1 mRNA, at least in the case of the barbiturate phenobarbital (PB) and the sedative 2-allyl-isopropylacetamide (AIA), has subsequently been shown to occur primarily at the transcription level in rat liver (Srivastava *et al.*, 1988 ; Yamamoto *et al.*, 1988 ; Srivastava *et al.*, 1990), chick embryo liver (Hansen *et al.*, 1989) and primary cultures of chick embryo hepatocytes (Hamilton *et al.*, 1991). This induction of ALAS-1 mRNA by chemicals that induce CYP apoprotein synthesis is not restricted to the liver but has also been observed in the testis following chorionic gonadotropin hormone treatment (Srivastava *et al.*, 1988), in the adrenal glands following ATCH administration (Rao *et al.*, 1990) and in the kidneys following AIA or PB treatment (Srivastava *et al.*, 1989a). In all cases this tissue-specific induction in ALAS-1 mRNA is accompanied by an increase in the mRNA for of a subset of CYP proteins (Srivastava *et al.*, 1988 ; Srivastava *et al.*, 1989a and 1989b ; Hansen and May, 1989 ; Drew and Ades, 1989). Presumably, the rationale behind the induction of ALAS by these compounds is to supply heme for the newly synthesised CYP apoproteins. In addition, certain of these inducers cause the autocatalytic destruction of the CYP proteins heme moiety during their metabolism, so that the induction of ALAS may serve a second role, in counterbalancing this loss (De Matteis, 1988).

Granick (1966) was the first to demonstrate that the drug-inducible increase in ALAS activity could be prevented by exogenous heme and therefore he proposed that ALAS synthesis is subject to a negative feedback repression by heme. Heme has subsequently been shown to regulate ALAS-1 levels by several mechanisms in the liver and presumably in other tissues as well. For example heme can repress the level of

ALAS-1 mRNA and inhibit the import of the ALAS-1 precursor protein into the mitochondria. The key questions to be answered are therefore; how does heme regulate ALAS-1 levels and how does a vast range of structurally diverse compounds induce ALAS-1 levels. These questions will be addressed in the following sections.

1.6.6: Mechanisms of Heme Repression of ALAS-1.

(i) Heme Lowers ALAS-1 mRNA Levels.

Heme has been shown to lower the steady state level of ALAS-1 mRNA in drug- and non-induced rat liver (Srivastava *et al.*, 1988 ; Yamamoto *et al.*, 1988), chick-embryo liver (Hamilton *et al.*, 1988) and chick primary hepatocytes (Ades *et al.*, 1987 ; Hamilton *et al.*, 1988). Furthermore, Srivastava *et al.* (1988) demonstrated that heme or its precursor ALA, could repress ALAS-1 mRNA levels in all tissues examined except erythroid spleen, implying that heme probably regulates basal ALAS-1 mRNA levels in all non-erythroid tissues. However, the current experimental data suggests that the repression of ALAS-1 mRNA by heme occurs by two different mechanisms in rats and chickens.

The level at which heme regulates ALAS-1 mRNA has been investigated in rat liver. Nuclear run-on experiments demonstrated that heme or ALA, could decrease the transcription rate of the ALAS-1 gene in drug- and non-induced rat liver (Srivastava *et al.*, 1988 and 1990). In a separate study by Yamamoto *et al.* (1988) it was also concluded that heme repressed the transcription of the ALAS-1 gene in rat liver. In this study intravenous heme administration was shown to rapidly decrease ALAS-1 mRNA levels in drug-induced rat liver. Since heme did not significantly affect the kinetics of ALAS-1 mRNA turnover, it was concluded that heme must be inhibiting the transcription of the ALAS-1 gene. An additional finding of this study was that the half-life of the ALAS-1 mRNA in rat liver was approximately 20 minutes, indicating that the mRNA is extremely unstable for a mammalian mRNA. A search for the sequences responsible for this instability form part of this thesis. Further characterisation of the

elements involved in the heme-mediated repression of ALAS-1 transcription has been hampered by the lack of a suitable *in vitro* culture system to study this phenomena. To date we have been unable to find a transformed cell line in which heme inhibits the transcription of the endogenous ALAS-1 gene (Healy, 1990 ; Braidotti, 1992). Recently, heme has been shown to repress the basal and drug-induced ALAS-1 mRNA levels in primary rat hepatocytes, cultured on the extracellular matrix "matrigel", but the level at which heme acts remains to be determined (Sinclair *et al.*, 1990). In contrast, to the results obtained by Yamamoto *et al.* (1988) analysis of the regulation of ALAS-1 mRNA levels by heme in rat hepatoma cells has also implicated heme in regulating the turnover of the ALAS-1 mRNA (this Thesis). The reason for this difference is discussed in detail in chapter three.

In chickens, heme appears to primarily affect the stability of the ALAS-1 mRNA. Evidence for an effect of heme on the turnover of the ALAS-1 mRNA was first presented by Drew and Ades (1989b) in drug-induced primary chick embryo hepatocytes. In this study, Drew and Ades showed that heme increased the turnover of the ALAS-1 mRNA by inhibiting transcription with α -amanitin. Subsequently we have shown by nuclear run on experiments, in 17-day drug-treated chick-embryos that the transcription of the ALAS-1 gene is unaffected by heme, whereas the level of ALAS-1 mRNA is rapidly decreased (Hahn, 1991). Similar studies have been performed in chick embryo hepatocytes (Hamilton *et al.*, 1991). In this study heme was shown to decrease the half-life of the ALAS-1 mRNA about 2.5 fold without affecting the transcription rate of the gene.

(ii) Heme inhibits ALAS-1 Translocation to the Mitochondria.

ALAS, is nuclear encoded and thus must be synthesised in the cytoplasm as a precursor prior to its import into the mitochondrion. Targeting of the ALAS precursor protein to the mitochondria, like the majority of nuclear-encoded products is dependent on an N-terminal presequence. During the import of the ALAS precursor this

presequence is removed by a matrix peptidase. For a review of import of proteins into the mitochondria the reader is referred to Schwarz and Neupert (1994).

Hayashi *et al.* (1972) were the first to observe that the administration of heme to drug-induced rats caused ALAS to accumulate in the cytosol of rat liver, while ALAS levels in the mitochondria rapidly decreased. Subsequently Kikuchi and Hayashi (1981) proposed a novel negative feedback mechanism in which heme inhibits the import of rat ALAS precursor into the mitochondrion. A similar mechanism has since been described in chick embryo livers (Srivastava *et al.*, 1983 ; Ades, 1983 ; Hayashi *et al.*, 1983). This inhibition of ALAS translocation occurs at physiological concentrations of heme (Yamamoto *et al.*, 1981) and appears to be specific for ALAS-1 as the import of an unrelated mitochondrial enzyme pyruvate carboxylase, was unaffected by heme (Srivastava *et al.*, 1983). Since the half-life of ALAS in the mitochondria has been estimated to be about 35 minutes (Hayashi *et al.*, 1980), inhibition of ALAS import would led to a rapid depletion of the enzyme and hence a rapid decrease in the rate of heme synthesis, when heme levels are high.

Although the specific mechanism underlying this heme-mediated inhibition of ALAS-1 translocation has not been fully elucidated, a recent study on the translocation of the erythroid-specific ALAS-2 enzyme has implicated two conserved amino acid motifs in the ALAS presequence in this phenomena (Lathrop and Timko, 1993). These motifs resemble the heme binding sequence (HRM) required for the heme activation of the yeast transcription factor HAP-1 (Pfiefer *et al.*, 1989) and are thought to bind heme directly (Zhang and Guarente, 1995). Since these sequences identified by Lathrop and Timko, are conserved between the ALAS-1 and ALAS-2 proteins it seems likely that these motifs are also involved in the regulation of the ALAS-1 precursor translocation.

1.6.7: Induction of ALAS-1 mRNA by Xenobiotics.

The mechanisms by which a vast range of compounds increase the amount of ALAS activity has attracted considerable attention, particularly since many of these compounds are known to precipitate attacks of acute porphyria in genetically susceptible

individuals (Kappas *et al.*, 1989). It has been noted that all these compounds that induce ALAS also induce the synthesis of one or more CYP proteins. Moreover, the induction of ALAS-1 occurs in the same tissue-specific pattern as that observed for the CYP proteins (May *et al.*, 1986). Thus the induction of ALAS appears to be to supply heme for the newly synthesised CYP apoproteins.

This increase in ALAS activity is accompanied by a rise in the amount of ALAS-1 mRNA. In rat liver, a range of compounds known to induce different classes of CYP proteins also increase ALAS-1 mRNA levels (Srivastava *et al.*, 1989b). However, although each compound strongly induced the expression of a particular subset of CYP genes, the level of induction of ALAS-1 mRNA varied significantly between the different compounds. For instance, when the rats were administered PB or 2-allyl-isopropylacetamide (AIA), which induce the PB-inducible class of CYPs, the level of ALAS-1 mRNA increased markedly. In contrast, dexamethasone, β-naphthoflavone and clofibrate which induce different classes of CYPs, caused a more moderate increase in ALAS-1 mRNA levels. Whether these differences in the induction of ALAS-1 mRNA reflect differences in the requirement of heme for CYP formation in each case, or differences in the ALAS-1 induction mechanism is not known. Induction of ALAS-1 mRNA by succinylacetone, a specific inhibitor of *de novo* heme synthesis was also observed in this study. Interestingly this induction of ALAS-1 mRNA occurred in the absence of a detectable rise in CYP synthesise and could be prevented by heme. In addition, when the rats were treated with drugs together with succinylacetone, the level of ALAS-1 mRNA showed a greater increase than with either compound alone. Thus in rat liver ALAS-1 mRNA levels appear to be positively regulated by drugs and negatively regulated by heme.

As mentioned, this induction of ALAS-1 mRNA by chemicals that induce CYP apoprotein synthesis is not restricted to the liver but has also been observed in the testis following chorionic gonadotropin hormone treatment (Srivastava *et al.*, 1988), in the adrenal glands following ATCH administration (Rao *et al.*, 1990) and in the kidneys following AIA or PB treatment (Srivastava *et al.*, 1989a). In all cases this tissue-specific

induction in ALAS-1 mRNA is accompanied by an increase in the mRNA levels of a subset of CYP proteins (Srivastava *et al.*, 1988 ; Srivastava *et al.*, 1989a and 1989b).

Transcriptional run on experiments have established that PB and AIA increase the rate of transcription of the ALAS-1 gene in rat liver (Srivastava *et al.*, 1988 and 1990). These drugs also induce the transcription of a specific subset of CYP genes but the mechanism for this induction remains elusive. Dexamethasone has also been reported to cause a modest 2-fold increase the transcriptional rate of the ALAS-1 gene in rat liver (Srivastava *et al.*, 1992) but whether other inducers of ALAS-1 mRNA also increase the transcription rate of the ALAS-1 gene has not been investigated. Similarly PB, AIA or 2-propyl-2-isopylacetamide (PIA) rapidly increase the transcription rate of the ALAS-1 gene in chick embryo liver (Hansen *et al.*, 1989 ; Dogra *et al.*, 1993) and primary chick embryo hepatocyte cultures (Hamilton *et al.*, 1991). Accompanying this rise in ALAS-1 transcription is a rise in the transcription of the major PB-inducible CYP genes in chicken liver; CYP2H1 and CYP2H2 (Hobbs *et al.*, 1986)

Two models have been proposed to explain the induction of ALAS-1 mRNA by these compounds. In the first model originally proposed by Granick (1966), drugs act directly on the ALAS-1 gene to increase its transcription rate. This hypothesis necessitates that the regulation of the ALAS-1 gene be complex. As mentioned ALAS is co-ordinately induced by any compound that induces CYP expression and since the elements involved in the induction of the different classes of CYP genes are distinct it therefore appears that the ALAS-1 gene must contain all the regulatory elements required for its co-ordinate induction by the different class of inducers.

For example, the induction of the aryl hydrocarbon inducible CYPs is dependent on the intracellular aryl hydrocarbon receptor (AhR). The Ah receptor functions as a ligand dependent DNA binding protein and activates transcription by binding to specific cis-acting control sequences termed Xenobiotic response elements (XREs) in the promoter of the target gene. The AhR receptor normally binds to DNA as a complex with the auxiliary factor Arnt (Whitelaw *et al.*, 1993).

A specific receptor is also involved in the induction of the hypolipidemic-inducible CYP genes. The hypolipidemic agents such as clofibrate and Wy-14,643

activate gene expression by binding to a specific member of the steroid hormone receptor superfamily, termed the peroxisome proliferator-activator receptor (PPAR). PPAR activates gene expression in a ligand dependent manner by binding to an imperfect direct repeat of the canonical hormone response element (AGGTCA). In the case of PPAR it binds to direct repeats separated by a single nucleotide (Muerhoff *et al.*, 1992). Recently, it has been suggested that the PPAR binds to its response element as a heterodimer with the retinoid X receptor (Isseman *et al.*, 1993).

The induction of members of the CYP3A gene family by glucocorticoids and antiglucocorticoids on the otherhand is not as well understood. Transfection studies by Burger *et al.* (1992) has defined a 164bp sequence in the promoter of the CYP3A1 gene that activates transcription in response to the synthetic glucocorticoid dexamethasone (Dex) and the glucocorticoid agonist pregnenolone-16α -cabonitrile (PCN). This 164bp fragment does not contain a traditional glucocorticoid response element. This fact, together with other differences in the glucocorticoid regulation of the CYP3A1 gene compared to genes regulated by the classical glucocorticoid response element, has led to the proposal that the CYP3A family is induced by glucocorticoids by a non-classical gluocorticoid receptor-mediated process (Schuetz *et al.*, 1986 ; Burger *et al.*, 1992).

Specific elements have also been implicated in the induction of the steroidogenic CYPs in the adrenal gland. The rate of steroidogenesis in the adrenal cortex is regulated primarily by the peptide hormone ATCH which is produced by the anterior pituitary. One action of ATCH is to co-ordinately increase the transcription of the steroid hydroxylases, thus maintaining optimal steroidogenesis. The action of ATCH appears to be mediated via cAMP and thus these genes were thought to contain cAMP responsive elements. The elements involved in this induction has been determined for two of these steroid hydroxylases, these are the microsomal CYPs; CYP17 and CYP21. Identification of the elements in the promoter of the bovine CYP17 and human CYP21 genes responsible for ATCH induction demonstrated that these two genes contained unique and unrelated cAMP responsive sequences (CRS) both of which are distinct from the consensus cAMP responsive element (CRE) found in other genes. Moreover, these elements are bound by different *trans*-acting factors (Zanger *et al.*, 1992).

The *cis*-acting sequences involved in mediating the induction of the PB-inducible class of CYPs has not yet been identified. However, several studies have identified promoter regions involved in the activation of the major chicken and rat PB-inducible genes (Hahn *et al.*, 1991 ; Ramsden *et al.*, 1993). Currently, the drug-responsive cis-acting elements in these regions are being sought.

In the alternative model, proposed by May *et al.* (1986), the transcription of the ALAS-1 gene is regulated solely by intracellular heme levels. According to this hypothesis, chemical inducers activate the transcription of the ALAS-1 gene indirectly by lowering a regulatory pool of free heme in the cell. This lowering of intracellular heme levels has been proposed to occur in several ways. Firstly, the increase in CYP apoprotein, caused by the activation of the CYP genes by drugs, is thought to sequester much of this free heme. In addition, several strong inducers of ALAS-1 such as AIA and DDC can also cause the autocatalytic destruction of the heme moiety of the CYP protein during their metabolism. This modified heme molecule is then released from the CYP protein and may in fact inhibit ferrochelatase. The resultant apo-CYP protein, itself, appears to be undamaged by this reaction and can go on to sequester more heme (Ortiz de Montellano and Carreia, 1983 ; De Matteis, 1988). Consistent with this proposal, the level of "free" cytoplasmic heme does appear to drop during drug-induction as measured by the degree of heme saturation of the cytoplasmic enzyme tryptophan pyrrolase (Badawy and Evans, 1975 ; Yamamoto *et al.*, 1981). Presumably, any regulatory heme pool in the nucleus will be equilibrium with this "free" cytoplasmic heme.

However, recent experimental data on the induction of the ALAS-1 gene in response to inducers of the CYP2H gene family (PB-inducible) in the chicken is inconsistent with this model. A key prediction in this heme-derepression model of drug-induction is that a reduction in the level of heme must occur prior to de-repression of the ALAS-1 gene. In the case of drugs that induce CYP genes, it can be predicted that the synthesis of CYP apoprotein must precede the induction of ALAS-1 transcription in order to lower heme and derepress ALAS-1 transcription. However, in chick embryo's, PB and AIA appear to simultaneously induce the transcription of the ALAS-1, CYP2H1 and CYP2H2 genes (Hansen *et al.*, 1989), whereas in the heme-repression model, an

increase in CYP transcription would have been expected to occur prior to ALAS-1 activation. Also pretreatment of chick embryo's with the translational inhibitor cycloheximide, did not inhibit the induction of ALAS-1 transcription by PB, but in fact caused a superinduction of ALAS-1 gene transcription (Dogra *et al.*, 1993). Since the concentration of cycloheximide used inhibited translation in the liver by at least 90%, it seemed unlikely that enough apo-CYP was synthesised to lower the regulatory heme pool sufficiently, to account for the rapid induction of ALAS-1 transcription. Lastly, we and others have been unable to demonstrate an effect of heme on the transcription rate of the ALAS-1 gene either in chick embryo liver (Hahn, 1991) or in primary chick embryo hepatocytes (Hamilton *et al.*, 1991). As mentioned, the reduction of ALAS-1 mRNA in these cells can be accounted for by an increase in the turnover rate of the mRNA. Therefore in chick embryo liver it would appear that, at least for the inducers of the CYP2H family (PB-inducible CYPs), these compounds act directly upon the ALAS-1 gene to increase its transcription. Heme, meanwhile controls the amount of ALAS-1 enzyme activity and hence the rate of heme synthesis, by regulating the half-life of the mRNA and by inhibiting the import of the precursor into the mitochondria. This latter step appears to be particularly important in chick embryo liver, since heme concentrations that completely suppress the drug-mediated induction of mitochondrial ALAS activity only inhibit the increase in ALAS-1 mRNA level by about 50% (Hamilton *et al.*, 1988).

As mentioned, in rats, heme can apparently inhibit the PB or AIA induced transcription of the ALAS-1 gene (Srivastava *et al.*, 1988 and 1990 ; Yamamoto *et al.*, 1988). It should also be noted that cycloheximide pretreatment of primary rat hepatocytes, unlike the situation in chick embryo's, abolishes the induction of ALAS-1 mRNA by a range of different drugs, but whether this is due to the suppression of CYP synthesis or some other mechanism is unknown (Burger *et al.*, 1990). Although both these observations in rats are consistent with the indirect heme-repression model of ALAS-1 drug-induction, they are not inconsistent with a direct effect of PB or AIA on the transcription of the ALAS-1 gene. Whether heme is the sole regulator of ALAS-1 transcription during drug-induction or whether drugs induce ALAS-1 transcription

directly in rats, awaits the identification of the elements responsible for heme-repression and drug-induction. Currently we are attempting to define regions in the ALAS-1 gene promoter that are responsible for these phenomena.

To date drugs that activate the PB-inducible and Dex-inducible CYPs have been investigated at the molecular level and so a point yet to be addressed is how do inducers of other classes of CYP genes, induce ALAS-1 mRNA?

1.6.8: Basal ALAS-1 Levels May be Co-ordinated with Mitochondrial Biosynthesis and Respiration.

In many tissues a significant portion of the heme produced goes to supply the respiratory cytochromes b, c_1, c, a and a_3. These cytochromes form part of the mitochondrial oxidative phosphorylation system, which consists of five multi-subunit complexes. These complexes are assembled from at least 60 different polypeptides which are encoded for by both nuclear and mitochondrial genes. In mammals, nuclear genes encode for the majority of the respiratory subunits as well as for all the enzymes and structural proteins required for mitochondrial transcription, translation and replication, while the mitochondrial genome contribute 13 subunits to the respiratory complex along with the ribosomal and transfer RNAs required for mitochondrial translation (Reviewed Altardi and Schatz, 1988). Several observations suggest that in vertebrates, expression of the nuclear and mitochondrial genes are co-ordinated with mitochondrial biogenesis and respiration to ensure efficient complex assembly. For example, a co-ordinated increase in the mRNA for several electron transport chain proteins has been observed in response to various metabolic and physiological stimuli, known to increase mitochondrial biogenesis and respiration (Williams *et al.*, 1987 ; Scarpulla *et al.*, 1986). In addition, the expression of some of these nuclear-encoded genes are also growth regulated, as shown by the increase in ADP/ATP translocase mRNA when quiescent cells are stimulated by serum or growth factors. This increase is thought to reflect the increased energy demand of proliferating cells (Battini *et al.*, 1987).

Since heme is required as a prosthetic group for the respiratory cytochromes in all cells it was possible that basal expression of the ALAS-1 gene may be co-ordinated with the synthesis of these components of the oxidative phosphorylation system. In support of this proposal, ALAS activity is rapidly increased in muscle in response to conditions that increase mitochondrial cytochrome production such as exercise (Essig *et al.*, 1990), thyroxine treatment (Holloszy and Winder, 1979) and development (Essig and McNabney, 1991).

Characterisation of the control elements in the promoter of the ALAS-1 gene required for its basal expression has provided further support for this notion (Braidotti, 1992 ; Braidotti *et al.*, 1993). Sequence comparisons of the human, chicken and rat promoter regions revealed several putative cis-acting sequences which have been implicated in the expression of other nuclear genes, required for mitochondrial biogenesis, respiration and growth.

Adjacent to the TATA box in the rat, chicken and human ALAS-1 promoters is a sequence that resembles a thyroid hormone response element (TRE). Thyroid hormone is a well known physiological effector of respiration rates, biosynthesis of respiratory proteins and overall mitochondrial biogenesis (Scarpulla *et al.*, 1986). Currently, we have some evidence to indicate that thyroid hormone can modulate ALAS-1 expression, since co-transfection of COS-1 cells (COS-1 cells lack thyroid hormone receptor) with rat ALAS-1 promoter constructs, carrying this region, and a thyroid hormone receptor expression clone resulted in an increase in the expression of the reporter gene. However, it is unclear at this stage if the increase in the expression of the reporter gene observed is due to a direct interaction between the thyroid hormone receptor and the ALAS-1 promoter. Experiments are currently being performed to investigate this.

Also present in all three ALAS-1 promoters are binding sites for the transcription factor; nuclear respiratory factor-1 (NRF-1), which has been shown to be required for the expression of a number of other nuclear encoded mitochondrial proteins (Evans and Scarpulla, 1990). The rat ALAS-1 promoter contains two NRF-1 sites at -59bp and -88bp, mutagenesis of these sites followed by transient transfection studies demonstrated that both these sites contribute to the basal transcription rate of the gene.

Moreover, disruption of both sites resulted in almost complete loss of expression indicating that the NRF-1 sites were essential for basal expression. Gel shift analysis using crude nuclear extract from a number of different cell lines detected a widely distributed protein that bound to these NRF-1 motifs. This complex showed the same mobility and thermostability as an authentic NRF-1 complex. Furthermore, the binding of this protein to the ALAS-1 NRF-1 sites could be abolished in a competition assay with an authentic (cytochrome c) NRF-1 site confirming that NRF-1 bound to these sites (Braidotti, 1992 ; Braidotti *et al.*, 1993).

NRF-1 was initially identified as a trans-activator of the cytochrome c gene (Evans and Scarpulla, 1989) and has subsequently been shown to be important for the transcription of at least one subunit in each of the respiratory complexes III, IV and V (Evans and Scarpulla, 1990 ; Chau *et al.*, 1992). NRF-1 sites have also been shown to be important for the transcription of two nuclear genes linked with mitochondrial transcription and replication. These are the mitochondrial RNA processing (MRP) endonuclease, thought to cleave the light-strand transcript to form primers for heavy-strand DNA replication and the mitochondrial transcription factor A (mtTFA) involved in the initiation of transcription from the heavy- and light-strand promoters (Evans and Scarpulla, 1990 ; Virbasius *et al.*, 1993 ; Virbasius *et al.*, 1994). This has led to the proposal that NRF-1 is important in co-ordinating the expression of nuclear and mitochondrial genes during organelle biogenesis. In this model NRF-1 sites in the ALAS-1 promoter serve to co-ordinate the level of heme synthesis with the synthesis of the respiratory chain subunits.

Recent data has suggested that NRF-1 may also have a more global function, since a systematic search for potential NRF-1 binding sites identified a number of potential target genes which encode products not directly involved in respiration or mitochondrial biogenesis (Varbasius *et al.*, 1993). Generally these potential target genes are ubiquitously expressed and code for either proteins involved in a rate-limiting step in a metabolic pathway or in other general metabolic functions such as signal transduction, chromosome maintenance and nucleic acid metabolism. Therefore it appears NRF-1 has the potential for integrating the expression of a number of proteins

with diverse functions required for cell maintenance, growth and proliferation. Although the function of these putative NRF-1 binding sites has not been investigated in most cases, the NRF-1 site in the gene for eukaryote initiation factor-2α (eIF-2α) has been shown to be functional (eIF-2α forms part of the translation initiation factor eIF-2 which catalyses the rate-limiting step in the initiation of translation). This site was initially identified in the promoter of the eIF-2α gene by Jacob *et al.* (1989) who subsequently termed the factor that bound to this site α-Pal. Whether, α-Pal and NRF-1 were identical or related proteins has recently been resolved by the purification and cloning of both factors (Virbasius *et al.*, 1993 ; Efiok *et al.*, 1994). Comparison of the nucleotide and amino acid sequence indicated that NRF-1 and α-Pal were identical.

Lastly, the rat and human ALAS-1 promoters contain a region that resembles the OXBOX element (Braidotti, 1992). The OXBOX has been found in several nuclear encoded oxidative phosphorylation genes and has been implicated in their increased expression in heart and skeletal muscle (Chung *et al.*, 1992). A protein which bound to a oligonucleotide corresponding to the rat ALAS-1 sequence was detected in nuclear extracts from the muscle myoblast cell line C_2C_{12} but not in hepatoma (HepG2) and kidney (COS-1) nuclear extracts, suggesting that the rat ALAS-1 OXBOX-like element may bind a muscle specific factor. The function of this element and identity of the protein which binds is currently being pursed in our laboratory.

Thus the striking feature of the regulatory elements identified in the ALAS-1 promoters so far is that they have all been implicated as regulators of genes involved in respiration and/or the growth of the cell. Therefore it appears that basal expression of ALAS-1 and hence heme synthesis is co-ordinately regulated with these processes.

1.7: Heme Catabolism.

The rate of heme metabolism in cells is dependent on both the rate of heme synthesis and its degradation. Therefore, the intracellular heme concentration will be determined largely by the relative levels of the two rate-limiting enzymes in the

respective synthetic (ALAS) and degradative (heme oxygenase) pathways. Research has therefore tended to focus on the regulation of these two enzymes. Although this thesis is concerned with the regulation of ALAS-1 by heme, the relationship between intracellular heme levels and the regulation of heme oxygenase (HO) warrants a brief review on HO.

Since excess heme appears to be toxic to cells, resulting in lipid and protein oxidation, probably all cells are capable of degrading heme (Muller-Erberhard and Fraig, 1993 ; Balla *et al.*, 1991). Heme oxygenase is the first and rate-limiting enzyme in heme catabolism. The enzyme is located in the endoplasmic reticulum and catalyses the cleavage of heme with the elimination of a porphyrin methene bridge as carbon monoxide and the formation of the bile pigment, biliverdin. This reaction is dependent on the transfer of electrons by NADPH cytochrome P450 reductase. Iron is released in this reaction, which is then re-utilised. Biliverdin is subsequently converted to bilirubin by biliverdin reductase and the bilirubin rendered more water soluble by conjugation with glucuronic acid in a reaction catalysed by UDP-glucuronyl-transferase, prior to its excretion in bile (Reviewed Brown *et al.*, 1990).

Heme oxygenase exists as two isozymes, heme oxygenase-1 (HO-1) and heme oxygenase-1(HO-2), which are the products of two separate genes (Cruse and Maines, 1988 ; McCoubrey and Maines, 1994). These genes are regulated quite differently and it has recently become apparent that the biological role of these two isozymes may be distinct.

HO-1 is expressed ubiquitously, but is present at high levels in the liver and spleen where it is responsible for the degradation of haemoglobin heme. A number of studies have now demonstrated that the HO-1 isozyme is induced by a number of oxidative-stress associated agents including heme, heavy metals, hyperthermia and UV radiation (Reviewed Maines, 1992 ; Shibahara, 1994). This induction of HO-1 by oxidative stress has been proposed to represent an antioxidant defence system (Stocker, 1990). Induction of HO-1 is thought to function as an antioxidant defence by decreasing the level of the potential pro-oxidants heme and heme-containing proteins as well as

increasing the concentration of the anti-oxidant bilirubin. Bilirubin has recently been proposed to function as a physiological antioxidant (Stocker *et al.*, 1987).

Presumably, the induction of HO-1 by heat stress serve's to destroy heme released from denatured hemoproteins (Raju and Maines, 1994). Although a role for the induction of HO-1 by agents that cause oxidative stress has not been fully elucidated, there appears to be an inverse relationship between the level of cellular glutathione, an important cellular antioxidant, and HO-1 induction (Lautier *et al.*, 1991). This study found that the depletion of cellular glutathione levels led to an increase in the basal expression of the HO-1 isozyme as well as increasing its induction in response to other agents, such as sodium arsenite and UV radiation, suggesting that HO-1 may play an antioxidant role. A protective role for HO-1 *in vivo* against oxidative stress has recently been suggested by the finding that pre-induction of HO-1 provides a rapid protective response to kidney failure and morbidity in the rat model for rhabdomyolysis syndrome (Nath *et al.*, 1992). Rhabdomyolysis syndrome is a disease instigated by muscle necrosis and is dominated by kidney failure. Renal failure is thought to be caused by tissue oxidation catalysed by the heme moiety of myoglobin. This syndrome can be mimicked in rats by intramuscular injections of hypertonic glycerol. In this rat model of the syndrome a single dose of haemoglobin heme, which induced HO-1, was sufficient to substantially reduce kidney failure and rat morbidity while conversely addition of HO-1 inhibitors increased the severity of the symptoms, suggesting that HO-1 induction may play an important protective role *in vivo*.

Heme and other inducers of HO-1 dramatically induce the transcription of the HO-1 gene, which in the case of heme is prevented by cycloheximide, suggesting that a labile protein may be required for induction (Alam and Smith, 1992). Several regulatory elements have been identified in the promoter of the HO-1 gene that are responsible for its induction by various agents.

Alam *et al.* (1994) has identified a heme and metal responsive enhancer region, termed SX2, approximately 4kb upstream of the CAP site of the mouse HO-1 gene. SX2 was previously demonstrated to function as a basal and TPA inducible enhancer, apparently through the binding of the transcription factor, AP-1, to two AP-1 binding

sites (Alam and Den, 1992). Takeda *et al.* (1994) have also recently identified a region in the human HO-1 promoter that is responsible for the cadmium-mediated induction of the human HO-1 gene (Takeda *et al.*, 1994). Transient transfection analysis of human HO-1 promoter constructs defined a 500bp region located between 4.5kb and 4kb upstream of the CAP site which was able to respond to cadmium treatment. Apart from being located a similar distance from the CAP site as the SX2 region identified by Alam *et al.* (1994), sequence analysis showed that the 3'-end of the human region shared substantial (64%) sequence similarity with SX2. Deletion and gel mobility analysis, further localised the cadmium responsive region to 20bp region. As yet the *cis*-acting sequence has not been fully elucidated but on the basis of their preliminary mutagenesis Takeda *et al.* (1994) has proposed that the sequence TGCTAGATTT forms the cadmium responsive element. The mouse cadmium inducible region, SX2, contains a similar sequence aGacAGATTT but whether this element is functional is not known. Interestingly, the 20bp cadmium responsive region is unable to respond to other inducers of HO-1, including heme, cobalt protoporphyrin and sodium arsenite, implying that the cis-acing elements responsible for the induction by these molecules must lie elsewhere. The sequence of this cadmium inducible element (CdRE), is distinct from the previously characterised metal-regulatory elements (MREs) found in the heavy metal inducible metallothionein genes (Culutta and Hamer, 1989). In addition, unlike the metallothionein MREs, the CdRE appears to be specific for cadmium and is unresponsive to zinc, although the HO-1 gene is strongly induced by this metal (Takeda *et al.*, 1995). Therefore, at least two types of metal responsive elements must exist in the human HO-1 promoter.

The molecular mechanism by which thermal stress induces the rat HO-1 gene has also been investigated (Okinaga and Shibahara, 1993). Sequence analysis of the rat HO-1 promoter identified two potential heat-shock elements (HSE1 and HSE2). Transient transfection analysis demonstrated HSE1 was sufficient to confer heat-shock upon the reporter gene while HSE2 by itself, could not, however the inclusion of HSE2 increased the magnitude of the response to heat-shock by HSE1. Gel mobility shift assays indicate that both elements bind a heat-shock activated protein while HSE2, but

not HSE1, also binds a constitutive factor. The role each of these proteins play in the regulation of heat-shock activation of the HO-1 gene has not been fully defined.

Lastly, several groups have examined the binding of proteins to a number of putative binding sites for known transcription factors found in the human HO-1 promoter. Extracts were prepared from non-induced and induced cells in the hope of finding a change in the binding pattern of these factors under the different conditions which may implicate a specific factor in the induction process (Lavrovsky *et al.*, 1994 ; Nascimento *et al.*, 1993). Lavrovsky *et al.* (1994) found that heme treatment of K562 cells specifically increased the binding of the transcription factors NF-κB and AP-2 to their putative binding sites in the human HO-1 promoter, suggesting that these factors may play a role in the activation of the HO-1 gene in response to heme. Of particular interest was the identification of a putative NF-κB site in the promoter, since this transcription factor is known to be involved in the activation of other genes in response to stress-stimuli (Liou and Baltimore, 1993) and to be under redox control (Mihm *et al.*, 1995). This last point is particularly provocative given that many of the agents that induce HO-1 expression can potentially modify the redox potential of the cell (Applegate *et al.*, 1991). In the second study, Nascimento *et al.* (1993) found that UVA irradiation of cells, strongly induced the binding activity of a protein to a sequence which included the core consensus sequence for the upstream stimulatory factor (USF). However, in both studies, a functional role for these binding sites in the activation of HO-1 transcription has yet to be demonstrated.

Thus like the regulation of the rate-limiting enzyme in heme biosynthesis (ALAS) the regulation of HO-1 appears to be complex, presumably to allow the fine control of heme levels under various metabolic and physiological conditions.

Unlike HO-1, HO-2 expression is not induced by heme or any of the other inducers of HO-1 but rather is constitutively expressed in many tissues, with the highest expression levels detected in the brain and testis. The biological role of HO-2 has been unknown for many years but recently it has been implicated in the generation of carbon monoxide in the brain. Carbon monoxide has been postulated to act as a neural messenger in the brain by activating guanyl cyclase to produce cyclic GMP, through its

binding to the heme moiety of the enzyme (Verma *et al.*, 1993 ; Maines, 1993). Since carbon monoxide is generated in the body by the action of heme oxygenase, and *in situ* hybridisation of brain slices revealed discrete neuronal co-localisation of guanyl cyclase, HO-2 and NADPH cytochrome P450 reductase mRNA, it was proposed that HO-2 may be involved in generating the carbon monoxide messenger (Verma *et al.*, 1993). In the same study high levels of ALAS mRNA were also co-localised with the other mRNAs, suggesting that ample heme was being generated in these cells to act as a substrate for HO-2. A role for carbon monoxide and HO-2 as part of a messenger system in the brain, was supported by the finding that inhibition of heme oxygenase activity prevented an increase in cyclic GMP levels in olfactory neuron's following odorant stimulation (Verma *et al.*, 1993), and also retarded the induction of long-term potentiation, a learning-linked process (Stevens and Wang, 1993).

1.8: mRNA Stability and Degradation in Eukaryotes.

Since, the majority of the work in this thesis deals with the regulation of the turnover of ALAS-1 mRNA, a brief review on eukaryotic mRNA degradation is presented in the following section. For recent reviews on this subject the reader is referred to Sachs (1993) and Decker and Parker (1994).

In eukaryotes mRNA turnover is a highly regulated process that can have a profound impact on the level and time period that a protein is produced. In mammalian cells, mRNA half-lives can vary over several orders of magnitude, for instance the half-life of the mRNA for the proto-oncogenes c-fos and c-myc is about 10 to 30 minutes in most cells, while in the same cell the α- and β-globin mRNAs may have half-lives of between 24 to 60 hours (Kabnick and Housman, 1988 ; Weiss and Liebhaber, 1994). The existence of highly unstable mRNAs is thought to allow for rapid and precise fluctuations in the level of a transcript and hence protein level, following an alteration in the rate of transcript synthesis or turnover (Hargrove and Schmidt, 1989). Examples of this include the transient expression of c-fos mRNA following serum stimulation of

quiescent cells (Zinn *et al.*, 1988) and the rapid fluctuations in the level cyclin B1 mRNA during the different phases of the cell cycle (Maity *et al.*, 1995). Conversely, many proteins that either need to be produced in large amounts or whose translation is regulated have stable mRNAs, as exemplified by the continued synthesis of globin chains in reticulocytes after the extrusion of the nucleus in mammals and the translation of maternal mRNAs in the egg during development (Sheets *et al.*, 1994).

The importance of mRNA decay in determining the overall level of gene expression is also exemplified by several human diseases which have been associated with changes in the stability of specific mRNAs. For example, a number of mutations that stabilise the c-fos and c-myc mRNAs have been associated with the transformed phenotype (Schiavi *et al.*, 1992). Also mutations that destabilise either the α- or β-globin mRNAs have been implicated in certain types of α- or β-thalassemias respectively (Weiss and Liebhaber, 1995 ; Lim *et al.*, 1992 ; Kugler *et al.*, 1995).

Searchers for RNA determinants involved in this differential decay of eukaryotic mRNAs have demonstrated that these determinants are comprised of discrete sequences located within the RNA. These elements can be roughly divided into two types. The larger group contains elements that function as destabilising sequences while the smaller group contain elements involved in the stabilisation of mRNAs in response to certain physiological stimuli. Destabilising elements were initially identified from highly unstable mRNAs by their ability to confer destabilisation to normally stable mRNAs (Shaw and Kamen, 1986 ; Owen and Kuhn, 1987 ; Yen *et al.*, 1988 ; Kabnick and Housman, 1988 ; Shyu *et al.*, 1989 ; Wisdom and Lee, 1991 ; Bernstein *et al.*, 1992 ; Nanbuet *et al.*, 1994 ; Alberta *et al.*, 1994). Many of these highly labile mRNAs have been found to contain several instability elements, which function independently, so that deletion of one element, often does not stabilise the mRNA (Shyu *et al.*, 1989).

Apart from the 5'CAP and 3' poly(A) tail structures found at the termini of nearly all eukaryotic mRNAs, and which together with the proteins that bind to them are thought to provide a general mechanism for preserving the integrity of mRNAs (Sachs and Wahle, 1993 ; Coutts and Brawerman, 1993 ; Rhoads, 1993) several elements involved in the stabilisation of specific mRNAs have also been identified. These

stabilising sequences have so far been identified in mRNAs whose stability changes in response to certain physiological stimuli such as iron depletion (Binder *et al.*, 1994), hormone levels (Dodson and Shapiro, 1994) and differentiation (Weiss and Liebhaber, 1995). Analysis of the function of these stabilising elements has suggested that they function by silencing a nearby destabilisation element. Furthermore, in no case has a stabilising sequence, by itself, (ie. when separated from its native destabilising element) been able to confer stability to a heterologous mRNA.

For example, the binding of the IRE-BP to the IREs found in the 3'UTR of the human transferrin receptor mRNA under iron starvation conditions, leads to the stabilisation of the mRNA. This was demonstrated by the expression of mutant transferrin receptor genes in mouse fibroblasts, carrying single base changes in the loop of the IREs, known to abolish IRE-BP binding *in vitro* (Casey *et al.*, 1989). These constructs expressed a mRNA which was constitutively unstable irrespective of the iron status of the cell. However, several mutations introduced into the iron-regulatory region, which did not disrupt IRE-BP binding, resulted in mRNA species that were constitutively stable even in the presence of high iron levels. This data was interpreted as evidence for a separate rapid turnover determinant in this region whose activity can be inhibited by the binding of the IRE-BP to adjacent IREs. This notion is supported by the fact that IRE/IRE-BP complexes in other genes, such as in ferritin mRNA, do not seem to be associated with increased stability of the mRNA (Koeller *et al.*, 1991). Recent evidence suggests that this rapid turnover determinant is a target for a site specific endo-ribonuclease and thus binding of the IRE-BP to adjacent sequences are thought to prevent this endonucleolytic attack on the rapid turnover determinant (Binder *et al.*, 1994). Similar mechanisms have been proposed to explain the stabilisation of the α -globin mRNA during erythroid differentiation and the stabilisation of the vitellogenin mRNA during estrogen administration. In the case of α -globin the formation of a specific protein complex on sequences present in the 3'UTR of the mRNA (Wang *et al.*, 1994) is thought to stabilise the mRNA by protecting it from attack by an erythroid-specific RNase (Weiss and Liebhaber, 1995). An estrogen-inducible protein that binds to the 3'UTR of the vitellogenin mRNA has also been implicated in the stabilisation of

this mRNA by estrogen (Dodson and Shapiro, 1994). This protein binds directly adjacent to a region of the 3'UTR which resembles a sequence implicated in the turnover of apolipoprotein II, another estrogen-stabilised mRNA (Binder *et al.*, 1989). This region of the apolipoprotein II mRNA contains multiple AAU sequences within a single stranded region which appear to be a target for an endonuclease. This has led to the proposal that the binding of this estrogen-inducible protein to its site next to this region in the vitellogenin 3'UTR may interfere with the recognition of these endonuclease sensitive sites (Dodson and Shapiro, 1994).

These data, namely; i) the characterisation of destabilising elements capable of functioning in a heterologous mRNA; ii) the existence of multiple destabilising elements within highly labile mRNAs and iii) the inability to detect stabilising sequences able to function independently of their native context, has led to the proposal that mRNAs are inherently stable within a cell, in the absence of specific destabilising elements (Sachs, 1993). Thus the decay rate of any particular mRNA will be principally determined by the number and strength of its destabilising sequences.

1.8.1: Eukaryotic Instability Determinants.

To date, instability elements have been found in the coding and 3'-untranslated region of mRNA, but none as yet have been definitively localised to the 5'UTR. While some of these instability determinants are found in several different mRNAs, suggesting that the decay of these mRNA may be co-ordinated, the majority appear to be unique for each particular mRNA. In addition, the activity of many of these instability determinants can be regulated by adjacent sequences or by the modification of the *trans*-acting factors that recognise them. For example, the binding of the IRE-BP to the 3'UTR of the transferrin receptor mRNA inhibits the activity of the rapid turnover determinant (Binder *et al.*, 1994) while the phorbal ester-mediated stabilisation of the GM-CSF (Malter and Hong, 1991 ; Rajagopalan and Malter, 1994) and ribonucleotide reductase R2 (Amarat *et al.*, 1994) mRNA has been correlated with a change in the binding

activity of the *trans*-acting factors that bind to these mRNAs instability determinants. Thus, like the control of mRNA synthesis by the many different transcription factors, the decay machinery may also be as equally complex, helping to ensure the appropriate level as well as temporal and tissue-specific expression of the mRNA and hence protein product.

Most of our understanding of mRNA turnover in mammalian cells has come from the study of highly unstable mRNAs and in particular the highly labile mRNAs for the proto-onogenes c-fos and c-myc. In addition, the study of mRNA turnover in yeast has provided many insights into the mechanisms by which the body of the mRNA is degraded. A few of these instability elements will now be discussed below.

Two mammalian mRNAs that have been studied extensively for destabilising sequences are the highly labile c-fos and c-myc mRNAs. Searches for instability elements in these two mRNAs demonstrated that these mRNAs contained at least two discrete instability determinants one located in the coding region (Shyu *et al.*, 1989 ; Wisdom and Lee, 1991) and one in the 3'UTR (Shyu *et al.*, 1989 ; Herrick and Ross, 1994). These elements appear to function independently of one another and may be active under different conditions (Shyu *et al.*, 1989). The 3'UTR instability determinant for both mRNAs consists of an adenosine-uridine rich element (ARE), which has been implicated in the rapid turnover of a number of other unstable mRNAs including several cytokines, lymphokines and other proto-oncogenes (Shaw and Kamen, 1986 ; Chen and Shyu, 1994), suggesting that the turnover of these mRNAs is co-ordinated in some way. In contrast, the coding region determinants of these two mRNAs are unique and apparently function quite differently to one another.

A regulatory role for the ARE was first suggested by Caput *et al.* (1986), who noted the existence of AREs in the 3'UTR of many highly unstable mammalian mRNAs that encoded inflammatory mediators. Further to this Caput *et al.* (1986) proposed that the 8 base consensus (UUAUUUAU) was the functional element. Shaw and Kamen (1986) subsequently showed that a 51 base pair ARE region of the granulocyte-monocyte colony stimulating factor (GM-CSF), which contained several copies of this sequence, caused the destabilisation of the normally stable rabbit β -globin mRNA.

Since then AREs have been shown to directly effect the stability of the c-fos (Shyu *et al.*, 1989), c-myc (Alberta *et al.*, 1994), β -interferon (Whittemore and Maniatis, 1990), urokinase-type plasminogen activator (Nanbu *et al.*, 1994) and interleukin-3 (Stoecklin *et al.*, 1994) mRNAs. Two structural features common to almost all the known AREs is the presence of at least one copy of the pentamer AUUUA and a high content of adenosine and uridine nucleotides. However, since these natural AREs varied markedly in their size and sequence composition the exact role each of these feature in an ARE remained unresolved. The AUUUA pentamer has been implicated in the function of both the GM-CSF and c-fos AREs, since mutations that disrupt these sequences also substantially increase the stability of hybrid mRNAs carrying these mutant AREs (Shaw and Kamen, 1986 ; Shyu *et al.*, 1991). However, these studies did not address the function of sequences outside of the AUUUA pentamer, so it was unclear if the AUUUA element formed part of a larger destabilising sequence, or was itself, sufficient for destabilising activity.

In an attempt to define the minimal core sequence required for ARE destabilising activity Lagnado *et al.* (1994) and Zubiaga *et al.* (1995) analysed synthetic ARE elements for destabilising activity. Using this approach the nonamer UUAUUUAUU was proposed to be the minimal sequence able to function as an efficient destabilisor. Moreover, the inclusion of additional adenosine or uridine residues to either side of this element did not increase the potency of this element any further suggesting that this consensus represents the optimal functional sequence. The proposal that this sequence forms the core functional sequence of the ARE is supported by the finding that this element occurs, at least once, in the 3'UTR of many labile cytokine and proto-oncogene mRNAs (Lagnado *et al.*, 1994). Some degree of mismatches to this consensus is tolerated, since nonamers carrying single changes in either the first or last two positions of the consensus still function, albeit less efficiently. However, double mismatches or changes in the AUUUA core cannot be tolerated in these synthetic AREs. In addition multiple copies of this element increased the potency of the synthetic ARE. This data suggests that the overall strength of an ARE will partly

reflect the number of copies and their degree of mismatch to the consensus and this may explain the range of half-lives observed for the ARE containing mRNAs.

However, analysis of naturally occurring AREs such as the c-fos ARE suggests that these elements may be more complex (You *et al.*, 1992 ; Chen *et al.*, 1994 ; Chen and Shyu, 1994). Shyu and co-workers, propose that the c-fos ARE is made up of two regions which they have termed domain I and domain II (Chen *et al.*, 1994 ; Chen and Shyu, 1994). Domain I contains one perfect copy of the UUAUUUAUU sequence and two imperfect copies UaAUUUAUU and aUAUUUAUa, while domain II is highly uridine rich and does not contain any AUUUA sequences. Domain I can function independently of Domain II, to destabilise a heterologous mRNA, albeit less efficiently than the intact ARE, while domain II cannot. Therefore, although domain II cannot act as a destabilisor itself, its presence appears to enhance the destabilising activity of domain I at least two fold. You *et al.* (1992) has characterised protein binding sites in domain II, which appear to be important for domain II activity since mutations that disrupt factor binding also abolish the enhancement function of domain II. In addition, an examination of AU-rich elements in a number of other unstable early-response-genes for instability activity, identified a novel type of AU-rich instability element that does not contain a match to the proposed consensus (Chen and Shyu, 1994).

However, deletion of the ARE element from the c-fos mRNA does not produce a stable mRNA and this led to the identification of a second instability element in the c-fos coding region (Kabnick and Housman, 1988 ; Shyu *et al.*, 1989).To date seven other examples of protein coding region instability determinants (CRDs) have been identified in yeast and higher eukaryotes. Each of these CRDs have unique properties indicating the existence of at least several different decay pathways in the cell. For example, the rapid degradation of β -tubulin mRNA, under conditions of tubulin monomer excess, appears to be dependent on the recognition of a specific N-terminal tetrapeptide (MREI) encoded by the first 12 nucleotides of the β -tubulin mRNA (Theodorakis and Cleveland, 1992 ; Bachurski *et al.*, 1994), while the c-fos CRD is still functional when translated out of frame suggesting that it is recognised as an RNA sequence (Wellington *et al.*, 1993). The yeast MATα1 mRNA CRD on the other hand appears to require the

pausing of the ribosome at several rare codons located within this sequence for its activity (Caponigro *et al.*, 1993). The c-myc CRD also requires translation but in this case translation appears to stimulate the recognition of the CRD as an endonucleolytic cleavage site (Bernstein *et al.*, 1992).

One common theme to have emerged from the study of the turnover of unstable mRNAs, such as the c-fos and c-myc mRNAs, is the requirement for translation for their decay. This was first suggested by the finding that protein synthesis inhibitors such as cycloheximide and pactinomycin, stabilised these normally short-lived mRNAs (Dani *et al.*, 1984 ; Rahmsdorf *et al.*, 1987). Since then diverse experimental evidence has suggested that the degradation of many eukaryotic mRNAs is coupled to the translation process. For example, protein synthesis inhibitors inhibit the turnover of other highly labile mRNAs such as the transferrin receptor (Mullner and Kuhn, 1988), yeast MATα1 (Cagonigro *et al.*, 1993) and β-tubulin (Theodorakis and Cleveland, 1992) mRNAs. Since the instability determinants of these highly labile mRNAs are quite different it appear that translation may be an important requirement for many of the decay pathways in the cell. This coupling of mRNA degradation to translation is not just limited to mRNAs which turnover rapidly, and has also been observed to be important for the turnover of some stable mRNAs as well. For example, in yeast cycloheximide treatment was found to stabilise a number of long-lived as well as short-lived mRNAs (Herrich *et al.*, 1990).

Since these early experiments effected translation globally they could not distinguish between the requirement for either direct translation of the mRNA for its decay or for the need to synthesise a highly unstable protein involved in the decay of the mRNA. Precedents for a requirement for both translation in *cis* and in *trans* in mRNA decay now exist. For instance a requirement for translation in *trans* has been observed for the degradation of the maternally derived Eg2 mRNA in xenopus embryos (Bouvet *et al.*, 1991). The Eg2 mRNA is one of several maternal mRNAs which are de-adenylated after fertilisation but remain stable as poly(A)$^-$ mRNAs until the mid blastula transition (MBT), after which they are rapidly degraded. The region responsible for this rapid degradation has been mapped to the 3'UTR of the Eg2 mRNA. However,

cycloheximide treatment leads to the stabilisation of this de-adenylated form of the Eg2 mRNA during the MBT indicating translation is required for its rapid turnover. Since, de-adenylation has been associated with the cessation of translation of maternally derived mRNAs in frog embryos (Wickens, 1992 ; Sheets *et al.*, 1994) the action of cycloheximide has been proposed to inhibit the translation of another maternally derived mRNA required for the turnover of the Eg2 mRNA. In support of this, the effect of cycloheximide on Eg2 mRNA decay could not be mimicked by specifically inhibiting the translation of chimeric mRNAs carrying the Eg2 instability determinant.

Interestingly, the majority of unstable mRNAs require translation in *cis* for their decay, suggesting a fundamental linkage between the translational machinery and mRNA decay. However, currently this requirement for the coupling of mRNA turnover to translation in *cis* appears to vary from one mRNA to another. For example, the rapid decay of β-tubulin mRNA, mediated by β-tubulin monomer excess, is dependent on the in-frame translation of the mRNA itself, since the degradation mechanism appears to involve the recognition of the nascent amino-terminal tetrapeptide as it emerges from the ribosome (Theodorakis and Cleveland, 1992). In contrast, the decay of the yeast MATα1 appears to require the pausing of the ribosome at a cluster of rare codons within the mRNA. This was demonstrated by showing that the replacement of this region of the MATα1 mRNA with a heterologous sequence did not affect the turnover of the mRNA provided the heterologous RNA contained several in frame rare codons, whereas, replacement of this sequence with heterologous RNA devoid of rare codons resulted in the substantial stabilisation of the mRNA (Caponigro *et al.*, 1993).

In other situations the need for coupling mRNA to translation in *cis* is more obscure. For instance, the rapid decay mediated by the GM-CSF ARE appears to require translation in *cis*, although this element is located in the 3'UTR. Indeed placement of the ARE within a translated portion of RNA appears to inhibit its activity (Savant-Bhonsale and Cleveland, 1992). However, the role translation plays in ARE function has not been fully resolved since several experiments which examined the relationship between the rapid decay mediated by different AREs and translation, have yielded differing results.

Aharon and Schneider (1993) examined the requirement for translation in *cis* for the decay of mRNAs carrying the GM-CSF ARE element in their 3'UTR. In this study, translation was specifically blocked by the presence of a strong secondary structure in the 5'UTR of the hybrid mRNA carrying the GM-CSF ARE in their 3'UTR. In this context, the activity of the ARE appeared to be severely retarded compared to control mRNAs which lacked the secondary structure in their 5'UTR. To demonstrate that this stabilisation was due to the inhibition of translation, the polio virus internal ribosome entry site (IRES) element was placed downstream of the secondary structure to restore translation. The polio virus IRES is a large RNA sequence element used by the virus to promote efficient translation of its mRNAs that lack 5'CAP structures by promoting the internal binding of ribosomes and initiation of translation (Jackson, 1988). When translation was restored by the placement of an IRES downstream of the secondary structure the activity of the ARE increased markedly resulting in an unstable mRNA.

In similar experiments, Koeller *et al.* (1991) examined the effect of specifically inhibiting the translation of hybrid mRNAs whose decay was driven by the c-fos ARE. In this work, translation was regulated by the insertion of an IRE into the 5'UTR of the mRNA. Thus the specific regulation of translation could be achieved by modulating iron levels without altering the general metabolism of the cell. Iron starvation, while inhibiting translation of these mRNAs by at least 95%, nevertheless did not alter the rapid turnover of these mRNAs. Cycloheximide treatment on the otherhand increased the stability of this mRNA several fold. This data therefore suggested that the function of the c-fos ARE was not coupled to translation, in *cis*. Likewise, the ARE located in the 3'UTR of the c-myc mRNA does not appear to need translation for its activity, since hybrid mRNAs carrying this element in their 3'UTR were not stabilised by cycloheximide treatment (Wisdom and Lee, 1991). Rather, the dependence on translation for the turnover of c-myc mRNA mapped to the c-myc CRD.

The reason for this difference between the GM-CSF, c-fos and c-myc ARE elements is not known. This data, could indicate a fundamental difference in the way these supposedly functionally equivalent AREs act. Alternatively, this finding may simply reflect a difference in the systems used to block translation. For example,

perhaps wild type levels of translation are not required for destabilisation, thus if translation was blocked more efficiently in one study than in another, a differential effect of translation may be observed. If this is so, it would be consistent with a model in which the requirement of translation in *cis* for mRNA decay, actually represents the recruitment of ribosome-associated decay factors to the mRNA (Sachs, 1993).

In support of this model, several factors implicated in the turnover of the mRNAs for c-myc (Bernstein *et al.*, 1992) and histone (Caruccio and Ross, 1994) mRNAs have been proposed to be ribosome-associated. In the case of c-myc degradation, the c-myc CRD appears to function only when ribosomes translate through this element. On the basis, that polysome bound c-myc mRNA in an *in vitro* decay system undergoes specific endonucleolytic attack and that this endonucleolytic cleavage is inhibited when ribosomes are prevented from traversing this region, Ross and co-workers propose that the endo-nuclease responsible must be associated with the ribosome (Herrick and Ross, 1994).

A ribosome associated ribonuclease has also been implicated in the decay of histone mRNAs. In cells and cell-free systems, the turnover of polysome bound histone mRNA apparently begins at the 3'-end of the mRNA and then proceeds in a 3' to 5' direction. In both systems the earliest detectable decay intermediate appears to be mRNA species lacking 5 or 12 bases from their 3'-ends. This results in the disruption of a stem-loop structure found a the 3'-end of all histone mRNAs, which has been associated with many aspects of histone mRNA metabolism, including 3'-end formation, nuclear export and cytoplasmic stability (Williams *et al.*, 1994). Whether these initial decay intermediates represent endonucleolytic attack of the stem-loop or pause sites for the 3' to 5' exonuclease was not determined. Since the decay of the polysome bound histone mRNA *in vitro* was essentially identical to the decay of the mRNA *in vivo* the 3' to 5' exonuclease responsible for histone mRNA turnover was proposed to be polysome associated (Ross *et al.*, 1986). Recently, a candidate for the 3' to 5' exonuclease involved in histone mRNA turnover has been purified from human polysomes (Caruccio and Ross, 1994). This ribonuclease display, all the properties associated with the histone

RNase and can accelerate the *in vitro* decay of histone mRNA in RNase depleted polysomes (high salt-washed).

Lastly, at least some of the factors involved in the rapid turnover of yeast mRNAs carrying premature translational-termination codons must interact with the ribosome (Peltz *et al.*, 1994). In the current model for this decay mechanism, the ribosome after terminating translation at a nonsense mutation codon scans downstream of this codon. If it encounters a specific downstream sequence, the ribosome is thought to pause and this somehow acts as a signal for rapid decay (Peltz *et al.*, 1993 ; Zhang *et al.*, 1995 ; Hagan *et al.*, 1995). Recognition of this signal then triggers mRNA de-capping which in turn promotes 5' to 3' exonucleolytic decay, most likely catalysed by the XRN 1 gene product (Muhlrad and Parker, 1994). Several factors involved in this pathway have now been cloned which should led to a better understanding of the relationship between the ribosome and this specific decay pathway (Peltz *et al.*, 1993 ; Cui *et al.*, 1995 ; He and Jacobson, 1995).

1.8.2: Mechanism of Eukaryotic mRNA Decay.

A key question is how do the various mRNA instability elements influence the course and kinetics of mRNA decay. Specifically; how are these elements recognised and by what; how do these elements function to bring about mRNA decay; and do these decay pathways converge at some point? The answer emerging from a number of studies of both the turnover of mammalian and yeast mRNAs is that these elements are invariably recognised by specific RNA-binding proteins and this recognition can then trigger one of three initial events. These initial events are the de-adenylation of the poly(A) tail, site-specific endonucleolytic cleavage and de-adenylation independent de-capping of the mRNA (Sachs, 1993 ; Decker and Parker, 1994). In this section only the decay of mRNA species that are normally polyadenylated will be discussed.

(i) De-adenylation Dependent Pathway.

Since the demonstration that the poly(A) tail of eukaryotic mRNAs undergoes progressive shortening in the cytoplasm, removal of the poly(A) tail has been proposed as an early step in the decay of many mRNAs (Sheiness and Darnell Jr., 1977). The first evidence that de-adenylation may precede the decay of unstable mRNAs came from the examination of c-myc decay *in vitro* (Brewer and Ross, 1988). In this system the shortening of the poly(A) tail was the first detectable decay intermediate. Laird-Offringa *et al.* (1990) found de-adenylation may be involved in the turnover of c-myc mRNA *in vivo*. In this study, stabilisation of c-myc mRNA by cycloheximide was found also to inhibit the shortening of the c-myc poly(A) tail. However, c-myc mRNAs carrying short poly(A) tails, due to the incubation of cells with the adenosine analogue 3'-deoxyadenosine (3'-dA), were insensitive to cycloheximide treatment and turned over rapidly. Although, 3'-dA affect other aspects of cellular metabolism such as transcription it is thought to primarily prevent poly(A) tail formation in cells by blocking elongation (Zeevi *et al.*, 1982). This data, suggested poly(A) tail shortening may be a prerequisite for c-myc turnover.

In both these early studies, steady state levels of c-myc mRNA were examined, but unfortunately in this situation the length of the poly(A) tail will be heterogeneous, making it difficult to demonstrate a requirement for de-adenylation prior to degradation of the transcribed portion of the mRNA (ie. the mRNA body). However, in the groundbreaking study of Wilson and Treisman (1988) this problem was addressed. Wilson and Treisman (1988) made use of the c-fos promoter, which produces a transient burst of transcription following serum stimulation of quiescent fibroblasts (Greenberg and Ziff, 1984), to produce a population of mRNAs with a relatively homogenous poly(A) tail. Using this system they were able to demonstrate that mRNAs carrying the c-fos ARE accumulated as de-adenylated species prior to the decay of the mRNA body. This data thus provided the first evidence that an instability element may function by promoting the rapid de-adenylation of the mRNAs poly(A) tail. Using a similar system Shyu *et al.* (1991) have also observed that the c-fos mRNA is de-adenylated prior to the

decay of the transcribed portion of the mRNA. These workers demonstrated that the regions responsible for this de-adenylation co-localised with the regions previously identified as c-fos instability determinants, namely the c-fos ARE and coding region determinant (CRD). However, the complete de-adenylation of the mRNA did not appear to be required to initiate decay. Rather the decay of the mRNA body appears to be initiated when the length of the poly(A) tail is shortened to about 30 residues. In contrast, the poly (A) tail of stable mRNAs such as the β -globin underwent very slow de-adenylation and retained at least 60 residues up to 8 hours after the repression of its synthesis (Shyu *et al.*, 1991 ; Schiavi *et al.*, 1994 ; Chen *et al.*, 1994 ; Zubiaga *et al.*, 1995). Thus two structurally distinct instability determinants were shown to facilitate the rapid removal of the mRNAs tail, suggesting that de-adenylation may be a general mechanism for targeting an mRNA for decay.

De-adenylation in yeast also appears to be a common mechanism for targeting mRNA for degradation. This was first demonstrated for the decay of the unstable MFA2 mRNA (Muhlrad and Parker, 1992). Subsequently, de-adenylation was found to precede the decay of four different yeast mRNAs that represented both unstable and stable mRNAs (Decker and Parker, 1993). In this study, a time lag was observed between the repression of transcription and the initiation of decay of the mRNA body. The length of this lag corresponded to the time required to shorten the poly(A) tail from around 50 to 60 adenosine residues to an oligo(A) tail of about 10 residues. Specifically, for unstable mRNAs this time lag was short and corresponded to rapid de-adenylation, while in stable mRNAs this time lag was longer and corresponded to a slower rate of de-adenylation. Thus in yeast de-adenylation appears to be a common event in the decay of many mRNAs.

One key question that has not been resolved is how do these elements promote de-adenylation? In mammals the mechanism is not well understood, although much effort has gone into determining the critical features of the instability elements themselves and identifying factors that may bind to them. For instance it has been demonstrated that the c-fos CRD is; i) recognised by the decay machinery as an RNA sequence, since it can function when translated out of frame (Wellington *et al.*, 1993);

ii) requires ribosomes to move across it for its destabilising activity (Schiavi *et al.*, 1994) and iii) binds at least two proteins, one polysome associated the other cytosolic (Chen *et al.*, 1992). The role these proteins play in c-fos CRD activity is not known. Although, deletion of the region, to which these proteins binds in the c-fos CRD abolishes its activity, this fragment is in itself unable to function as a destabilising sequence.

Likewise, the mechanism by which the ARE element promotes de-adenylation is not understood. Numerous groups have identified factors that bind to ARE elements, however due to a lack of a functional assay in most cases the role these proteins play in ARE-mediated decay is not known (Malter, 1989 ; Malter and Hong, 1991 ; Vakalopoulou *et al.*, 1991 ; Bohjanen *et al.*, 1991 ; Bohjanen *et al.*, 1992 ; You *et al.*, 1992 ; Bickel *et al.*, 1992 ; Savant-Bhonsale and Cleveland, 1992 ; Huang *et al.*, 1993 ; Hamilton *et al.*, 1993 ; Zaidi *et al.*, 1994 ; Ehrenman *et al.*, 1994 ; Henics *et al.*, 1994 ; Nagy and Rigby, 1995). There is some controversy to whether some of the factors identified in the *in vitro* RNA binding experiments represent true ARE binding proteins (Sachs, 1993). Firstly, some of these factors appear to bind to the uridine-rich portions of the ARE and have equal affinity for poly(U), implying that the ARE may not be their *in vivo* substrate (You *et al.*, 1992). Furthermore with the recent suggestion that the nonamer UUAUUUAUU forms the core ARE sequence it may be expected that factors involved in ARE activity bind to this site (Lagnado *et al.*, 1994). Indeed, some factors do appear to have specificity for AUUUA containing RNA making them good candidates for functional ARE binding proteins (Vakalopoulou *et al.*, 1991 ; Bohjanen *et al.*, 1992). This issue has become more complex with the identification of some of these factors which bind to AREs *in vitro*. For instance, two proteins that have been identified as ARE binding proteins by *in vitro* UV cross-linking experiments are the heterogenous nuclear ribonucleoproteins A1 and C (Hamilton *et al.*, 1993) which are generally thought to be involved in pre-mRNA splicing in the nucleus (Mayeda and Krainer, 1992). This finding raises the question as to whether these factors are true ARE binding proteins *in vivo*. In addition, a recent report indicated that a 36kDa protein that bound to AU-rich sequences including the c-myc and IL-2 AREs was the enzyme

glyceraldehyde-3-phosphate dehydrogenase (Nagy *et al.*, 1995). Interestingly, there is some evidence for GAPDH behaving as an RNA binding protein *in vivo*, since GAPDH co-purifies with the polysome fraction of cells. In addition, it appears from the sedimentation of polysome bound mRNAs containing the GM-CSF ARE, that ARE activity may be mediated by a large 20S protein complex *in vivo* (Savant-Bhonsale and Cleveland, 1992). In this study, polysomes were fractionated through a sucrose sedimentation gradients and the fractions containing the reporter mRNA assayed by RNase protection. Reporter mRNAs known to be unstable *in vivo*, due to the presence of a functional ARE element were consistently found in fractions representing larger polysomes, than the same reporter mRNAs that lack a functional ARE, and are stable *in vivo*. This size difference appeared to correspond to a 20S complex indicating that a multi-protein complex probably associates with the ARE *in vivo*.

Recently, some progress has been made in assigning functions to specific ARE binding proteins using *in vitro* decay reactions. For example, Zhang *et al.* (1993) have purified two polypeptides of 37 and 40kDa, that specifically bind to the c-myc and GM-CSF 3'UTR and selectively accelerate the decay of c-myc mRNA in a cell-free decay system, indicating that these factors are involved in the ARE-mediated instability. In contrast, Rajagopalan and Malter (1994) have used an *in vitro* decay system to show that the previously identified ARE-binding protein AUBF (Malter, 1989) stabilises the GM-CSF mRNA by binding to the ARE instability element.

In summary, the finding of i) multiple ARE-binding proteins involved in either the activation or inactivation of ARE activity, ii) the apparent differential affinity of some of these proteins for certain ARE elements and iii) the apparent need for a multi-protein complex to form on the ARE for its activity may help explain the apparent differential decay of ARE containing mRNAs *in vivo* (Bohjanen *et al.*, 1992 ; Savant-Bhonsale and Cleveland, 1992 ; Rajagopalan and Malter, 1994 ; Henics *et al.*, 1994).

Two important questions that remain to be answered are i) how do these instability elements promote de-adenylation and ii) how does the removal of the poly(A) tail promote the degradation of the mRNA body. Presumably the formation of specific RNA-protein complexes at the instability elements interact in some way with the

poly(A) tail to enhance its removal. Two basic models have been proposed to explain this interaction in mammalian cells (Schiavi *et al.*, 1992 ; Decker and Parker, 1994) In the first model, the interaction of the instability element with the poly(A) tail somehow displaces the poly(A) binding protein from the poly(A) tail. Normally, the poly(A) tails of eukaryotic mRNAs are associated with a specific RNA-binding protein, termed the poly(A) binding protein or PABP, which is thought to sterically protect the poly(A) tail from nucleolytic attack (Sachs and Wahle, 1993). Thus the removal of PABP from the poly(A) tail could led to rapid de-adenylation.

Alternatively, the RNA-protein complex at the instability determinants may interact with the poly(A) nuclease stimulating its activity directly without the need for PABP removal. Such a situation appears to be the case in yeast. In yeast, a nuclease associated with the shortening of the poly(A) tail has been cloned (Sachs and Deardorff, 1992). Interestingly, this nuclease PABP-dependent poly(A)ribonuclease or PAN, only functions when the poly(A) tail is bound by PABP. Characterisation of PAN activity *in vitro* has provided an important insight into how destabilising sequences may enhance poly(A) tail removal (Lowell *et al.*, 1992). In this system, the rate of de-adenylation of poly-adenylated substrates, catalysed by PAN, was found to be influenced by upstream sequences. Specifically, mRNAs carrying the MFA2 instability element were de-adenylated much faster than RNA derived from more stable mRNAs. Further analysis of the kinetics of this reaction indicated that the MFA2 instability element appears to enhance poly(A) tail removal by switching PAN from being a distributive to a more processive enzyme. However, whether a similar system exists in mammals awaits the purification and cloning of the poly(A) nuclease from such a source.

Once the mRNA is de-adenylated what happens next? Once again the events following de-adenylation in mammals are poorly defined, although evidence from the *in vitro* decay of polysome bound c-myc mRNA suggests the decay proceeds in a 3' to 5' direction, following de-adenylation (Brewer and Ross, 1988). In contrast, much progress has been made in understanding the series of events that occur following the de-adenylation of yeast mRNAs (Decker and Parker, 1993 ; Hsu and Stevens, 1993 ; Muhlrad *et al.*, 1994 ; Muhlrad *et al.*, 1995). Several studies have made use of stable

secondary structures that block the activity of yeast exonucleases (Vreken and Rue, 1992) to trap decay intermediates of both unstable (MFA2) and stable (PGK1) mRNAs. Decay intermediates trapped by this process were found to mainly lack sequences from the 5' end of the molecule to the start of the secondary structure, suggesting the involvement of a 5' to 3' exonuclease in the decay of the mRNA body. In the case of the PGK1 mRNA some addition decay intermediates were detected that were consistent with 3' to 5' exonucleolytic decay. However, this pathway appeared to be much slower than the 5' to 3' pathway in these experiments, indicating it may only represent a minor decay pathway in yeast (Muhlrad *et al.*, 1995). In an earlier study on the decay of the yeast PGK1 mRNA, it was reported that following the de-adenylation of the mRNA, the rate limiting step in the decay of the mRNA was an endonucleolytic cleavage in the coding region (Vreken and Raue, 1992). Like the studies of Parker and co-workers, stable secondary structures that block the activity of yeast exonucleases were used to trap decay intermediates of PGK1 mRNA. This cleavage event was not observed in the latter studies (Decker and Parker, 1993 ; Muhlrad *et al.*, 1995). The reason for this discrepancy between the studies is not known. However, since in the earlier study the PGK1 decay intermediates were detected under steady state conditions it was not possible to determine the order of events in the decay of the mRNA. For instance, the endonucleolytic cleavage may not have been directly stimulated by the de-adenylation of the mRNA but rather may have been the result of endonucleolytic attack on the stabilised poly(A)$^-$ species, which is normally rapidly degraded. The absence of similar species from the study of Parker and co-workers may also reflect the relative abundance of the endonuclease in the different strains of yeast used in each study.

Two 5' to 3' exonucleases have now been characterised in yeast and these are XRN1 (Larimer and Stevens, 1990) and HKE1 (Kenna *et al.*, 1993). Analysis of the turnover of the MFA2 and PGK1 mRNAs in XRN1$^-$ yeast strains, found that these mRNAs underwent normal de-adenylation but persisted as full length poly(A)$^-$ species which lacked the 5'CAP structure, indicating that the first event following de-adenylation may be de-capping of the transcript, followed by 5' to 3' exonucleolytic decay, most likely carried out by the XRN1 protein. In support of this notion is the

finding that XRN1 is normally blocked by the 5' CAP structure (Stevens, 1978). Thus for two mRNAs with quite different stabilities, but equally reliant on de-adenylation for their decay, the major decay pathway appears to involve de-capping followed by 5' to 3' exonucleolytic decay.

Interactions between the 5' and 3' termini of mRNA have been proposed previously to explain the effect of the poly(A) tail and 3'UTR sequences on the initiation of translation in eukaryotes (Sachs and Davis, 1989 ; Jackson and Standart, 1990 ; Jackson, 1993 ; Wickens, 1992). Furthermore, there is some physical evidence for the termini interacting directly (Rubin *et al.*, 1994 ; Christensen *et al.*, 1987). Rubin *et al.* (1994) found that the radiolabelled poly(A) tail of the rabbit β -globin mRNA was co-captured, following ribonuclease V1 treatment, on a aminophenylboronate-agarose column, which specifically captures the CAP structure of a mRNA. This suggested that the 5' and 3' ends of the molecule must bind to each other (the captured fragments were sized to ensure that the captured products were not the native molecules). The interaction of the 5' and 3' termini of mRNA molecules has also been suggested by the finding of circular polysome structures in electron micrographs (Christensen *et al.*, 1987).

On this basis, Decker and Parker (1994) proposed that the poly(A) tail and 5'CAP structure normally interact to form a specific mRNA-protein complex that is involved in the initiation of translation. This complex also serves to inhibit de-capping, perhaps by efficiently recruiting translation initiation factors to the 5'UTR and CAP, thereby denying access of the de-capping enzyme to the 5'-CAP. De-adenylation of the mRNA would disrupt this interaction, thus allowing access of the de-capping enzyme to the CAP. Furthermore, given the role the poly(A) tail and its associated factors have in the initiation of translation, de-adenylation may also decrease the rate of initiation of translation leading to a more accessible CAP structure.

Whether de-adenylation promotes a similar decay mechanism in mammals is not known. However, it should be pointed out that a 5' to 3' exonuclease has recently been partially purified from mouse ascites cells, where this enzyme appears to be the major cytoplasmic nuclease (Coutts and Brawerman, 1993 ; Coutts *et al.,* 1993). Interestingly,

the enzyme can degrade protein-free RNA molecules containing 5'CAP structures but cannot degrade these same molecules in crude cytoplasmic extracts, implying that the action of the nuclease may be normally regulated by the binding of factors to the 5'-termini. Thus the potential exists in mammalian cells for alterations in the protein-RNA complex at or near the 5'-CAP, perhaps due to changes in the interaction of the 5' and 3' ends of the mRNA to lead to 5' to 3' exonucleolytic decay.

(ii) Site Specific Cleavage.

A second mechanism for regulating the turnover of a mRNA involves the site-specific cleavage of the mRNA by an endo-ribonuclease. Endonucleolytic cleavage was first implicated in the turnover of certain mRNAs by the detection of mRNA fragments *in vivo* that corresponded to both the 5' and 3' portions of the transcript. Such fragments have been identified for the chicken 9E3 (Stoeckle and Hanafusa, 1989), chicken apolipoprotein II (Binder *et al.*, 1989), xenopus Xlhbox2B (Brown and Harland, 1990), insulin like growth factor II (Nielson and Christiansen, 1992) and human transferrin receptor (Binder *et al.*, 1994) mRNAs. Consistent with these fragments being generated in the initial event in the turnover of these mRNAs was the finding that the 3'-end fragment retained its poly(A) tail. Further evidence that these truncated species represent decay intermediates was the observation that the production of these fragments fluctuate under conditions in which the turnover of the full length mRNA alters. For instance, a good correlation was observed between an increase in the amount of truncated 9E3 mRNA species relative to the amount of full length transcript in conditions were the 9E3 mRNA is least stable (Stoeckle and Hanafusa, 1989). However, it has yet to be shown that this cleavage is responsible for determining the half-life of the 9E3 mRNA. Presumably, following the endonucleolytic cleavage of these mRNAs the truncated species produced are rapidly degraded by exonucleases from the unprotected termini.

The best evidence for instability determinants regulating mRNA turnover by promoting endonucleolytic cleavage has come from the study of the turnover of the xenopus Xlhbox2B and human transferrin receptor mRNAs. In xenopus oocytes, the mRNA for the homeo-box protein Xlhbox2B appears to be specifically destabilised during the latter stages of development (Wright *et al.*, 1987). After the injection of *in vitro* synthesised radiolabelled Xlhbox2B mRNA into oocytes, specific decay intermediates were isolated that were consistent with site-specific endonucleolytic cleavage (Brown and Harland, 1990). This cleavage appeared to be specific for the Xlhbox2B mRNA since cleavage was not seen with two control RNAs. Using a series of Xlhbox2B fragments, the endonuclease cleavage site was mapped to a 19 base element in the 3'UTR of the mRNA. Deletion of this region , abolished cleavage of the injected RNA and this was correlated with an increase in stability of the injected RNA. In contrast, the transfer of this region to another, normally stable mRNA, caused this RNA to be cleaved in a homologous manner to the Xlhbox2B RNA in oocytes, and this appeared to correlate with a decrease in the stability of the RNA. In support of this notion that the turnover of the Xlhbox2B mRNA may be regulated by an endonuclease. Brown *et al.* (1993) have characterised a factor present in the cytoplasm of oocytes which can regulate this reaction. In an *in vitro* decay system, the cleavage of the labelled substrate can be enhanced by the addition of increasing amounts of unlabelled specific competitor, suggesting the presence of a sequence specific inhibitor factor, which is present in limiting amounts. Interestingly, the amount or activity of this inhibitor appears to vary during oocyte development, as judged by the amount of cleavage detected *in vitro*, using oocyte lysate prepared from different stages of their development. Specifically, cleavage activity was low in lysates from early stage oocytes and little enhancement of the reaction is observed with increasing amounts of cold specific competitor, suggesting high levels of inhibitor activity at these stages. In contrast, cleavage activity is higher in lysates prepared from later stage oocytes and this activity can be substantially increased by the addition of the same amounts of cold specific competitor. This difference in the activity of the cleavage reaction *in vitro*

mimics the differential stability of the Xlhbox2B mRNA observed *in vivo*, implicating this endonucleolytic cleavage in the regulating the turnover of this mRNA Xlhbox2B.

In mammals, the turnover of the transferrin receptor appears to be regulated by an endonucleolytic cleavage (Binder *et al.*, 1994). The turnover of the human transferrin receptor (TfR) mRNA is regulated by intracellular iron levels (reviewed Klausner *et al.*, 1993). When iron is abundant the TfR mRNA has a relatively short half-life (~45 min), however when iron levels fall in the cell, the half-life of the TfR mRNA is specifically increased to greater than 3hr. The net result of which is an increase in the number of transferrin receptors on the cells surface and a greater uptake of iron-transferrin complex from the circulation.

The determinants involved in this iron-regulated stability have been mapped to a 250 base region of the TfR mRNA 3'UTR, which is sufficient to confer iron-regulated instability to a reporter mRNA (Mullner and Kuhn, 1988 ; Casey *et al.*, 1989; Koeller *et al.*, 1991). This region contains three iron-responsive elements and an as yet poorly defined rapid turnover determinant (Casey *et al.*, 1989). Binding of the cytosolic IRE-BP to the IRE under low iron conditions has been shown to be important in the stabilisation of the mRNA, since mutations which result in the disruption of IRE-BP binding result in a constitutively unstable mRNA. This suggested the presence of an iron-independent instability element in this region (Casey *et al.*, 1989 ; Koeller *et al.*, 1991). Further evidence for a separate instability element in this region was obtained by the finding of addition mutants, which resulted in a constitutively stable mRNA. In these mutants the binding of the IRE-BP to the IREs was unaffected. Thus a model for the regulation of TfR mRNA stability has been proposed in which the binding of the IRE-BP to the IRE elements mask the rapid turnover determinant (Reviewed Klausner *et al.*, 1993).

Binder *et al.* (1994) has documented the appearance of truncated TfR RNA species which are consistent with endonucleolytic cleavage within this previously defined iron-regulatory region. For instance, cleavage products representing both the 5' and 3' ends of the TfR mRNA are detectable in several cell lines. The existence of both 5' and 3' fragments is most consistent with an endonucleolytic cleavage. Moreover, the

3'-end fragment appeared to retain a long poly(A) tail, indicating de-adenylation was probably not a prerequisite for the decay of the TfR mRNA. Analysis of the TfR mutant constructs synthesised by Casey *et al.* (1989) provided further support for the stability of the TfR mRNA being regulated by an endonucleolytic cleavage event. Specifically, the appearance of the truncated species correlated with the rapid turnover of the TfR mRNA. In mouse fibroblasts expressing an iron-regulated human TfR mRNA, the appearance of the truncated species was only seen when the cells were treated with an iron source, and this appearance of the truncated species correlated with a decrease in the stability of the mRNA. In contrast, the truncated species were always detectable, irrespective of the iron status of the cell, in mouse cells expressing a constitutively unstable human TfR mRNA, whereas the truncated species were never detected in cells expressing a constitutively stable form of the TfR mRNA. Mapping of the termini of the truncated fragments was most consistent with a single cleavage event next to a G nucleotide that lies just 3' to one of the IRE elements (IRE C) in the iron regulatory region. This G nucleotide is located in a portion of the RNA which is conserved between human, rat (Roberts and Griswold, 1990) and chicken (Koeller *et al.*, 1989) TfR mRNAs. Indeed, site directed mutagenesis of a 7 base region containing this G nucleotide was sufficient to render the human TfR mRNA constitutively stable with a corresponding loss of the truncated RNA species indicating the importance of this region in the turnover of the mRNA.

(iii) De-adenylation Independent De-capping.

The third type of instability element appears to promote the de-capping of the mRNA, leading to 5' to 3' exonucleolytic degradation. Currently, this mode of mRNA decay has only been observed for the rapid decay promoted by certain nonsense mutation in yeast (Muhlrad and Parker, 1994). However decay intermediates lacking a small portion of the 5' end have also been detected in transgenic mice expressing a unstable mutant β -globin mRNAs that contain nonsense mutations (Lim *et al.*, 1992).

These truncated β -globin mRNAs could indicate a similar de-capping in mammalian cells, alternatively these species could be generated by an endonucleolytic cleavage at the 5' end of the molecule. However, more recent data on the turnover of nonsense-codon containing mRNAs in mammals suggests that the turnover of these mRNAs occurs mainly in the nucleus (See next section).

1.8.3: RNA Turnover in the Nucleus.

Although mRNA degradation is thought to mainly occur in the cytoplasm, there is now some evidence for mRNA turnover in the nucleus. This surprising conclusion has come from two sources which are discussed below.

It has been known for some time that the presence of nonsense mutations in mRNA (ie. mutations that cause premature translational termination) result in a decrease in the abundance of this mutant species in both mammals (Reviewed Cooper, 1993) and yeast (Reviewed Peltz *et al.*, 1994). In several cases this has been documented as an enhancement of the turnover of this mRNA species. In yeast, this decay occurs in the cytoplasm and has been intimately linked to translation of the mRNA. A combination of genetic and biochemical approaches have resulted in identification of many of the sequences and *trans*-acting factors involved in this decay (Peltz *et al.*, 1993 ; Cui *et al.*, 1995 ; He and Jacobson, 1995 ; Zhang *et al.*, 1995) as well as the mechanism (Muhlrad and Parker, 1994). In yeast nonsense mutations were found to trigger the de-capping of the mRNA which subsequently resulted in the 5' to 3' decay of the molecule (Muhlrad and Parker, 1994).

However, characterisation of this nonsense mediated decay phenomena in mammalian systems has proved to be more controversial. In several cases, such as for the triosephosphate isomerase (Cheng and Maquat, 1993 ; Belgrader *et al.*, 1994), Igκ light chain (Lozano *et al.*, 1994) and β-globin (Kugler *et al.*, 1995) genes, this decrease in mRNA levels cannot be correlated with any change in the transcription rate of the gene or the cytoplasmic stability of the mutant mRNA. Rather this reduction in total mRNA levels correlates with a reduction in the level of fully spliced mutant mRNA

within the nucleus without affecting the level of mutant pre-mRNA (Cheng and Maquat, 1993 ; Belgrader *et al.*, 1994 ; Kugler *et al.*, 1995). This been interpreted as a increase in the degradation of the mutant mRNA in the nucleus. Paradoxically, translation appears to be involved in this process, since this nonsense-codon mediated decay can be abrogated in *cis* by inhibiting the translation of the mRNA or in *trans* by the co-expression of an amber suppressor tRNA (Belgrader *et al.*, 1993 ; Kugler *et al.*, 1995). Moreover this amber suppressor tRNA effect was specific for amber nonsense-mutations (Belgrader *et al.*, 1993). Due to technical limitations it is currently unknown if the recognition and decay of nonsense-containing mRNAs occurs prior to export or concomitantly with mRNA export (Belgrader *et al.*, 1994).

The intranuclear turnover of mRNA has also been implicated in the down-regulation of fibronectin mRNA levels by the Ha-ras oncogene product (Chandler *et al.*, 1994) and the turnover of the drosophila nucleus-specific Hsr-omrga-n transcript (Hogan *et al.*, 1994).

1.8.4: Summary mRNA turnover.

In summary, over the last few years it has become increasingly apparent that the regulation of mRNA turnover is an important control point in eukaryotic gene expression. For instance the half-lives of many mRNAs are modulated in response to exogenous stimuli and the developmental and differentiation state of the cell. These modulations show a good correlation with the steady state level of the mRNA and this in turn often determines the net amount of protein produced. From the study of unstable mRNAs it has become apparent that all the information needed to determine a mRNAs stability lies in discrete regions within the mRNA. These sequences can be found through out the mRNA and may be redundant. In a few cases, sequences that stabilise a mRNA have also been identified and these seem to function by regulating the activity of a nearby instability determinant. This has led to the proposal that the half-life of a mRNA will determined primarily by the number, strength and regulation of its

instability elements. Much progress has been made in identify these instability elements. In contrast much less is known about how these elements function, and the trans-acting factors that recognise them. The study of mRNA turnover has also raised many interesting and important points. For instance, in many cases translation and mRNA turnover appear to be intimately linked but the reason for this is unknown. Also, many instability elements appear to act over large distances to influence events at the termini of the mRNA molecule, for example the c-fos CRD somehow enhances the removal of the mRNAs poly(A) tail. Lastly, from a number of studies, particularly in yeast it has become apparent that interactions between the 5' and 3' termini of the mRNA molecule play an important role in many processes including translation and mRNA turnover.

Therefore, the challenge in this field is to understand how these instability elements function, namely; i) how are translation and mRNA turnover linked; ii) how can instability elements influence events over large distances and iii) what is the nature of the interactions between the 5' and 3' termini of the mRNA molecule and how do instability elements alter this? Hopefully, the purification of the trans-acting factors that interact with these instability elements, combined with *in vitro* decay systems that mimic *in vivo* decay will provide a better understanding of these events. In addition, genetic approaches in yeast have been useful in identifying factors and events involved in the turnover of several mRNAs. Since some of the early events observed in the decay of these yeast mRNAs, such as de-adenylation of the mRNA prior to decay of the transcribed portion of the mRNA, are conserved between yeast and mammals, the challenge is now to see if similar pathways exist for the turnover of mammalian mRNAs.

1.9: Aims of this Thesis.

One of the major aims of the work in this laboratory is to understand the molecular control of heme biosynthesis in animals. As ALAS is the first and rate limiting enzyme of the heme biosynthetic pathway in the liver and probably most other tissues, attention has focused on the understanding the molecular basis for the control of

ALAS. Of particular interest is how the ubiquitous isozyme ALAS-1 is negatively regulated by the end product of the pathway, heme and the relationship between this regulation and the induction of ALAS in the liver by a large number of substances that induce the synthesis of the CYP system.

Evidence from our laboratory and others indicates that heme can decrease the steady state level of ALAS-1 mRNA in the liver and other tissues. We are investigating this negative regulation in rats as a model for the regulation of ALAS-1 mRNA in human liver. In an attempt to understand at the molecular level how heme regulates ALAS-1 mRNA levels, the mechanism of heme repression was examined in the fetal rat hepatoma cell line FRL 4.1. This led to the conclusion that heme modulates the stability of the ALAS-1 mRNA.

The second aim of this study, was therefore to determine how heme can increase the turnover of the ALAS-1 mRNA. To this end hybrid genes were constructed between the rat ALAS-1 gene and the stable β-globin gene and these genes were expressed in the FRL 4.1 cell line in an attempt to define regions of the ALAS-1 mRNA important for the heme-mediated degradation of the mRNA.

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER 2: MATERIALS AND METHODS.

2.1 MATERIALS.

2.1.1 Drugs, Chemicals and Reagents.

Hemin (Ferriprotoporphyrin IX chloride) and tin mesoporphyrin IX Dichloride were purchased from Porphyrin Products Inc. USA. Desferrioxamine (Desferal®) was a kind gift from Ciba-Giegy.

The following products were obtained from Sigma Chemical Co: Acrylamide, actinomycin D, agarose (Type 1), ampicillin, bisacrylamide (N,N'-methylene-bis-acrylamide), bovine serum albumin (BSA), chloramphenicol, cycloheximide, deoxyribonucleotide triphosphates (dNTPs), tRNA (Baker's Yeast), dithiothreitol (DTT), ethidium bromide, ethylenediaminetetra-acetic acid (EDTA), N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (Hepes), ribonucleotide triphosphates (rNTPs), o-nitrophenyl-B-D-galactopyranoside (ONPG), salmon sperm DNA, sodium dodecyl sulphate (SDS), spermidine, spermine, Tris-base, phenylmethylsulfonyl fluoride (PMSF), δ-aminolevulinic acid (ALA), 4,6-dioxoheptanoic acid (succinyl acetone), 12-o-tetradecanoylphorbol-13-acetate (TPA) and 2-aminopurine (Nitrate salt).

Sources of other important reagents were as follows : phenol: Wako pure chemicals; polyethylene glycol 6000: BDH chemicals; N,N,N',N'-tetramethethylethenediamine (TEMED): Tokyo Kasei; trichloroacetic acid (TCA): Univar Pty. Ltd; guanidine thiocyanate: Merck.

Kits for *in vitro* synthesis of RNA were obtained from Biotechnology Research Enterprises of South Australia (Bresatec). Sequanase Version 2.0 sequencing kit was purchased from United States Biochemical Corporation. Oligolabelling of DNA was performed using Amershams Inc. Megaprime DNA labelling kit. RNA protection assay

kits were purchased from Ambion, Inc. All other chemicals and reagents were of analytical grade.

2.1.2 Radiochemicals.

D-threo-[dichloroacetyl-1-^{14}C] chloramphenicol (57 mCi/mmol) were purchased from Amersham. [α-^{32}P] dATP (1800 Ci/mmol), [α-^{32}P] dCTP (1800 Ci/mmol), [γ-^{32}P] dATP (>2000 Ci/mmol) and [α-^{32}P] UTP (1500 Ci/mmol) were purchased from Bresatec.

2.1.3 Enzymes.

Restriction enzymes were purchased from Pharmacia or New England biolabs.
Other enzymes were obtained from the following sources:
Calf intestinal phosphatase : Boeringher Mannheim.
E. coli DNA polymerase I (Klenow fragment) : Bresatec.
proteinase K : Boeringher Mannheim.
ribonuclease A (RNase A) : Sigma. The stock solution (10mg/ml) was incubated at 100^{o}C for 10 min to inactivate any DNase activity.
RNase H : Promega.
T4 DNA ligase : Bresatec.
T4 DNA polymerase : Boeringher Mannheim.

2.1.4 Buffers.

Denhardt's solution : 0.1%(w/v) Ficoll, 0.1%(w/v) polyvinylpyrrolidine, 0.1%(w/v) BSA.
NET : 100mM NaCl, 1mM EDTA, 10mM Tris HCl (pH 7.5).

SSC : 150mM NaCl, 15mM sodium citrate.

SSPE : 150mM NaCl, 10mM NaH_2PO_4, 1mM EDTA.

TAE : 40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA, (pH 8.2).

TBE : 90mM Tris, 90mM boric acid, 2.5mM EDTA, (pH 8.3).

TE : 10mM Tris-HCl (pH 7.5), 0.1mM EDTA.

TES : 25mM Tris-HCl (pH 8.0), 10mM EDTA, 15% sucrose.

3x urea loading buffer : 4 M urea, 50% sucrose, 50 mM EDTA, 0.1%bromophenol blue

All buffers were sterilised by autoclaving or, where necessary, by filtration through a Sartorius™ Minisart NML 0.2µm filter.

2.1.5 Cloning Vectors.

pBluesript SK/KS were purchased from Statagene. pSP 64 and pSP 72 were purchased from Bresatec.

2.1.6 Cloned DNA Sequences.

The following cloned DNA sequences, used as probes throughout this study, were generous gifts from the following :

pHFβ-Actin (Gunning *et al.*, 1983) was kindly provided by Dr. L. Mattschoss, Stanford University, California.

The plasmid pNEORßG^{GC} (Shaw and Kamen, 1986) was kindly provided by Dr. L. Vakalopoulou, Schering Aktiengesellschaft Berlin.

pRH01 a full length cDNA clone of rat heme oxygenase-1 (Shibahara *et al.*, 1985) was provided by Prof. Abraham.

pB7 a cDNA clone of rat CYP2B1 (Affolter *et al.*, 1986) was provided by Dr. Adesnik.

pRGAPDH a cDNA of rat glyceraldehyde-3-phosphate deydrogenase (Piechaczyk *et al.*, 1984) was provided by Dr. Klinken.

pRSVN.06 was provided by Dr. A. Robbins.

pBKhßGLOBIN (Lawn *et al.*, 1980) was provided by Dr. Booker.

pBc-MYC (Stanton *et al.*, 1984) was provided by Barry Powell.

2.1.7 Synthetic Oligonucleotides.

Synthetic DNA primers were obtained from Bresatec. The primer sequences are listed below:

Primer 1 : 5'-dGGCGCCGGGTACCGCGGCCTG-3'

Primer 2 : 5'-dAGGGCGGTACCATCTATTGCTTAC-3'

Primer 3 : 5'-dCCCACAAGTAGGCCTAAGCTCGC-3'

P1 : 5'-dAGGGACTCGGGATAAGAATGGGC-3'

M13 Universal sequencing primer (17mer) : 5'-dGTAAAACGACGGCCAGT-3'

M13 Reverse sequencing primer (25mer) : 5'-dCACACAGGAAACAGCTATGACCATG-3'

RS175 : 5'-dCGGAATTCATATAATTTACTTCAC-3'

SP6 primer : 5'-dGATTTAGGTGACACTATAG-3'

T3 primer : 5'-dATTAACCCTCACTAAAGGGA-3'

T7 primer : 5'-dTAATACGACTCACTATAGGG-3'

TS-1 : 5'-dCACTCTCCATGGTCAGGAAGTATGC-3'

#5393 : 5'-dGCCTGGTCATCAACTCATCA-3'

2.1.8 Bacterial Strains.

The following *E.coli* K12 strains were used :

(1) *E.coli* BB4 : supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1 tonA ΔlacU169 F'[proAB$^+$ lacIq lacZΔM15 Tn10 (tet^r)] host for recombinant plasmids and M13 bacteriophage.

(2) *E.coli* DH5α: supE44 ΔlacU169 (p80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 host for recombinant plasmids, obtained from the *E. coli* Genetic Stock Centre, Yale University, New Haven.

(3) *E.coli* XL1-Blue : supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac^- F'[$proAB^+$ $lacI^q$ lacZΔM15 Tn10 (tet^r)] host for recombinant plasmids and M13 bacteriophage, purchased from Stratagene.

(4) *E. coli* CJ236 : dut, ung, thi, rel A; pCJ105 (Cm^r).

(5) *E. Coli* GM119 : dam3, dcm6, met B1, gal K2, gal T22, lac Y1, tsx 7 and Sup E44.

Stock cultures of these (and plasmid transformed bacteria) were prepared by dilution of an overnight culture with an equal volume of 80% glycerol and stored at either -20°C, or -80°C for long term storage. Single colonies of bacteria, obtained by streaking the glycerol stock onto agar plates of suitable medium (Section 2.1.9) were used to inoculate liquid growth medium, and the bacterial cultures were grown at 37°C with continuous shaking to provide adequate aeration.

2.1.9 Bacterial Growth Media.

Growth media were prepared in double-distilled water and sterilised by autoclaving, antibiotics and other labile chemicals were added after the solution had cooled to 50°C.

(1) Luria (L) broth : contained 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH. Agar plates were prepared by adding 1.5% (w/v) Bacto-agar (Difco) to the L broth. Ampicillin (50 μg/ml) or tetracycline (10 μg/ml) were added where appropriate for growth of transformed bacteria, to maintain selective pressure for the plasmid.

(2) 2 x YT Medium : 1.6% Bacto-tryptone, 1% yeast extract, 0.5% NaCl, adjusted to pH 7.5 with NaOH.

(3) Psi (ψ) Broth : 2% Bacto-tryptone, 0.5% yeast extract, 0.5% $MgSO_4$,adjusted to pH 7.6 with 0.1M KOH

(4) Solid Media : Agar plates were prepared by supplementing the above media with 1.5% Bacto-agar. Soft overlays were 0.7% agar in L broth or 2xYT medium.

2.1.10 Tissue Culture Cell Lines.

FRL 4.1 a cell line derived from the *in vitro* transformation of fetal rat hepatocytes was kindly provided by Dr. G. Yeoh (Yeoh *et al.*, 1990).

2.1.11 Tissue Culture Media.

Phosphate buffered saline (PBS) : 136mM NaCl, 2.6mM KCl, 1.5mM KH_2PO_4 and 8mM Na_2HPO_4, (pH 7.4), was sterilised by autoclaving (20 psi for 25 minutes at 140^oC).

Trypsin/EDTA solution : 0.1% trypsin (Difco) and 1x EDTA Versene buffer solution (CSL), was sterilised by filtration through a 0.2μm filter (Whatman).

Dulbecco's minimal essential medium, (DMEM) (Gibco), 28mmol/l $NaHCO_3$, 19mmol/l glucose, and 20mmol/l Hepes, (pH 7.3), was supplemented with 50,000 Units/l of Gentamycin (Gibco), and filter sterilised as described above.

Ham's F12 with L-glutamine (Gibco), and 28mmol/l $NaHCO_3$ (pH 7.4), was supplemented with 50,000 Units/l of Gentamycin (Gibco), and filter sterilised as described above.

Williams E (Gibco), and 28mmol/l $NaHC0_3$, (pH 7.4), was supplemented with 50,000 Units/l Gentamycin and was filter sterilised as described above.

Foetal Calf Serum : CSL .

Nu Serum™ : Collaborative Research Inc.

2.1.12 Miscellaneous.

3MM paper : Whatman Ltd.

Nitrocellulose (BA 85) and Nytran 0.45μm : Schleicher and Schuell.

X-ray film : either Fuji Photo Film Co. Ltd, Tokyo, Japan or Kodak Diagnostic film X-Omat AR, USA.

2.2 RECOMBINANT DNA METHODS

2.2.1 General DNA Methods.

The following methods were performed essentially as described in "Molecular Cloning; *A laboratory Manual*" Maniatis *et al.* (1982) : Growth, maintenance and preservation of bacterial; quantitation of DNA and RNA; autoradiography; agarose and polyacrylamide gel electrophoresis; DNA and RNA precipitation's; phenol/chloroform extractions; end-filling or end labelling of DNA fragments using the Klenow fragment of *E.coli* DNA polymerase I.

All manipulations involving viable organisms which contained recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

2.2.2 Plasmid DNA Preparation.

The rapid alkaline hydrolysis procedure of Birnboim and Doly (1979) was used for the isolation of plasmid DNA from 2ml overnight cultures for analytical restriction digests. This method was also employed for the bulk preparation of plasmid cDNAs from 50ml cultures for use as probes for radiolabelling in Northern hybridisation analysis (Section 2.3.2).

DNA used for transfection of tissue culture cell lines, probes for nuclear run-ons and *in vitro* RNA transcription reaction were routinely grown up in 250ml cultures inoculated with 100µl from a 5ml overnight culture. The plasmid was extracted using the alkaline lysis procedure described above and further purified by caesium-chloride density gradient centrifugation in a Beckman TL-100 benchtop ultracentrifuge and TLA-100.2 rotor.

2.2.3 Restriction Enzyme Digestions of DNA.

In analytical digests, 0.5-1μg of DNA was incubated with 2-5units each of the appropriate restriction enzyme(s) for a minimum of 2 hours in the buffer conditions specified by the manufacturer. Reactions were terminated by the addition of a 1/3 volume of urea load buffer and electrophoresed on 1% mini-agarose gels in TBE buffer.

In preparative digests, 5 μg of DNA was restricted in a reaction volume of 30μl, and the desired DNA fragments were isolated as detailed below.

2.2.4 Preparation of Cloning Vectors.

Plasmids were linearized with the appropriate restriction enzyme(s). To prevent self-ligation of the vector, 5' terminal phosphate groups were removed by incubation in 50mM Tris-HCl (pH 9.0), 1mM $MgCl_2$, 0.1mM $ZnCl_2$, with 1 units of calf intestinal phosphatase (CIP), in a final volume of 50μl for 1 hr at 37^{o}C for sticky ends, or 15 minutes at 37 ^{o}C followed by 15 minutes at 56^{o}C for blunt ends. The vector DNA was isolated after electrophoresis on a 1.0% agarose TBE gel using a Geneclean™ II kit according to the manufacturers' instructions. The DNA was resuspended at a concentration of 20-50 ng/μl, for use in ligation reactions.

2.2.5 Preparation of DNA Restriction Fragments.

DNA was incubated with the appropriate restriction enzyme(s) as described above (Section 2.2.3) and restriction fragments were isolated from either a horizontal 0.8%-2.0% agarose gel or a vertical 8% polyacrylamide gel, depending on the size of the restriction fragment(s). Bands representing restriction fragments were visualised under UV light following staining with ethidium bromide, and the appropriate fragment(s)

excised from the gel. DNA fragments from agarose gels were isolated using the Geneclean™ II protocol. Fragments isolated from polyacrylamide gels were eluted from the gel slice by incubation in 400µl of 0.5M ammonium acetate, 0.1% SDS, at 37°C for 16 h. The DNA was precipitated by the addition of 2.5 volumes of 100% ethanol, washed in 70% ethanol, air dried and resuspended in 10-20µl of 0.1mM EDTA.

2.2.6 Ligation of DNA.

A 10µl reaction contained 20ng of vector DNA, a 3 molar excess of the insert DNA, 50mM Tris-HCl (pH 7.4), 10mM $MgCl_2$, 1mM DTT, 1mM ATP, and 1unit of T4 DNA ligase. The reactions were incubated for either 4 hours at room temperature, or overnight at 4°C. A control ligation with vector only was set up and included in the subsequent transformation to determine background levels of uncut or recircularised vector DNA.

2.2.7 Transformation of *E.coli* with Recombinant Plasmids.

E. coli cells were made competent by the method the rubidium chloride method, briefly a single colony of the *E.coli* host strain was inoculated into 5ml of Psi (y) broth (where appropriate the ψ-broth was supplemented with an antibiotic) and the culture incubated overnight at 37°C with continuous shaking. 3.3ml of the overnight was subcultured into 100 ml of ψ broth an incubated at 37°C until the culture reached an O.D. of ~ 0.6. 50mls of this culture was then used to inoculate 1.0L of ψ-broth and again the culture was grown up to an O. D. of ~ 0.6. The cells were then chilled on ice for 5 minutes before being pelleted at 2500xg for 5 minutes at 4°C. The cells were then resuspended in 0.4 volumes of Tfb1 (30mM KAc, 100mM RbCl, 10 mM $CaCl_2$, 50mM MnCl, 15% glycerol, adjusted to pH 5.8, with 0.2M acetic acid), incubated on ice for 5 minutes and pelleted at 2500xg, at 4°C for 5 minutes. The cells were then resuspended in .04 volumes of Tfb2 (10mM MOPS free acid, 10mM RbCl, 75mM $CaCl_2$, 15% glycerol adjusted to pH 6.5, with 0.1 M KOH), and incubated on ice for a further 15

minutes, before being stored at -80°C in 500µl aliquot's. 100-150µl of this cell suspension was mixed with 10µl of the DNA ligation reaction mix (Section 2.2.6) and left on ice for 40 minutes. The cells were then heat shocked at 42°C for 2 minutes, L-broth containing 20mM glucose was added (1ml if the overlay method of plating was used or 100µl if the cells were spread directly onto the agar plates), and the cells were incubated at 37°C for at 20 minutes. The transformed cells were then plated onto L-agar containing 100µg/ml of ampicillin, either by spreading with a wire spreader, or the cells were mixed with 3ml of 0.7% L-agar overlay, and poured onto the plates. The agar plates were routinely incubated at 37°C overnight.

2.2.8 Dideoxy-Chain Sequencing Analysis.

Sequencing was performed by the Sanger (1977) dideoxy method, using the sequencing reagents supplied in the USB Sequenase® V2.0 kit.

Double stranded sequencing was performed using plasmid DNA purified by alkaline lysis. 5-10µg of plasmid was used per reaction. The DNA was denatured in 0.2M NaOH, 0.2mM EDTA for 15 minutes at 37°C. The mixture was then neutralised by passage through a TE equilibrated spin-column Murphey *et al.* (1988). 7µl of the elutant (~2µg) was used in a sequencing reaction using [^{35}S]-dATP in accordance with the protocol accompanying the Sequenase® V2.0 kit.

2.2.9 Gel electrophoresis of DNA for Sequence Analysis.

Sequencing reactions (1µl) were electrophoresed on 6% polyacrylamide gels containing 7M urea in 1 x TBE buffer at 1800 V. After electrophoresis, gels were transferred to whatman® 3MM chromatography paper and dried down under vacuum. Autoradiography was for 16-24 hours at room temperature.

2.2.10 Preparation of [^{32}P]-Labelled DNA Probes.

(i) Oligo-Labelling of DNA.

Linearized plasmids or isolated cDNA inserts were labelled with [α-^{32}P]-dATP using the Megaprime® DNA Labelling kit (Amersham). Briefly 50-100ng of DNA was denatured in the presence of random nonamers in a total volume of 25µl at 100°C for 4 minutes. Reactions were set up at room temperature as per the instruction manual, using 50µCi of a [α-^{32}P]-dATP and 2U of Klenow fragment in a total volume of 50µl. The reactions were then incubated at 37°C for 10 minutes before being terminated by the addition of EDTA (final concentration 20mM). The probes were then denatured by incubation at 100°C for 4-5 minutes before being added to hybridisation solution.

(ii) 5' End-Labelling of Synthetic DNA Oligonucleotides.

The synthetic DNA oligonucleotides used as probes were [^{32}P] labelled at the 5' end using [γ-^{32}P] ATP and T4 polynucleotide kinase. The reaction mixture contained 10mM $MgCl_2$ 50mM Tris-HCl (pH 7.4), 5mM DTT, 0.1mM spermidine, 0.1mM EDTA, 100µCi [γ-^{32}P] ATP and 2 units of T4 polynucleotide kinase in a final volume of 20µl. This was incubated at 37°C for 30 minutes. Following the addition of 10µl formamide loading buffer, the reaction was run on a 20% polyacrylamide gel at 18mA for 60 minutes to separate the [^{32}P]-labelled oligomer from unincorporated label. The labelled oligomer was localised by autoradiography, excised from the gel and eluted in TE buffer at 37°C for 16 h. This solution was then used directly for hybridisation.

2.2.11 Oligonucleotide Site-Directed Mutagenesis.

Site directed mutagenesis was performed on double stranded DNA, using a modification of the Uracil selection method of Kunkel *et al.* (1987). Briefly, plasmids to be mutagenised were transformed into the *E. coli* strain CJ236 dut^-, ung^- and incubated overnight in 2YT supplemented with 33µg/ml Chloramphenicol, 100µg/ml Ampicillin and 5µg/ml uridine. The plasmid was then isolated via the alkaline lysis method resulting in DNA that contained several uracil residues in place of thymidine residues.

5-10µg of the template was then denatured as for double stranded sequencing in a final concentration of 0.2M NaOH, 0.2mM EDTA, for 15 minutes at 37°C , before being neutralised by passing through TE equilibrated spin columns (section 2. 2. 8.) . 7µl (~1-2µg) of the resulting elutant was incubated with 100ng of cold kinased mutagenic primer, 2µl of 5x Sequenase buffer [200mM Tris-HCl (pH 7.5), 100mM $MgCl_2$, 250mM NaCl], in a total volume of 10µl. The reaction was heated to 68°C for 2 minutes, the heating block was then turned off and the reactions allowed to cool slowly to room temperature. The reaction was placed on ice and made to 500µM dNTP, 1mM ATP, 1mM DTT, 60mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 25mM NaCl with 3U T4 DNA polymerase and 3U T4 DNA ligase. The reactions were left on ice for 5 minutes, transferred to 24°C for 5 minutes before being incubated for 90 minutes at 37°C. 5-10µl of the mutagenesis reaction was used to transform XL-1-Blue cells. This procedure resulted in about a 20-30% mutation efficiency, thus allowing direct screening for mutants by restriction digestion analysis, without the need for TMACl washing. All mutants were sequenced for both the desired mutation as well as for any random mutations introduced during the synthesis reaction.

2.2.12 Polymerase Chain Reaction.

(i) For PCR from RNA : 1µg of total RNA was heated for 5 minutes to 65°C with 4µl (50µg/ml) oligo d(T) in a total volume of 11µl and placed on ice. To this was

added 2µl dNTP mix (10mM each), 2µl RNasin (40,000)U/ml), 4µl of 5x BRL reverse transcriptase buffer and 200U BRL Mu MMLV reverse transcriptase, and the reaction incubated for 40 minutes at 37°C.

10µl of this cDNA synthesis reaction was then amplified in a 50µl reaction [50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 25pmol specific 5'and 3' primers, 200µM dNTP's, 0.01% gelatine and 1.1U Taq DNA polymerase] for 30 cycles (96°C 45 seconds, 55°C 45 seconds, and 72°C 1minute 45 seconds).

(ii) For PCR from plasmids : 10ng of plasmid was used in a standard 50µl PCR reaction for 30 cycles (5 minutes 96°C, 55°C 45s, 72°C 1min 45s, followed by 29 cycles 96°C 45 seconds, 55°C 45 seconds, and 72°C 1minute 45 seconds).

2.2.13 Southern Blot Analysis.

(i) Isolation of genomic DNA from tissue culture cells: Genomic DNA was prepared from tissue culture cells as described in Ausbel *et al.* (1989).

(ii) Southern blot analysis: 20µg of genomic DNA or 1-2µg of cloned DNA was digested with the appropriate restriction enzymes and electrophoresed on 0.8-1.0% agarose gels in 1x TBE. Following staining with ethidium bromide, the gels were visualised under UV light, and photographed. When large fragments were expected (ie larger than 5kb) the gels were treated with 0.25M HCl for 5 minutes to partially depurinate the DNA prior to the denaturation and neutralisation steps. Denaturation, neutralisation and capillary transfer of the DNA to Nytran™ filters (Schleicher and Schuell) was as described in the manufacturers protocol, except the transfer was carried out in 20x SSPE. Filters were pre-hybridised for 4-16 hours at 42°C in 50% formamide, 5 x SSC, 5 x Denhardt's solution, 0.1% SDS, 0.05% sodium pyrophosphate, and 200 µg/ml of sonicated salmon sperm DNA in roller bottles. Hybridisations were carried out for 18-24 hours under the same conditions, except for the addition of radiolabelled

probe (1-5 x 10^8 cpm/μg). Filters were washed twice in 2x SSPE, 0.1% SDS and 0.5% sodium pyrophosphate at 65°C for 10 minutes each, followed by one wash in 0.1-0.5x SSPE, 0.1% SDS and 0.5% sodium pyrophosphate at 60°C-65°C for 40 minutes depending on the degree of homology between the probe and the target message.

2.2.14 Colony Screening.

Rapid screening of bacterial colonies was performed using Nytran™ membranes according to the "microwave lysis" protocol (Buluwela *et al.*, 1989). Screening for recombinant clones used standard prehybridisation, hybridisation and washing conditions as described in section 2.2.13.

2.3 METHODS FOR ISOLATION AND ANALYSIS OF RNA.

2.3.1 Preparation of Total RNA from Rat Tissue.

Total RNA was extracted from 2g of tissue using the guanidinium isothiocyanate procedure described by Chomczynski and Sacchi (1987), modified in the following way. Firstly, the volumes used in the published extraction procedure were scaled up to 10ml/g of tissue. Secondly, when RNA is prepared from liver, glycogen tended to co-purify with the RNA and this was removed by precipitation of the RNA with 3 volumes of 4M Na acetate at 0°C overnight. The RNA was recovered by centrifugation at 8000x g for 15 minutes at 4°C, and resuspended in 0.1mM EDTA.

2.3.2 Isolation of RNA from Tissue Culture Cells.

(i): Total RNA was extracted from tissue culture cells, essentially as described by Chomczynski and Sacchi (1987).

(ii): Cytoplasmic RNA was prepared from FRL 4.1 cells as described by Gough (1988).

Following RNA extraction, the absorbance values at 260 nm and 280 nm of each RNA sample were determined on a Shimadzu UV-160A spectrophotometer. The A_{260}/A_{280} ratio of the RNA samples were consistently in the range 1.8-2.0. The relationship of one A_{260} unit equal to 40 µg/ml RNA was used in the calculation of RNA concentrations.

2.3.3 Northern Hybridisation Analysis of RNA.

(i) Northern Blots:

RNA for Northern blots (10-20µg) was denatured in 10mM sodium phosphate (pH 7.4), 50% formamide, 2.2M formaldehyde, 0.5mM EDTA at 65 ^{o}C for 5 minutes, prior to electrophoresis on 1% agarose gels containing 1.1M formaldehyde and 10mM sodium phosphate (pH 7.4). The RNA was transferred onto either BA85 nitrocellulose or Nytran™ filters (Schliecher and Schuell) using capillary transfer. Following transfer, the filters were irradiated with 120mJoules of UV radiation in a Stratagene UV Stratalinker™ 1800 which results in the RNA being covalently crosslinked to the filter (manufacturer's instruction manual). Filters were pre-hybridised for 4-16 hours at 42^{o}C in 50% formamide, 5x SSPE, 5x Denhardt's solution (section 2.1.4), 0.1% SDS, 0.05% sodium pyrophosphate, and 200µg/ml of sonicated salmon sperm DNA in roller bottles. Hybridisations were carried out for 18-24 hours under the same conditions, except for the addition of radiolabelled probe (1-5 x 10^{8} cpm/µg). Filters were washed twice in 2x SSPE, 0.1% SDS and 0.5% sodium pyrophosphate at 65^{o}C for 10 minutes each, followed by one wash in 0.1-0.5x SSPE, 0.1% SDS and 0.5% sodium pyrophosphate at 60^{o}C-65^{o}C for 40 minutes depending on the degree of homology between the probe and the target message.

(ii) Probe Removal:

Northern Blots were stripped of probe for re-use by washing blots in 5mMTris-HCl (pH 8.0), 0.2mM EDTA, 0.05% sodium pyrophosphate and 0.1x Denhardt's solution for 1-2 hours at 65°C-67°C. Filters were then rinsed in 2x SSPE and either re-used immediately or stored at 4°C between clean pieces of Whatman 3M paper.

(iii) RNase H Cleavage Protocol for Determining the Length of an mRNA Poly(A) Tail:

RNase H treatment of mRNA was performed using the hybridisation conditions of Mercer and Wake (1985). Samples of total RNA (50-130µg) were annealed with 1µg of the synthetic oligonucleotide #5393 (5'-dGCCTGGTCATCAACTCATCA-3'), which is complimentary to the sequence +1742 to +1762 of the rat ALAS-1 mRNA. To generate *in vitro* de-adenylated products, oligo$(dT)_{12\text{-}18}$ (0.5µg Pharmacia) was also included in one of the the hydridisation reactions. Hybrids were cleaved by incubating the reactions with 1.5U RNase H (Promega) in 28mM $MgCl_2$ 25mM KCl, 0.5mM EDTA and 20mM Tris-HCl (pH 8.0), at 37°C for 30 minutes. The RNA was then recovered by the method of Chomczynski and Sacchi (1987) and electrophoresed on a 1.3% agarose gel containing 1.1M formaldehyde and 10mM sodium phosphate (pH 7.4). Northern blotting, prehybridisation, hybridisation and washing conditions were identical to those used for standard Northern blots (Scetion 2.3.3I). Size of the bands were estimated by comparison to ethidium bromode stained RNA size markers (Promega) run on the same gel.

2.3.4 *In Vitro* Synthesis of [^{32}P]-Labelled RNA.

10-20µg of template DNA was linearized with the appropriate enzyme, separated from uncut vector by electrophoresis and the template recovered by the Geneclean™ protocol. 2µg of this DNA was added to a reaction mix containing 10mM

DTT, 40mM.Tris-HCl (pH 7.6), 6mM $MgCl_2$, 500μM of each ATP, CTP and GTP, 50μM UTP, 1μg/ml BSA, 100μCi [α-^{32}P] UTP and 4 units of SP6 RNA polymerase or T7 RNA polymerase, in a total volume of 20μl. Following incubation at 37°C for 60 minutes, the reaction mix was treated with 1U of RNase free DNase 1 for 10 minutes at 37°C, full length products were then separated on a 6-8% sequencing gel, and the wet gels autoradiographed for 30 seconds. The band corresponding to the full length RNA transcript was cut out and eluted 4-6 hours in 400μl of elution buffer (0.5M ammonium acetate, 1mM EDTA, and 0.1% SDS) at 37°C. The buffer containing the probe was collected and stored at -20 °C for up to 1 week.

2.3.5 RNA Protection Analysis.

10-20μg of the test RNA and 50,000-100,000 cpm of the single stranded RNA probe were combined in a 1.5ml Eppendorf tube and pelleted by centrifugation. RNase protections were carried out using the RPA II™ ribonuclease protection kit from Ambion Inc as per the manufacturer protocol. The protected RNA was then dissolved in formamide loading buffer (Maniatis *et al.,* 1982), denatured by heating at 85°C for 2 minutes and analysed by electrophoresis on a 6-8% sequencing gel. Hpa II cut pUC19 plasmid DNA, which had been end-filled with [α-^{32}P] dCTP, was used as size markers. The gel was autoradiographed at -80°C for approximately one week using Kodak X-Omat sensitive film, or overnight on storage phosphor screens.

2. 4. METHODS FOR THE ISOLATION OF NUCLEI AND TRANSCRIPTIONAL RUN-ON ASSAY

2.4.1 Isolation of Nuclei from Mammalian Tissue Culture Cells.

Approximately 1x 10^8 cells per treatment were used to isolate nuclei. The protocol used was similar to that descried by Ausubel *et al.* (1989). The media was removed from the tissue culture flask, and the cells washed with 20ml PBS. The cells were then trypsinised and collected in 10ml of media containing 5% FCS to inactivate the trypsin. The cells were pelleted at 1000xg for 5 minutes at 4°C, resuspended and washed twice in ice cold PBS. The supernatant was removed and the cell pellet loosened by vortexing at half maximal speed for 10 sec. prior to the addition of 4ml of NP-40 lysis buffer [10mM Tris-HCl (pH 7.4), 10mM NaCl, 3mM $MgCl_2$, 0.5% NP-40 and 0.1mM PMSF]. The vortexing was continued as the buffer was added and for 10 sec afterwards. The cells were incubated on ice for 5 mins. and centrifuged for 5 mins. at 500x g, at 4°C.

The nuclei pellet was resuspended in a further 4ml of NP-40 lysis buffer by vortexing as above, and centrifuged 5 mins. at 500x g, at 4°C. The supernatant was discarded, and the nuclei were resuspended in 1.5ml of glycerol storage buffer [20mM Tris-HCl pH (7.9), 75 mM NaCl, 0.5mM EDTA, 50% glycerol, 1mM DTT, and 0.1% PMSF], and transferred to a small glass homogeniser. Large clumps of nuclei were broken up by several strokes of the homogeniser, and the nuclei transferred to an eppendorf tube. The nuclei were pelleted for 15 sec at 4°C in an eppendorf centrifuge and counted using a haemocytometer. Nuclei were adjusted to a concentration of 1x 10^8 nuclei per ml with storage buffer and snap frozen in liquid nitrogen and stored at -80°C.

2.4.2. Isolation of Nuclei from Rat Liver.

Nuclei were isolated from rat liver by the method of Schibler *et al.* (1983). 2g of liver was homogenised in 20ml of homogenising buffer [0.3 M sucrose, 15 mM Hepes (pH7.5), 2mM EDTA, 0.5 mM EGTA, 60mm KCl, 15mM NaCl, 0.5 mM spermidine, 0.15mM spermine, 5mM DTT, and 0.1mM PMSF], with 10-20 strokes of a glass-teflon Potter-Elvehjem homogeniser (clearance 0.23mm), motor-driven at about 500 rpm.

The homogenate was filtered through 4 layers of cheesecloth, and layered over 10 ml of 30% sucrose in homogenising buffer and centrifuged for 10 min. at 2 500 rpm (750x g) in a HB-4 rotor, at 4°C. The pellet was resuspended in 8ml of Buffer B [2M sucrose, 15mM Hepes (pH 7.5), 0.1mM EGTA, 0.1mM EDTA, 60mM KCl, 15mM NaCl, 0.5mM spermidine, 0.15mM spermine, 5mM DTT, and 0.1mM PMSF], layered over 4ml of buffer B and centrifuged for 20 min. at 20,000 rpm (75,000x g) in a SW-41 rotor at 4°C.

The supernatant was aspirated and the nuclei resuspended in 1.5 ml of storage buffer [20mM Tris-HCl (pH 7.9), 75 mM NaCl, 0.5mM EDTA, 50% glycerol, 1mM DTT, and 0.1% PMSF], clumps removed by several strokes in a glass-glass homogeniser, and the nuclei pelleted at 12, 000 x g for 15 sec at 4°C, in an eppendorf centrifuge. The pellet was resuspended in an equal volume of storage buffer, and a 1µl aliquot was diluted in 400µl of storage buffer and counted using a haemocytometer. The concentration was adjusted to 3x 10^8 nuclei per ml. The nuclei were snap frozen in liquid nitrogen and stored at -80°C.

2.4.3. Nuclear Run-On Assay.

Transcription reactions contained 100mM Tris-HCl (pH 7.9), 50mM NaCl, 0.4mM EDTA, 5mM $MgCl_2$, 1.5mM $MnCl_2$, 1.2mM DTT, 30% glycerol, 1mM each of ATP, rCTP and GTP, 2µM of unlabelled UTP, 0.1mM PMSF, 100µM [α-^{32}P] rUTP and 1.5 x 10^7 nuclei, in a final volume of 150µl. Reactions were incubated for the

required time at 26°C. To monitor incorporation of the [α-^{32}P] UTP, TCA precipitable radioactivity of 1-5 µl aliquot's was determined in triplicate's by a modification of the method described in section 2.6.1. Carrier RNA was not required, and nuclei were first lysed in 100 µl of 10% SDS , 10mM EDTA. This results in a much lower background radioactivity (Marzluff *et al.,* 1978).

2.4.4. Preparation of ^{32}P-RNA .

^{32}P-RNA was prepared as descried by Vannice *et al.* (1984). Reactions were terminated by the addition of 750µl of 0.48% SDS, with 100µg of *E. coli* tRNA as carrier. After gentle mixing, 900µl of 100mM sodium acetate (pH 5.0), 20mM EDTA was added and the RNA was incubated on ice for 15 min. The RNA was then extracted by the addition of 1.8ml of water saturated acidic phenol, and centrifugation at 10, 000 rpm (16, 000x g) for 20 min at 4°C in a HB-4 rotor. The top phase was collected, 3M Na acetate (pH 5.5), was added to a final concentration of 0.2M and the RNA precipitated by the addition of 2.5 volumes of ethanol. The pellet was washed with 70% ethanol, air dried and resuspended in 200µl of 1mM EDTA. The TCA-precipitable radioactivity in 1µl was determined in triplicates as described in section 2.6.1.

2.4.4. Hybridisation of ^{32}P-RNA to Immobilised DNA.

5µg of double-stranded plasmid clones was applied to a nylon filter using a slot-blot apparatus (Schleicher and Schuell). For each slot, 5µg of DNA in 200µl of TE buffer was incubated with 20µl of 3M NaOH for 30 min. at 65°C, the mixture kept on ice and was neutralised with 220µl of 2M ammonium acetate just prior to loading. The nylon membrane was treated by soaking first in H_2O, followed by 2M ammonium acetate for 10 minutes each. The DNA in a final volume of 440 µl was applied to the filter under gentle suction and the DNA covalently cross-linked to the filter using UV treatment (section 2.2.3.)

Filters were prehybridised in 5ml of 50% formamide, 5 x SSPE, 10mM Tris-HCl (pH 7.6), 1mM EDTA, 0.1% sodium pyrophosphate, 0.1% SDS, 100µg/ml *E. Coli* tRNA and 0.2% each of ficoll, polyvinylpyrrolidone and bovine serum albumin, at 52°C overnight. Hybridisations were carried out in the same solution with $2x10^6$ cpm of [^{32}P]-RNA , for 72 hr. at 52°C. Filters were washed twice at 65°C for 15 min. in 2xSSC, 0.1% SDS, 0.1% sodium pyrophosphate, then once in 0.5xSSC 0.1% SDS, 0.1% sodium pyrophosphate at 65°C for 60 min. The filters were then treated with 10 µg/ml of RNase A in 70 ml of 1xSSC for 30 min at 37°C, washed in 1xSSC for 30 min. at room temperature before being exposed to X-ray film or storage phosphor screens.

2.5 METHODS FOR TRANSIENT EXPRESSION OF PROMOTER CONSTRUCTS IN TISSUE CULTURE CELL LINES

2.5.1 Cell Maintenance.

Fetal rat hepatoma (FRL 4.1) cells were routinely maintained in 150 cm^3 flasks (Costar) in a 1:1 ratio of DMEM/Hams F12, 5% FCS at 37°C in an atmosphere of 5% CO_2 and were subcultured every 3-4 days. To subculture or harvest the cells the culture media was removed and the cells washed in PBS before the addition of 2.5ml of trypsin solution (0.1% trypsin, 0.02% EDTA). The cells were left at room temperature until they began to detach from the flask. 10ml of culture media was added and the flask washed to remove any remaining cells. The cells were routinely subcultured by splitting 1:6, into fresh media and incubating at 37°C. For harvesting, the cells were washed twice in 10ml of PBS, and pelleted by centrifugation at 1200x g for 5 minutes, before resuspending in the appropriate buffer.

2.5.2. Serum Free Conditions.

For serum free conditions cells were plated in 10mm petri-dishes at a density of 9x 10^6 / dish in William's E media supplemented with 10% Nuserum (a serum reduced supplement), and the cells were left overnight to attach. The next morning the cells were washed once with PBS and the media changed to William's E with no serum supplement, cells were left for 30hr before the media was again changed to William's E +/- drugs or hemin.

2.5.3. Drug Treatments.

A 1mM stock of hemin or tin mesoporphyrin was prepared freshly each time, by dissolving the hemin in 0.25% calcium carbonate and adjusting the pH to 7.4, by the addition of 1M HCl. Hemin was used at a concentration of 2μM for all experiments unless stated otherwise in the text, this resulted in a final concentration of calcium carbonate of 5x 10^{-4} %. ALA was dissolved in PBS at a concentration 100 fold greater than the final concentration required in the culture media. TPA was dissolved in DMSO to a concentration of 300nM. 2-Aminopurine (Nitrate salt) was dissolved in tissue culture media as a 10x stock and was neutralised with 0.1M NaOH. Actinomycin D was dissolved in 100% ethanol at 5mg/ml. Its OD at 440nm was then determined and the concentration adjusted to 1.9mg/ml. The absorbance of a 1mg/ml solution of Actinomycin D is 0.182 at 440nm. The final concentration of ethanol added to the tissue culture dish was therefore 0.53%. Iron was added as Ferric ammonium citrate at a final concentration of 200μM iron in PBS. The iron chelator Desferal® was dissolved in PBS and used at a final concentration of 100μM.

2.5.4. Transfection of Fetal Rat Hepatomas Cells by Electroporation.

Transfection of FRl 4.1 cells by electroporation was performed by a modification of the method of Chu *et al.* (1987).

(i) Transient transfections: Cells were harvested by trypsin treatment, washed 2x in ice cold Hepes buffered saline (HBS) [20mM Hepes (pH 7.05), 137mM NaCl, 5mM KCl, 700µM Na_2HPO_4, 60mM Dextrose] and finally resuspended at a concentration of 1 x 10^7 cells / ml, in HBS supplemented with 250µg / ml salmon sperm DNA. 500µl aliquot's of cells were mixed with 50µl of FCS and 7.5-15µg of linearized construct in Bio-rad electroporation cuvettes and left at 4°C for 10 min. before being exposed to a single pulse of 230 volts, 960µfd capacitor, in a Bio-rad Gene-pulsar electroporator. Cuvettes were left at room temperature for 10 min. after electroporation before 500µl of media was added and the cells gently plated in 10mls DME/Hams F12 5% FCS. The plates were left overnight to attach, in the morning cells were washed once in PBS, fresh media was added and the cells incubated for a further 48hr. before harvesting.

(ii) Stable transfections: Stable transfections were performed by the same procedure modified in the following way, 7.5µg of linearized construct was used to transfect a total of 5 x 10^5 cells, which were then plated onto four 5mm petri dishes. The cells were again left overnight to attach, washed with PBS and fresh media added. After 48hr the media was changed to DME:F12 (1:1 vol/vol) 5% FCS supplemented with 100µg /ml G418. Selection with G418 was maintained for at least one week in which time all mock transfected cells were killed. Several hundred colonies were then pooled to give a heterogenous population, and aliquot's frozen at -80°C.

2.5.5 Assay for Chloramphenicol Acetyltransferase (CAT) Activity.

Transfected cells were harvested using a rubber policeman and washed twice in PBS. The cell pellet was resuspended in 100ml of 250mM Tris-HCl buffer, (pH 7.6). The cells were lysed by three cycles of freeze-thawing and the lysate was centrifuged for 5 minutes to remove cellular debris. The protein concentration of the supernatant was determined using the Bio-Rad Bradford protein assay. To the cell lysate EDTA was added to a final concentration of 5mM prior to incubation at 65^{o}C for 10 minutes and then centrifugation for 5 minutes to remove deacetylase activity (Adrisani *et al.*, 1987). CAT activity in the supernatant was then assayed as described by Gorman *et al.* (1982). To 130µl of 250mM Tris-HCl, (pH 7.6), containing 20-100µg of protein was added 10µl of 10mM acetyl-coenzyme A, 39µl of water and 1µl of [^{14}C] chloramphenicol. The reaction mixture was incubated at 37^{o}C for 1 h after which 10ml of acetyl-conenzyme A was added and the incubation continued for another hour. To stop the reaction, 1ml of ethyl acetate was added and the solution vortexed to extract the chloramphenicol. The upper, organic phase was transferred to a clean Eppendorf tube and the ethyl acetate was evaporated. The residue was dissolved in 10ml of ethyl acetate and spotted onto silica plates (Merck). Acetylated [^{14}C] chloramphenicol was resolved by thin layer chromatography in a solvent of chloroform : methanol (9:1). The silica plates were air dried and autoradiographed at -80^{o}C for 16 hours. After autoradiography, the spots corresponding to acetylated chloramphenicol were cut out and the amount of radioactivity quantitated by liquid scintillation counting.

2.5.6 Bradford Protein Assay.

The protein content of the cell extracts was determined using 5µl of cell extract and the Bio-Rad protein microassay procedure according to the manufacturer's instructions. Bovine serum albumin was used as the protein standard.

2.6 MISCELLANEOUS METHODS.

2.6.1 Measurement of TCA-Precipitable Radioactivity.

1-5µl of sample was added to 100µl of H_2O, with 100µg of carrier (denatured salmon sperm DNA for DNA samples, or *E coli* tRNA for RNA samples). 1.5ml of ice cold 5% TCA containing 1% sodium pyrophosphate was added and the samples kept on ice for 30 minutes. The precipitate was collected by filtration through a Whatman GF/A glass fibre disc and this was washed with 25ml of ice cold 5% TCA, 1% sodium pyrophosphate followed by 10ml of ethanol. The discs were dried and counted in 3ml of Optiscint™ (Pharmacia) scintillation fluid in a LKB Wallac 1214 Rackbeta liquid scintillation counter.

2.6.2 Densitometric Quantitation of Bands on Autoradiograms.

Quantitation of bands on autoradiograms was performed on a Molecular Dynamics computing laser densitometer, using their ImageQuant® data analysis software. Exposure times were adjusted so that the signals were within the linear range of the film used. In addition to autoradiography, radioactivity was also detected by using storage Phosphor technology. Radiolabel detection was carried out on a Molecular Dynamics Phosphorimager, using Molecular Dynamics ImageQuant® data analysis software.

2.6.3 Computer Analysis.

Conserved motifs in the 3'UTRs of the chicken, rat and human ALAS-1 mRNAs were searched for using SPCOMP (Dr. A. Sivaprasad, Biochemistry Dept. Uni. Adelaide), a modified version of DBCOMP (Staden, 1982).

The programs GAP anf Fold used in chapter 4 form part of the suite of programs from the Genetics Computing Group of the University of Wisconsin (Devereux, 1984).

CHAPTER THREE

THE EFFECT OF HEME ON ALAS-1 mRNA LEVELS IN FRL 4.1 CELLS.

CHAPTER 3: THE EFFECT OF HEME ON ALAS-1 mRNA LEVELS IN FRL 4.1 CELLS.

3.1 Introduction.

The regulation of heme biosynthesis has been the focus of our research group for a number of years. Of particular interest is the molecular basis for the negative feedback regulation by heme on the housekeeping ALA Synthase (ALAS-1) isozyme. As discussed in section 1.6.6., ALAS-1 levels can be controlled by heme in two ways. Firstly, heme can block the import of the precursor form of the enzyme into the mitochondria and secondly heme can repress the level of ALAS-1 mRNA. The molecular basis for heme's ability to repress the level of ALAS-1 mRNA is the subject of this chapter. Several experiments (Srivastava *et al.,* 1988 ; Srivastava *et al.*, 1990) demonstrated that heme could repress the transcription of the ALAS-1 gene in rat liver, however a similar effect of heme on ALAS-1 transcription is not observed in chickens (Hamilton *et al.,* 1991) or in the human hepatoma cell line HepG2 (Healy, 1990). Furthermore, we have been unable to demonstrate an effect of heme on the transcription of a reporter gene carrying up to 2.7kbp of the 5'-flanking region of the rat ALAS-1 gene in the rat hepatoma cell line H4-II-E-C3 (Braidotti, 1992). One explanation for this is that the *cis*-acting sequences required for heme repression are lacking from these constructs. Alternatively ALAS-1 transcription may not be repressed by heme in H4-II-E-C3 (H4) cells. This has recently been demonstrated in the human hepatoma cell line HepG2 and in 17-day chicken primary hepatocytes raising the possibility that heme regulates ALAS-1 mRNA levels by a post-transcriptional mechanism in cultured cells. The transcriptional regulation by heme of the ALAS-1 gene observed in rat liver (Srivastava *et al.,* 1988 ; Srivastava *et al.*, 1990) has also been questioned by Hamilton *et al.* (1991) raising the possibility that this phenomena is an artefact of the high doses of heme used.

In this study I have concentrated on examining the regulation of ALAS-1 mRNA by heme in another rat hepatoma cell line termed FRL 4.1. We chose to examine the regulation of ALAS-1 mRNA in FRL 4.1 cells rather than in H4 cells for several reasons. Firstly, we found that FRL 4.1 cells can grow in serum free media such as William's E, enabling an examination of the effect of heme addition on ALAS-1 mRNA levels without the added complication of exogenous heme already being present in the serum. Secondly, we wanted to compare the effect of heme on the regulation of ALAS-1 mRNA in several different cell lines to determine if ALAS-1 mRNA regulation occurs in a similar manner. Establishing the regulation of ALAS-1 mRNA *in vitro* will allow us determine if this regulation also occurs *in vivo* or whether true differences in the heme repression of ALAS-1 mRNA exist in culture compared with *in vivo*.

3.2 RESULTS.

3.2.1 Northern Analysis of ALAS-1 mRNA levels in FRL 4.1 cells.

The fetal hepatoma cell line FRL 4.1 was derived by Yeoh *et al.* (1990) from the *in vitro* chemical transformation of 19-day gestation fetal rat hepatocytes followed by the passage of these cells in immunodeficient nude mice to produce tumours. The cell line FRL 4.1 was one cell line produced from such tumours, and it retains a number of liver specific markers (Yeoh *et al.*, 1990). In tissue culture cells, grown in media supplemented with fetal calf serum, ALAS-1 mRNA levels are generally very low (Healy, 1990). This is thought to be caused by free heme present in the serum repressing ALAS-1 mRNA levels. To avoid this problem FRL 4.1 cells were cultured in serum free conditions prior to RNA isolation. Serum free conditions consisted of plating cells in William's E (a defined media) supplemented with 10% NuSerum a reduced serum substitute. Cells were allowed to attach overnight in this media prior to being washed in

PBS (Phosphate buffered saline). The cells were then incubated in William's E media alone, for a further 30hr before heme[2] was added and RNA isolated.

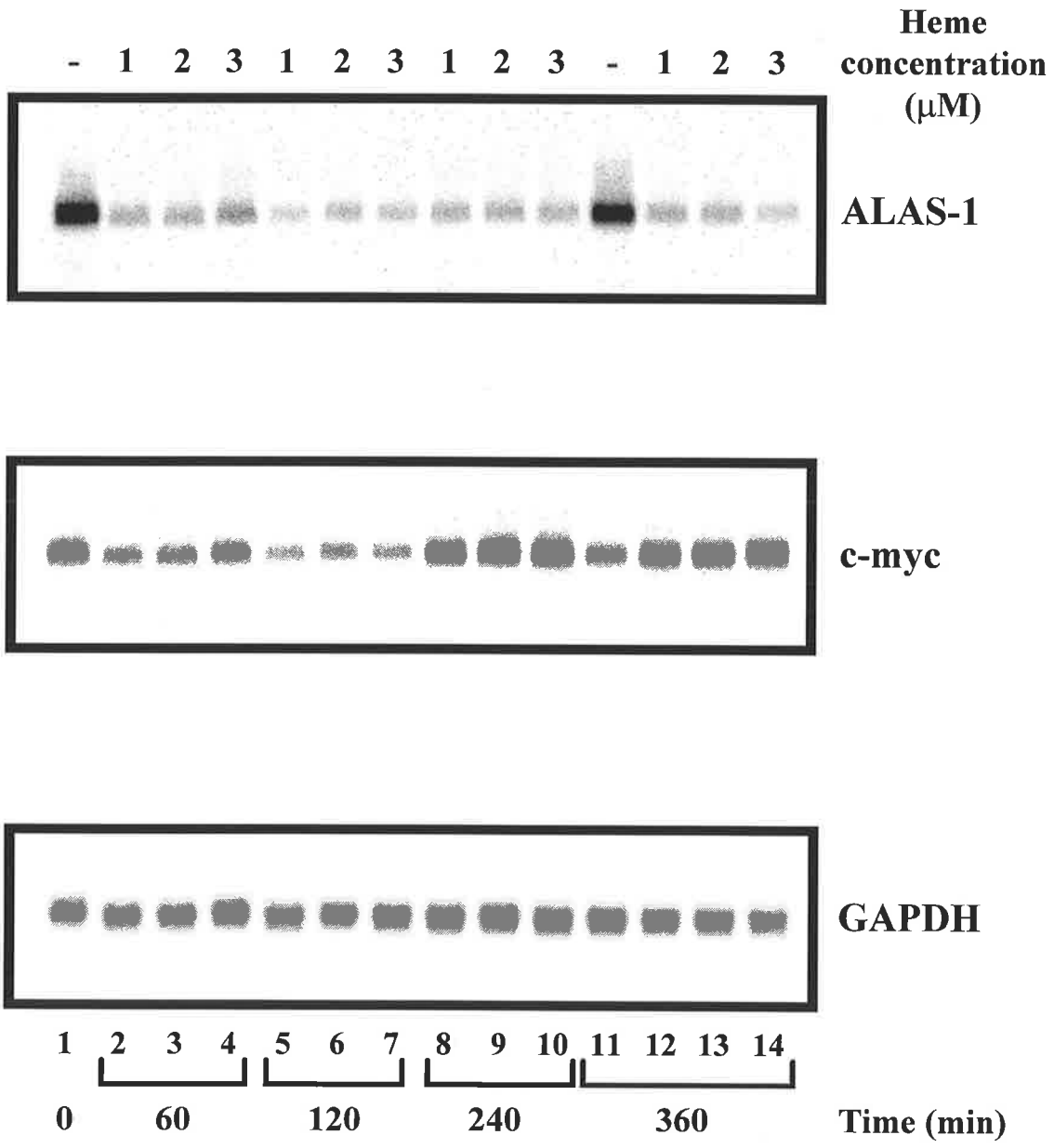
Preliminary experiments clearly demonstrated that ALAS-1 mRNA levels in FRL 4.1 cells decreased upon heme treatment (data not shown). To establish the concentration of heme required to repress ALAS-1 mRNA levels, and to investigate the kinetics of the response, a time course of ALAS-1 mRNA after heme treatment was performed. FRL 4.1 cells were treated with 1, 2 or 3μM heme and total RNA was isolated at zero, 60, 120, 240 and 360 minutes after heme addition. Total RNA (20μg) from each time point was then subjected to Northern blot analysis and the filter probed sequentially for ALAS-1, c-myc and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) mRNA. Filters were washed and exposed to a molecular dynamics storage phosphor screen. The levels of the three mRNAs were quantitated using the molecular dynamics ImageQuant software. Levels of the ALAS-1 and c-myc mRNAs were corrected for loading and transfer variations by normalising to GAPDH mRNA levels which have been shown not to vary with heme treatment.

ALAS-1 mRNA levels rapidly decreased in response to heme treatment (fig 3.1), declining to 20% of the untreated sample after 60 minutes. This level further declined to 10% of the untreated control after 2 hr, and this level was maintained for at least 6 hr after the addition of heme. All three concentrations of heme used produced similar results, demonstrating that as little as 1μM is sufficient to obtain maximum repression of ALAS-1 mRNA levels. Hamilton *et al.* (1991) have similarly shown that 1μM heme is sufficient to obtain maximum inhibition of ALAS-1 mRNA levels in drug induced chick-embryo hepatocytes. To determine whether this effect on ALAS-1 mRNA levels was specific or a more general toxic effect, the mRNA level of the proto-oncogene c-myc was examined. The half life of the c-myc mRNA is known to be very short (Laird-Offringa, 1992) and therefore any non-specific toxic effect caused by the addition of exogenous heme, should be rapidly reflected in the steady state level of c-myc mRNA. Initially c-myc mRNA does show a slight decline in its steady state level in response to

[2] Heme is ferroprotoporphyrin IX and is the form that exists in hemoproteins inside the cell. Heme is readily auto-oxidised *in vitro* to form ferriprotoporphyrin IX which is termed hemin. Experimentally, hemin was used instead of heme but to avoid confusion both forms have been termed heme in the text.

Figure 3.1: **Northern Analysis of ALAS-1 mRNA Levels in FRL 4.1 Cells Treated with Heme.**

FRL 4.1 cells were maintained in serum free conditions for at least 30hr prior to the addition of heme. The media was then changed to serum free media containing no heme (lanes 1 and 11), 1μM heme (lanes 2, 5, 8 and 12), 2μM heme (lanes 3, 6, 9 and 13) or 3μM heme (lanes 4, 7, 10 and 14). Total RNA was then isolated at the times indicated by the method of Chomzynski and Sacchi (1987). 20μg of total RNA from each sample was then separated on a 1% agarose gel containing 1.1M formaldehyde. Following Northern transfer to Nytran (section 2.3.3), the filter was initially probed with a random primed [^{32}P] labelled rat ALAS-1 cDNA probe pGEM Rat 1 (Srivastava *et al.*, 1988). The filter was exposed to a molecular dynamics PhosphorImager screen and the image produced is shown. Sebsequently the filter was stripped of ALAS-1 probe (according to the manufactors instructions) and reprobed with cDNA clones for c-myc and GAPDH.


Heme concentration (μM)
- 1 2 3 1 2 3 1 2 3 - 1 2 3
ALAS-1
c-myc
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14
0 60 120 240 360
Time (min)

heme reaching a minimum of about 50% of the untreated control at 2hr, but subsequently c-myc mRNA levels recover to be as high or higher than the untreated control levels. The mRNA for the housekeeping gene GAPDH remains constant throughout the time course and was used as a loading control.

Clearly basal ALAS-1 mRNA levels are negatively regulated by heme in FRL 4.1 cells. This implies that not only does heme negatively control the drug mediated increase in ALAS-1 mRNA levels observed in rat liver, but that heme may also control the normal basal level of ALAS-1. Two possibilities exist to explain how heme can affect the level of ALAS-1 mRNA in FRL 4.1 cells. Firstly heme could act to decrease the transcription of the ALAS-1 gene, or alternatively but not mutually exclusively, heme could alter the half life of the ALAS-1 mRNA. These possibilities were investigated.

3.2.2 The Effect of Heme on the Transcription rate of the ALAS-1 Gene

In order to determine how heme was repressing ALAS-1 mRNA levels, the relative rate of transcription of the ALAS-1 gene was assayed by nuclear run-on experiments. In an *in vitro* nuclear run-on assay, nuclei are isolated from cells under conditions that cause RNA polymerase to stall. The nascent RNA molecules are then extended *in vitro* in the presence of [α-^{32}P] UTP, the subsequent labelled RNA isolated and the amount of a particular mRNA quantitated by hybridisation to an excess of its corresponding cDNA probe immobilised to a filter. Under these conditions no new initiation of transcription occurs. Therefore the relative number of RNA polymerases transcribing the gene and thus the rate of initiation of transcription can be determined by quantitating the number of counts bound to the filter.

Nuclei were prepared from FRL 4.1 cells according to the method of Ausubel *et al.* (1989) as described in detail in Section 2.4.1. To ascertain if the nuclei prepared in this manner were capable of extending nascent RNA molecules *in vitro*, a time course of [α-^{32}P] UTP incorporation was performed. Since nuclei were isolated from both heme and non-heme treated cells, to assay for differences in the level of transcription of the

ALAS-1 gene, it was important to compare the overall rate of incorporation of [α-^{32}P] UTP in nuclei from both types of nuclei (fig 3.2). As can be seen the incorporation of [α-^{32}P] UTP was very similar in nuclei isolated from both heme and untreated cells and therefore it appears that heme treatment does not affect the overall transcription rate of the cell. Importantly this incorporation occurs linearly for at least 30 minutes, suggesting there is no significant degradation of the RNA molecules produced in this time period and therefore the nuclei were able to be used in the nuclear run-on assay. In subsequent experiments, nuclei were incubated for 25 minutes to ensure good incorporation while minimising potential RNA degradation. In our laboratory the conditions required to obtain quantitative hybridisation of the [α-^{32}P] labelled RNA to its corresponding filter bound DNA probe have been well established (Srivastava *et al.,* 1988). These conditions consist of using between 2-5x 10^6 cpm of TCA precipitable counts of labelled RNA, and 5μg of plasmid DNA to ensure an excess of filter bound complementary DNA.

The relative transcription rate of the ALAS-1 gene was then determined, under the conditions outlined in Section 2.4, using nuclei isolated from FRL 4.1 cells grown under serum-free conditions. To one group of cells 2μM heme was added 3hr prior to nuclei isolation, while the control group received the calcium carbonate vehicle alone for 3hr. Under these conditions a maximal effect on the steady state level of ALAS-1 mRNA is observed (fig 3.1). Two independent experiments were performed and the results quantitated by phosphor-imaging. As can be seen in (fig 3.3) the transcription rate of the ALAS-1 gene is very low, but is detectable. To measure any non-specific hybridisation caused by plasmid sequences pBluescript was included on the filter (all the cDNA probes were subcloned into pBluescript). The signal generated from the pBluescript control was then subtracted from the specific probes. Quantitation of the results from two experiments demonstrated that heme treatment did not significantly affect the transcription rate of the ALAS-1 gene, nor that of the control gene GAPDH. Heme treatment however did significantly increase the transcription rate of the heme oxygenase-1 gene, indicating that FRL 4.1 cells were able to respond transcriptionally to heme addition. The ability of heme to increase the transcription rate of the heme

Figure 3.2: **Time Course of [^{32}P] RNA Synthesis in Nuclei Isolated from FRL 4.1 Cells.**

The time course of [^{32}P]-UTP incorporation into RNA was produced from nuclei isolated from FRL 4.1 cells grown in either serum free media or serum free media containing 2μM heme for 3hr. FRL 4.1 cells were maintained in serum free conditions for 30hr prior to the experiment. Aliquots of the reaction were removed in triplicate at 5 minute intervals and the amount of TCA precipitable radioactivity was determined as described in section 2.6.1.

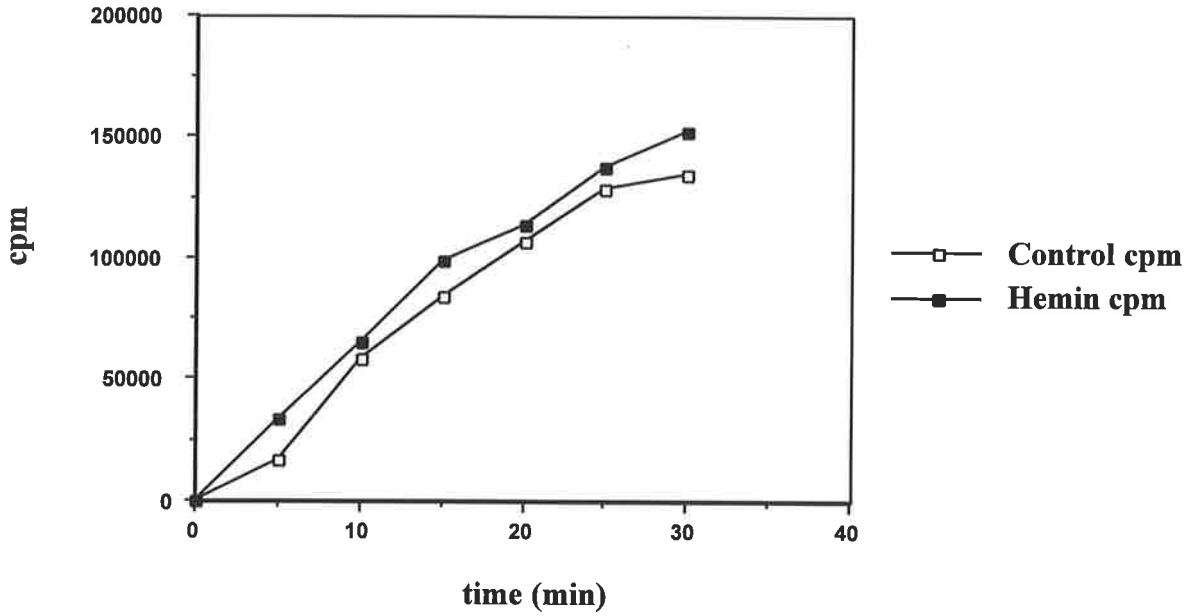
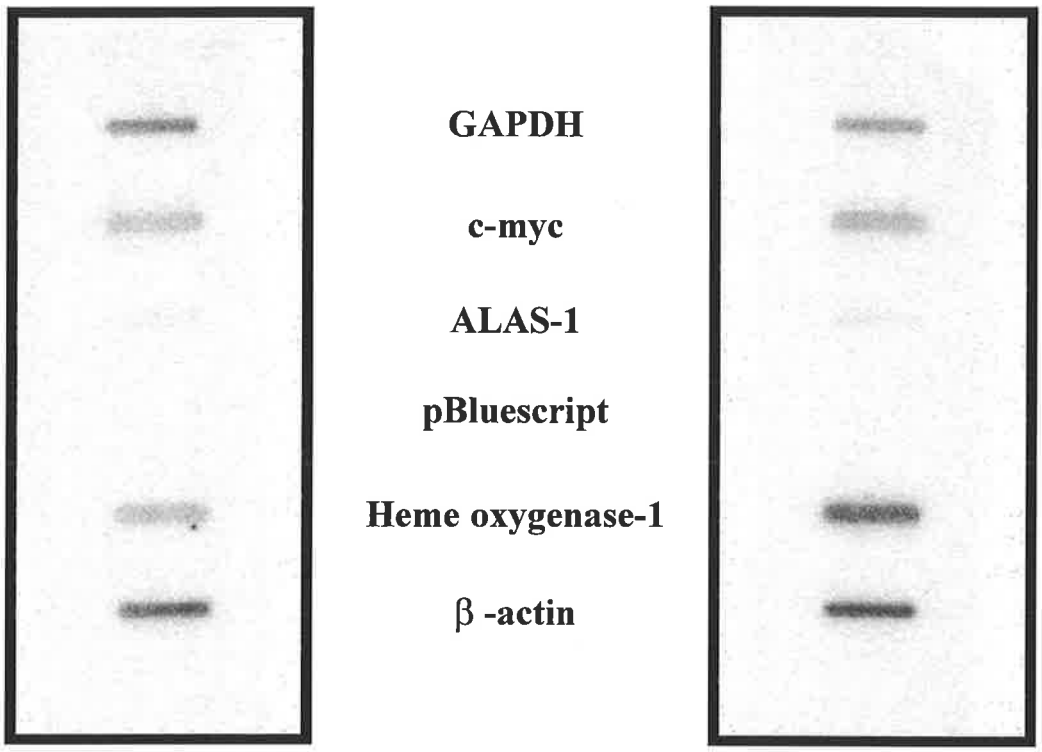

200000
150000
100000
50000
0
cpm
0
10
20
30
40
time (min)
Control cpm
Hemin cpm

Figure 3.3: **The Effect of Heme on the Transcription Rate of the ALAS-1 Gene in FRL 4.1 Cells.**

FRL 4.1 cells were maintained in serum free conditions for 30hr prior to the addition of heme. Nuclei were isolated from cells which had either been grown in serum free media or media containing 2μM heme for 3hr. Nuclei were then allowed to transcribe *in vitro* in the presence of [α-^{32}P]-UTP. [^{32}P] labelled RNA was isolated (section 2.4.4) and hybridized to slot blots containing 5μg of each of the indicated cDNA probes. The vector pBluescript was included as a background control. Filters were exposed to PhosphorImager screens for 1 week.



3 Hrs
no heme

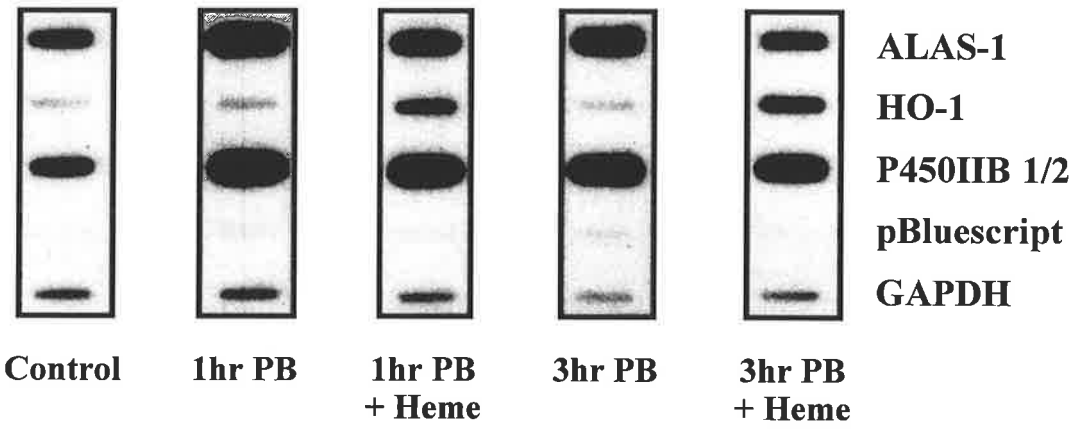
3 Hrs
+ heme (2μM)

oxygenase-1 gene is well documented (Alam *et al.*, 1989). The addition of heme to FRL 4.1 cells also caused a slight increase in the transcription rate of the c-myc and β-actin genes (1.8 and 1.6 fold respectively). However the transcription rates of these two genes were only determined once and therefore need to be repeated, although the increase in c-myc transcription correlates with the increase in c-myc mRNA seen at 4hr (fig 3.1). These results clearly demonstrate that heme does not affect the basal rate of transcription of the ALAS-1 gene in FRL 4.1 cells. This result was surprising in the light of the results of Srivastava *et al.* (1988 and 1990) and Yamamoto *et al.* (1988) where heme had been implicated to primarily affect the transcription rate of the ALAS-1 gene. However the physiological relevance of the results obtained by Srivastava *et al.* (1988 ; 1990) have been questioned by Hamilton *et al.* (1991) on the basis that the amount of heme administered to the rats in their studies may have been excessive.

To further investigate whether heme can affect the transcription rate of the ALAS-1 gene in rat liver, a preliminary study using a much lower dose of heme has been performed. Rats were simultaneously given a single dose of phenobarbital (80mg/kg) intraperitoneally, and heme (2mg/kg) intravenously and nuclei isolated 1 and 3hr after drug induction. This is in contrast to the study of Srivastava *et al.* (1990) in which rats were twice administered heme (40mg/kg) intraperitoneally during the 18 hour time period prior to sacrifice. The transcription rates of various genes were then measured by the *in vitro* nuclear run on procedure (fig 3.4). As can be seen phenobarbital caused a rapid increase in the transcription rates of the genes for ALAS-1 and the major phenobarbital inducible cytochrome P450s CYP2B1 and CYP2B2. This increase in the transcription rate of the ALAS-1 gene was partially inhibited at 1hr and almost completely abolished at 3hrs by heme treatment. The drug mediated induction of CYP2B1 and CYP2B2 gene transcription rates on the other hand were unaffected by heme treatment. Heme levels were raised in the livers of these rats as heme treatment led to a rapid increase in the transcription rate of the heme oxygenase-1 gene. Although it is still unknown if 2mg/kg of heme mimics a physiological relevant increase in heme levels in the liver, this experiment provides evidence that low doses of heme are able to inhibit the drug mediated induction of ALAS-1 transcription in the rat liver.

Figure 3.4: **The Effect of Phenobarbital and Heme on the Transcription Rate of the ALAS-1 Gene in Rat Liver.**

Male wistar rats (200g) were given a single dose of phenobarbital (80mg/kg) intraperitoneally and heme (2mg/kg) intravenously. The rats were then sacrificed at 1hr and 3hr after the administration of the drugs and nuclei prepared from the liver as described in Srivastava *et al.* (1988). Control rats did not receive any drugs. Nuclei were then allowed to transcribe *in vitro* in the presence of [α-^{32}P]-UTP. [^{32}P] labelled RNA was isolated and hybridized to slot blots containing 5µg of each of the indicated cDNA probes. The vector pBluescript was included as a background control.


ALAS-1
HO-1
P450IIB 1/2
pBluescript
GAPDH
Control
1hr PB
1hr PB + Heme
3hr PB
3hr PB + Heme

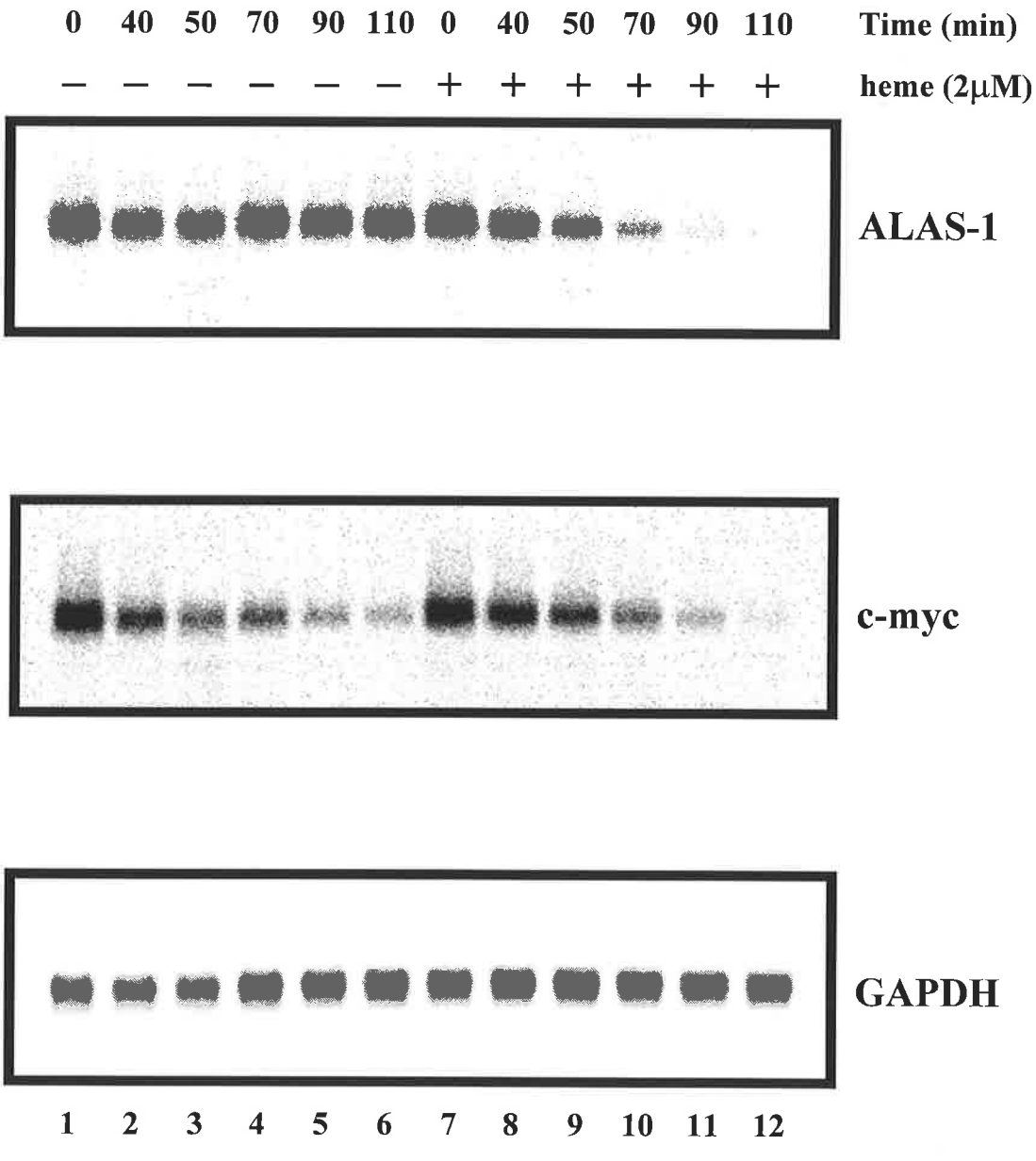
Although there was conflicting data on how heme regulates ALAS-1 mRNA levels between rats and transformed cell lines, it was decided to further characterise the mechanism by which heme causes the destabilisation of the ALAS-1 mRNA in FRL 4.1 cells. As mentioned in section 1.6.6.i the half-life of the ALAS-1 mRNA in rat liver is extremely short and we assumed that the *cis*-elements and the *trans*-acting factors that bind to them and which are responsible for the rapid turnover of the ALAS-1 mRNA in FRL 4.1 cells would also be responsible for the extremely short half-life of the mRNA in rat liver. Since the regulation of mRNA stability has become increasingly apparent as an important process that determines the level of expression of a gene (Sachs, 1993), an understanding of the regulation of ALAS-1 mRNA turnover would contribute to the overall understanding of the control of heme synthesis in rat liver. Furthermore the mechanisms by which mammalian mRNAs turnover have only begun to be characterised, thus the decay of ALAS-1 mRNA provided an interesting model for investigating mRNA turnover in mammalian cells.

3.2.3 The Effect of Heme on ALAS-1 mRNA Half-life.

Since heme did not have an effect on the transcription rate of the ALAS-1 gene in FRL 4.1 cells, it was of interest to determine whether heme could decrease the half-life of the ALAS-1 mRNA. Drew and Ades (1989) and more recently Hamilton *et al.* (1991) have shown that heme reduces the half-life of phenobarbital-induced ALAS-1 mRNA in primarily hepatocytes cultured from 17-day chick embryos. However, Yamamoto *et al.* (1988) could find no evidence that heme reduced the half-life of the ALAS-1 mRNA in drug induced rat livers thus raising the possibility that the two species regulate ALAS-1 mRNA levels by different mechanisms. To determine if heme has an effect on the half-life of ALAS-1 mRNA in rat hepatoma cells, FRL 4.1 cells were treated with actinomycin D to block further RNA synthesis, and the level of ALAS-1 mRNA determined in the presence or absence of added heme. Fig 3.5 shows a typical time course of such an experiment. Treatment of cells with 2μM heme results in a rapid disappearance of ALAS-1 mRNA, becoming virtually undetectable by 90

Figure 3.5: **The Effect of Heme on the Decay of the ALAS-1 mRNA.**

FRL 4.1 cells were maintained in serum-free conditions 30hr prior to the media being changed to serum-free media containing 10μg/ml actinomycin D (lanes 1 to 6) or 10μg/ml actinomycin D plus 2μM heme (lanes 7 to 12). Total RNA was then isolated at the indicated times after the addition of actinomycin D and 20μg of total RNA was subjected to Northern blot analysis. The filter was then sequentially probed for ALAS-1, c-myc and GAPDH mRNAs.


0 40 50 70 90 110 0 40 50 70 90 110
Time (min)
− − − − − − + + + + + +
heme (2μM)
ALAS-1
c-myc
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12

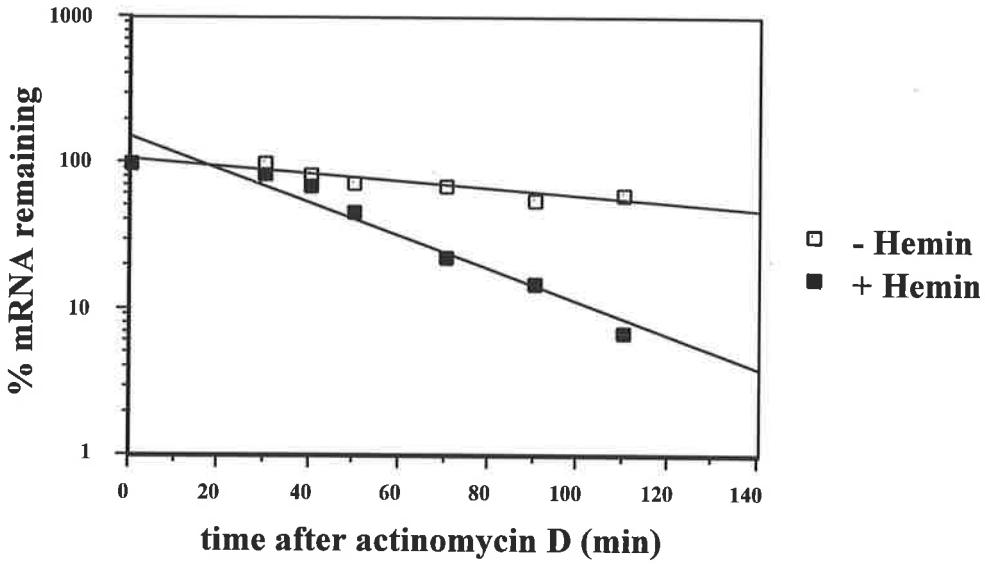
minutes, while substantial amounts of ALAS-1 mRNA remain in the cell treated with actinomycin D alone. To determine the specificity of this heme-mediated response the mRNA for c-myc was also examined. As can be seen in fig 3.5 the decay rate of the c-myc mRNA was unaffected by heme treatment, implying that heme is acting specifically to destabilise the ALAS-1 mRNA. The short half-life of the c-myc mRNA is consistent with findings in other cells (Laird-Offringa, 1992) and demonstrates that the actinomycin D was functioning in both heme and untreated cells. Again GAPDH mRNA levels did not vary significantly and were used to normalise loading variations. In order to estimate the half-life of the ALAS-1 mRNA, relative levels were quantitated using the ImageQuant software package. Data from three independent experiments was then used to create a plot of % mRNA remaining versus time and linear regression analysis was used to estimate the half-life of ALAS-1 mRNA (fig 3.6a). The half-life of the ALAS-1 mRNA in untreated cells was found to be 131 minutes while heme treatment reduced this about 3-fold to 42 minutes. This half-life is considerably longer than that estimated by Yamamoto *et al.* (1988) in rat liver, but this may be a consequence of using a transformed cell line. Similar half-life estimates for the c-myc mRNA were performed (fig 3.6b). In FRL 4.1 cells c-myc mRNA had a half-life of 43 minutes irrespective of heme treatment, confirming that heme is acting specifically on ALAS-1 mRNA. Interestingly in FRL 4.1 cells the half-life of ALAS-1 mRNA in the presence of heme is similar to that of c-myc mRNA, a mRNA whose stability is normally tightly regulated.

During the last several years some progress has been made in determining how mRNAs are degraded. Firstly, it is clear that unstable mRNAs contain discrete *cis*-acting sequences that target the mRNA for selective degradation (Sachs, 1993). Secondly, studies in yeast using the translational inhibitor cycloheximide have established that translation is important in many cases for mRNA degradation (Herrick *et al.*, 1990). More recently a number of groups have shown that mRNAs containing the ARE (adenosine/uridine rich) instability element from GM-CSF, require translation of the mRNA for the ARE to be functional (Aharon and Schneider, 1993 ; Savant-Bhonsale and Cleveland, 1992). In the case of the chicken ALAS-1 mRNA Hamilton *et al.* (1991)

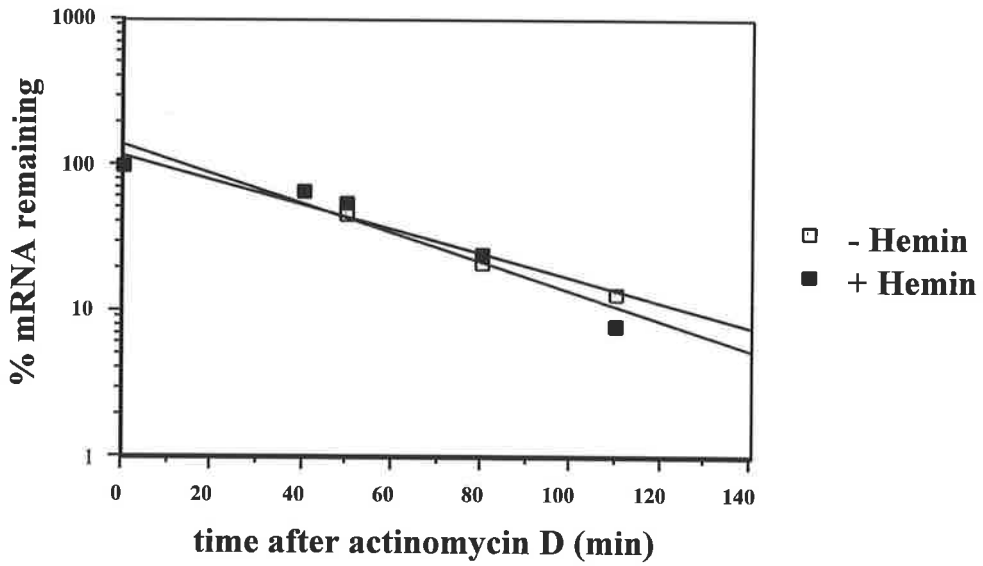
Figure 3.6: **Half-Life Determinations of ALAS-1 and c-myc mRNA in FRL 4.1 cells Treated with Heme.**

Data from 3 independent experiments, as described in fig 3.5, was used to plot the % mRNA remaining versus time. Lines represent the best fit as determined by regression analysis of the data ($R^2 > .87$), and were used to estimate the half-life of ALAS-1 and c-myc mRNA under each treatment regiment.

A: ALAS-1



B: c-myc



have demonstrated that cycloheximide blocks the heme-mediated decrease in the half-life of the ALAS-1 mRNA. Therefore it was of interest to determine if the rat ALAS-1 mRNA also required translation for the heme-mediated instability.

3.2.4 The Effect of Cycloheximide on Rat ALAS-1 mRNA Stability.

Initially FRL 4.1 cells were treated with various doses of cycloheximide and total protein synthesis measured by [C^{14}]-leucine incorporation (Hamilton *et al.*, 1992) at 60 or 90 minutes post drug treatment. Table 1 shows the results of such an experiment. Maximal inhibition of protein synthesis occurred at 2μM and incubating the cells for 90 minutes only gave a very slight increase in inhibition over 60 minute incubations. It should be noted at this point that inhibition of protein synthesis as measured by [C^{14}]-leucine incorporation was not 100% and so a small amount of translation is still occurring.

To determine the effect of translation on the heme-mediated instability of the rat ALAS-1 mRNA, cells were initially pretreated with 2μM cycloheximide for 60 minutes before the addition of fresh media containing 2μM cycloheximide and either actinomycin D, or actinomycin D plus 2μM heme. RNA was then isolated from cells at various times and the level of ALAS-1, c-myc and GAPDH mRNAs determined via Northern blot analysis (fig 3.7). Blocking protein synthesis in FRL 4.1 cells with cycloheximide, did not cause a stabilisation of the ALAS-1 mRNA. ALAS-1 mRNA levels from cycloheximide treated cells closely matched the levels seen in the control cells (lanes 1 to 7 versus 8 to 14). ALAS-1 mRNA levels from two experiments were quantitated, normalised to GAPDH levels, and plotted as % mRNA remaining versus time (fig 3.8a). As can be seen the decay rate of ALAS-1 mRNA in both control and heme treated FRL 4.1 cells was unaffected by blocking protein synthesis with cycloheximide. As a control for cycloheximide mediated inhibition of protein synthesis, the stability of the mRNA for c-myc was examined. The filters were stripped of ALAS-1 probe and reprobed for c-myc mRNA (fig 3.7). The instability of the c-myc mRNA is known to be dependent on translation (Wisdom and Lee, 1991 ; Brewer and Ross, 1989). In agreement with this when FRL 4.1 cells were treated with cycloheximide the

Table 3.1

Treatment	(%) inhibition of [^{14}C]-leu incorporation	
Cycloheximide (μM)	60 min	90 min
1μM	78	87
2μM	82	85
3μM	82	85

Table 3.1: The Effect of Cycloheximide on Protein Synthesis in FRL 4.1 Cells

FRL 4.1 cells were maintained in serum-free conditions for 30 hr prior to the medium being changed to either fresh medium containing 0.25μCi [^{14}C]-leu or fresh medium containing 0.25μCi [^{14}C]-leu plus the indicated concentrations of cycloheximide. Cells were harvested at the indicated times and the amount of [^{14}C]-leu incorporated into TCA precipitable protein determined as described by Hamilton *et al.* (1992)

Figure 3.7: **The Effect of Cycloheximide on the Decay of ALAS-1 mRNA.**

FRL 4.1 cells were maintained in serum-free conditions for 30hr prior to the media being changed to either fresh serum free media (lanes 1 to 7) or serum free media containing 2μM cycloheximide (lanes 8 to 14). Cells were then left for 60 min. prior to the media being changed to fresh media containing 10μg/ml actinomycin D (lanes 1 to 4), 10μg/ml actinomycin D plus 2μM heme (lanes 5 to 7), 2μM cycloheximide plus 10μg/ml actinomycin D (lanes 8 to 11) and 2μM cycloheximide, 10μg/ml actinomycin D and 2μM heme (lanes 12 to 14). Total RNA was isolated at the indicated times after the addition of actinomycin D. 20μg of total RNA was then subjected to Northern analysis and the filter sequentially probed for ALAS-1, c-myc and GAPDH mRNAs.

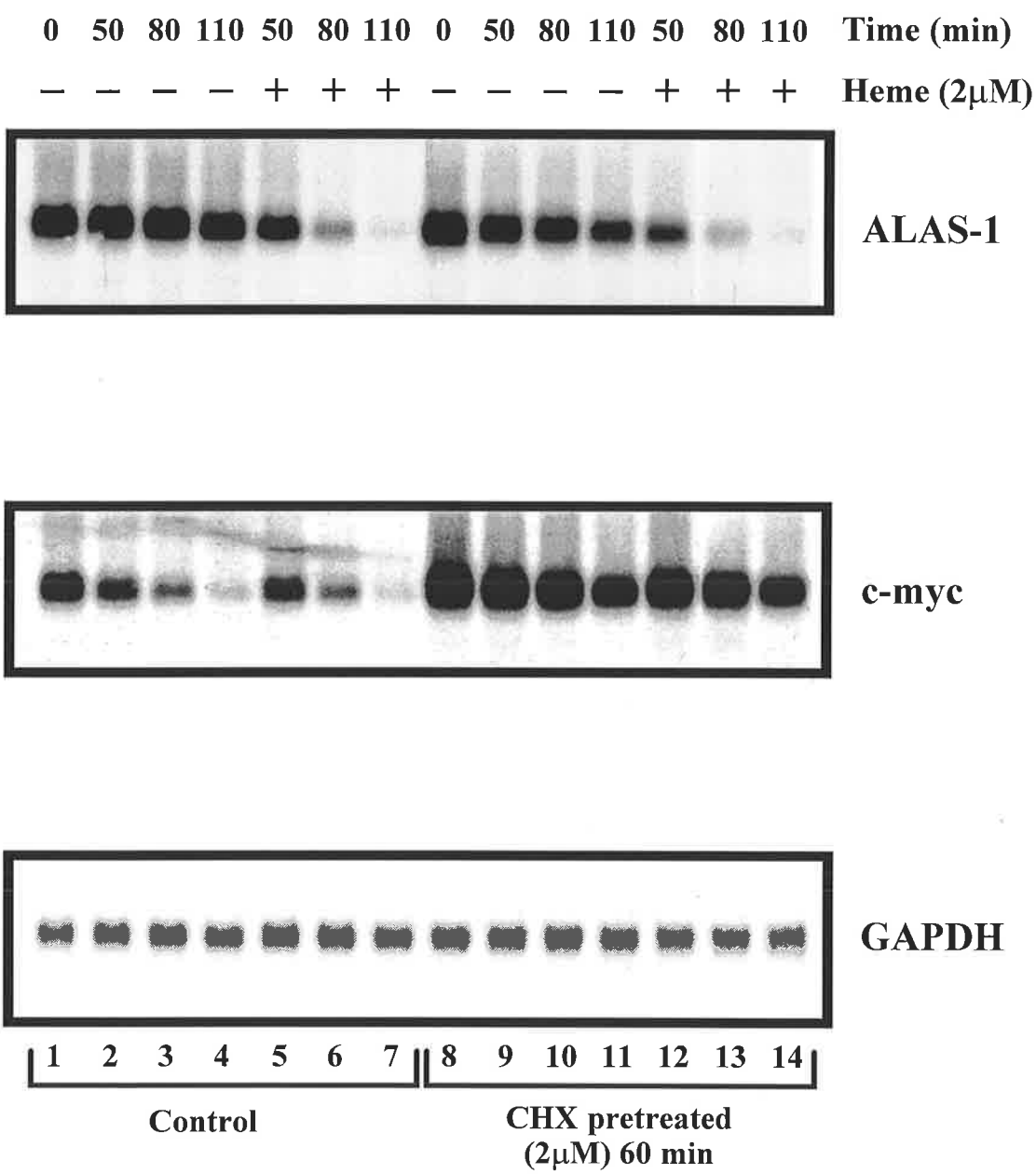
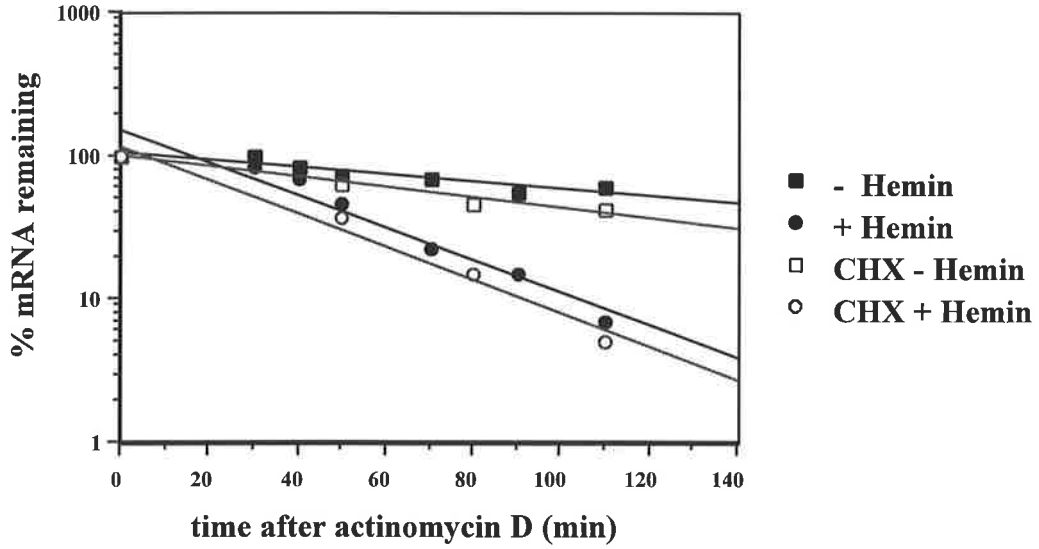

0 50 80 110 50 80 110 0 50 80 110 50 80 110 Time (min)
− − − − + + + − − − − + + + Heme (2μM)
ALAS-1
c-myc
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Control
CHX pretreated
(2μM) 60 min

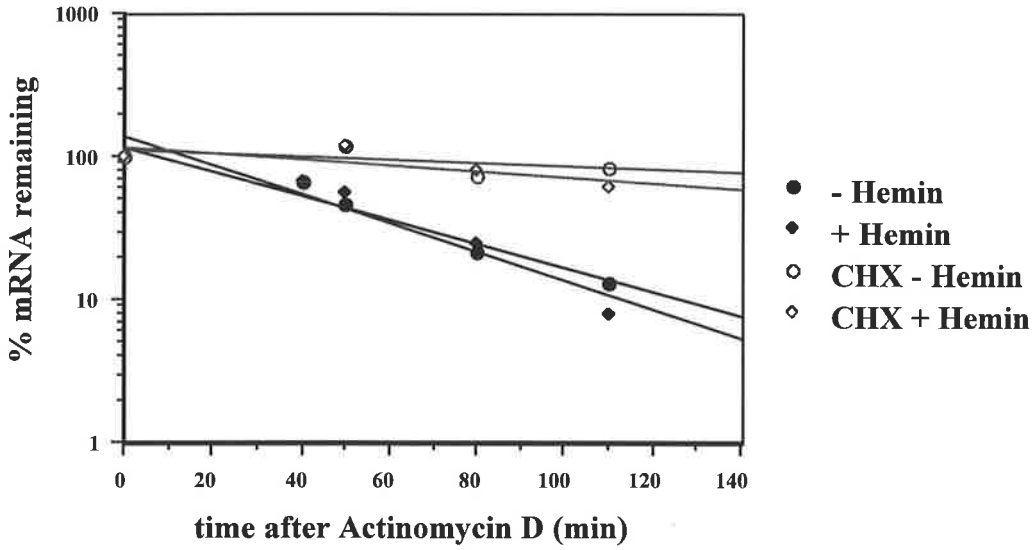
Figure 3.8: **Half-Life Determinations of ALAS-1 and c-myc mRNA in FRL 4.1 cells Treated with Cycloheximide and Heme.**

Data from 2 independent experiments, as described in fig 3.7 was used to plot the % mRNA remaining versus time. Lines represent the best fit as determined by regression analysis of the data ($R^2 > .87$), and were used to estimate the half-life of ALAS-1 and c-myc mRNA under each treatment regiment.

A: ALAS-1



B: c-myc



c-myc mRNA was stabilised (lanes 1 to 7 versus 8 to 14) in both control and heme treated cells. The level of GAPDH mRNA was unaffected by cycloheximide treatment.

3.2.5 Is De-adenylation of the Poly(A) Tail the First Step in the Heme-mediated Decay of the ALAS-1 mRNA?

De-adenylation, or shortening of the poly(A) tail has been implicated as an important first step in the decay of a number of short-lived mRNAs (Reviewed Decker and Parker, 1994). Moreover, the same sequences that have been previously implicated to promote the rapid decay of these mRNAs are also involved in the rapid de-adenylation of the mRNAs. These elements include the AU-rich (ARE) of c-fos (Shyu *et al.*, 1991), c-myc (Laird-Offringa *et al.*, (1990), the c-fos coding region determinate (CDR) (Shyu *et al.,* 1991) and the instability regions of the yeast mating factor A2 (MFA2) 3'UTR (Muhlrad and Parker, 1992). Furthermore, studies by (Decker and Parker, 1993 ; Muhlrad *et al.*, 1995) have implicated de-adenylation as the first step in the decay of four yeast mRNAs. Interestingly, their data indicated that both stable and unstable mRNA species underwent de-adenylation to an oligonucleotide tail prior to the decay of the body of the mRNA. These results suggest that de-adenylation may therefore be part of a common pathway used in the decay of eukaryotic mRNAs.

Therefore it was of interest to determine if heme could stimulate the de-adenylation rate of the ALAS-1 mRNA poly(A) tail and whether this de-adenylation could be correlated with a decrease in the level of the ALAS-1 mRNA. To facilitate this measurement the ALAS-1 mRNA was subjected to oligonucleotide directed RNase H cleavage, prior to Northern blot analysis to generate smaller fragments which could be resolved easier by gel electrophoresis. This procedure is based upon the method used by Shyu *et al.* (1991) to determine the length of the c-fos poly(A) tail following serum stimulation of fibroblasts. Briefly, an oligonucleotide (#5393) complementary to a specific region (+1742 to + 1762), located 364 nucleotides from the poly(A) tail addition site, was hybridised to the ALAS-1 mRNA. The RNA portion of the RNA:DNA duplex was then cleaved by RNase H to form two products, a 1741

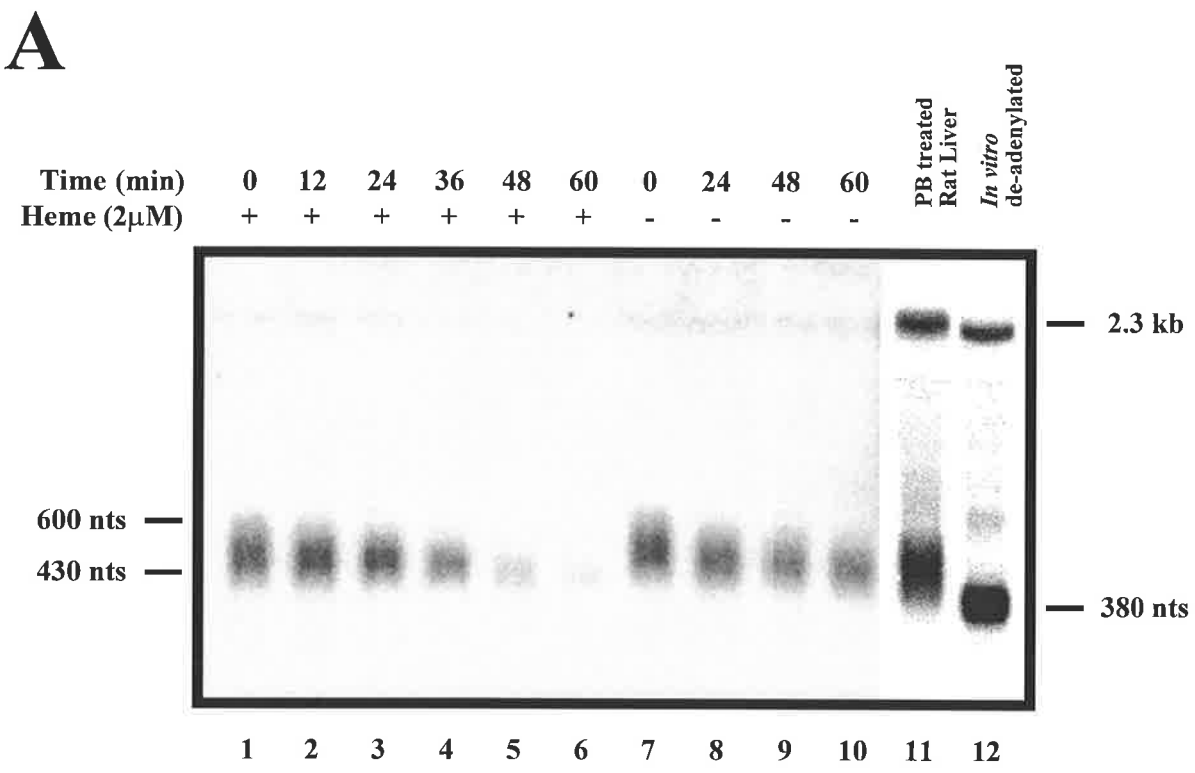
nucleotide fragment which contains the 5'-end of the mRNA and a smaller fragment which corresponds to the 3'-terminal 364 nucleotides of the ALAS-1 mRNA plus the poly(A) tail. The products of the cleavage reaction were then subjected to Northern blot analysis. In this way fragments containing the 3'-end of the mRNA are generated which differ in size only by the length of their poly(A) tail. The relative length of these fragments and therefore the length of their poly(A) tail can be determined by comparing their sizes with a fragment which has been fully de-adenylated *in vitro*. Fully de-adenylated fragments are generated *in vitro* by adding oligo d(T) as a second primer to the RNA prior to the RNase H cleavage step.

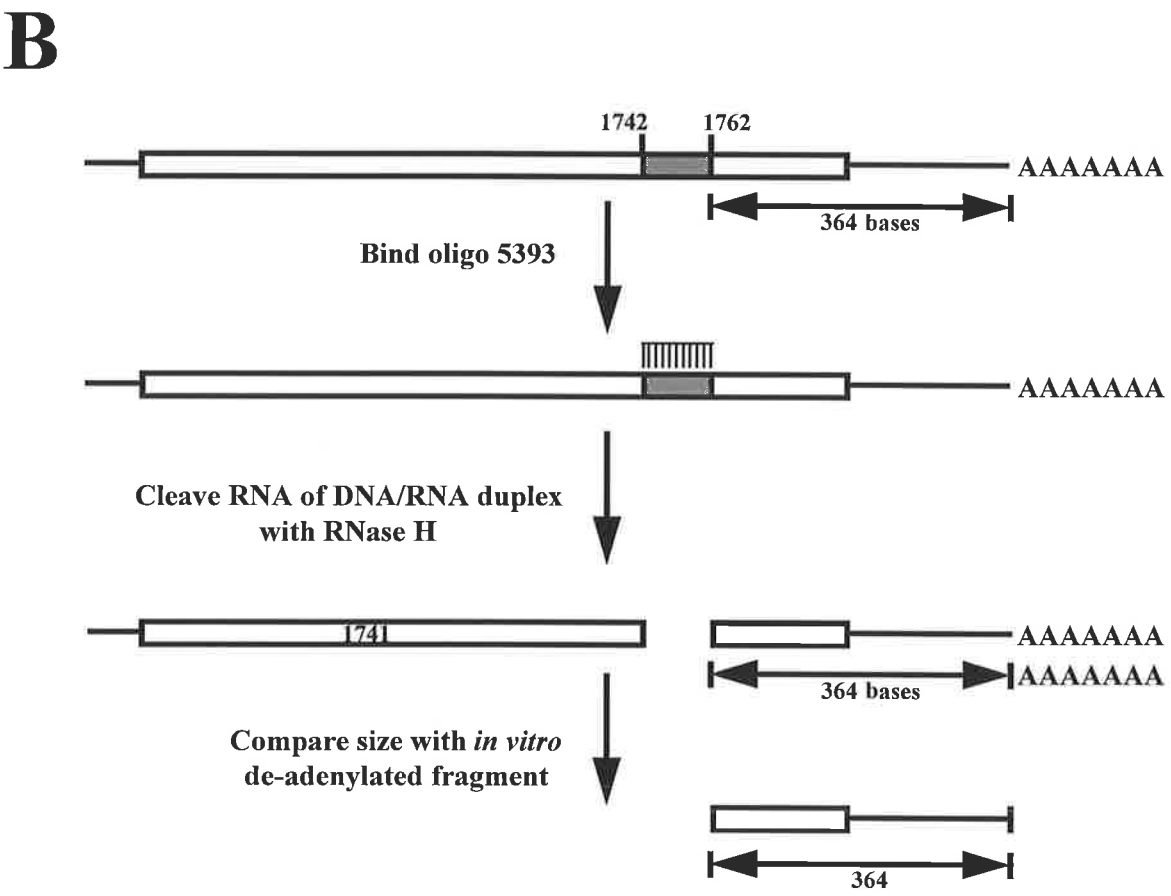
The relative length of the ALAS-1 poly(A) tail was determined by Northern blot analysis in FRL 4.1 cells treated with actinomycin D or actinomycin D plus heme (fig 3.9). Due to the very low level of ALAS-1 mRNA and the small size of the fragment generated, 131µg of total RNA was used in the Northern blot (as up to 60µg of RNA gave an almost undetectable signal). As a positive control 50µg of phenobarbital induced total rat liver RNA was used in one of the cleavage reactions; the same RNA was also used to generate the *in vitro* de-adenylated product (lanes 11 and 12). The oligonucleotide-directed RNase H cleavage procedure detected a single product in all the lanes containing FRL 4.1 RNA when using a 3'-specific probe. A larger product of approximately 2.3kb in size was also observed in the tracks containing the PB-induced rat liver RNA (lanes 11 and 12). The size of this band corresponds to the size of the uncleaved ALAS-1 mRNA and is assumed to be a result of inefficient RNase H cleavage. The size of the fragments generated were estimated by the comparison of the electrophoretic mobility of the 3'-end fragment with the migration of RNA molecular weight markers (section 2.3.3). The size of the *in vitro* de-adenylated 3'-species was estimated by this method to be approximately 380 nucleotides in length which is in good agreement with the known fragment length of 364 nucleotides implying that the products observed were the result of the specific oligonucleotide-directed cleavage of the ALAS-1 mRNA. A diffuse band is observed at the zero minute time point in both control and heme treated cells (lane 7 and 1). The top edge of this band was estimated to correspond to an RNA of size 600 nucleotides and therefore the poly(A) tail was

Figure 3.9 **The Effect of Heme on the Length of the ALAS-1 Poly(A) Tail.**

A): Total RNA was isolated at the indicated times after the the addition of actinomycin D (lanes 7 to 10) or actinomycin D plus (2μM) heme (lanes 1 to 6) and samples (131μg) were subjected to oligonucleotide-directed RNase H cleavage and Northern blotting. As a positive control 50μg of phenobarbital-induced rat liver RNA was also subjected to the RNase H cleavage procedure (lane 11). A de-adenylated 3'-fragment of ALAS-1 was prepared *in vitro* by annealing a sample (50μg) of PB-induced rat liver RNA with two oligonucleotides; the ALAS-1 specific oligo 5393 and oligo $d(T)_{12}$, prior to RNase H digestion. The size of the fragments produced were estimated by comparing their migration with the migration of RNA markers run on the same gel.

B): A schematic of the oligonucleotide directed RNase H cleavage procedure showing the sizes of the expected products.


A
Time (min) 0 12 24 36 48 60 0 24 48 60
Heme (2μM) + + + + + + - - - -
PB treated Rat Liver
In vitro de-adenylated
2.3 kb
600 nts
430 nts
380 nts
1 2 3 4 5 6 7 8 9 10 11 12


B
1742
1762
AAAAAAA
364 bases
Bind oligo 5393
AAAAAAA
Cleave RNA of DNA/RNA duplex with RNase H
1741
AAAAAAA
AAAAAAA
364 bases
Compare size with in vitro de-adenylated fragment
364

estimated to be approximately 220 nucleotides in length. The bottom edge of this band runs just above the top edge of the de-adenylated product (lane 12) which corresponded to a RNA of size 430 nucleotides and therefore the poly(A) tail is estimated to consist of about 50 adenosine residues. Since the ALAS-1 mRNA is constitutively expressed, until actinomycin D treatment, the heterogenous size of the poly(A) tail from 50-220 nucleotides most likely represents mRNAs which have been transcribed and transported to the cytoplasm at different times. It is well documented that the poly(A) tail is progressively shortened in the cytoplasm (Sheiness and Darnell, 1973). Over time the top edge of this band contracts downwards in both heme treated (lanes 1 to 6) and control cells (lanes 7 to 10), indicating that the poly(A) tail is being progressively shortened. As expected heme treatment also markedly reduced the amount of product detected (lanes 1 to 6) compared with the control cells, indicating that the decay of the ALAS-1 mRNA was occurring at the same time as de-adenylation. To ascertain if this decrease in the signal was also true for the larger 5'-species produced from the cleavage reaction, the filter was stripped and re-probed with the a fragment that can hybridise to both cleavage products. The level of the 5'-fragment showed a very similar pattern of decay to that observed for the 3'-fragment , with heme causing a more rapid decrease in the amount of the 5'-species than seen in the control (data not shown). However, there was a substantial disruption of the signal by the 18S ribosomal RNA (18S rRNA) which co-migrates with the 1741 base 5'-fragment in the 1.3% agarose gel. This interference was most likely due to the large amount (131µg) of RNA analysed on the Northern blot. To establish conclusively the effect of heme on the abundance of the 5'-fragment and therefore the time at which heme alters the level of this species in relation to the de-adenylation state of the mRNA, smaller amounts of RNA need to be run on a separate gel to allow this species to be detected properly.

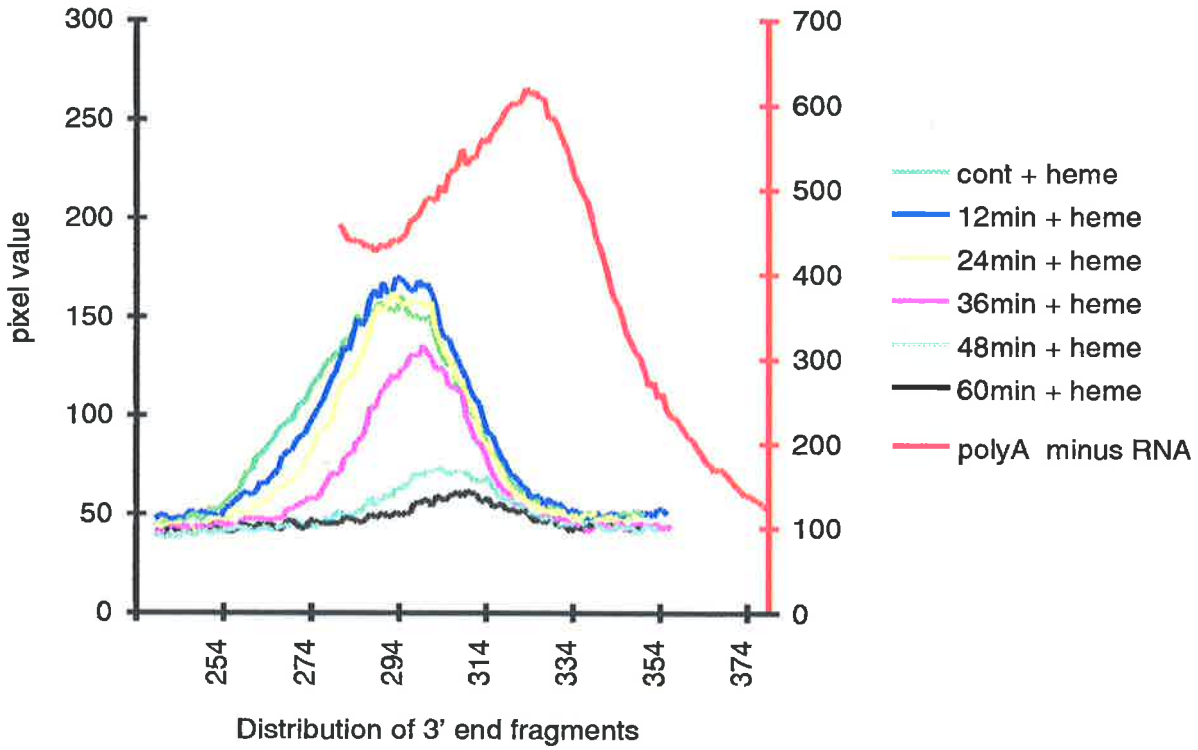
To investigate whether heme caused an increase in the de-adenylation rate of the ALAS-1 mRNA poly(A) tail, the distribution of the 3'-fragments was compared between heme and control cells. Individual lanes from (fig 3.9) were scanned with a PhosphorImager and the pixel value, which corresponds to the relative amount of product, for each coordinate on the y-axis was plotted for the various time points (fig

Figure 3.10: **The Effect of Heme on the Rate of ALAS-1 Poly(A) Tail Shortening in FRL 4.1 Cells.**

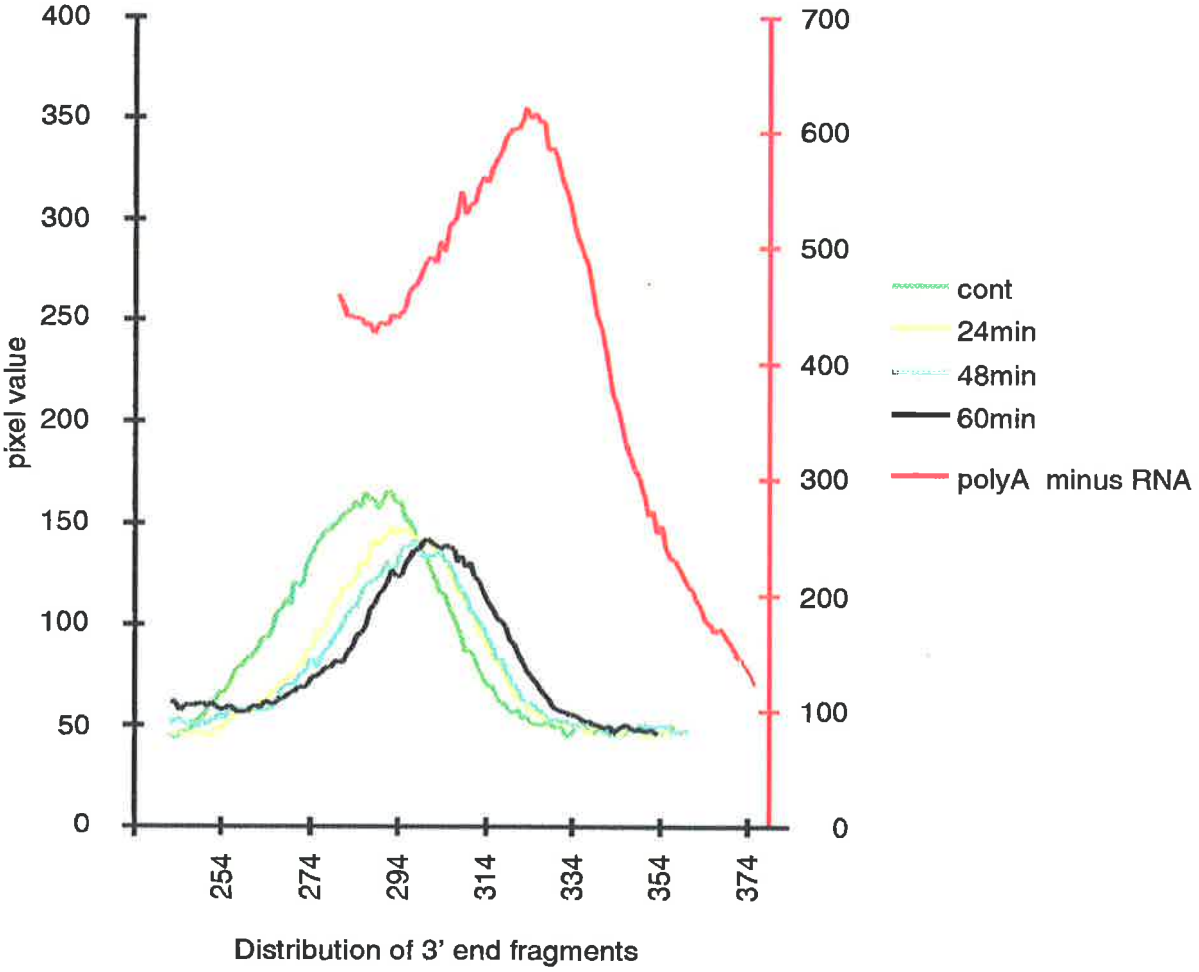
A and B): The distribution profile of ALAS-1 3'-fragments was generated by scanning individual lanes from fig 3.9 with a PhosphorImager and plotting the pixel value obtained for each co-ordinate on the y-axis of a line drawn through the band. The different colours denote the distribution profiles for different time points. The top graph represents data obtained from heme treated cells while the bottom graph plots the data obtained from Ca_2C0_3 (vehicle) treated cells.

C): The graph of the disappearance of the body of the 3'-fragment versus time for heme treated and vehicle treated cells.

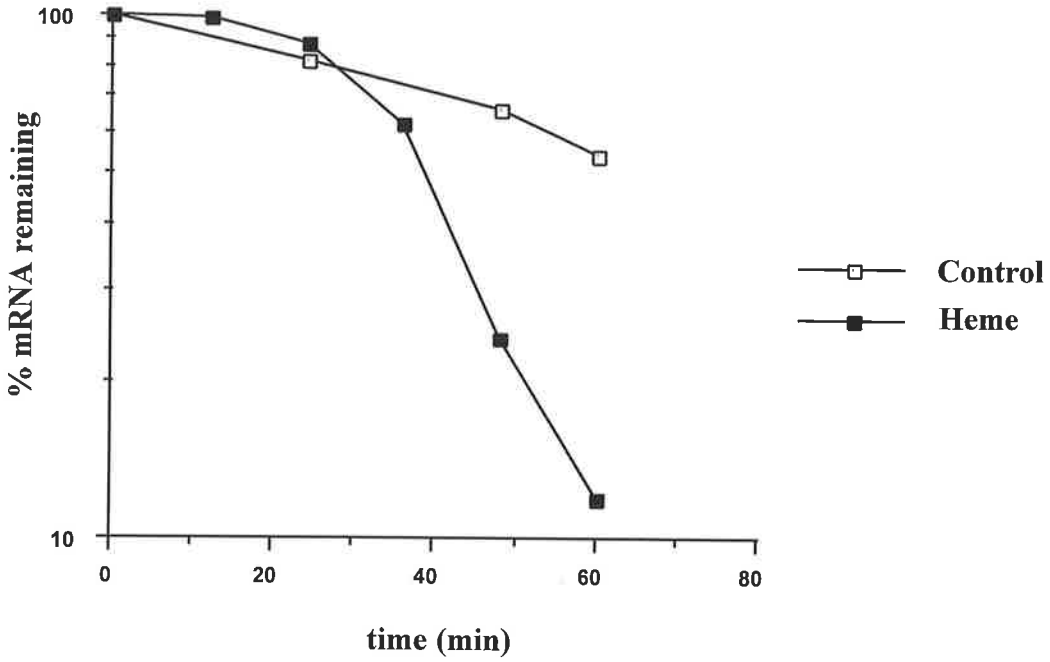
A


300
250
200
150
100
50
0
pixel value
700
600
500
400
300
200
100
0
254
274
294
314
334
354
374
Distribution of 3' end fragments
cont + heme
12min + heme
24min + heme
36min + heme
48min + heme
60min + heme
polyA minus RNA

B


400
350
300
250
200
150
100
50
0
pixel value
700
600
500
400
300
200
100
0
254
274
294
314
334
354
374
Distribution of 3' end fragments
cont
24min
48min
60min
polyA minus RNA

C



3.10a). Secondly, the decay rate of the ALAS-1 mRNA body was compared with the de-adenylation state of the 3'-species. In both heme treated and control cells, the profile of the distribution of 3'-fragments contract and shift towards the right (fig 3.10a) indicating a decrease in the poly(A) tail length in the population of ALAS-1 molecules over time. However, most of the remaining population retains a substantial poly(A) tail, estimated to be greater than 50 nucleotides in length over the 60 minutes assayed. When the profiles obtained for the 48 and 60 minute time points were compared it appeared that heme caused a slightly larger shift in the profile to the right, as measured by the shift in the mean distribution. This difference was not apparent at the earlier 24 minute time point. The significance of this shift is unknown. Although the difference in the profiles at 48 and 60 minutes could be interpreted to mean that heme increased the rate of de-adenylation of the ALAS-1 mRNA, this interpretation needs to be treated cautiously. Firstly, very little product remained in the heme treated samples at 48 and 60 minutes making an accurate quantitation of the distribution of the products extremely difficult due to the substantially decreased signal to noise ratio. It should also be noted that this experiment has only been performed once and needs to be repeated to obtain an accurate picture on the de-adenylation rate of the poly(A) tail under the two conditions. Secondly, if heme stimulated the de-adenylation rate of the ALAS-1 mRNA it would be predicted that the profile of the distribution of the 3'-fragment would show a more rapid constriction as the poly(A) tail is rapidly removed. This does not appear to be the case with a relatively broad range of poly(A) tail length still observed even at 48 and 60 minutes. Therefore the significance of this shift is currently unknown but more experiments should resolve this.

The distribution profile of the 3'-species was then compared to the decay of the body of this fragment, to determine if any correlation existed between the decay of the mRNA body and the shortening of the poly(A) tail. Only a slight drop in the level of the 3'-species is observed at 24 minutes, after which a rapid decline in the level of this species is observed in heme treated cells compared with control cells (fig 3.10b). At 24 minutes the mean of the distribution of the ALAS-1 fragments are very similar implying that there is little difference in the range of poly(A) tail length prior to the onset of the

heme-mediated decay of the ALAS-1 mRNA. This suggests that the heme-mediated decay of the ALAS-1 mRNA occurs simultaneously with the normal cytoplasmic shortening of the poly(A) tail.

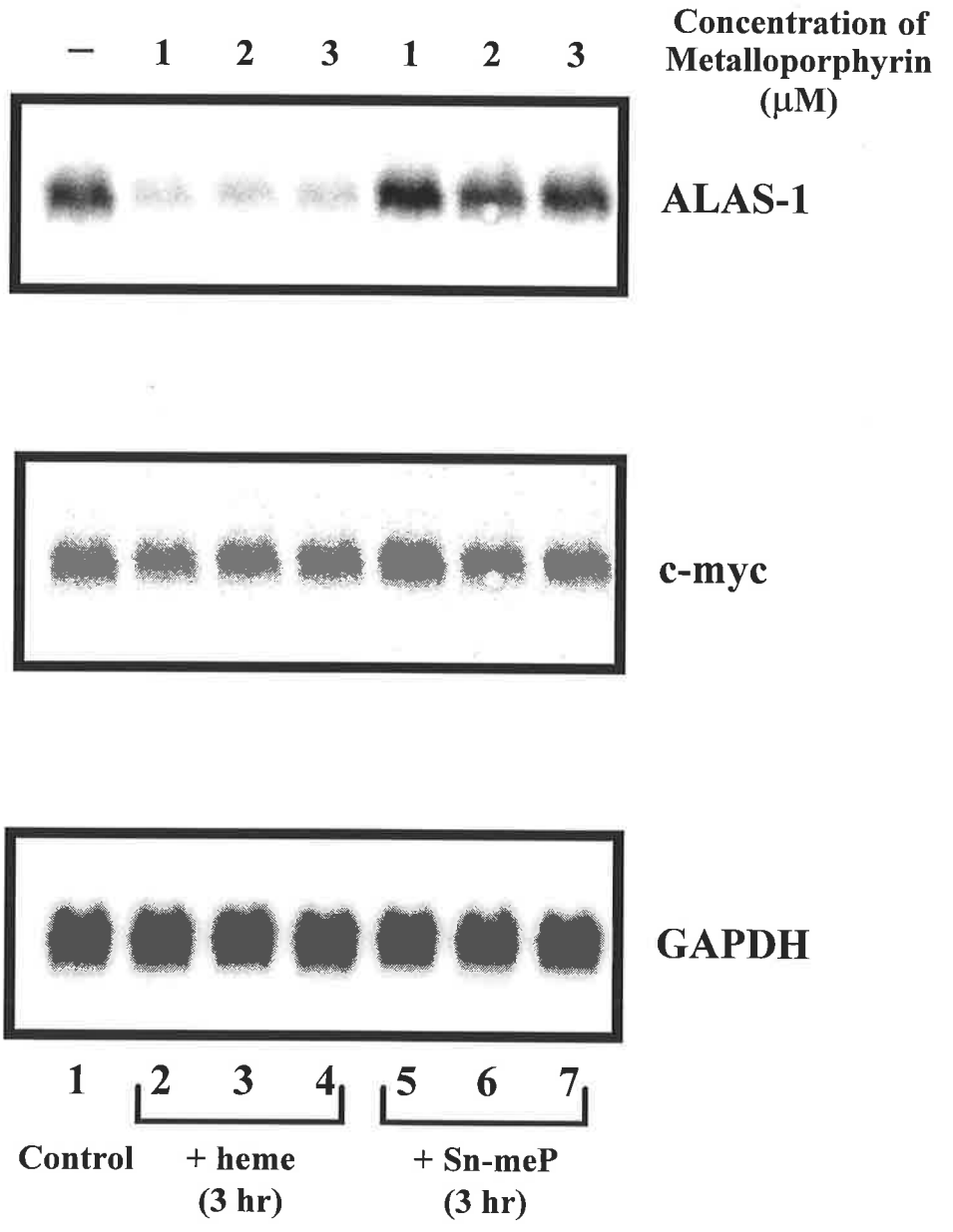
This is in contrast to the results obtained for the decay of a number of unstable mRNAs in mammals including c-myc (Laird-Offringa *et al.*, 1990), c-fos (Shyu, *et al.*, (1991) and gro-α (Stoeckle, 1992), where shortening of the poly(A) tail to a length of < 30 nucleotides precedes the decay of the body of the mRNA. Therefore if de-adenylation was an important first step in the heme-mediated destabilisation of the ALAS-1 mRNA it would be predicted that a rapid shift in the number of 3'-end fragments carrying very small to no poly(A) tails would occur prior to the decay of the mRNA. Since this is not observed and given that heme may only cause a minor increase, if any, in the rate of poly(A) shortening it would appear that the heme-mediated destabilisation of the ALAS-1 mRNA occurs independently of poly(A) shortening. The degradation of the transferrin receptor has also recently been shown to be independent of poly(A) tail shortening and has been shown to involve an endonucleolytic cleavage within the 3'UTR (Binder, *et al.*, 1994). Thus the heme-mediated destabilisation of the ALAS-1 mRNA more closely resembles, at least superficially, the iron-mediated destabilisation of the transferrin receptor, although the mechanism and the *cis*-acting sequences involved in the decay of the body of the ALAS-1 mRNA are still unknown.

3.2.6 Is Heme Specifically Required for ALAS-1 mRNA Instability?

Some metalloporphyrins other than heme, such as tin-protoporphyrin (Sn-PP) and tin-mesoporphyrin (Sn-meP) can also induce heme oxygenase 1 synthesis (Eisenstein *et al.*, 1991 ; Sardana and Kappas, 1987) and therefore it was of interest to determine if these metalloporphyrins could also regulate ALAS-1 mRNA levels. FRL 4.1 cells grown in serum free conditions were treated with 1, 2 or 3μM heme or Sn-meP and RNA isolated 3hr later. Sn-meP was used in preference to Sn-PP as the meso form has a more potent *in vivo* effect on heme oxygenase induction (Drummond *et al.*, 1987). The Northern blot analysis in fig 3.11 clearly shows that Sn-meP did not affect the

Figure 3.11: **The Effect of Heme and Heme Analogues on ALAS-1 mRNA Levels in FRL 4.1 Cells.**

FRL 4.1 cells maintained in serum-free conditions for 30hr were changed to either fresh media containing solvent; lane 1, heme; lanes 2 to 4, or tin-mesoporphyrin (Sn-meP), lanes 5 to 7. Total RNA was isolated 3hr later and 20μg used in a Northern blot. The filter was then probed sequentially for ALAS-1, c-myc and GAPDH mRNA. The three different concentrations of the metalloporphyrins used in this experiment are indicated.


Concentration of Metalloporphyrin (μM)
– 1 2 3 1 2 3
ALAS-1
c-myc
GAPDH
1 2 3 4 5 6 7
Control
+ heme (3 hr)
+ Sn-meP (3 hr)

steady state level of ALAS-1 mRNA, while the same concentrations of heme readily decreased ALAS-1 mRNA levels. The mRNAs for c-myc and GAPDH were similarly unaffected by Sn-meP treatment. This data provides evidence that iron-protoporphyrin IX (heme) is an obligatory factor in the destabilisation of ALAS-1 mRNA.

3.2.7 Is Iron Required for ALAS-1 mRNA Instability?

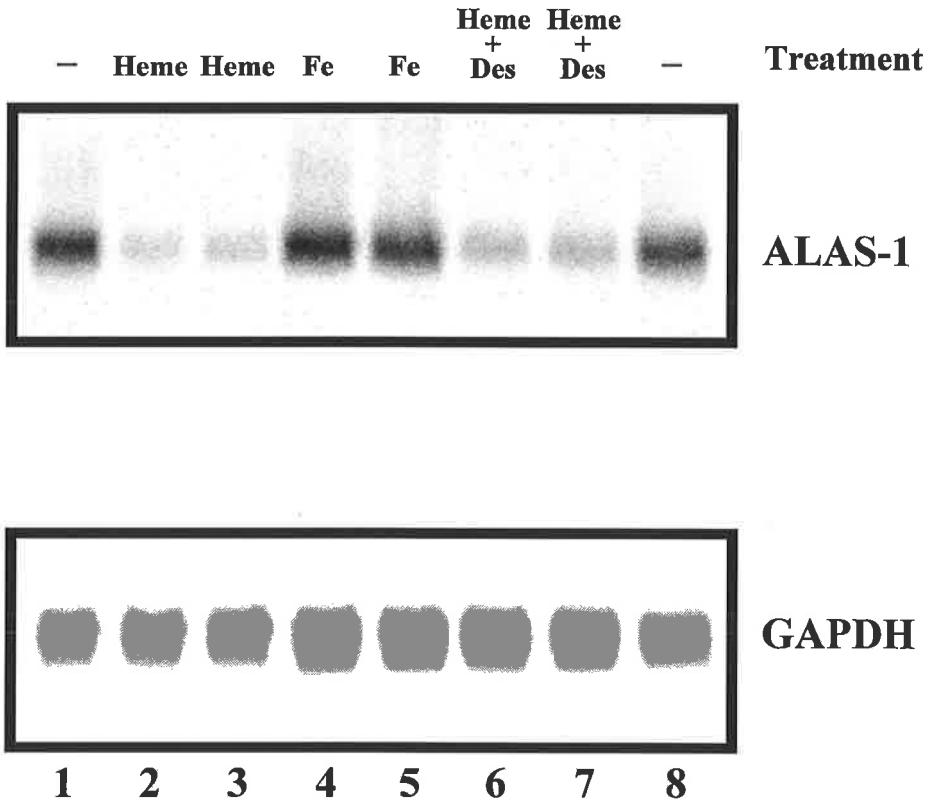
The addition of heme to FRL 4.1 cells caused an increase in the transcription rate of the heme oxygenase gene (fig 3.3) and in the amount of its mRNA (data not shown). Therefore heme entering cells could be rapidly degraded by heme oxygenase to release iron and it may be this build up of iron which affects the decay of the ALAS-1 mRNA. Iron is known to alter the stability of the transferrin receptor mRNA via iron responsive elements (IREs) located in the 3' untranslated region (UTR) of the transferrin receptor mRNA (Mullner and Kuhn, 1988 ; Casey *et al.*, 1989). Examination of the nucleotide sequence of the rat and human ALAS-1 mRNAs did not reveal any sequences with homology to an IRE, and iron would therefore have to destabilise ALAS-1 mRNA by a different mechanism.

To determine if iron was required for ALAS-1 mRNA instability, FRL 4.1 cells grown in serum free conditions, were treated with iron ammonium citrate, heme or heme plus the iron chelator desferrioxamine (fig 3.12). The addition of heme to FRL 4.1 cells as expected caused a rapid drop in the steady state level of ALAS-1 mRNA, which was not prevented by the addition of the iron chelator desferrioxamine (Bridges and Cudkowicz, 1984). Desferrioxamine is taken up by cells and at the concentrations used can suppress the heme mediated increase in ferritin translation, presumably by binding iron released by heme degradation (Eisenstein *et al.*, 1991 ; Rogers and Munro, 1987). The high concentration of iron used in this experiment also caused a small drop (25%) in ALAS-1 mRNA levels but the effect was much less than that observed with heme. Lower concentrations of iron ammonium citrate did not affect ALAS-1 mRNA levels (data not shown). The level of GAPDH mRNA was unchanged by either treatment.

Figure 3.12: **The Effect of Iron on ALAS-1 mRNA Levels in FRL 4.1 Cells.**

FRL 4.1 cells maintained in serum free conditions for 30hr were changed to fresh media containing Ca_2CO_3 vehicle (lanes 1 and 8), 2μM heme (lanes 2 and 3), 200μM iron[1] (lanes 4 and 5) or 100μM desferrioxamine and 2μM heme (lanes 6 and 7). Total RNA was then isolated at 90min (lanes 1, 2, 4 and 6) or 180min. (lanes 3, 5, 7 and 8) and 20μg of each sample subjected to Northern analysis. The filter was then sequentially probed for ALAS-1 and GAPDH mRNA.

1: Iron was added as iron ammonium citrate the concentration of which was calculated to give a final concentration of 200μM iron.


–
Heme
Heme
Fe
Fe
Heme + Des
Heme + Des
–
Treatment
ALAS-1
GAPDH
1
2
3
4
5
6
7
8

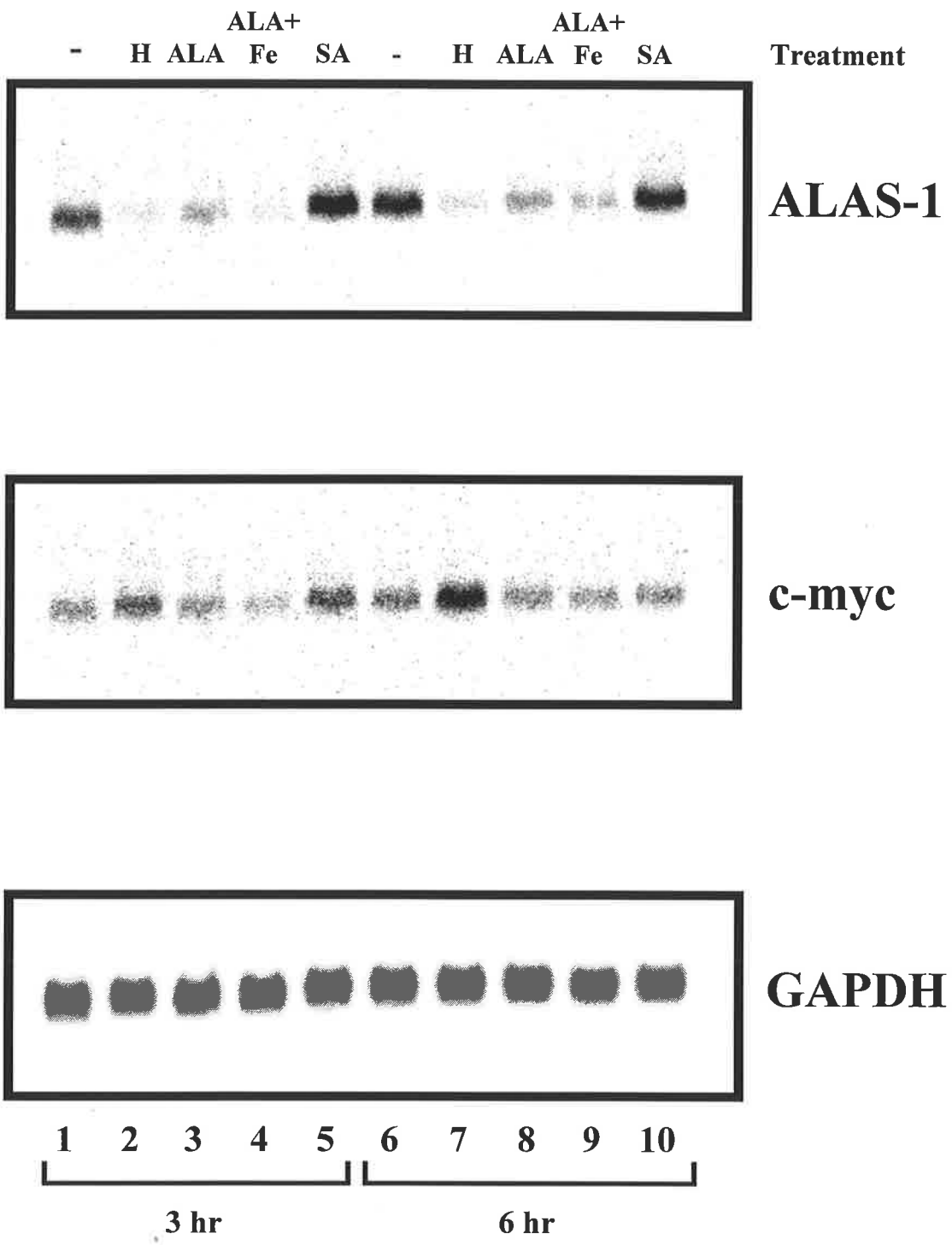
These results suggest that heme and not iron is required for ALAS-1 mRNA destabilisation as much higher amounts of non-heme iron, than the amount of iron provided by 2μM heme, produced only a small effect on ALAS-1 mRNA levels. Secondly the iron chelator desferrioxamine could not prevent heme's ability to decrease ALAS-1 mRNA levels. However the relative ability of heme and iron to affect the translation of ferritin can vary between cells (Mattia *et al.*, 1986) implying that differential transport rates of heme-iron and non-heme iron into cells may influence the ability of these two molecules to act as an efficient intracellular iron source. This does not appear to be the case in this instance as desferrioxamine could not prevent the decrease in ALAS-1 mRNA levels by heme. The small effect of high iron concentrations on ALAS-1 mRNA levels may therefore reflect a general toxic effect due to the generation of free radicals by iron, via the fenton reaction (Sadzadeh *et al.*, 1984).

3.2.8 Does the In Vivo Rate of Heme Synthesis Affect the Stability of the ALAS-1 mRNA?

The experiments performed to date have examined the effect of exogenously added heme on ALAS-1 mRNA levels. To determine whether changes in the *de novo* rate of heme synthesis could lead to changes in ALAS-1 mRNA levels, FRL 4.1 cells were treated with 5-aminolevulinic acid (ALA) or succinylacetone (SA). ALA is the product of the ALAS enzyme and therefore the addition of ALA by-passes the rate limiting step in heme synthesis, and should lead to an increase in heme biosynthesis (Anderson *et al.,* 1981). Succinylacetone is a potent inhibitor of the second enzyme of the heme synthesis pathway aminolevulinic acid dehydratase (ALA-D) and therefore the addition of SA to cells should lead to a decrease in heme synthesis (Ebert, 1979). FRL 4.1 cells grown in serum free conditions were treated with 2μM heme, 500μM ALA, 500μM ALA and iron ammonium citrate or 500μM succinylacetone. Iron was included with ALA as it has been reported in human hepatoma (Hep G2) cells that ALA treatment alone led to an accumulation of protoporphyrins resulting in increased cell

Figure 3.13: **The Effect of ALA, Iron, Succinylacetone and Heme on ALAS-1 mRNA Levels in FRL 4.1 Cells**

FRL 4.1 cells maintained in serum free conditions were changed to fresh media containing Ca_2CO_3 vehicle (lane 1 and 6), 2μM heme (lanes 2 and 7), 500μM ALA (lanes 3 and 8), 500μM ALA plus 200μM iron (lanes 4 and 9), or 500μM succinylacetone (lanes 5 and 10). Total RNA was then isolated 3hr (lanes 1 to 5) or 6hr (lanes 6 to 10) later. 20μg of total RNA was subjected to Northern blot analysis and the filter sequentially probed for ALAS-1, c-myc and GAPDH mRNA.


ALA+
ALA+
- H ALA Fe SA - H ALA Fe SA
Treatment
ALAS-1
c-myc
GAPDH
1 2 3 4 5 6 7 8 9 10
3 hr
6 hr

toxicity and iron prevented this (Iwasa *et al.*, 1989). The level of the mRNAs for ALAS-1, c-myc and GAPDH were quantitated by Northern blot analysis (fig.3.13).

Exogenous heme treatment lowered the level of ALAS-1 mRNA to 30% of that found in the untreated control cells, while ALA plus iron and ALA alone reduced the level of ALAS-1 mRNA to 40% and 50-60% of that found in the control cells respectively. Therefore it appears that exogenous heme is more effective in lowering ALAS-1 mRNA levels in FRL 4.1 cells than either ALA plus iron or ALA alone. In addition ALA plus iron proved to be more potent than ALA alone in decreasing ALAS-1 mRNA levels. Succinylacetone produced only a slight increase in ALAS-1 mRNA levels (130% of untreated control) at both time periods. The steady state level of c-myc mRNA also fluctuated with the different treatments with heme increasing the level of c-myc mRNA 147% and 179% compared with the untreated control cells at 3hr and 6hr respectively. Treatment with ALA or ALA plus iron initially caused the steady state level of c-myc mRNA to decrease but the level subsequently recovered to be close to the that detected in the untreated control cells at 6hrs. This initial drop in c-myc mRNA was not as great nor as prolonged as the decrease observed in ALAS-1 mRNA. A similar effect on c-myc mRNA was observed with heme addition (fig 3.1) but occurred earlier than the fluctuations produced by ALA addition. This effect on c-myc mRNA observed with heme did not appear to be due to a decrease in the stability of the mRNA (fig 3.6b) but was presumably due to fluctuations in c-myc transcription (fig 3.3). Since ALA should be converted to heme inside the cell, the fluctuations in ALAS-1 and c-myc mRNA levels are probably due to the build up of heme. Heme would then alter ALAS-1 mRNA stability and c-myc transcription. The effect of ALA may be expected to occur at a later time point than heme depending on how quickly ALA is converted to heme within FRL 4.1 cells.

The reason ALA and ALA plus iron caused less of an effect than exogenous heme on ALAS-1 mRNA levels in FRL 4.1 cells is not known. Presumably the free heme pool in the cell was not increased as greatly nor as rapidly by ALA addition compared with exogenous heme. This could have been caused by a second enzyme of the heme biosynthesis pathway becoming rate limiting in these conditions, resulting in

insufficient heme being synthesised during the time period of the experiment to maximally reduce ALAS-1 mRNA levels. In agreement with this proposal was the observation that the fluctuations in c-myc mRNA seen in heme treated cells was delayed by several hours in ALA treated cells. The relative levels of the other enzymes of the heme pathway although apparently in excess in rat liver and other tissues is unknown in tissue culture cells. For instance it is known that ALA-D activity declines markedly when rat liver hepatocytes are cultured *in vitro* (Guzelian *et al.*, 1984).

3.2.9 Is Phosphorylation Involved in the Heme Mediated Destabilisation of ALAS-1 mRNA?

An important process for regulating cellular functions involves the phosphorylation and dephosphorylation of proteins, catalysed by the protein kinase and phosphatase enzymes. To date over 70 protein kinases have been described and many of these are involved in regulating gene expression. The phosphorylation of proteins by protein kinases can influence the expression of genes at a number of steps including initiation of transcription (Angel *et al.*, 1987 , Lee *et al.*, 1987), translation (Chen and London, 1995) and mRNA stability (Malter and Hong, 1991).

Heme can affect the activity of at least two mammalian protein kinases. One termed the heme-regulated inhibitor (HRI) is involved in co-ordinating protein synthesis with heme availability in reticulocytes. HRI is an erythroid specific (Crosby *et al.*, 1994), cyclic AMP-independent protein kinase which specifically phosphorylates the 38-kDa α-subunit (eIF-2α) of eukaryotic initiation factor 2 (eIF-2), resulting in a complex forming between eIF-2α and eIF-2B leading to an inhibition of protein synthesis. HRI is active when cellular heme levels are low, but is inactivated when cellular heme levels are high (Yang *et al.,* 1992). A second potential heme responsive protein kinase has been characterised in Hela cells. Following heat shock in Hela cells, protein synthesis is inhibited presumably by the activation of a protein kinase (De Benedetti and Corrado, 1986). The activation of this protein kinase correlates with an increase in the phosphorylation of the eIF-2α subunit and interestingly this

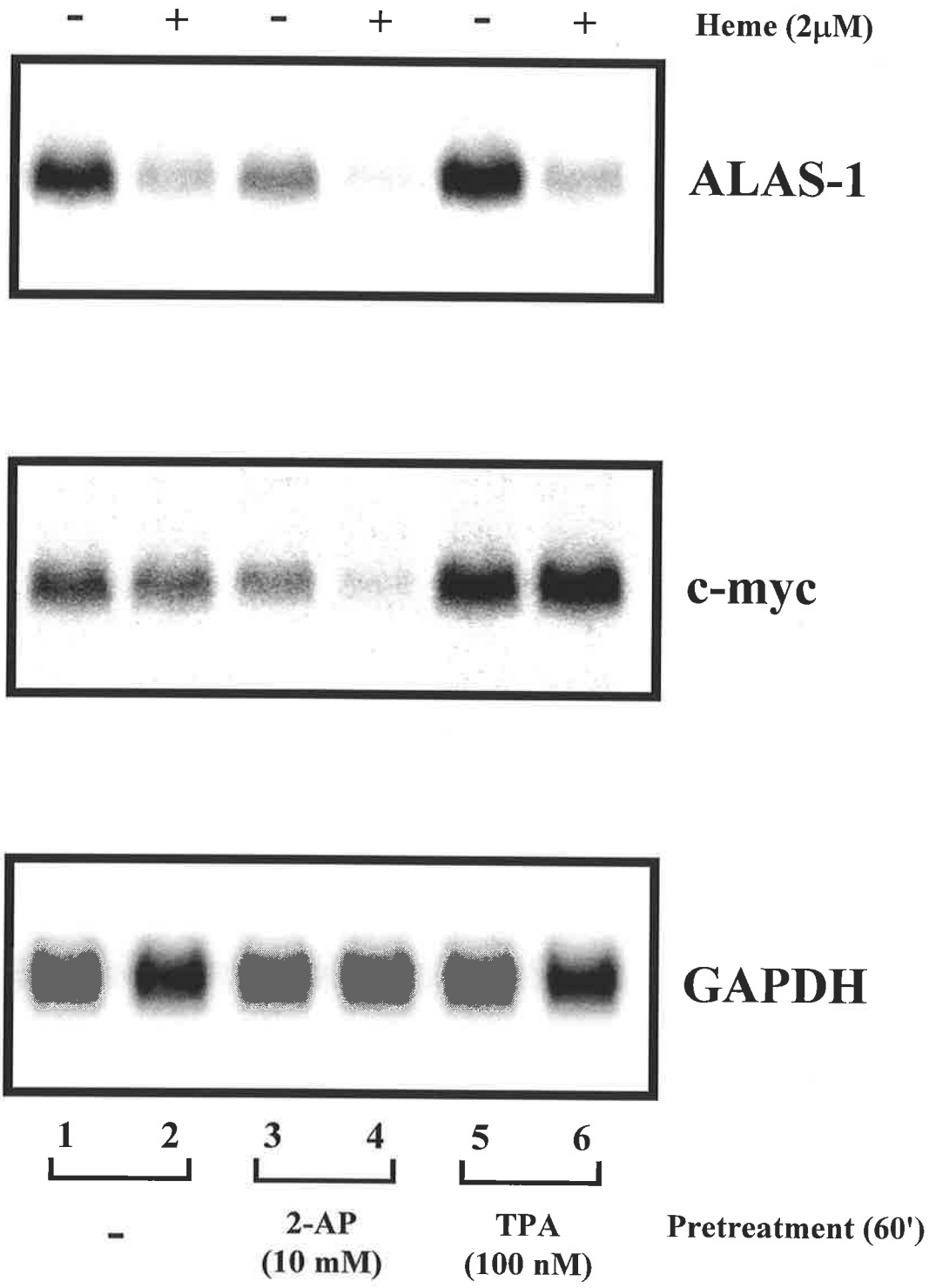
phosphorylation is inhibited by heme addition both *in vitro* and *in vivo* (De Benedetti and Corrado, 1986). Since the previously characterised HRI kinase is erythroid specific (Crosby *et al.*, 1994) these results imply that at least two protein kinases exist whose activities are modulated by cellular heme levels. In support of this a heme-sensitive kinase has recently been cloned from the rat brain (Mellor *et al.*, 1994), this kinase shows substantial homology at both the nucleotide (81%) and amino acid (82%) level to the HRI kinase purified from rabbit reticulyocites (Chen *et al.*, 1991). Whether this kinase is indeed the rat homologue of the rabbit erythroid-specific heme-regulated inhibitor (HRI) or a closely related species awaits the cloning of a HRI cDNA from rat reticulocytes. In the study of Crosby *et al.* (1994) trace amounts of HRI signal were detected by PCR in other tissue's including the brain, but this was thought to have been due to contamination of the tissue samples by blood, as the erythroid-specific transcription factor NF-E2 could also be amplified from these tissues. In contrast Mellor *et al.* (1994) reported significant amounts of the rat brain HRI mRNA in a number of non-erythroid tissues. The reason for this apparent discrepancy between the two studies has not been resolved but the finding of Mellor *et al.* (1994) raises the possibility of either additional roles for the HRI enzyme in the rat or a family of heme-responsive kinases.

To determine whether phosphorylation may be involved in the destabilisation of the ALAS-1 mRNA in response to heme, the effect of the protein kinase inhibitor 2-aminopurine (2-AP) on ALAS-1 mRNA levels was examined. The mechanism by which 2-AP blocks protein kinase activity is not well understood but is believed to involve the modifications of serine and threonine residues (Carrier *et al.*, 1992). The effect of the tumour promoter phorbol 12-myristate 13-acetate (TPA) on heme repression was also examined. Phorbol esters are functional analogues of diacylgycerol the endogenous activator of protein kinase C (Blumberg, 1988).

FRL 4.1 cells grown under serum free conditions were pretreated for 60 minutes with 10mM 2-AP or with 100nM of the protein kinase C activator TPA prior to the addition of 2μM heme or vehicle alone. Total RNA was isolated 3hr later and the level of ALAS-1, c-myc and GAPDH mRNA determined by Northern blot analysis (fig 3.14)

Figure 3.14: **The Effect of 2-Aminopurine (2-AP) and TPA on the Heme Mediated Decrease in ALAS-1 mRNA in FRL 4.1 Cells.**

FRL 4.1 cells maintained in serum free conditions for 30hr were changed to fresh media (lanes 1 and 2), or fresh media containing either 10mM 2-AP (lanes 3 and 4), or 100nM TPA (lanes 5 and 6). After 60min. the media was again changed to fresh media (lane 1), or fresh media containing 2μM heme (lane 2), 10mM 2-AP (lane 3), 10mM 2-AP plus 2μM heme (lane 4), 100nM TPA (lane 5), and 100nM TPA plus 2μM heme (lane 6). Total RNA was isolated after 3hr and 20μg used in a Northern blot.


- + - + - +
Heme (2μM)
ALAS-1
c-myc
GAPDH
1 2 3 4 5 6
-
2-AP
(10 mM)
TPA
(100 nM)
Pretreatment (60')

The dose of TPA and 2-AP used have been shown to be sufficient to maximally affect the phosphorylation state of proteins in the mouse fibroblast cell line C3H 10T1/2 within 30 minutes (Mahadevan *et al.*, 1990).

The steady state level of ALAS-1 mRNA fluctuated with 2-AP and TPA treatment. 2-AP treatment caused the level of ALAS-1 mRNA to decline to approximately half the level found in untreated cells (lane 1 versus 3), while TPA treatment reproducibly caused a slight increase in ALAS-1 mRNA levels (lane 1 versus 5). The basis of these fluctuations is unknown, although three copies of the consensus sequence for the transcription factor AP-1 are located in the first intron of the rat ALAS-1 gene (Braidotti, 1992). The transcription factor AP-1 has been shown to be able to mediate the induction of the transcription of a number of genes in response to Phorbol esters (Lee *et al.*, 1987) through the modulation of its phosphorylation state (Adate *et al.*, 1993). Heme treatment caused a 80% drop in the steady state level of ALAS-1 mRNA regardless of 2-AP and TPA pretreatment. To determine whether this effect was specific to ALAS-1 mRNA the mRNAs for c-myc and GAPDH were also examined. Contrary to the result observed in fibroblasts, 2-AP treatment also caused a decrease in the basal level of c-myc mRNA in FRL 4.1 cells (Zinn *et al.*, 1988). Heme treatment caused a further reduction in c-myc levels to about 40% of the level detected in the 2-AP alone treated cells. The reason for this drop is unknown but could imply that the concentration of heme used combined with 2-AP was toxic to the cell, leading to a general repression of transcription or increased mRNA degradation. As reported in other hepatoma cells (Duronio *èt al.*, 1990) c-myc mRNA levels increase only slightly with short term TPA treatment. Heme had no effect on TPA induced c-myc mRNA levels. The level of GAPDH mRNA remained unchanged by all treatments. Together these results imply that phosphorylation does not play a role in the destabilisation of ALAS-1 mRNA in response to heme.

3.3 DISCUSSION.

Since Granick (1966) first observed that heme could prevent the drug induced increase in ALAS activity in chick embryo hepatocytes thus demonstrating that ALAS-1 in the liver is subject to negative feedback, much effort has been concentrated on how heme achieves this feedback inhibition. Later studies extended this finding to chick embryo liver, different rat tissues and tissue culture cells (Reviewed May *et al.*, 1995). Heme has been shown to repress ALAS by several mechanisms. Firstly, heme can act post-transcriptionally to inhibit the import of the ALAS precursor protein into mitochondria thus denying the enzyme access to its substrates (Section 1.6.6). Secondly, heme has been shown to decrease the level of ALAS-1 mRNA in drug induced chick embryo hepatocytes (Ades *et al.*, 1987), and in drug induced rat liver (Srivastava *et al.*, 1988 ;1990). Furthermore, Srivastava *et al.* (1988) demonstrated that heme or its precursor ALA, could repress basal ALAS-1 mRNA levels in all tissues examined including liver, kidney, heart, lung, testis and brain. These results implied that heme is not only involved in controlling the rate of heme synthesis during drug induction but also in regulating normal cellular heme levels. The mechanism by which heme represses ALAS-1 mRNA levels was also examined. Nuclear run on experiments demonstrated that heme or its precursor ALA, could decrease the transcription rate of the ALAS-1 gene in drug treated and control rat livers (Srivastava *et al.,* 1988 ; 1990). Yamamoto *et al.* (1988) has also studied the heme mediated repression of ALAS-1 mRNA in rat liver. In this study rats treated with the drug AIA (2-ally-2-isopropylacetamide) for 3.5hr, to induce ALAS-1 mRNA levels, were then injected with the transcriptional inhibitors actinomycin D or α-amanatin 1hr prior to being sacrificed. Half the α-amanatin treated rats were also given heme (4mg/kg) at the same time as the α-amanatin injections. Quantitations of the level of ALAS-1 mRNA in the liver showed there was a rapid loss of ALAS-1 mRNA after actinomycin D or α-amanatin treatment and that heme did not significantly increase this rate of loss. These authors therefore concluded that the heme mediated suppression of ALAS-1 mRNA must be occurring only at the level of transcription and not by an accelerated decay of the ALAS-1 mRNA. An additional

finding of this study was that in rat liver ALAS-1 mRNA was very unstable, with an estimated half life of 20 minutes making ALAS-1 mRNA one of the most unstable mammalian mRNAs known.

The experiments in this chapter were performed in order to begin to understand at the molecular level how heme could negatively regulate ALAS-1 mRNA levels, and also to investigate the mechanism that causes ALAS-1 mRNA to be highly labile. Due to the difficulties associated with performing these studies in whole animals the regulation of ALAS-1 mRNA levels was examined in the rat hepatoma cell line FRL 4.1.

Experiments demonstrated that ALAS-1 mRNA levels are rapidly repressed following heme treatment of FRL 4.1 cells, however contrary to the results obtained in rat liver (Srivastava *et al.,* 1988 and 1990), heme did not appear to affect the transcription of the ALAS-1 gene. Instead heme caused a specific increase in the degradation rate of the ALAS-1 mRNA. This approximate 3 fold decrease in the half life of the message was sufficient to cause the rapid decline in the steady state level of ALAS-1 mRNA. A similar effect of heme on the decay rate of the ALAS-1 mRNA in the chicken has been observed. In cultured chick embryo hepatocytes heme has been shown to decrease the half life of the ALAS-1 mRNA from 3.5 hr to 1.2 hr (Hamilton *et al.*, 1991 , Drew and Ades, 1989b). Hamilton *et al.* (1991) also demonstrated that in these cells heme did not alter the transcription rate of the chicken ALAS-1 gene. Furthermore it has been found that when 17 day chick embryos are administered heme, the level of ALAS-1 mRNA in the liver is lowered, but the rate of ALAS-1 gene transcription is essentially unaffected (Hahn, 1991), supporting the notion that in chickens ALAS-1 mRNA levels are negatively controlled by heme at the level of mRNA stability.

The reason for the difference in the regulation of ALAS-1 mRNA by heme in rat liver versus rat hepatoma cells is unknown. As the effect of heme on the stability of the ALAS-1 mRNA is very specific in FRL 4.1 cells, and since heme also affects the half life of the ALAS-1 mRNA in chickens, it seems likely that a similar mechanism occurs in rat liver. However Yamamoto *et al.* (1988) could find no evidence for a heme

mediated effect on ALAS-1 mRNA stability in rat liver. The lack of an effect, in rat liver, on the stability of the ALAS-1 mRNA in response to heme could be explained in several ways. Hamilton *et al.* (1991) suggested that endogenous heme levels in the livers of these rats were already greatly increased, due to the induction of ALA synthase by AIA treatment, prior to the administration of α-amanatin. They proposed that this increased level of endogenous heme is sufficient to fully destabilise the ALAS-1 mRNA in the α-amanatin alone treated rats therefore giving similar half life determinations to the heme treated rats. However studies performed by (Yamamoto *et al.*, 1981) that attempted to measure the free heme pool found that although ALA synthase activity was greatly increased in liver mitochondria of AIA treated rats, cellular heme levels actually drop. These authors measured heme levels indirectly, by assaying the degree of heme saturation of tryptophan pyrrolase. Tryptophan pyrrolase has been proposed to be a sensitive marker of heme concentrations in the liver, since the degree of heme saturation of this enzyme can vary considerably, increasing with heme treatment and decreasing after the administration of porphyrinogenic drugs such as AIA and DDC (Badawy *et al.*, 1975 ; Badawy and Evans, 1973). The drop in cellular heme levels following AIA administration is thought to be due the ability of AIA to cause the destruction, at a high frequency, of the cytochrome P450s heme group, during the oxidation of the drug (Ortiz de Montellano and Carreia,1983). However if the heme responsive factor involved in regulating the stability of the ALAS-1 mRNA, has a higher affinity for heme than tryptophan pyrrolase, estimating heme levels by this method may not be relevant. Indeed an effect of heme on the import of ALA Synthase into the mitochondria is observed in conditions where tryptophan pyrrolase is not fully saturated. Also the effect of blocking transcription, by the highly toxic compounds actinomycin D or α-amanatin, on heme levels or mRNA metabolism in the whole animal prior to RNA isolation is unknown. An effect of these drugs on general mRNA metabolism in the liver cannot be ruled out as Yamamoto *et al.* (1988) do not appear to have examined the decay of any other mRNA.

An important question raised by these studies is that if heme negatively controls the transcription rate of the ALAS-1 gene in the rat why is this not observed in the rat

hepatoma cell line FRL 4.1 or in a variety of other transformed cell lines? Two possibilities can be proposed to explain this. Firstly it is possible that FRL 4.1 cells being a transformed cell line may have lost the ability to regulate the transcription of the ALAS-1 gene in response to heme, perhaps by the loss of expression of the heme responsive transcription factor. It is well known that the transformed phenotype can cause the loss of many differentiated features of the cell (Ibsen and Fishman, 1979). It is noteworthy in this respect that no transformed mammalian hepatoma cell line has been described which supports the phenobarbital mediated induction of ALAS-1 transcription (May *et al.*, 1995). Recently a primary rat hepatocyte culture system has been developed that supports, albeit at a lower level, the phenobarbital mediated increase in ALAS-1 mRNA. Furthermore this induction was at least partly inhibited by ALA or heme addition. Non-induced levels of ALAS-1 mRNA were also repressed by ALA or heme addition suggesting that primary rat hepatocytes may provide the best *in vitro* model to investigate the *in vivo* situation (Sinclair *et al.*, 1990). However the level at which heme is acting upon ALAS-1 mRNA levels in these cells remains to be determined.

An alternative view has been put forward by Hamilton *et al.* (1991). These researchers have suggested that the doses of ALA and heme used in the studies of Srivastava *et al.* (1988 ; 1990) to determine the effect of heme on ALAS-1 transcription were unphysiological. In fact the total dose of heme administered to the rats was 80mg/kg, a dose 26 times that needed to prevent ALA Synthase induction (Hayashi *et al.*, 1972). Hamilton *et al.* (1991) did not comment on the study of Yamamoto *et al.* (1988) whose results support heme regulation of ALAS-1 transcription. However, it should be pointed out that ALA and heme were given intraperitoneally in the studies of Srivastava *et al.* (1988 and 1990) while heme was administered intravenously in the studies performed by Hayashi *et al.* (1972). Therefore, a direct comparison of heme concentrations used is probably not valid, since factors such as heme uptake into the blood stream from the intra peritoneal cavity must be considered. Secondly, Anderson *et al.* (1981) have shown that at least in chick embryos, only 1-2% of ALA injected could be accounted for as protoporphyrin in the liver. Therefore, without a means of measuring the heme concentration in the liver it is unclear if the doses used by

Srivastava *et al.* (1988 ; 1990) caused a physiological relevant increase or not in heme levels in the liver.

To begin to address this issue a preliminary investigation on the transcription rate of the ALAS-1 gene was performed in rats which had been treated with much lower doses of heme. The dose used (2mg/kg) was just sufficient to cause the maximal inhibition of mitochondrial ALAS activity in rat liver (Hayashi *et al.*, 1981) when delivered intravenously. This low dose of heme was found to be sufficient to specifically repress the phenobarbital-mediated increase in the transcription rate of the ALAS-1 gene, suggesting the results obtained by Srivastava *et al.* (1988) were not due to an artefact of the high heme concentrations used. It is unknown if the lower dose of heme used in this experiment can also affect the basal rate of ALAS-1 transcription. Experiments to investigate this will be carried out in the future. This result although preliminary supports the notion that mammalian transformed cell lines may have lost the ability to respond transcriptionally to heme.

We decided to continue to pursue the regulation of ALAS-1 mRNA stability in FRL 4.1 cells with two aims in mind. Firstly, we assumed that the *cis*-elements and the *trans*-acting factors that bind to them, responsible for the rapid turnover of the ALAS-1 mRNA in FRL 4.1 cells would also be responsible for the extremely short half-life of the mRNA in rat liver. Since the regulation of mRNA stability has become increasingly apparent as an important process that determines the level of expression of a gene (Sachs, 1993), an understanding of the regulation of ALAS-1 mRNA turnover would contribute to the overall understanding of the control of heme synthesis in rat liver. Furthermore the mechanisms by which mammalian mRNAs turnover have only begun to be characterised, thus the turnover of ALAS-1 mRNA provided an interesting model for investigating mRNA turnover in mammalian cells.

The second aim was to identify the mechanism for the heme-mediated instability of the ALAS-1 mRNA in hepatoma cells. On the basis, of other mRNAs whose decay is regulated such as the transferrin receptor mRNA, we predict that heme treatment will alter the binding pattern of specific *trans*-acting factors to these instability determinants. We further predict that the *cis*-elements involved in the heme-mediated turnover of

ALAS-1 will be identical to the elements required for the rapid turnover of the mRNA in rat liver. These *trans*-acting factors can then be looked for in rat liver extracts, and the effect of heme on their binding activity determined. This data should allow an assessment of the likelihood of heme regulation of ALAS-1 mRNA turnover in rat liver.

Initially, the requirement for translation for ALAS-1 mRNA destabilisation was examined. Although a clear understanding of the relationship between translation and mRNA decay have yet to be fully achieved, it appears that many mRNAs require translation for their decay. This dependence on translation can be divided into two classes. In one class, are mRNAs whose degradation is dependent on translation of the mRNA itself, the highly unstable mRNA for the lymphokine GM-CSF belongs to this class (Aharon and Schneider, 1993). In the second class are mRNAs whose decay appear to be dependent on a labile polypeptide and therefore this polypeptide must be continuously synthesised for the decay of these mRNAs to occur. The human transferrin receptor mRNA appear to fall into this class (Koeller *et al.*, 1991). In an attempt to ascertain if translation was required for ALAS-1 mRNA degradation, FRL 4.1 hepatoma cells were treated with the translation inhibitor cycloheximide. This series of experiments demonstrated that the decay of the ALAS-1 mRNA in the presence or absence of heme was unchanged, while the decay of the mRNA for c-myc was, as expected, substantially inhibited by cycloheximide treatment (Wisdom and Lee, 1991). However protein synthesis was not completely abolished in these experiments raising the possibility that sufficient translation was still occurring to maintain the normal decay rate of the ALAS-1 mRNA. In this scenario the decay of the ALAS-1 mRNA needs to be less sensitive to the inhibition of translation than the mRNA for c-myc as the latter is stabilised by the same concentration of cycloheximide. Perhaps the decay of the mRNA for ALAS-1 is mediated through a labile protein and therefore its decay will be unaffected until the protein reaches a critical level (Hamilton *et al.*, 1991), while the decay of the mRNA for c-myc appears to be dependent on translation of the c-myc mRNA itself (Wisdom and Lee, 1991). To conclusively determine if translation is required for the destabilisation of the rat ALAS-1 mRNA these experiments need to be repeated under conditions where a greater inhibition of protein synthesis is achieved.

The effect of specifically blocking the translation of the ALAS-1 mRNA could be examined by placing a stable stem loop structure in the 5'UTR of the mRNA to inhibit ribosome binding. This approach of specifically blocking protein synthesis has been used previously to determine the role of translation in the decay of the mRNAs for c-fos and transferrin receptor Koeller *et al.* (1991) and for the decay of GM-CSF mRNA (Aharon and Schneider, 1993). However on the basis of the results obtained so far it would appear that the decay of ALAS-1 mRNA in rats is not dependent on translation. This result is in contrast to that observed in chick embryo hepatocytes where two groups have demonstrated that the decay of the mRNA for ALAS-1 is dependent on translation (Hamilton *et al.,* 1991 ; Ryan and Ades,1991). Hamilton *et al.* (1991) found that cycloheximide inhibited the heme mediated decrease in the half life of the ALAS-1 mRNA but did not affect the half life of the mRNA in control cells. On this basis they proposed that a highly labile protein was involved in the heme-mediated effect on the half life of ALAS-1 mRNA. In contrast the study of Ryan and Ades (1991) found that cycloheximide also significantly inhibited the disappearance of ALAS-1 mRNA from α-amanatin alone treated cells, suggesting translation is required for ALAS-1 mRNA decay in general and not just for the heme effect. Therefore it is unclear at this stage if a labile protein or translation of the ALAS-1 mRNA itself is required for the decay of the chicken ALAS-1 mRNA. The reason why the heme regulated instability of the mRNA for ALAS-1 differs in its dependence on translation between species is currently unknown but could simply reflect different stabilities of the proteins involved or a more fundamental difference in the mechanism of ALAS-1 mRNA decay.

A differential dependence on translation has been reported in other systems. Several studies examining the function of the ARE (Adenosine/Uridine rich) element have found evidence for a differential dependence on translation. The ARE is a complex element found in the 3'UTR of several highly labile mRNAs (section 1.8) but not in the 3'UTR of the ALAS-1 mRNA. The length and sequence composition of an ARE appears to vary between mRNAs but it is usually uridine rich and almost always contains at least one copy of the pentamer AUUUA. For example, the instability of hybrid mRNAs containing the GM-CSF ARE were demonstrated by Aharon and Schneider (1993) to be

dependent on their translation, while the ARE located in the 3'UTR of c-myc is not involved in c-myc's mRNA co-translational instability (Wisdom and Lee, 1991). The dependence for translation for c-myc mRNA instability has instead been mapped to a region in the coding portion of the mRNA (Wisdom and Lee, 1991). In addition the 3'UTR of c-fos which contains a functional ARE was able to confer instability to the transferrin receptor mRNA regardless of the translational status of the hybrid mRNA (Koeller *et al.*, 1991).

The role of the poly(A) tail in the heme-mediated destabilisation of the ALAS-1 mRNA was also examined. Using the oligonucleotide directed cleavage procedure, changes in the length of the ALAS-1 poly(A) tail were determined in FRL 4.1 cells which had been treated with actinomycin D or actinomycin D plus heme. Although the ALAS-1 mRNA poly(A) tail became progressively shorter during the 60 minutes assayed, heme did not appear to have an significant effect on this shortening. Also, the change in the length of the poly(A) tail of ALAS-1 mRNA in heme treated cells versus control cells did not appear to correlate with the rapid decrease in the main body of mRNA with heme. These results therefore suggest that de-adenylation of the poly(A) tail is not a prerequisite for the heme-mediated destabilisation of the ALAS-1 mRNA. However a potential problem with this experiment is that the 3'-fragments which are detected are the surviving population of transcripts. Therefore, if very rapid degradation occurs following poly(A) tail shortening no build up of mRNA molecules with very short poly(A) tails will be observed prior to the decay of the ALAS-1 mRNA body. This potential problem is also increased by the fact that the population of ALAS-1 mRNA molecules at time zero was markedly heterogeneous in the length of poly(A) tail making it more difficult to detect changes in the poly(A) tail length in the ALAS-1 mRNA population. This heterogeneity is most likely due to the constitutive expression of the ALAS-1 mRNA, leading to a population of ALAS-1 mRNA molecules which have been in the cytoplasm for differing lengths of time. These facts make it difficult to determine whether the decay of the main body of the mRNA occurs primarily on mRNA molecules containing no or very short poly(A) tails or whether the decay of the ALAS-1 mRNA can occur independently of prior poly(A) tail shortening.

This problem could be partially overcome by the use of an inducible/repressible promoter system driving the expression of the ALAS-1 gene. Transient induction of this promoter would lead to a pulse of newly synthesised transcripts which would be homogeneous in the length of their poly(A) tail. The discrete starting size of the poly(A) tail would then allow a better determination of the relationship between the shortening of the poly(A) tail and the degradation of the ALAS-1 mRNA. The promoter of the c-fos gene which is transiently expressed following serum stimulation of serum-starved cells (Greenberg and Ziff, 1984) has been used extensively for this purpose (Wilson and Treisman, 1988 ; Shyu *et al.,* 1991; Wellington *et al.*, 1993; Schiavi *et al.*, 1994 ; Chen *et al.*, 1994 ; Chen and Shyu, 1994, Lagnado *et al.*, 1994 ; Zubiaga *et al.*, 1995).

An interesting point to note from this experiment is the approximately 24 minute time lag between the addition of heme and a noticeable change in the decay rate of the ALAS-1 mRNA body in heme treated cells versus control cells (fig 3.10b). Two possible explanations exist to account for this. Firstly, the poly(A) tail may need to be shortened to a minimum length prior to the decay of the mRNA body. Such a phenomena has been implicated in the decay of chimeric transcripts between c-fos and β-globin, where the shortening of the poly(A) tail to between 25-60 adenosine residues is observed prior to the decay of the mRNA body (Shyu *et al.,* 1991 ; Schiavi *et al.*, 1994). However, this does not appear to be the case in the heme-mediated decay of the ALAS-1 mRNA as there was no significant difference in the distribution of 3'-ALAS-1 fragments between heme treated and control cells prior to the decay of the mRNA. If the poly(A) tail needed to be shortened to a minimum length prior to the decay of the ALAS-1 mRNA body a difference in the distribution of 3'-fragments at the 24 minute time point would have been expected as the decay of the ALAS-1 mRNA body differs in heme treated versus control cells after this time point. Also the majority of the 3'-species still retain a relatively long (greater than 100 adenosines) poly(A) tail at 24 minutes, which by analogy to the decay of the c-fos/β-globin chimeric transcripts is too long to target the molecules for rapid decay (Shyu *et al.,* 1991 ; Schiavi *et al.,* 1994 ; Chen *et al.,* 1994). Therefore, the timing of the heme-mediated decay of the ALAS-1 mRNA body and the shortening of the poly(A) tail does not appear to correlate.

Alternatively, the lag in the decay of the mRNA could be caused by a slow rate of heme uptake by the cell. If heme uptake is the rate-limiting factor, a time lag may be expected between the addition of heme and the time when heme levels are increased sufficiently in the cell to initiate the heme-mediated destabilisation of the ALAS-1 mRNA.

To truly address the question of whether the decay of the main body of the ALAS-1 mRNA occurs independently of poly(A) tail shortening, decay intermediates need to be analysed for the presence or absence of a poly(A) tail. Although, the detection of decay intermediates has proven in many cases to be very difficult to isolate, intermediates have been isolated for several genes including rat apolipoprotein II (Binder *et al.*, 1987), human transferrin receptor (Binder, *et al.,* 1994), c-fos and c-myc (Laird-Offringa *et al.,* 1990), chick fibroblast 9E3 (Stoeckle and Hanafusa, 1989), human gro-α (Stoeckle, 1992), Xenopus Xlhbox 2B (Brown and Harland, 1990) and yeast (MFA2) mating factor A2 (Muhlrad *et al.*, 1994). In the case of the human transferrin receptor (Binder, *et al.,* 1994), chick fibroblast 9E3 (Stoeckle and Hanafusa, 1989) and the Xenopus Xlhbox 2B (Brown and Harland, 1990) truncated species have been isolated which carry a substantial poly(A) tail. This finding implies that there is a class of mRNA species whose decay is independent of shortening of their poly(A) tail and thus the ALAS-1 mRNA appears to belong to this class of mRNA species.

An examination was initiated to characterise the nature of the signal that causes the accelerated degradation of the ALAS-1 mRNA in conditions of high heme. Another metalloporphyrin (tin mesoporphyrin) was unable to decrease ALAS-1 mRNA levels at least at the same concentrations as heme. This suggests that the coordination chemistry of iron may be important in the interaction of heme with the presumptive heme responsive factor(s) since another metal could not substitute for iron in the porphyrin ring. However, this study on the requirement for iron in the porphyrin ring was by no means exhaustive and needs to be repeated with a number of other metalloporphyrins. A recent study by Cable *et al.* (1994) also found that tin-mesoporphyrin was ineffectual in destabilising ALAS-1 mRNA in cultured chick embryo hepatocytes; they did however show that another metalloporphyrin, zinc mesoporphyrin IX (ZnMeP), could promote the destabilisation of the ALAS-1 mRNA. Indeed, in their hands ZnMeP appeared to be

a more effective promoter of ALAS-1 mRNA turnover than equivalent doses of heme, perhaps because ZnMeP did not induce HO-1 levels. Therefore it would be interesting to determine the effect of ZnMeP and a range of other metalloporphyrins on the turnover of rat ALAS-1 mRNA in FRL 4.1 cells.

Alternatively the inability of tin-mesoporphyrin to alter the steady state level of ALAS-1 mRNA may reflect a poor uptake of the molecule by FRL 4.1 cells. This possibility was raised by a recent study of Smith *et al.* (1993) which found that free tin-protporphyrin (Sn-PP) was an ineffective inducer of heme oxygenase-1 (HO-1) mRNA in the mouse hepatoma cell line Hepa-1, while cobalt protoporphyrin and heme were effective inducers. This is in contrast to rats were Sn-PP is a potent inducer of HO-1 mRNA in rat liver (Sardana and Kappas, 1987). Interestingly, the complex formed between Sn-PP and hemopexin was an effective inducer of HO-1 mRNA in Hepa-1 cells. Hemopexin is a plasma glycoprotein involved in the transport of heme *in vivo* , principally to the liver (Reviewed Smith, 1990a). The binding of heme or heme analogues such as Sn-PP and Co-PP to hemopexin causes a conformational change promoting the interaction between the heme-hemopexin complex and its membrane bound receptor (Smith *et al.*, 1988 ; Smith *et al.,* 1993). The heme-hemopexin-receptor complex can then enter the cell via endocytosis, where upon release of its heme, hemopexin is recycled back to the plasma (Smith *et al.*, 1990b). The result of Smith *et al.* (1993) raises the possibility that Sn-PP is unable to cross the plasma membrane as efficiently as heme or Co-PP and therefore requires hemopexin mediated transport for efficient uptake. Indeed Smith *et al.* (1993) found that complexing Sn-PP with hemopexin doubled the uptake of Sn-PP into Hepa-1 cells compared to free Sn-PP. Similar measurements using the same concentration of Co-PP also demonstrated an approximately 10-fold higher accumulation of Co-PP in the cell compared to free Sn-PP again implying that the uptake of Sn-PP is inefficient in Hepa-1 cells. Although free tin-mesoporphyrin has been used to induce HO-1 mRNA in rat-2 fibroblasts (Eisenstein *et al.* 1991) the concentration used was 25 times higher than that used by Smith *et al.* (1993) and 80 times higher than the concentrations used here. In my experiments and in the study of Eisenstein *et al.* (1991) the protoporphyrin ring was meso-substituted at the

vinyl groups at position 2 and 4. The effect of this substitution on the heme analogues uptake is unknown as is the degree of uptake of Sn-PP in cells other than mouse Hepa-1 cells. Unfortunately HO-1 mRNA levels were not examined in FRL 4.1 cells treated with Sn-meP in this study. To resolve this FRL 4.1 cells will be treated with Co-PP and Sn-meP and the effect of these heme analogues on ALAS-1 and HO-1 mRNA levels determined.

The possibility that heme was acting as a source of intracellular iron for the destabilisation of ALAS-1 mRNA was discounted. This was concluded from two experiments. Firstly non-heme iron provided to the cell as iron ammonium citrate did not alter the steady state level of ALAS-1 mRNA. Secondly, the iron chelator desferroxamine could not abolish the effect of heme on ALAS-1 mRNA levels. In addition, a decrease in ALAS-1 mRNA could be achieved by increasing heme levels *in vivo* by the addition of the heme precursor ALA.

Lastly an attempt to examine whether phosphorylation was involved in the heme mediated destabilisation of ALAS-1 mRNA was performed. A role for protein kinase C in the signal can probably be ruled out since the phorbol ester TPA did not interfere with the decrease in ALAS-1 mRNA levels following heme treatment. The protein kinase inhibitor 2 aminopurine (2-AP) was also used to determine if phosphorylation was involved. Although ALAS-1 mRNA levels decreased with 2-AP treatment, heme addition caused a further drop in ALAS-1 mRNA. This drop in ALAS-1 mRNA levels following heme addition was of the same magnitude (ie. 80%) irrespective of 2-AP treatment implying that 2-AP did not interfere with the cells ability to respond to heme. However the addition of 2-AP and heme to the cell also caused a significantly greater drop in c-myc levels than 2-AP alone suggesting that this combination of chemicals may be having a general effect on the cell. The level of GAPDH mRNA was unchanged by 2-AP and heme treatment but this may be expected as GAPDH is a highly stable mRNA and therefore any change in the steady state level of GAPDH mRNA will occur very slowly. The mRNA for c-myc on the other hand is highly labile and therefore will rapidly reflect any toxic effect on the cell. Although the possibility that the effect observed with 2-AP and heme on ALAS-1 mRNA levels is due to a toxic effect of the

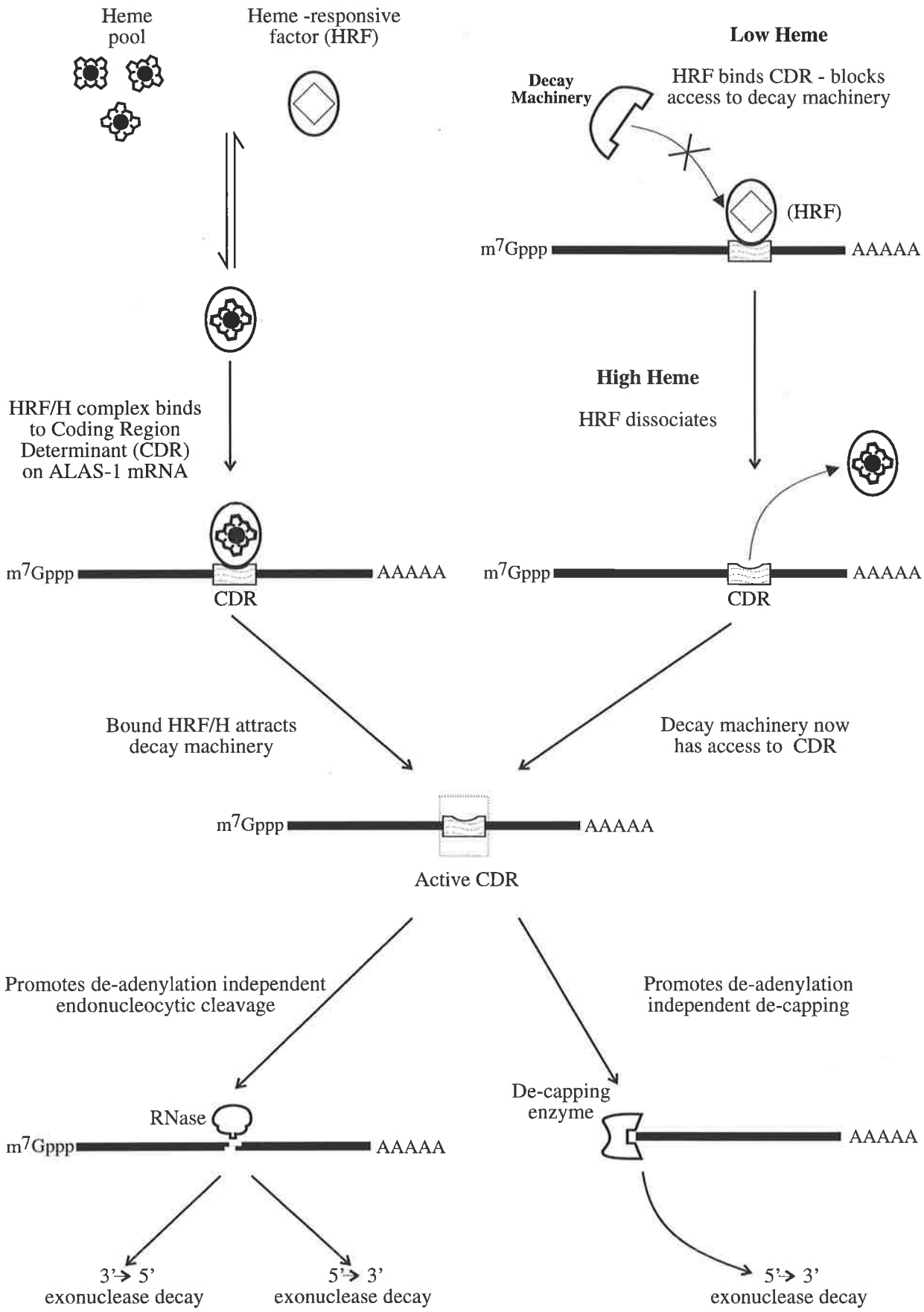
two chemicals cannot be ruled out this appears to be unlikely. In this scenario 2-AP would have to inhibit the heme mediated destabilisation of ALAS-1 mRNA and then with the addition of heme cause a toxic effect producing a drop in ALAS-1 mRNA levels of the same magnitude as seen in non 2-AP treated cells. A clearer picture of the effect of 2-AP and heme together on the cell will be obtained when the decay rate of the mRNAs for ALAS-1 and c-myc are measured. In this current study the steady state levels of the two mRNAs were measured and thus reflect both the transcription rate of the gene and the decay rate of the mRNA. The transcription rate of the c-myc gene is known to fluctuate in response to a variety of cellular signals and therefore an effect of 2-AP and heme on c-myc transcription and not on the stability of its mRNA cannot be ruled out at this stage (Luscher and Eisenman, 1990). Therefore, the role phosphorylation plays in the heme mediated destabilisation of ALAS-1 mRNA is still unclear. However it does appear that protein kinase C and protein kinases inhibited by 2-AP do not play a role. Whether 2-AP acts as a general or a specific protein kinase inhibitor is unclear. The effect of 2-AP seems to depend on the experimental system used. For example, 2-AP inhibits the phosphorylation of virtually all phosphoproteins in an *in vitro* reticulocyte lysate system (Farrell *et al.*, 1977). In contrast 2-AP appears to act relatively specifically on intact mouse fibroblasts and does not appear to alter the phosphorylation state of cellular proteins in general in these cells (Mhadevan *et al.*, 1990). To date 2-AP has only been shown definitively to affect the activity of two protein kinases; the heme regulated and dsRNA-dependent eIF-2α protein kinases (De Benedetti and Baglioni, 1983 ; Farrell *et al.*, 1977 ; Kaufman and Murtha, 1987). Therefore, a more conclusive investigation into the role phosphorylation plays in heme mediated destabilisation of ALAS-1 mRNA awaits the identification of the factors that target the mRNA for rapid decay.

In conclusion heme causes an increase in the decay rate of the rat ALAS-1 mRNA in transformed cell lines. Rapid mRNA degradation of the ALAS-1 mRNA, coupled with a block in the import of the ALAS-1 precursor into the mitochondria (section 1.6.6.ii), in response to high cellular heme levels provides a particular tight control on the synthesis of heme. The physiological reason for this extremely tight

regulation is however still unclear but presumably relates to a toxic effect caused by a build up of free heme (Muller-Eberhard and Fraig, 1993). The characterisation of highly labile eukaryotic mRNAs have demonstrated that their stabilities are determined by discrete *cis*-acting sequences found within the mRNA. Presumably these *cis*-acting sequences are then specifically recognised by *trans*-acting factors involved in the decay of the mRNA. The function of these *cis*-acting sequences and the proteins that recognise them can vary greatly (section 1.8). Several examples of regulated mRNA stability have now been documented (Schuler and Cole, 1988 ; Takahama and Singer, 1992 ; Klausner *et al.*, 1993), implying that the *trans*-acting factors required for destabilisation can be regulated.

On this basis a model for the mechanism by which heme could mediate the destabilisation of ALAS-1 mRNA can be proposed. Heme could influence the stability of the ALAS-1 mRNA by several mechanisms (fig 3.15). Heme could bind directly to a factor involved in the destabilisation of the ALAS-1 mRNA and alter its activity. The factor could then bind to the ALAS-1 mRNA instability sequence targeting the mRNA for decay. Alternatively the binding of heme to the protein may cause the disassociation of the factor from the ALAS-1 mRNA thus unmasking the instability sequence for recognition by the factors involved in mRNA decay. In a slight modification of this model heme may interact indirectly with the factor involved in ALAS-1 mRNA instability. In this "indirect" model increased heme levels in the cell may inhibit or activate a specific kinase leading to phosphorylation and modulation of the activity of the instability factor. Once the ALAS-1 mRNA has been recognised the main body of the mRNA appears to be degraded by a poly(A) independent pathway. This decay could involve an endonucleolytic cleavage within the ALAS-1 body, followed by exonucleolytic decay. Alternatively, the decay machinery bound to the ALAS-1 mRNA could promote de-capping of the mRNA followed by 5' to 3' exonucleolytic decay (Decker and Parker, 1994).

Figure 3.15: **Proposed Model(s) for the Heme-Mediated Decay of the ALAS-1 mRNA**


Heme pool
Heme -responsive factor (HRF)
Low Heme
Decay Machinery
HRF binds CDR - blocks access to decay machinery
(HRF)
m7Gppp
AAAAA
High Heme
HRF dissociates
HRF/H complex binds to Coding Region Determinant (CDR) on ALAS-1 mRNA
CDR
Bound HRF/H attracts decay machinery
Decay machinery now has access to CDR
Active CDR
Promotes de-adenylation independent endonucleocytic cleavage
Promotes de-adenylation independent de-capping
RNase
De-capping enzyme
3'→ 5' exonuclease decay
5'→ 3' exonuclease decay
5'→ 3' exonuclease decay

CHAPTER FOUR

ANALYSIS OF THE 3'UTR OF THE RAT ALAS-1 mRNA FOR DESTABILISING ACTIVITY.

CHAPTER FOUR: ANALYSIS OF THE 3'UTR OF THE RAT ALAS-1 mRNA FOR DESTABILISING ACTIVITY.

4.1 INTRODUCTION.

Searches for determinants of mRNA stability have revealed that these determinants are comprised of discrete sequences within a mRNA. Further work has established that, at least for the short-lived mRNAs, these *cis*-acting sequences may be functionally redundant, with a mRNA containing several sequences that are able to target it for rapid decay. To date these sequences have been identified experimentally by their ability to confer destabilisation to a normally stable mRNA. This ability of the destabilising sequence to act "dominantly" on a normally stable mRNA has led to the proposal that mRNAs are inherently stable within a cell in the absence of destabilising sequences. Therefore, the decay rate of any particular mRNA is determined by the number and strength of its destabilising sequence(s). This proposal is supported by the characterisation of known stabilising sequences which appear to function by regulating the activity of a nearby destabilising sequence (Binder *et al.*, 1994 ; Dodson and Shapiro, 1994 ; Sachs, 1993).

On this basis, it was predicted that the *cis*-acting sequences responsible for the heme-mediated decay of the ALAS-1 mRNA would be located at discrete sites within the ALAS-1 mRNA. Furthermore this heme-response should be transferable to a hybrid mRNA containing the appropriate region of the ALAS-1 mRNA. Initially the 3'-untranslated (3'UTR) of the ALAS-1 mRNA was investigated for the presence of destabilisation sequences. This approach was based on the fact that the majority of destabilising sequences characterised so far are located in the 3'UTR of their respective mRNAs (Sachs, 1993 ; Decker and Parker, 1994). Destabilising sequences have also been found in the coding region of unstable mRNAs but apart from β-tubulin mRNA (Theodorakis and Cleveland, 1992) and mRNAs carrying premature stop codons (Lim *et al.*, 1992 ; Peltz *et al.*, 1993) these coding region determinants are normally found in

mRNAs in conjunction with 3'UTR instability determinants, with the two determinants appearing to function independently (Shyu *et al.*, 1989 ; Herrick and Ross, 1994). Secondly, the rabbit β-globin expression vector, pNeoRβ-globin^{GC} was available and the insertion of the 3'UTR of the ALAS-1 gene into the β-globin gene could be accomplished by a simple cloning procedure.

This chapter describes the synthesis of the hybrid gene construct pNeoRβ-globin^{UTR} from the parental vector pNeoRβ-globin^{GC}. These two constructs were then stably transfected into the rat hepatoma cell line FRL 4.1 and the effect of heme on the decay of the two β-globin mRNAs determined.

4.2 RESULTS.

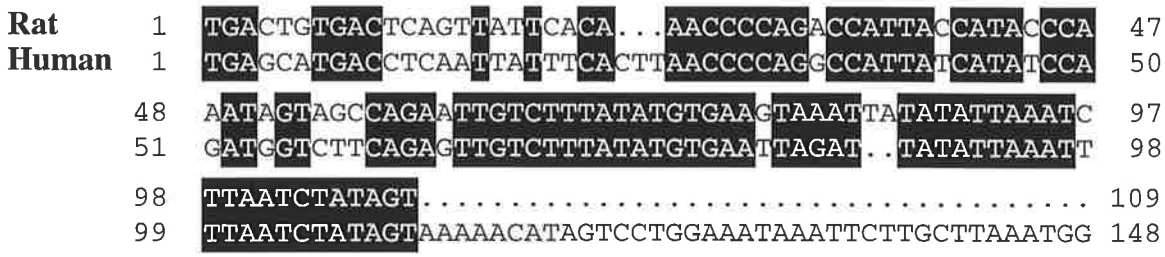
4.2.1 Sequence analysis of the 3'-untranslated region of the rat, human and chicken ALAS-1 mRNA

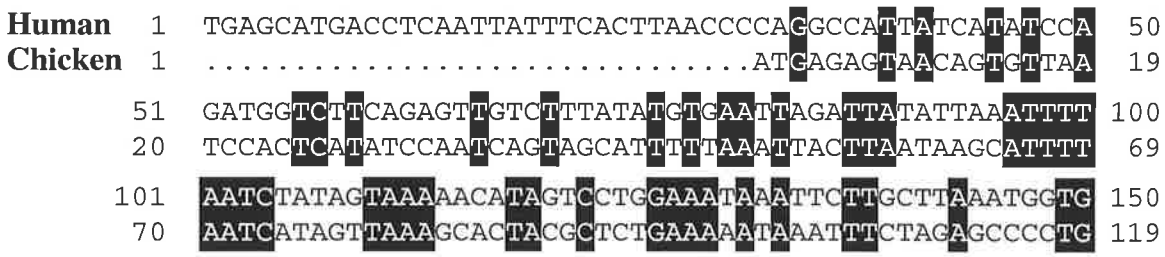
Since heme causes the destabilisation of the ALAS-1 mRNA for rat (previous chapter), chicken (Drew and Ades, 1989 ; Hamilton *et al.*, 1991) and human (Healy, 1990), it was predicted that the *cis*-acting sequences involved would be conserved between species. Such sequence conservation between the human and murine GM-CSF mRNA led to the identification of the ARE element (Caput *et al.*, 1986 ; Shaw and Kamen, 1986).

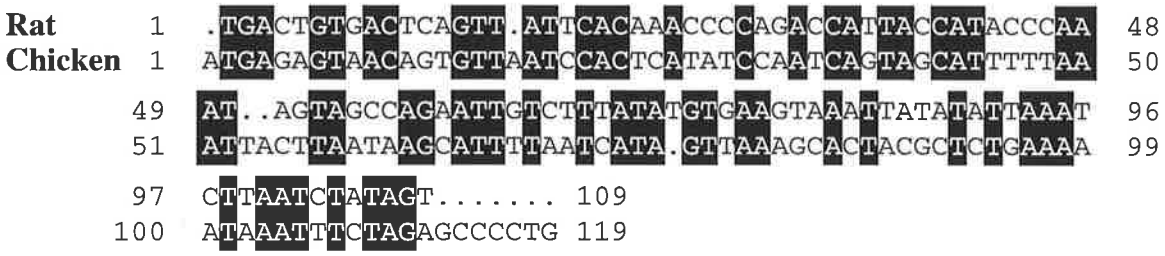
Initially the sequences of the chicken and human ALAS-1 3'UTR were aligned with the rat ALAS-1 3'UTR in order to detect regions which were conserved in the three species. The GAP program of the GCG software package (Section 2.6.3) was used to optimally align the chicken and human 3'UTRs with the rat sequence (fig 4.1). As can be seen the rat and human ALAS-1 3'UTR show a high degree (77%) of sequence similarity, suggesting that the sequence of the 3'UTR may have an important functional role (fig4.1). The significance of this high degree of homology, however is unclear. Firstly, this similarity is in keeping with the high degree of homology (86%) at the

Figure 4.1: **Alignment of the 3'UTR Sequence of the Rat, Human and Chicken ALAS-1 mRNAs.**

The 3'-untranslated regions of the rat, human and chicken ALAS-1 mRNAs were compared using the GAP program of the GCG software package. Conserved residues are boxed. The 3'UTRs are numbered from the start of the translational termination codon (1) to the end of the 3'UTR.


Rat 1 TGACTGTGACTCAGTTATTCACA...AACCCCAGACCATTACCATACCCA 47
Human 1 TGAGCATGACCTCAATTATTTCACTTAACCCCAGGCCATTATCATATCCA 50
48 AATAGTAGCCAGAATTGTCTTTATATGTGAAGTAAATTATATATTAAATC 97
51 GATGGTCTTCAGAGTTGTCTTTATATGTGAATTAGAT..TATATTAAATT 98
98 TTAATCTATAGT..... 109
99 TTAATCTATAGTAAAAACATAGTCCTGGAAATAAATTCTTGCTTAAATGG 148


Human 1 TGAGCATGACCTCAATTATTTCACTTAACCCCAGGCCATTATCATATCCA 50
Chicken 1 .....ATGAGAGTAACAGTGTTAA 19
51 GATGGTCTTCAGAGTTGTCTTTATATGTGAATTAGATTATATTAAATTTT 100
20 TCCACTCATATCCAATCAGTAGCATTTTTAAATTACTTAATAAGCATTTT 69
101 AATCTATAGTAAAAACATAGTCCTGGAAATAAATTCTTGCTTAAATGGTG 150
70 AATCATAGTTAAAGCACTACGCTCTGAAAAATAAATTTCTAGAGCCCCTG 119


Rat 1 .TGACTGTGACTCAGTT.ATTCACAAACCCCAGACCATTACCATACCCAA 48
Chicken 1 ATGAGAGTAACAGTGTTAATCCACTCATATCCAATCAGTAGCATTTTTAA 50
49 AT..AGTAGCCAGAATTGTCTTTATATGTGAAGTAAATTATATATTAAAT 96
51 ATTACTTAATAAGCATTTTAATCATA.GTTAAAGCACTACGCTCTGAAAA 99
97 CTTAATCTATAGT..... 109
100 ATAAATTTCTAGAGCCCCTG 119

nucleotide level found throughout the entire human and rat coding region, suggesting that this sequence conservation simply reflects the overall similarity between the two genes. Secondly, this high degree of homology in the 3'UTR is not shared between the rat and chicken (55%) or the human and chicken (38%) sequences. As the program GAP produces the optimal alignment over the entire length of the two sequences examined, a small highly conserved region which may form a binding site for a heme-responsive *trans*-factor may not be aligned.

Therefore the program SPCOMP (section 2.6.3) was used to search for small regions of homology conserved between all three species that may form a putative binding site for a heme-responsive factor. In this search several assumptions were made. Firstly, it was assumed that the primary sequence was the most important consideration for *trans*-factor binding and not secondary structure. Secondly, six bases were chosen for the minimum size for any putative *trans*-acting factor binding site and thus regions needed to show a homology of six bases or greater to be selected. This selection criteria therefore causes a bias towards *trans*-acting binding sites that are invariant whereas, at least, for the DNA binding *trans*-factor(s) the *cis*-acting sequence can show substantial variability and for example, the erythroid-specific transcription factor GATA-1 can bind to a number of sequences that vary from the consensus (A/T)GATA(A/G) (Ko and Engel, 1993).

Computer analysis, using the SPCOMP program revealed four sequences that are conserved in the 3'UTR of all three species (fig 4.2). The first (TCATATCCAA), located at position 25 of the chicken 3'UTR showed a nine out of ten and eight out of ten match with sequences found in the human (TCATATCCAg) and rat (cCATAcCCAA) 3'UTR respectively.

Also detected in the human and chicken 3'UTR was the sequence ATTTTAATC. The rat 3'UTR contains a similar sequence (ATcTTAATC) that differs by only 1 base from the human and chicken sequence. The chicken also contains a second sequence (ATTTTTAA) that resembles this conserved region, but this second motif is not present in the 3'UTR of the rat or human ALAS-1 mRNA. Interestingly, part of this sequence has been noted previously by Drew and Ades (1989) and Yamamoto *et*

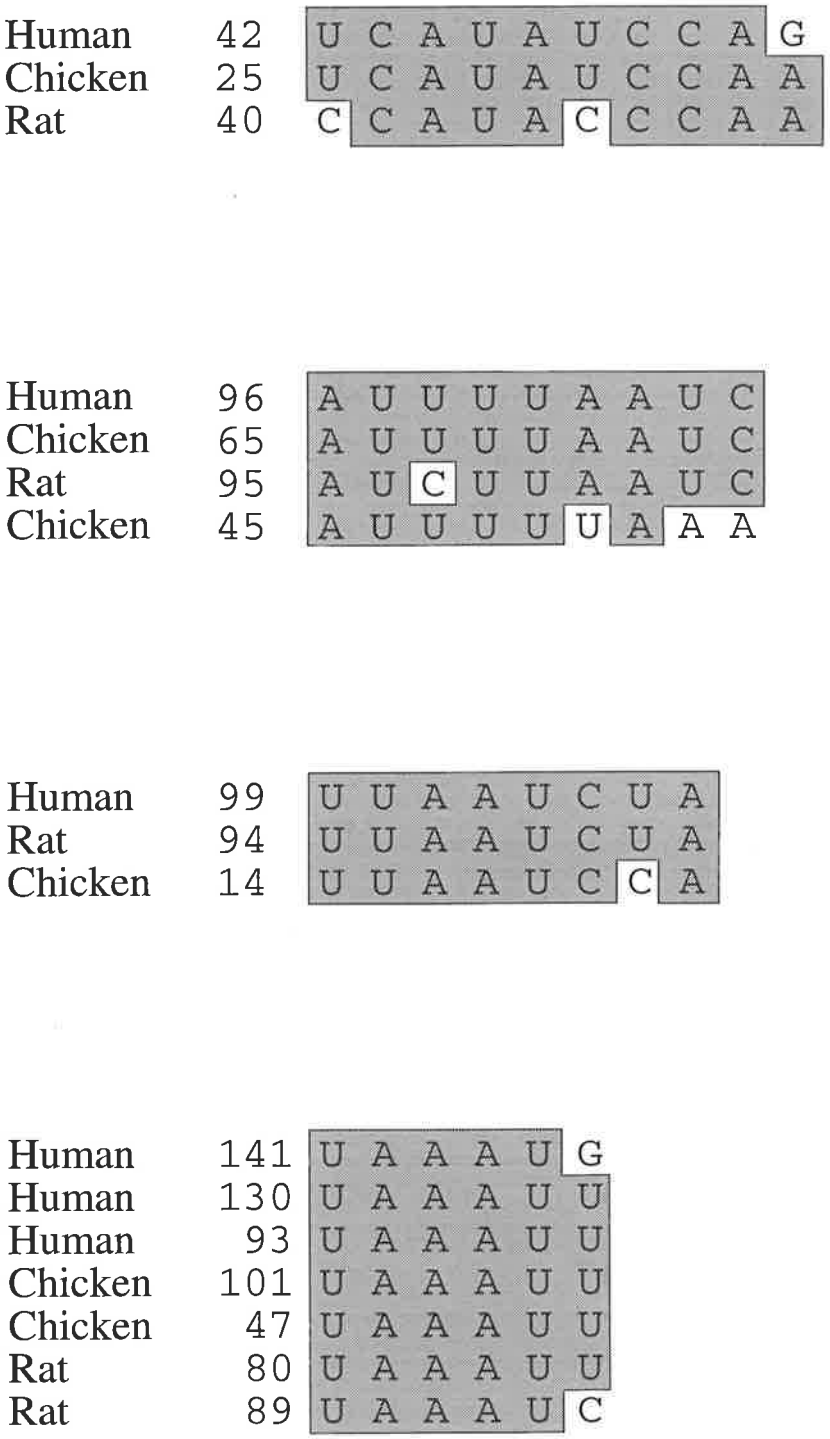


Fig 4.2 Conserved regions of the rat, chicken and human ALAS-1 3' UTR.

The sequence of the 3' UTR of the rat, chicken and human ALAS-1 mRNA was scanned by the SPCOMP program (Section 2.6.3) for sequences conserved in all three species. To be selected, the sequences needed to be at least 6 bases in length. The four sequences identified are shown above with matches shaded. The numbers give the distance of the first base of the conserved region from the start of the 3' UTR.

al. (1988) to resemble the AUUUA pentameric sequence which forms an integral component of most ARE instability elements. However, this sequence is unlikely to function as an ARE-like element. Firstly, even the presence of a perfect copy of the AUUUA pentamer in the 3'UTR of a mRNA is not always sufficient to act as an ARE, as demonstrated by the existence of such a motif in the 3'UTR of the long lived rabbit β-globin and human fibronectin mRNA (Dean *et al.*, 1988 ; Ross and Sullivan, 1985). Indeed the minimal sequence required for ARE function has recently been proposed to be UUAUUUA (U/A)(U/A) (Lagnado *et al.*, 1994 ; Zubiaga *et al.*, 1995). This extended sequence is not present in the conserved region identified in the 3'UTR of the ALAS-1 mRNA. Also arguing strongly against this ALAS-1 sequence functioning as an ARE element are the results of a number of studies which have demonstrated that mutations in the AUUUA pentamer itself are not tolerated and abolish instability function of the ARE. This data implies that the AU(U/C)UUA core of the ALAS-1 conserved region is unlikely to function as an ARE-like element (Lagnado *et al.*, 1994 ; Vakalopoulou *et al.*, 1991 ; Shyu *et al.*, 1991 ; Shaw and Kamen, 1986).

Overlapping the conserved region in the rat and human 3'UTR is the sequence TTAATCTA identified on the basis of its homology to a sequence (TTAATCcA) at position 14 of the chicken 3'UTR (fig 4.2). Lastly, the sequence TAAATT was detected in the 3'UTR of all three species. This motif is present as two perfect copies in the human and chicken 3'UTR and once in the rat 3'UTR. In addition the rat and human 3'UTR both contain an imperfect copy of this sequence that varies from the conserved sequence at the last position.

Although, the primary sequence plays the major-role in the recognition and binding of most *trans*-acting factors to their *cis*-acting target sequences, the single-stranded nature of mRNA allows for the *cis*-acting sequences to exist as a sequence/structure motif. The classical example of this is the recognition and binding of the iron-responsive element binding protein (IRE-BP) to the (IRE) iron-responsive element (Reviewed Klausner *et al.*, 1993). The consensus IRE is a stable stem-loop structure which consists of a) a six-membered loop, the first five bases of which are most often CAGUG and the sixth base most often a pyrimidine; b) an upper stem

usually of five base pairs, in which the bases can be any complementary pair of RNA base pairs; c) a bulge that is invariably a cytosine residue and d) a lower stem of variable length and sequence.

Therefore, the 3'UTRs of the rat, human and chicken ALAS-1 mRNAs were analysed for the existence of conserved secondary structures which could be candidates for the putative heme-responsive instability element. Initially, the program Fold (section 2.6.3) was used to display the optimal folded structure of the entire 3'UTR. Although all three sequences are capable of forming extensive secondary structures (fig 4.3), there does not appear to be any conserved structure formed by the three 3'UTRs. Importantly, and by analogy to the IRE structure, no conservation is observed in the size or sequence of the loop regions. The 3'UTRs of the rat, human and chicken ALAS-1 mRNA were also analysed for individual stem-loops by the program StemLoop (section 2.6.3) however no conserved stem-loop structures were identified (data not shown).

Taken together, the computer analysis suggests that if the 3'UTR is involved in the heme-mediated destabilisation effect the *cis*-acting target sequence(s) is most likely to be recognised by its sequence and not as a specific secondary structure. Secondly, several sequences, conserved in all three species, were identified that are candidates for any destabilisation activity detected. Attempts to determine whether the 3'UTR of the ALAS-1 mRNA contains destabilisation activity are described in the remainder of this chapter and the next chapter.

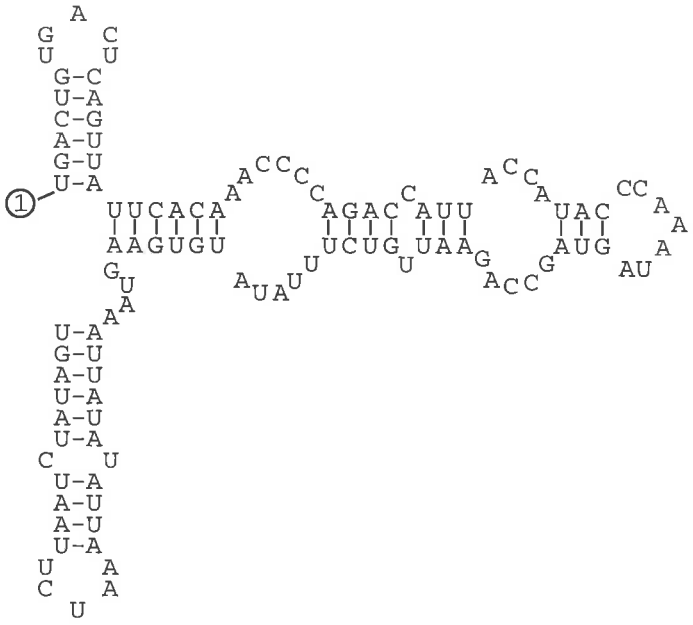
4.2.2 Construction of a hybrid rabbit β-globin mRNA containing the rat ALAS-1 3'UTR (pNeoRβ-globin^{UTR}).

To determine whether the 3'UTR of the rat ALAS-1 mRNA was sufficient to cause the heme mediated destabilisation of the ALAS-1 mRNA, a portion of the 3'UTR was cloned into the 3'UTR of the rabbit β-globin gene. The mRNA for β-globin is normally very stable in cells with a half-life greater than 17hr (Kabnick and Housman, 1988 ; Volloch and Housman, 1981) and has been used extensively to assay for

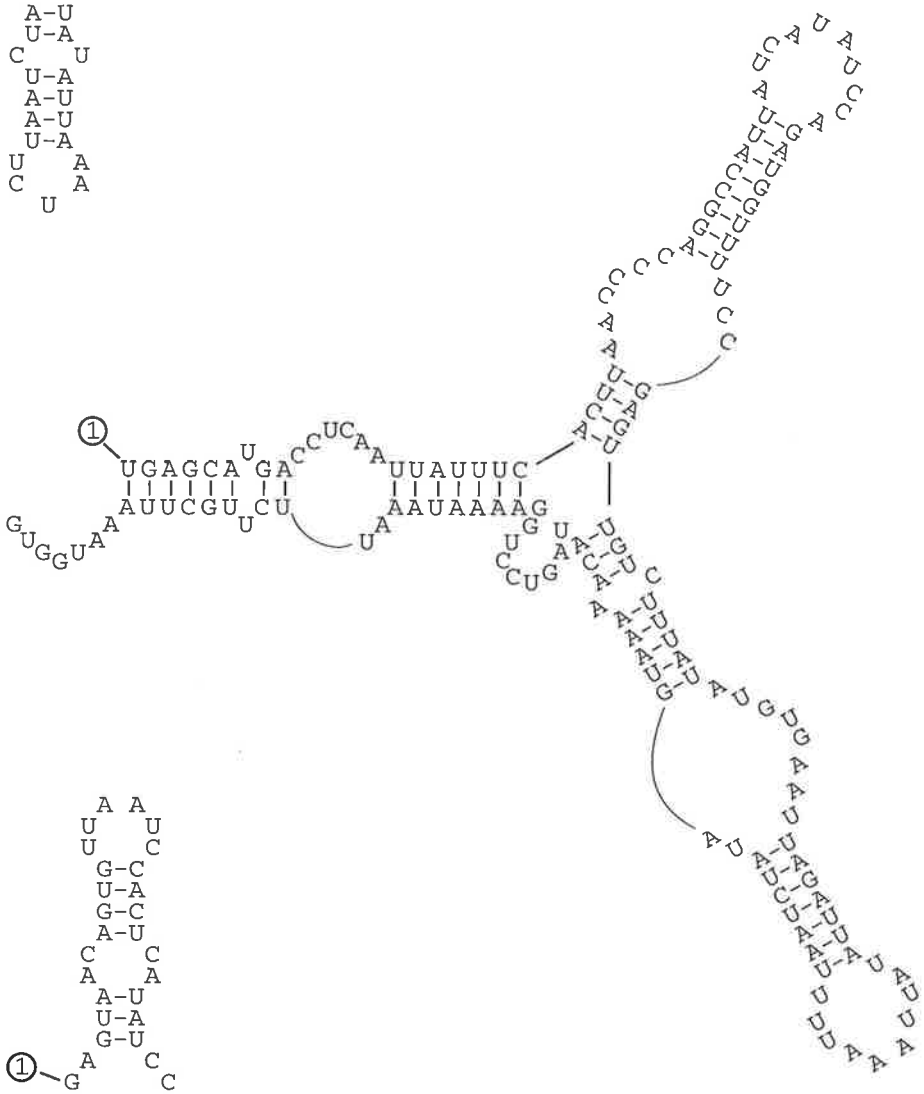
Figure 4.3: **Comparison of Potential Secondary Structures Formed by the Rat, Human and Chicken ALAS-1 3'-untranslated regions**.

Computer analysis of the 3'-untranslated regions of the rat (A), human (B) and chicken (C) ALAS-1 mRNAs for stable secondary structures revealed each could form an extensive stemloop structure. A diagrammatic representations of these structures are shown. The number 1 denotes the start of the 3'UTR.

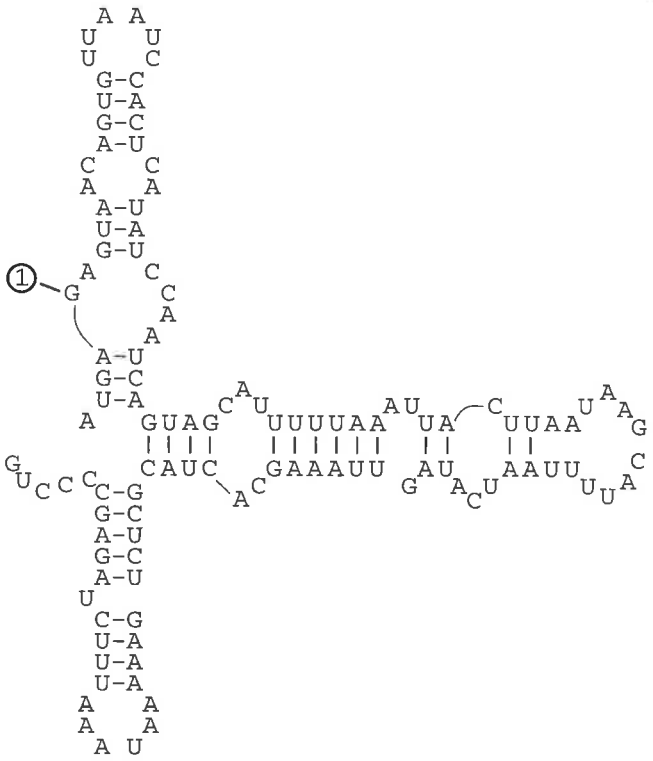
A: Rat



B: Human



C: Chicken



destabilising sequences (Shaw and Kamen, 1986 ; Vakalopoulou *et al.*, 1991 ; Shyu *et al.*, 1989). To assay for destabilising activity, putative sequences are cloned into the transcribed portion of the β-globin gene to produce a hybrid gene construct. These constructs are then stably introduced into cells and the ability of the putative destabilising sequence to affect the decay of the normally stable β-globin mRNA measured.

The rabbit β-globin expression vector pNeoRβ-globin^{GC} (Shaw and Kamen, 1986) was a kind gift from Dr. E Vakalopoulou. pNeoRβ-globin^{GC} contains a 62 base pair (bp) "GC" oligonucleotide inserted into the 3'UTR of the β-globin gene at the unique Bgl II site. This insertion has been previously demonstrated not to alter the stability of the β-globin mRNA (Shaw and Kamen, 1986 ; Vakalopoulou *et al.*, 1991). Contained within this oligonucleotide are the unique restriction site's Xba I and Sal I (fig 4.4). A schematic of the construction of pNeoRβ-globin^{UTR} from pNeoRβ-globin^{GC} is shown in fig 4.5. In this construct the Xba I to Sal I portion of the GC oligonucleotide is replaced by an 87 bp fragment of the ALAS-1 3'UTR, extending from the stop codon to immediately 5' to the poly-adenylation signal. This fragment was chosen over the entire 3'UTR since we did not want to include the rat ALAS-1 poly-adenylation signal in the construct, the inclusion of which may have resulted in its use, thereby generating hybrid molecules lacking part of the β-globin 3'UTR. The effect of removing part of the β-globin 3'UTR on the stability of the β-globin mRNA is unknown and therefore we wished to avoid this potential problem. Secondly, we wanted to compare the effect of heme on the stability of mRNAs produced from the parental construct pNeoRβ-globin^{GC} with mRNAs produced from the test construct pNeoRβ-globin^{UTR} and thus we wanted to generate mRNAs identical in all other respects.

Briefly, to generate the ALAS-1 3'UTR fragment, a Stu I to Pst I fragment from p101B1 (Srivastava *et al.*, 1988) containing the entire 3'UTR of rat ALAS-1 cDNA was cloned directionally into the Pst I and Pvu II sites of the vector pSP73 (Krieg and Melton, 1987). The Stu I site encompasses the ALAS-1 stop codon while the Pst I site flanks the cDNA insertion site. The polymerase chain reaction (PCR) was then used to generate the smaller 3'UTR fragment. One primer used RS 175 (section 2.1.7) binds

Figure 4.4: **Map of the β-globin Expression Vector pNeoRβ-globin^{GC}**

The β-globin expression vector pNeoRβ-globin^{GC} was a kind gift from Dr. Vakalopolou. In the vector the expression of the β-globin gene is directed by the polyoma virus early region promoter, while the transcription of the neomycin resistance gene (Neo) is driven by the SV40 virus early region promoter. Also shown is the unique Kpn I site used to linearize the vector and the Bgl II site used to insert the GC oligonucleotide into the 3'UTR of the β-globin gene. Indicated above the vector are the sequences for the GC oligonucleotide and the 87 bp rat ALAS-1 3'UTR fragment. The unique Xba I and Sal I sites in the "GC" insert used in the cloning of the ALAS-1 fragment are boxed. The conserved regions identified in the 3'UTR of the ALAS-1 mRNA (section 4.2.1) are underlined.

UTR-CTGACTGTGACTCAGTTATTCACAAACCCCAGACCATTACCATACCCAAATAGTAGCCAGAATTGTCTTTATATGTGAAGTAAATTATAT

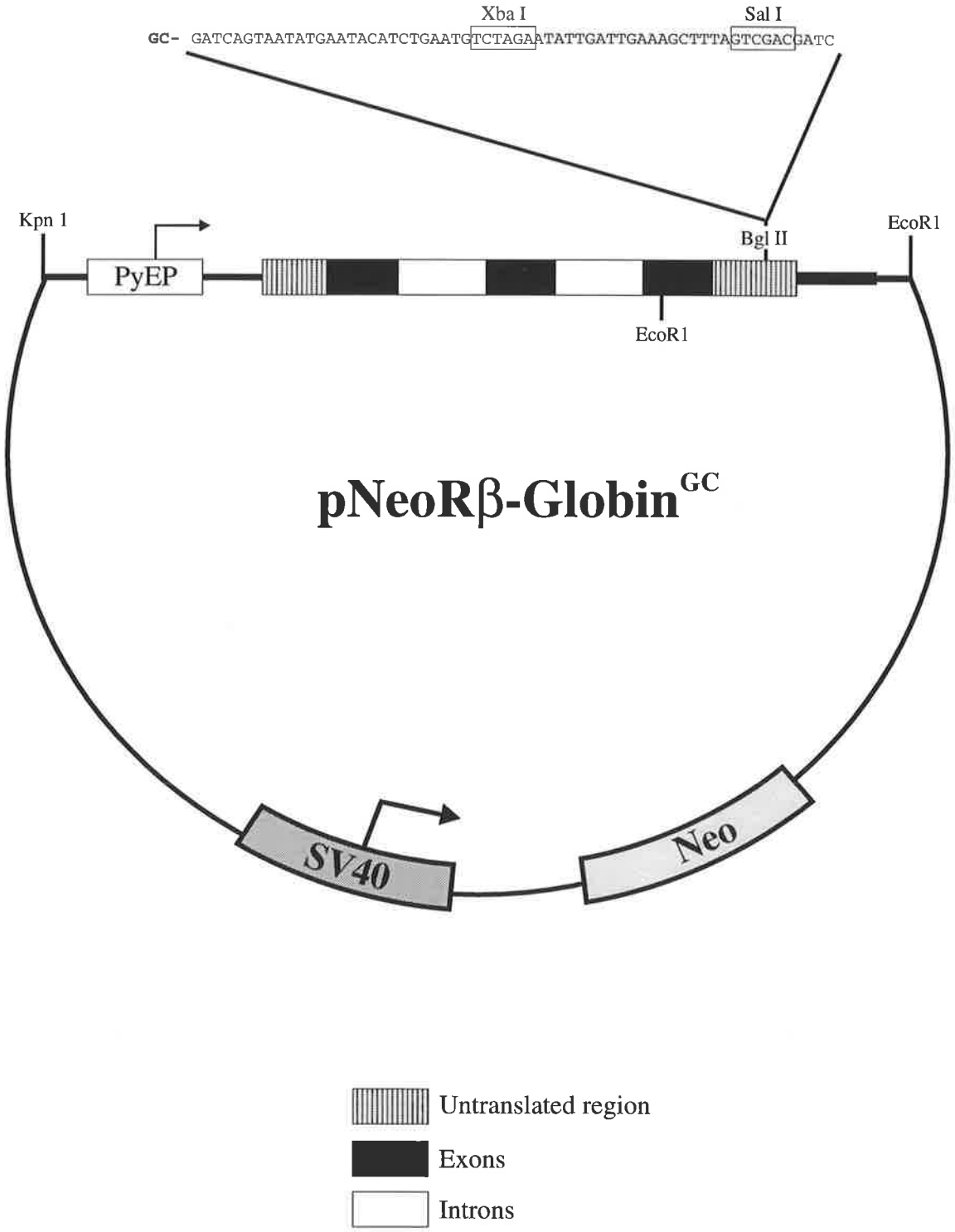
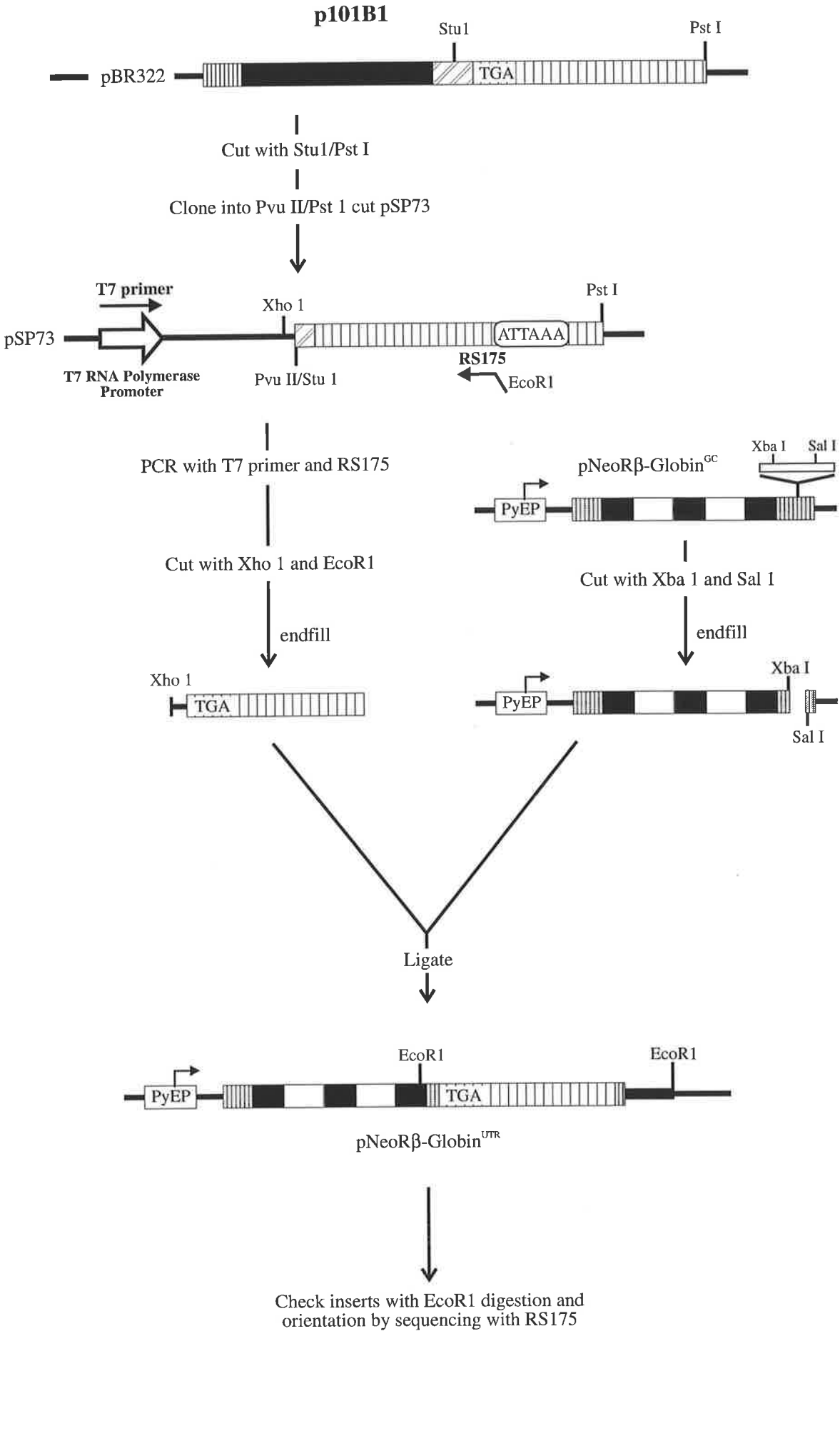


Figure 4.5: **Strategy Used to construct pNeoRβ-globin^{UTR}.**

The 160bp Stu I / Pst I fragment, containing the 3'UTR of the ALAS-1 cDNA clone p101B1, was cloned into the Pvu II and Pst I sites of pSP73. The polymerase chain reaction was then used to generate a smaller 87bp fragment, lacking the 3' end of the 3'UTR, including the poly adenylation signal. This PCR fragment contained an Eco RI and Xho I site at the 3' and 5' ends respectively. The PCR product was subsequently digested with Eco RI and Xho I, the 5' overhangs endfilled and cloned into the pNeoRβ-globin^{GC} which had been cut with Sal I and Xba I and endfilled. The Stu I site located immediately upstream of the TGA stop codon in the rat ALAS-1 gene is indicated by the hatched box. Also shown is the rat polyadenylation signal ATTAAA. The organisation of the rabbit β-globin gene is also shown with exons indicated by filled boxes, introns by open boxes and the untranslated regions by stripes.


p101B1
Stu1
Pst I
pBR322
TGA
Cut with Stu1/Pst I
Clone into Pvu II/Pst 1 cut pSP73
T7 primer
Xho 1
Pst I
pSP73
ATTAAA
RS175
T7 RNA Polymerase Promoter
Pvu II/Stu 1
EcoR1
PCR with T7 primer and RS175
pNeoRβ-Globin^GC
Xba I
Sal I
PyEP
Cut with Xho 1 and EcoR1
Cut with Xba 1 and Sal 1
endfill
endfill
Xho 1
TGA
Xba I
PyEP
Sal I
Ligate
EcoR1
EcoR1
PyEP
TGA
pNeoRβ-Globin^UTR
Check inserts with EcoR1 digestion and orientation by sequencing with RS175

immediately 5' to the rat poly-adenylation signal and contains an Eco RI site at its 5' end. The second primer used was complementary to the T7 RNA polymerase promoter (T7 section 2.1.7) located in the pSP73 vector. Following the PCR reaction, the product was purified, digested with Eco RI and Xho I, endfilled and ligated into pNeoRβ-globin^{GC} which had been digested with Xba I and Sal I and endfilled. The Xho I site used to cut the PCR product was located in the pSP73 poly-linker adjacent to the Pvu II used to clone in the 3'UTR of ALAS-1. Putative clones were identified by diagnostic Eco RI digestion (fig 4.4) and sequenced using primer RS 175 to orientate the insert with respect to the β-globin 3'UTR. To confirm the sequence of the insert, and its insertion site, the approximately 0.73kb Eco RI fragment of pNeoRβ-globin^{UTR} was cloned into the Eco RI site of the vector pTZ 19 and sequenced in both directions using the universal sequencing (USP) and T7 RNA pol primers (section 2.1.7).

4.2.3 Optimisation of FRL 4.1 Cell Transfection.

A large number of methods are available for the introduction of recombinant DNA molecules into mammalian tissue culture cells, common methods include, co-precipitation of DNA with calcium phosphate, cationic-liposome mediated transfection and electroporation. Electroporation has been used to introduce DNA into a wide variety of both plant and animal cells (Chu *et al.*, 1987 ; Andreason and Evans, 1988), including the rat hepatoma cell line H4-II-E-C3 (Braidotti, 1992). Therefore, electroporation was chosen to introduce the pNeoRβ-globin constructs into FRL 4.1 cells.

The technique of electroporation involves the exposure of a suspension of cultured cells to a pulsed electric field, which presumably creates transient pores in the cell membrane through which the DNA enters the cell (Andreason and Evans, 1988). A critical parameter for the successful transfection of cells is the strength of the electric field generated, and a number of researchers have shown that this parameter can vary greatly between different cell types (Knutson and Yee, 1987). Therefore, prior to introducing the β-globin constructs into FRL 4.1 cells, the optimal field strength for

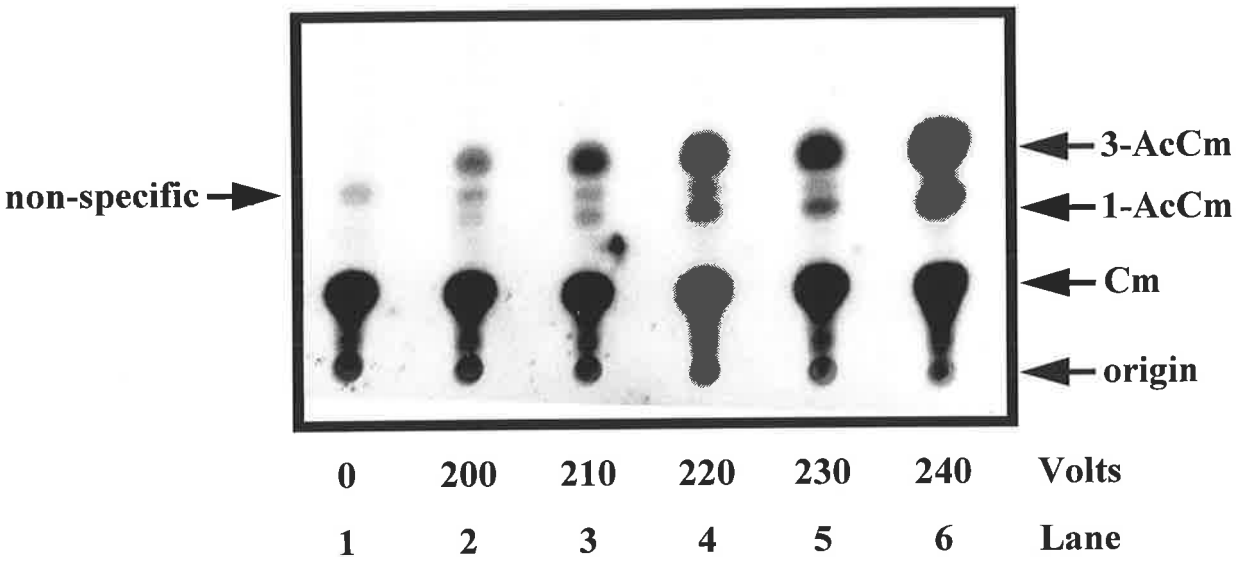
transfection was investigated. In a preliminary experiment FRL 4.1 cells were electroporated (section 2.4.4) at increasing voltages and capacitance to determine the voltage which caused approximately 50% cell death. Optimal transfection has been reported to occur under voltages which result in 50% cell death (Knutson and Yee, 1987 ; Boggs *et al.*, 1986). For FRL 4.1 cells this was found to occur from 200-220 volts using a 960μfD capacitor (Data not shown). To determine the optimal voltage for DNA uptake and expression, FRL 4.1 cells were transiently transfected, at increasing voltages, with a construct containing the chloramphenicol resistance (CAT) reporter gene under the control of the Rous sarcoma virus long terminal repeat sequence. It is well documented that the conditions optimised for transient expression are essentially identical with those required for stable transformations (Andreason and Evans, 1988). The CAT activity produced was then assayed as described in section 2.4.5 (fig 4.6). As can be seen there is an increase in the amount of CAT activity as the voltage increases up to 240 volts, however 230 volts was chosen for subsequent experiments as electroporation at 240 volts produced an unacceptably high cell mortality rate. After a long exposure of the TLC plate a non-specific band appears in all lanes including non-transfected FRL 4.1 cell extract (lane 1) and is indicated on the autoradiograph.

4.2.4 Transfection of FRL 4.1 Cells with the pNeoRβ-globin constructs.

The pNeoRβ-globin constructs were linearised by digestion with Kpn I and then 7.5μg of the linearised construct was introduced into FRL 4.1 cells by electroporation under the conditions optimised above. Cells were allowed to recover for 48hr before the addition of 100μg/ml of the neomycin analogue G418. The vector pNeoRβ-globin contains the bacterial neomycin resistance gene under the control of the SV40 virus early region and the presence of this gene therefore confers G418 resistance to the transfected cell. As a control an aliquot of FRL 4.1 cells was electroporated in the absence of DNA and cultured under the same conditions. Cells were maintained in 100μg/ml G418 for a minimum of 10 days during which time all non-stably transformed

Figure 4.6: **Transfection Efficiency Optimisation by Measurement of Cat Activity.**

The vector pRSVCAT was introduced into FRL 4.1 cells by electroporation at the indicated voltages. After 48 hours, the cells were harvested and 50μg of protein extract (where possible) was used to assay for CAT activity. An autoradiograph of the thin layer chromatography plate is shown. The position of the origin, unacetylated chloramphenicol (Cm) and the mono-acetylated forms of chloramphenicol (1-AcCm and 3-AcCm) are indicated. After long exposure times a non-specific radioactive product appears in all lanes, including non-transfected FRL 4.1 cell extract and is indicated by an arrow.


non-specific
3-AcCm
1-AcCm
Cm
origin
0
200
210
220
230
240
Volts
1
2
3
4
5
6
Lane

cells died, as judged by the viability of the control cells. Approximately 200 G418 resistant colonies for each construct were then pooled to give a heterogenous population of cells. A heterogenous population of cells allows a better comparison of the decay rates of the two β-globin mRNAs as it minimises the differences in expression of the two transcripts due to the transgene integration sites. Aliquots of these cells were then frozen at a low passage number. In subsequent experiments low passage number cells were always used to minimise any-one clone outgrowing the others due to a selection advantage.

4.2.5 The Expression of pNeoRβ-globin constructs FRL 4.1 cells.

The RNase protection assay (section 2.3.5) was employed to detect the amount of mRNA for Neo and β-globin produced in these stably transfected FRL 4.1 cells. As can be seen in fig 4.7 the mRNAs for Neo and β-globin were readily detectable in these cells using radiolabelled rabbit β-globin and Neo probes (Vakalopoulou *et al.*, 1991). Interestingly the mRNA for β-globin^{UTR} and β-globin^{GC} accumulate to very similar levels in these cells (lane 3 versus lane 4). This is in contrast to that observed when the GC insert is replaced by a functional ARE element, termed AT (Shaw and Kamen, 1986 ; Vakalopoulou *et al.*, 1991). In this case the presence of a functional destabilising element caused substantially less accumulation of the β-globin^{AT} transcript, presumably due to the mRNAs decreased half-life. The level of mRNA for the neo gene was also determined in these FRL 4.1 cells. As can be seen in fig 4.7 an equivalent amount of total RNA from the different cell populations contained approximately equivalent amounts of neo mRNA; this indicates that the vector copy number and the overall transcription rates from the constructs were approximately the same for both populations. These results implied that the 3'UTR fragment of ALAS-1 was insufficient to affect the half-life of the β-globin mRNA in FRL 4.1 cells at least in the absence of exogenously added heme. In the next experiment the effect of exogenously added heme on the half-life of the β-globin mRNAs was examined.

Figure 4.7: **Expression Levels of the Hybrid β-globin Transcripts in FRL 4.1 Cells**

RNase protection of total RNA (12μg) isolated from FRL 4.1 cell lines containing integrated copies of pNeoRβ-globin^{GC} and pNeoRβ-globin^{UTR}. Lanes 1 and 6: antisense RNA probes for β-globin and Neo respectively. Lanes 2 and 7: antisense probes plus RNase. Lanes 3,4 and 5 contain the β-globin protected product from FRL 4.1 cells transfected with pNeoRβ-globin^{GC} (lane 3), pNeoRβ-globin^{UTR} (lane 4) and non-transfected FRL 4.1 cells (lane 5). Lanes 8,9 and 10 contain the Neo protected product from FRL 4.1 cells transfected with pNeoRβ-globin^{GC} (lane 8), pNeoRβ-globin^{UTR} (lane 9) and non-transfected FRL 4.1 cells (lane 10).

1
2
3
4
5
Globin probe
6
7
8
9
10
Neo probe
Probe - RNase
Probe + RNase
pNeoβ-globin^GC
pNeoβ-globin^UTR
FRL
Probe - RNase
Probe + RNase
pNeoβ-globin^GC
pNeoβ-globin^UTR
FRL

4.2.6 The Effect of Heme on the Decay of pNeoRβ-globin^{UTR} and pNeoRβ-globin^{GC} mRNA in FRL 4.1 cells.

To determine if heme could alter the stability of the β-globin^{UTR} mRNA, total RNA was isolated at various times from FRL 4.1 cells expressing the hybrid β-globin mRNAs treated with actinomycin D or actinomycin D plus 4μM heme. The level of β-globin mRNA was then assayed for by RNase protection (fig 4.8). Due to the similar size of the protected products for Neo and β-globin the neo probe was not included in this assay. At time zero the level of β-globin mRNA produced from the pNeoRβ-globin^{UTR} construct was very similar to the amount produced from pNeoRβ-globin^{GC} (lanes 1 and 5 versus lanes 9 and 13). Indeed the stability of both mRNAs appear to be very similar, with no decrease in the amount of either β-globin^{GC} or β-globin^{UTR} mRNA over the 3.5 hours assayed. Moreover the addition of heme to the cells did not affect the level or decay rate of β-globin^{UTR} mRNA, implying that the 3'UTR of the rat ALAS-1 gene was insufficient to confer heme-mediated instability to the rabbit β-globin mRNA. As expected the β-globin transcript generated from pNeoRβ-globin^{GC} was stable (half-life > 3.5 hr), and furthermore the addition of heme to the cells did not significantly alter the decay rate of the β-globin^{GC} mRNA. To demonstrate that heme was still capable of destabilising ALAS-1 mRNA in these cells, the effect of heme on the level of endogenous ALAS-1 mRNA was determined. A Northern blot was performed using the same RNA (15μg total) as used in the RNase protection assay (fig 4.8) and the resultant filter sequentially probed for ALAS-1 and GAPDH mRNAs (fig 4.9). As expected, treatment of these cells with 4μM heme caused a rapid loss in ALAS-1 mRNA (lanes 5 to 8 and 13 to 16) compared with non-heme treated cells (lanes 1 to 4 and 9 to 12) in both β-globin^{UTR} (lanes 9 to 16) and β-globin^{GC} (lanes 1 to 8) expressing cells. The level of GAPDH mRNA did not vary in either cell population during this time course irrespective of heme or vehicle treatment. This result implies that neither the transfection/selection procedure nor the over-expression of β-globin

Figure 4.8: **The Effect of Heme on the Decay of the Hybrid β-globin mRNAs.**

Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pNeoRβ-globin^{GC} (lanes 1 to 8) or pNeoRβ-globin^{UTR} (lanes 9 to 16). At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D (lanes 1 to 4 and 9 to 12) or actinomycin D plus 4μM heme (lanes 5 to 8 and 13 to 16). 15μg of total RNA was then used in an RNase protection assay to detect the level of β-globin mRNA. Lane 18 shows the size of the β-globin anti-sense transcript used as the probe, while lane 17 contains the β-globin probe after RNase digestion. The sizes of the protected products were estimated by comparing their migration with the single stranded Hpa 1 cut pUC19 DNA markers (M).

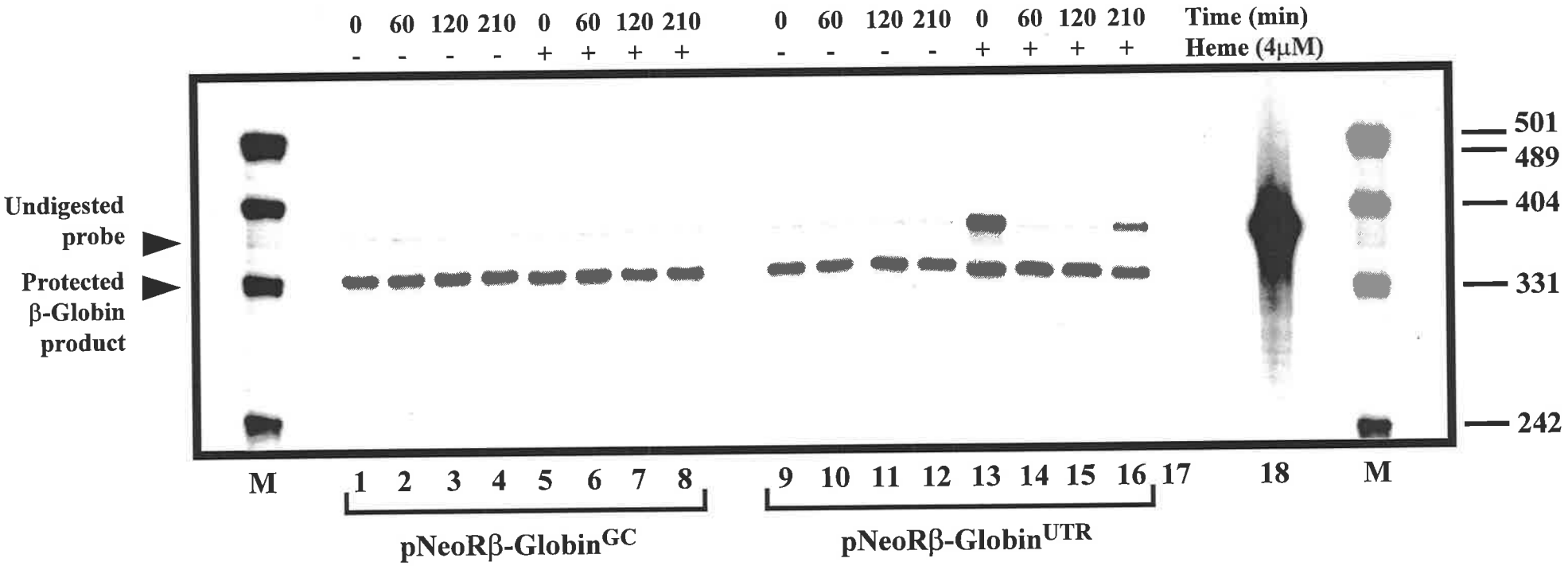
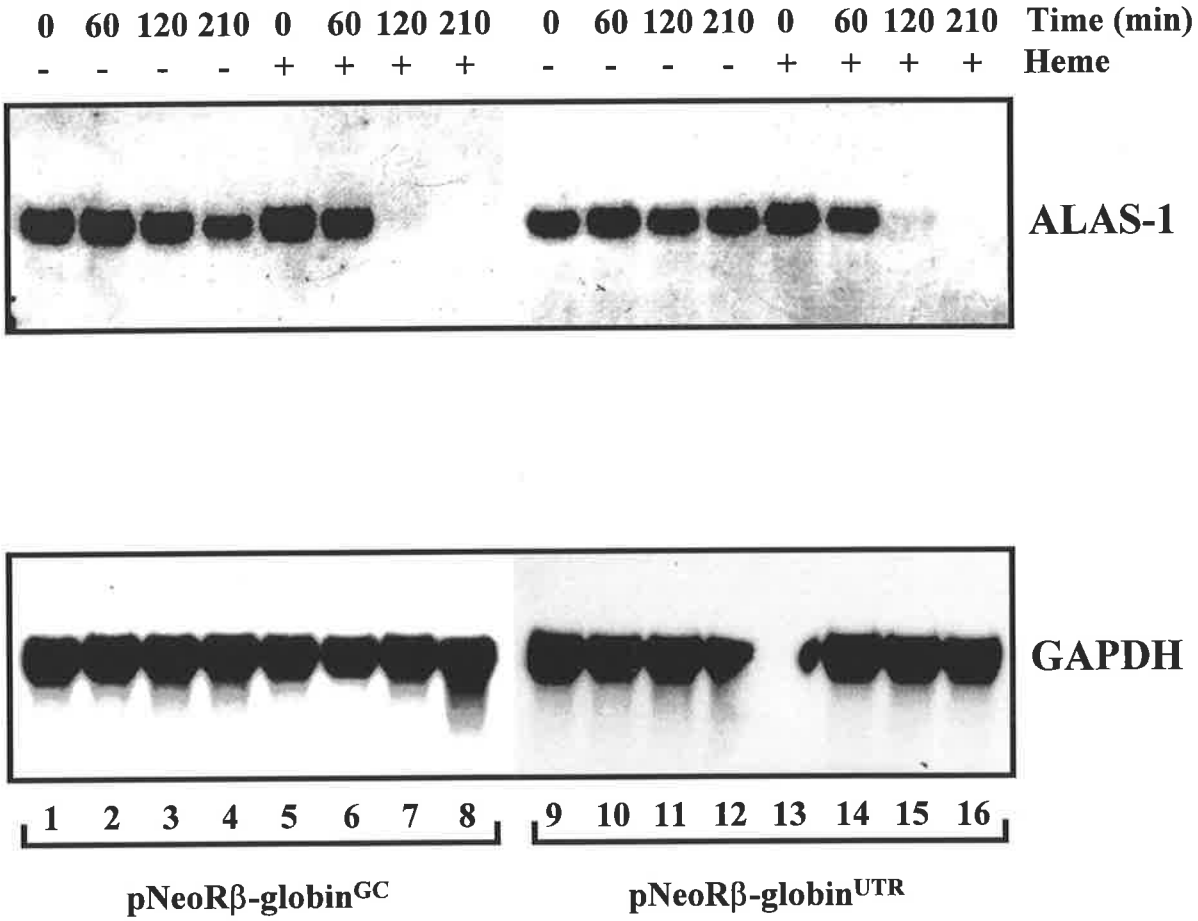

0 60 120 210 0 60 120 210 0 60 120 210 0 60 120 210 Time (min)
- - - - + + + + - - - - + + + + Heme (4μM)
Undigested probe
Protected β-Globin product
501
489
404
331
242
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M
pNeoRβ-Globin^GC
pNeoRβ-Globin^UTR

Figure 4.9: **Northern Blot Analysis of Endogenous ALAS-1 mRNA Levels in FRL 4.1 Cells Expressing the Hybrid β-globin mRNAs.**

A Northern blot was performed on 15μg of the same RNA as used in the RNase protection assay (fig 4.8). The resultant filter was sequentially probed for ALAS-1 and GAPDH mRNA. Lanes 1 to 8: RNA isolated from β-globin^{GC} expressing cells. Lanes 9 to 16: RNA isolated from β-globin^{UTR} expressing cells.


0 60 120 210 0 60 120 210 0 60 120 210 0 60 120 210 Time (min)
- - - - + + + + - - - - + + + + Heme
ALAS-1
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
pNeoRβ-globin^GC
pNeoRβ-globin^UTR

mRNA and protein in these cells, caused an inhibition of the heme-mediated destabilisation of the ALAS-1 mRNA. These results, therefore imply that the 3'UTR fragment used in this study was insufficient to confer heme-mediated destabilisation to the β-globin mRNA, suggesting the sequences responsible for the destabilisation of the ALAS-1 mRNA residue elsewhere in the mRNA.

4.3 DISCUSSION.

Differential decay rates among mRNA molecules contribute significantly to the control of gene expression in eukaryotes (section 1.8). Analysis of the determinants responsible for the differences in degradation rate has shown that these determinants are found at discrete sequences within the mRNA molecule. The experiments performed in this study were initiated to begin to characterise the *cis*-acting sequences located within the ALAS-1 mRNA required to specifically target the mRNA for rapid degradation in the presence of raised heme concentrations.

Initially the 3'-untranslated region (3'UTR) of the rat ALAS-1 mRNA was examined for destabilising activity. Hybrid gene constructs were generated in which the majority of the ALAS-1 3'UTR was inserted into the 3'UTR of the rabbit β-globin gene. However, no effect of heme on the half-life of the hybrid mRNA β-globin^{UTR} was observed. One explanation for this is that the *cis*-acting sequences required for the heme-mediated destabilisation effect reside elsewhere in the ALAS-1 mRNA. Alternatively, the lack of an effect may be due to the inability of the heme-responsive element to function in the context of the β-globin mRNA, or due to a problem in the model system used.

A potential problem with the model system used lies with the use of the β-globin gene as the reporter gene. The resulting cell populations produced are essentially over-expressing β-globin mRNA and presumably β-globin polypeptide. The β-globin polypeptide may then sequester the exogenously added heme, thus preventing a sufficient rise in the free heme pool to activate the putative heme-responsive *trans*-

acting factors. This appears unlikely for two reasons. Firstly, in the same cells, the endogenous ALAS-1 mRNA is still destabilised by heme, implying that over-expression of the β-globin mRNA or polypeptide does not interfere with the heme-mediated destabilisation of the ALAS-1 mRNA. Secondly, the concentration of heme used (4μM) in these experiments was 4 times higher than the amount of heme required to maximally repress the steady state level of the ALAS-1 mRNA, hence it appears unlikely that enough β-globin polypeptide was made to sequester sufficient heme to prevent the heme-mediated destabilisation of the ALAS-1 mRNA.

The failure of a putative heme-responsive element to function when inserted into the β-globin mRNA could also have been due to sequences within the β-globin mRNA inhibiting the function of the ALAS-1 destabilisation element. For example, the β-globin 3'UTR may contain a stabilising sequence that contributes to the extremely long half-life of the rabbit β-globin mRNA. The close proximity of this stabilising sequence to the ALAS-1 heme-responsive element may then inhibit the function of the heme-responsive region, perhaps by sterically hindering the binding of a heme-responsive *trans*-acting factor. However it should be noted that the ARE element of GM-CSF inserted into the same site of the rabbit β-globin gene functions normally, resulting in the rapid decay of the hybrid mRNA that mimicked the decay of the GM-CSF mRNA (Shaw and Kamen, 1986 ; Vakalopoulou *et al.*, 1991). The data therefore indicated that a destabilising sequence inserted into the Bgl II site in the rabbit β-globin 3'UTR will not be subjected to regulation by any potential stabilising element found in the β-globin mRNA. However, this result has only been demonstrated for the ARE destabilising sequence and may not be valid for the heme-responsive element. It is currently unknown if the β-globin mRNA contains any stability determinants in its 3'UTR, although an erythroid-specific determinant for α-globin mRNA stability has recently been described (Weiss and Liebhaber, 1994 ; Weiss and Liebhaber, 1992 ; Wang *et al.*, 1994). This stability determinant only appears to be important in erythroid cells, as mutants which disrupt the function of the element, such as the α-thalassemia's Constant-Spring, KD and Icaria, only have a detrimental affect on the half-life of the α-

globin mRNA in erythroid cells. In non-erythroid cells the half-life of the mutant and wild type α-globin mRNA's are both extremely long (Weiss and Leibhaber, 1994).

A similar erythroid-specific stabilisation of the mouse transferrin receptor mRNA has been reported in differentiating murine erythroleukemic (MEL) cells and this increased stability of the transferrin receptor mRNA has been proposed to be due to the presence of a similar element as found in the α-globin mRNA (Chan *et al.*, 1994). This has led to the proposal that many mRNAs, which encode proteins important for erythropoiesis, are stabilised by a common mechanism in erythroid cells. Therefore, it is possible that the β-globin mRNA also contains stabilising elements, and that these stability determinants are capable of regulating the activity of the heme-responsive element. The relevance of this is unclear, as presumably any specific β-globin stability determinant , like the stability determinants of α-globin and transferrin receptor, only functions in erythroid cells. Secondly, it is unknown if the α-globin, and therefore any potential β-globin stability determinant, can regulate the activity of a heterologous instability determinant. However, as FRL 4.1 cells were isolated from fetal liver from 19-day gestation embryos, a time at which the liver is the major site of erythropoiesis, it must be considered that FRL 4.1 cells may express the necessary proteins to stabilise erythroid-specific mRNAs. This however appears to be unlikely, as the FRL 4.1 cell line was selected for on the basis of expression of a number of liver-specific markers (Yeoh *et al.*, 1990) implying that the cell line is hepatic-like and not erythroid-like. Also FRl 4.1 cells do not express detectable levels of the mRNAs for β-globin or the erythroid-specific ALAS-2 isoenzyme again indicating the non-erythroid nature of the cell line.

Taken together the most likely interpretation of the data is that the 3'UTR fragment does not contain the heme-responsive instability element. One possibility is that the heme-responsive destabilisation sequence is located 3' to the poly(A) signal and thus was not included in the β-globin mRNA. Included in this missing region are two of the sequences, conserved in all three species, and thus, candidates for the putative heme-responsive *cis*-acting sequence. Whether this region is important in the heme-mediated destabilisation effect is examined in the next chapter.

Alternatively, the elements involved in the heme-mediated destabilisation effect may be located in regions other than the 3'UTR. One possibility is that the heme-responsive sequences reside in the 5'UTR of the mRNA. Although a role for specific sequences located in the 5'UTR in mRNA degradation has not yet been conclusively established, several studies have implicated the 5'UTR in the regulation of mRNA decay. For instance, the 5'UTR of the viral v-fos oncogene appears to be able to regulate the activity of the ARE element of c-fos and GM-CSF, in hybrid mRNAs containing the two elements (Roy *et al.*, 1992). Secondly, replacement of the β-globin 5'UTR with the 5'UTR of the c-fos gene results in an approximately 2.5 fold decrease in the half-life of the hybrid β-globin transcript (Kabnick and Housman, 1988), suggesting the c-fos 5'UTR contains an instability determinant. Lastly, an oestrogen-dependent polysomal protein which binds to the 5'UTR of the chicken vitellogenin II mRNA has been implicated in the oestrogen-mediated stabilisation of this mRNA (Liang and Jost, 1991). However, what remains to be determined is whether these effects of the 5'UTR, are brought about by the well documented ability of the 5'UTR to alter translation levels, and hence only affect mRNA turnover indirectly, or due to a more direct influence on mRNA degradation.

The second possibility, is that the heme-responsive elements reside in the coding region of the ALAS-1 mRNA. There are now a number of well defined examples of instability determinants located in the coding portion of the mRNA. These include the coding region determinants (CDR) of c-fos (Shyu *et al.*, 1989 ; Schiavi *et al.*, 1994), c-myc (Wisdom and Lee, 1991 ; Prokipcak *et al.*, 1994), β-tubulin (Theodorakis and Cleveland, 1992) and the yeast MATα1 mRNA (Parker and Jacobson, 1990). If the heme-responsive sequence does reside in the coding portion of the ALAS-1 mRNA, it would appear that it mediates the decay of the ALAS-1 mRNA by a novel pathway. Interestingly, the function of the other previously characterised coding region determinants have been coupled to translation, whereas experiments performed with cycloheximide (chapter 3) suggest that the heme-mediated decay of the ALAS-1 mRNA occurs independently of translation.

Lastly, it is possible that the heme-responsive *cis*-acting sequence is a composite element and may require the interaction of sequences found in more than region. Several examples have now been characterised in which RNA elements involved in mRNA decay can interact over large distances. The best characterised of these is the decay of the yeast *MFA2* mRNA (Mulhlrad *et al.*, 1994). In the decay of the MFA2 mRNA, sequences in the 3'UTR promote rapid de-adenylation, which in turn stimulates the removal of the 5' cap structure. One model developed to explain this, proposes that the 5' and 3' termini normally interact with one another to stimulate both translation (Sachs and Deardorff, 1992) and to prevent decapping (Decker and Parker, 1994). The instability sequences, by changing the adenylation state of the mRNA, alter or disrupt this interaction thus leading to de-capping and transcript decay. Although, it is unknown if such a mechanism exist in higher eukaryotes, the conserved nature of the poly(A) tail and 5' cap structures, suggest that such a mechanism could exist.

To further investigate the location of the heme-responsive element(s) in the ALAS-1 mRNA a series of hybrid constructs were made in which different regions of the human ALAS-1 mRNA were transposed with the corresponding region of the β-globin mRNA. This work is described in detail in the following chapter.

CHAPTER FIVE

LOCALISATION OF THE HUMAN ALAS-1 mRNA INSTABILITY DETERMINANT TO THE CODING REGION

CHAPTER FIVE: LOCALISATION OF THE HUMAN ALAS-1 mRNA INSTABILITY DETERMINANT TO THE CODING REGION.

5.1 INTRODUCTION

In the previous chapter the 3'-untranslated region of the rat ALAS-1 mRNA was examined for destabilising activity. Hybrid gene constructs were synthesised in which the majority of the rat ALAS-1 3'UTR was inserted into the 3'UTR of the rabbit β-globin gene. However, no effect of heme on the half-life of the hybrid mRNA was observed, suggesting that the *cis*-acting sequences required for the heme-mediated destabilisation effect reside elsewhere in the ALAS-1 mRNA.

Described in this chapter, is the construction and testing of a more extensive series of hybrid ALAS-1/β-globin gene constructs, designed to identify the region(s) of the ALAS-1 mRNA important for the heme-mediated destabilisation effect. Initially, the ALAS-1 mRNA was divided into three regions, the 5'-untranslated, coding and 3'-untranslated regions and the ability of each of these regions, whether individually or in combination, to confer heme-mediated instability to the hybrid mRNAs was then tested in stably transfected FRL 4.1 cells.

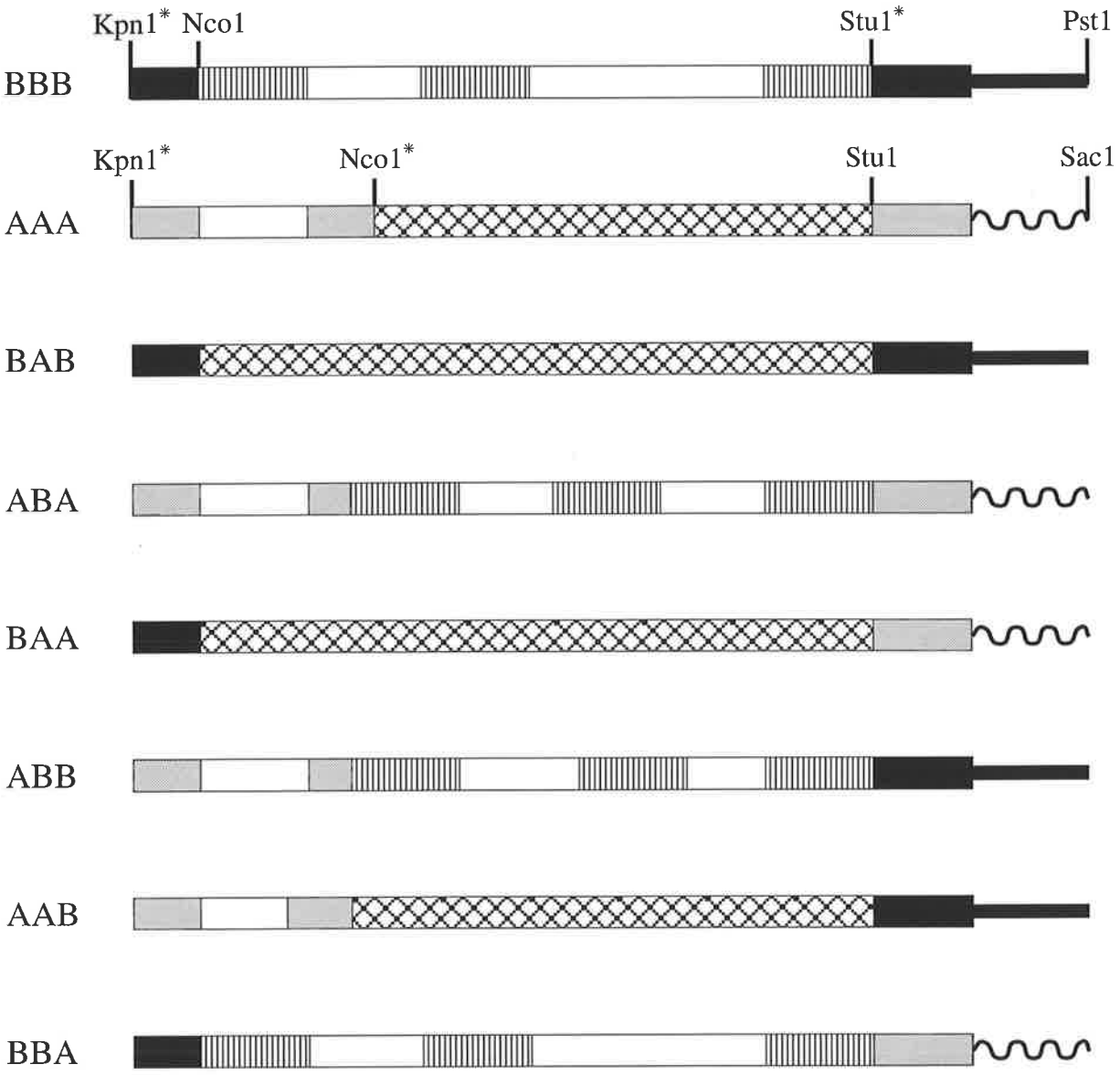
5.2 RESULTS.

5.2.1 Strategy for the Construction of ALAS-1/β-globin Hybrid Gene Constructs.

To assay for destabilising activity we wished to transpose the three regions of the ALAS-1 mRNA (ie. the 5'UTR, the coding and the 3'UTR) with the corresponding region of the β-globin mRNA, to form the eight genes depicted in figure 5.1. In this series of hybrid genes each region of the ALAS-1 mRNA can be tested for destabilising

Figure 5.1: **Chimeric Human ALAS-1/β-globin genes.**

The chimeric ALAS-1/β-globin gene constructs are shown opposite. The different regions of the mRNAs are indicated by black rectangles for β-globin untranslated regions; grey rectangles for ALAS-1 untranslated regions; stripped rectangles for β-globin coding regions; hatched rectangles for ALAS-1 coding region; open rectangles for introns; thick lines for β-globin 3'-flanking sequences and wavy lines for ALAS-1 3'-flanking sequences. Sites created to facilitate cloning steps are indicated by asterisks.


Kpn1* Nco1
Stu1*
Pst1
BBB
Kpn1*
Nco1*
Stu1
Sac1
AAA
BAB
ABA
BAA
ABB
AAB
BBA

activity, either individually or in combination with another region. Also synthesised were genes that resulted in the expression of the wild-type ALAS-1 (AAA) and β-globin (BBB) mRNAs to act as controls.

Unfortunately, in our laboratory only a partial clone of the rat ALAS-1 gene has been isolated from the screening of a rat genomic λ library (Braidotti *et al.*, 1993). This partial clone lacks a substantial portion of the 3'-end of the gene. At the same time, genomic clones for the human ALAS-1 gene were isolated from a human cosmid library. Since, a number of human clones were isolated it was likely that at least one of these contained the 3'-end of the human ALAS-1 gene. Therefore, we decided to use the human ALAS-1 gene, in the hybrid ALAS-1/β-globin gene constructs, instead of isolating and characterising more rat ALAS-1 genomic clones. We, reasoned that the use of the human ALAS-1 gene in the hybrid constructs would be acceptable, for the following reasons. Firstly, in the human hepatoma cell line HepG2, heme represses the steady state level of ALAS-1 mRNA at a post-transcriptional level (Healy, 1990). This data strongly supports the notion that the human ALAS-1 mRNA is also destabilised by heme. Secondly, the use of the human gene in the hybrid gene constructs, allowed for the differentiation of the signal produced from the construct and the endogenous rat gene in an RNase protection assay. Indeed by using human and rat specific ALAS-1 probes the change in the level of both transcripts (the endogenous and the hybrid) can be followed simultaneously, allowing for a better comparison of the effect of heme on the two mRNAs. At the nucleotide level, the human and rat ALAS-1 mRNAs are very similar, and thus any heme-responsive element found in the human mRNA is likely to be the same as the element found in the rat mRNA. The transfection experiments were again performed in the rat hepatoma cell line FRL 4.1 rather than human hepatoma cells, due to the formers ability to grow in the absence of serum and thus the results obtained are free of any influence of serum, and in particular any heme present in the serum.

Recently, the entire rat ALAS-1 gene has been isolated and was found to consist of eleven exons which encompassed more than 14kb (Yomogida *et al.*, 1993). Preliminary mapping of the human ALAS-1 gene also estimated the size of the human

gene to be greater than 13kb (Healy, 1990). The large size of the human gene makes it extremely difficult to manipulate and therefore it was decided to use the coding region of the ALAS-1 cDNA in the hybrid gene constructs instead of the equivalent genomic region.

In order, to begin to synthesise the hybrid β-globin/ALAS-1 constructs depicted in figure 5.1 full length copies of the human ALAS-1 5'UTR and 3'UTR needed to be isolated and several restriction sites incorporated into the ALAS-1 and β-globin genes to facilitate the transposition of the different regions

Firstly, we wished to isolate the ALAS-1 3'UTR as a genomic fragment which also contained 3'-flanking sequences. This was to ensure that the hybrid mRNAs produced are processed and polyadenlyated at the same position as the endogenous ALAS-1 molecule. In the case of mammalian mRNAs the creation of the 3'-end of the molecule is a multistage process that involves an endonucleolytic cleavage of the nascent transcript which is coupled, in most cases, with the synthesis of a poly(A) tail at the 3'-end of this processed transcript and finally the release of the elongating RNA polymerase II complex from the DNA template although, the processing of the 3'-end appears to be independent of RNA polymerase II termination (Wahle, 1992).

The signal and molecular events that promote the cleavage-poly adenylation reaction are now well defined. This signal consists of two elements, the sequence AAUAAA located 20-30 nucleotides before the end of the mRNA and a GU or U-rich sequence located within the first 50 nucleotides downstream of the 3'-end of the mature mRNA (Proudfoot, 1991 ; Wahle and Keller, 1992 ; Manley and Proudfoot, 1994).

The sequences and molecular events involved in the termination of transcription are still poorly defined. However, it is clear, that a functional poly(A) signal is essential for the normal termination of poly(A)$^+$ mRNAs (Whitelaw and Proudfoot, 1986 ; Logan *et al.*, 1987 ; Connelly and Manley 1988). Several recent studies, investigating the transcriptional termination between closely linked genes have also implicated sequences downstream of the poly(A) signal in the termination process. These downstream sequences appear to function as RNA polymerase II pause sites. The identification of the downstream sequences in closely linked genes has led to the proposal that the

termination signal for RNA polymerase II exists as a bipartite sequence, consisting of a poly(A) signal and a downstream pause site (Connelly and Manley, 1989 ; Ashfield *et al.*, 1991 ; Enriquez-Harris *et al.*, 1991). However the mechanism whereby the poly(A) signal and the transcriptional pause site cause termination is still unknown.

It is also unclear if the downstream pause site is required for the termination of most mRNA genes, which normally occurs at heterogeneous sites, located over hundreds of basepairs (Proudfoot, 1989). In the case of closely linked genes the cleavage and termination processes need to be closely coupled to prevent transcriptional interference of the downstream promoter by read-through of the RNA polymerase from the upstream gene (Cullen *et al.*, 1984 ; Proudfoot, 1986), and it has been proposed that the bipartite poly(A)-pause site prevents this (Eggermont and Proudfoot, 1993).

Secondly, a full length copy of the ALAS-1 5'UTR needed to be isolated as the human cDNA clones previously isolated in our laboratory are truncated in the 5'UTR. To isolate the full length 5'UTR either 5' RACE PCR could be attempted or the 5'UTR isolated from the genomic ALAS-1 clones. However, as described in section 1.5, the rat and human ALAS-1 5'UTR is interrupted by an intron of approximately 1kb in size. Promoter expression studies in which the ALAS-1 promoter and its 5'UTR (minus or plus this intron) were used to drive the expression of the CAT or growth hormone reporter genes have implicated this intron in the regulation of ALAS-1 expression (Braidotti *et al.*, 1993 ; Healy, 1990).

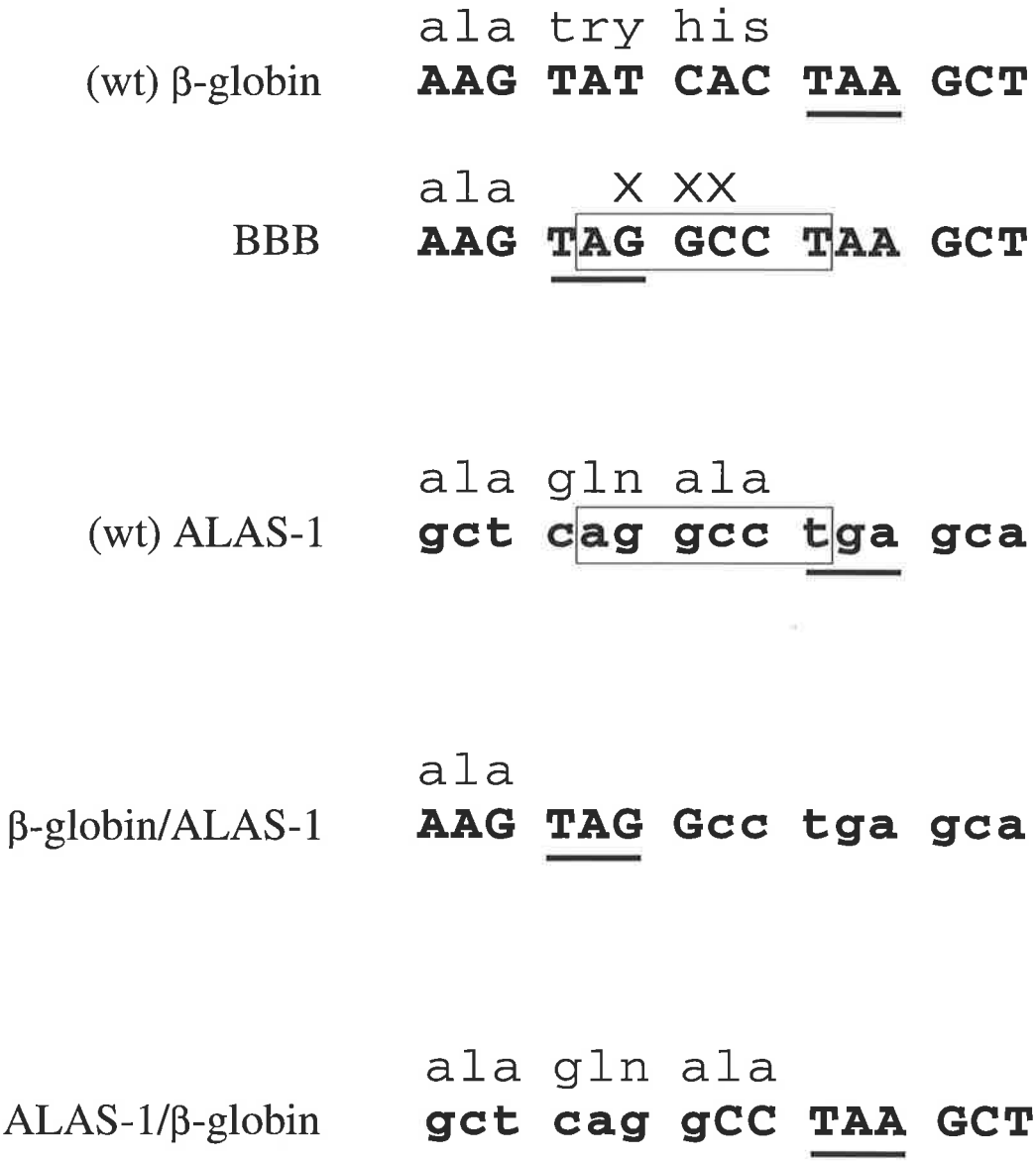
Introns have been implicated in the control of gene expression in a number of other genes. Some introns contain enhancer elements and stimulate gene expression by increasing the transcription rate of the gene, examples of this include the thyroid hormone α-receptor (Lazar *et al.*, 1994), mouse immunoglobulin chain (Gillies *et al.*, 1983) and the rat α2μ globulin (Choy *et al.*, 1989) genes. In addition, a number of studies have found that the insertion of an intron into an intronless gene construct leads to an increase in the level of the mRNA (Dewille *et al.*, 1988 ; Li *et al.*, 1991 ; Palmiter *et al.*, 1991). In many cases this increase in the steady state level of the mRNA is not reflected by any change in the transcription rate of the gene. Rather, this effect appears to be due to the presence of the intron as such, and not some specific sequence, as

foreign or chimeric introns produce the same effect as the native intron when inserted into the intronless gene (Korb *et al.*, 1993). This "general" stimulatory effect of introns on mRNA levels is still poorly understood but recent experiments have linked this effect with the splicing reaction (Buchman and Berg, 1988 ; Korb *et al.*, 1993). Perhaps the association of the splicing machinery with the nascent RNA transcripts targets the RNA for more efficient transport to the cytoplasm. A defined processing pathway has been proposed in the "RNA Track" model of RNA processing and transport from the nucleus to the cytoplasm (Reviewed Rosbash and Singer, 1993). The presence of the intron in the 5'UTR of the ALAS-1 gene would thus be an advantage as it may ensure the efficient processing and transport of the hybrid mRNAs from the nucleus to the cytoplasm (the presumed site of ALAS-1 mRNA degradation). This is particularly important for the hybrid mRNAs containing the ALAS-1 coding region, which lacks any intronic sequences. Therefore it was decided to isolate the ALAS-1 5'UTR as a genomic fragment which thus contained intron 1.

To allow the transposition of the different ALAS-1 and β-globin regions a number of restriction sites needed to be created at the boundaries between these regions (fig 5.1). To transpose the 5'UTR of the ALAS-1 and β-globin genes, a Kpn I site was created 15bp and 8bp upstream of the β-globin and ALAS-1 CAP sites respectively. In addition an Nco I site was created that spanned the ALAS-1 initiation codon; the human β-globin gene has a naturally occurring Nco I site at this position. Hence the 5'UTRs of the two genes could be transposed as Kpn I/Nco I fragments. To swap the 3'UTR of the two genes a Stu I site was created immediately 5'-prime to the β-globin stop codon; the ALAS-1 gene contains a natural Stu I site at this position. The placement of a Stu I site at this position in the β-globin gene created an in-frame stop codon upstream of the normal β-globin termination codon in both the parental β-globin (BBB) gene and those hybrid β-globin genes that contained the 3'UTR of the ALAS-1 gene. This in frame stop codon resulted in a β-globin polypeptide which lacked the last two amino acids normally found at the carboxyl-end of the β-globin polypeptide (fig 5.2) The hybrid ALAS-1 mRNAs containing the 3'UTR of the β-globin gene terminate translation at the normal position (fig 5.2).

Figure 5.2: **Nucleotide and Amino-Acid Sequence Surrounding the Termination Codon of the Hybrid genes.**

The creation of the Stu I site at the 3'-end of the human β-globin protein coding region caused the premature termination of translation in the parental β-globin BBB and hybrid BBA and ABA genes. Stu I sites are indicated by the box, with the bases changed in the wild type β-globin gene to form BBB indicated by crosses. In frame termination codons are underlined and the resultant amino acid sequence is shown above each nucleotide sequence. Nucleotides from the β-globin gene are shown as capitals, while those nucleotides derived from the ALAS-1 gene are shown as lower case letters.


ala try his
(wt) β-globin
AAG TAT CAC TAA GCT
ala X XX
BBB
AAG TAG GCC TAA GCT
ala gln ala
(wt) ALAS-1
gct cag gcc tga gca
ala
β-globin/ALAS-1
AAG TAG Gcc tga gca
ala gln ala
ALAS-1/β-globin
gct cag gCC TAA GCT

5.2.2 Construction of the parental ALAS-1 (AAA) and β-globin (BBB) genes.

Initially, the two parental genes (AAA) and (BBB) were synthesised to act as the templates for the generation of the remaining of the hybrid gene constructs depicted in figure 5.1. However as discussed, we firstly wished to isolate the human ALAS-1 3'UTR as a genomic DNA fragment which contained several hundred bases downstream of the 3'-end of the mature mRNA. This was to ensure that all of the sequences necessary for, at least, the proper processing of the nascent hybrid transcripts were present in the hybrid constructs.

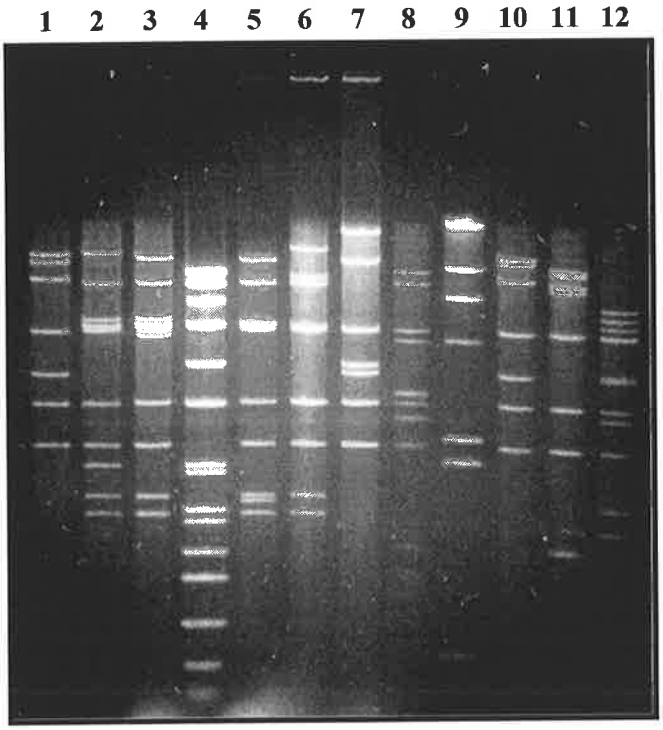
i) Isolation of the 3'-end of the human ALAS-1 gene.

Previously, in our laboratory 10 genomic clones were isolated from a human genomic cosmid library (Bawden unpublished), by hybridisation with a fragment (from the 5'-end of the cDNA to just downstream of the Bam HI site at +1564) of the human ALAS-1 cDNA (Bawden *et al.*, 1987), but the clones had not been analysed for the existence of the 3'-end of the human ALAS-1 gene. Therefore to determine which, if any, of these cosmid clones contained the 3'-end of the gene, the clones were digested with Eco RI and Southern blot analysis performed (Section 2.2.13). The resultant filter was then probed for the 3'-end of the gene with a fragment of the human ALAS-1 cDNA that extended from the Sac I site at +1796 to the end of the cDNA. This probe contained 216bp of coding region and 120bp of 3'UTR. As can be seen in fig. 5.3, six of the ten cosmid clones cross-hybridise to the 3'-end probe, including cosmid 2, 7, 9, 11, 13 and 21. In all cases the probe hybridised to a single Eco RI fragment of approximately 11kb in size for cosmids 2, 7, 9, 11 and 21 and 13kb for cosmid 13, suggesting that the 3'-end of the ALAS-1 gene is contained within this single Eco RI fragment. The reason for the size discrepancy in the Eco RI generated fragment, which cross-hybridises with the probe is unknown. It is possible that the 11kb Eco RI fragment of cosmids 2, 7, 9, 11 and 21 is located at one end of the genomic DNA insert and therefore this fragment is generated by Eco RI cleaving once in the genomic DNA and once in the cosmid vector

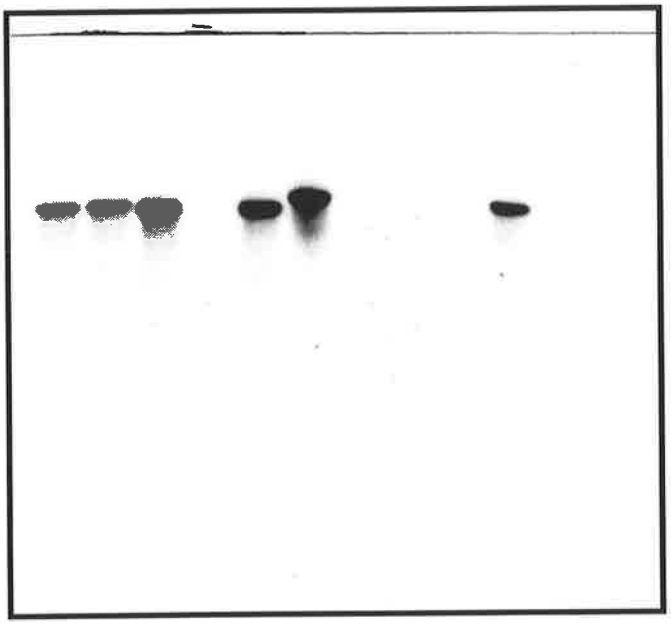
Figure 5.3: **Southern Blot Analysis of the Human ALAS-1 Genomic Clones, for the 3'-end of the gene.**

DNA from ten cosmid clones, previously isolated in our laboratory, was digested with Eco RI and electrophoresed on a 1.0% agarose gel. The DNA fragments were visualised after staining with ethidium bromide, prior to their transfer to a nylon membrane (Nytran) by Southern blot transfer (section 2.2.13). The resultant filter was then probed with a Sac I (+1796) to Eco RI (polylinker) fragment of the human ALAS-1 cDNA that represents the 3'-end of the ALAS-1 cDNA. (A) Photograph of the ethidium bromide gel. (B) Image from PhophorImager that shows the six cosmids which contained an Eco RI fragment that cross-hybridised with the probe; lane 1, cosmid 2; lane 2, cosmid 7; lane 3, cosmid 9; lane 5, cosmid 11; lane 6, cosmid 13; lane, 7 cosmid 14; lane 8, cosmid 19; lane 10, cosmid 21; lane 11, cosmid 24 and lane 12, cosmid 26. Lanes 4 and 9 contain Eco RI cut SPP-1 DNA and Hind III cut λ DNA molecular weight markers respectively. (C) Map of the human ALAS-1 cDNA in pTZ18.

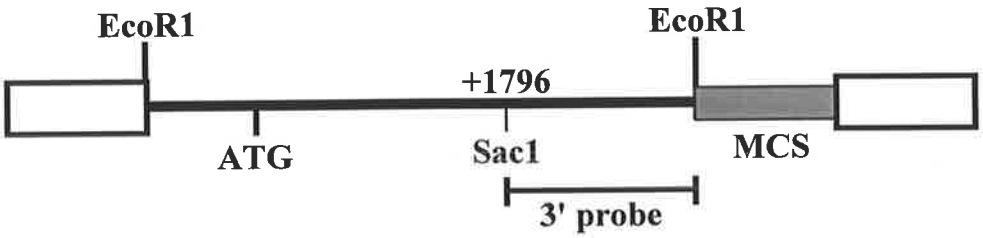
A


1 2 3 4 5 6 7 8 9 10 11 12
23.13
8.51

B


23.13
8.51

C


EcoR1
EcoR1
+1796
ATG
Sac1
MCS
3' probe

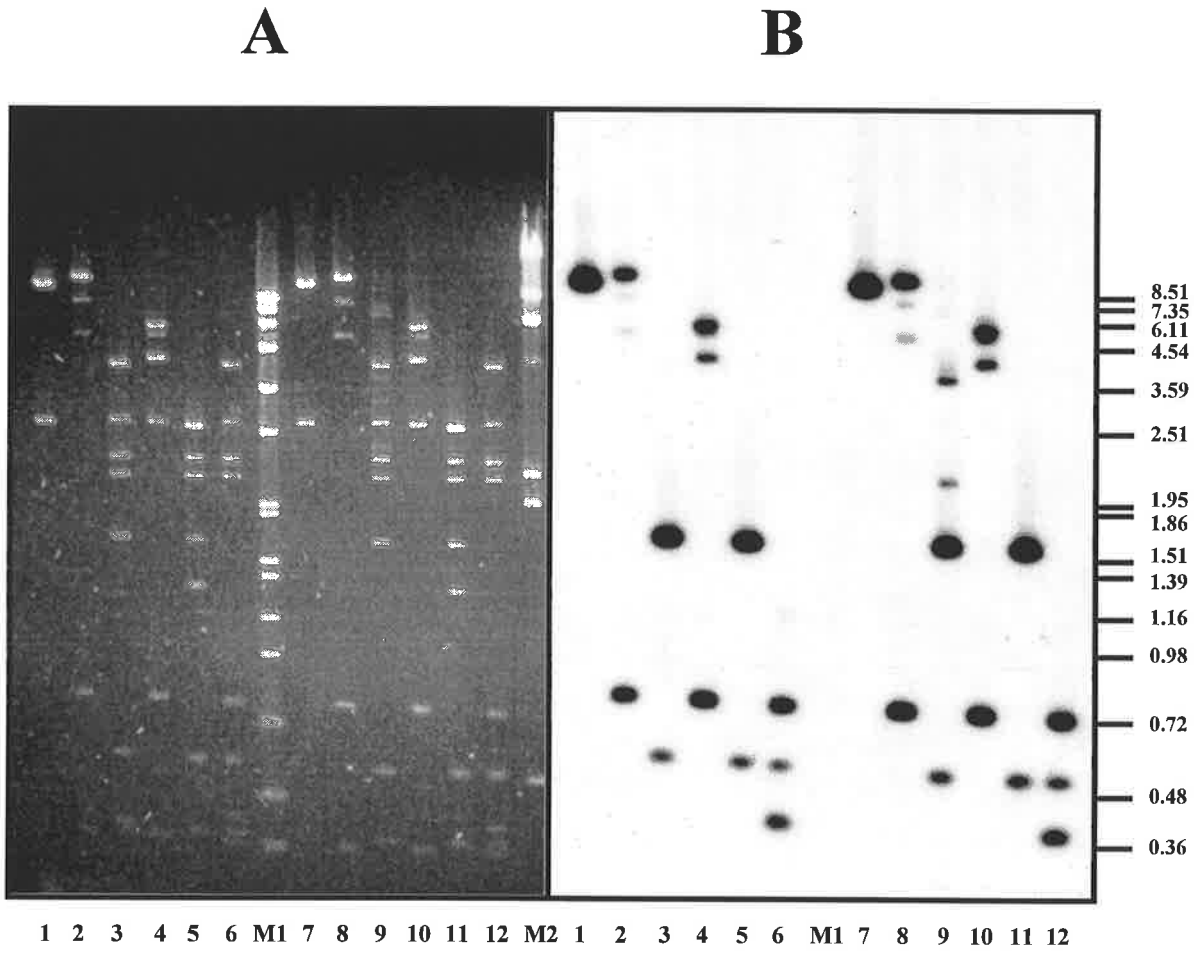
pAVCV007. The cosmid vector contains two Eco RI site's that flank the genomic DNA insertion site (Choo *et al.*, 1986).

To further characterise the 3'-end of the ALAS-1 gene the 11kb Eco RI fragment from cosmids 2, 7, 9, 11 and 21 and the 13kb fragment from cosmid 13 were isolated and cloned into Eco RI digested pBluescript KS$^-$ (pBKS$^-$). Several positive clones were isolated which contained the 11kb Eco RI fragment of cosmid 2 and these were termed pBK2.E11. To begin to map the 3'-end of the ALAS-1 gene by restriction digest analysis, Southern blot analysis was carried out on two pBK2.E11 clones. Southern blot analysis was performed on 2µg pBK2.E11 DNA digested with the restriction enzymes Eco RI, Stu I or Sac I individually or in combination, and the resultant filter probed with the Sac I/Eco RI 3'-end probe (fig 5.4). The 3'-probe hybridises to two fragments of size 1.6kb and 0.6kb (lanes 3 and 9) in Sac I digested pBK2.E11 and four bands of size 11, 8.2, 5.8 and 0.75kb in Stu 1 digested DNA (lanes 2 and 8). Since the Sac I site at position +1796 is unique in the ALAS-1 cDNA and forms the 5'-end of the probe only a single Sac I fragment was expected to hybridise to the probe if this region was contained within a single exon in the ALAS-1 gene. Therefore, the hybridisation of the probe to two fragments suggests that an intron exists between the Sac I site and the 3'-end of the gene and that this intron must contain a Sac I site. Analysis of the intensity of fluorescence of the 8.2 and 5.5kb Stu I restriction fragments on the ethidium bromide stained gel (fig5.4 A; lane 2 and 8) suggested that Stu I had only partially digested the DNA, making it difficult to ascertain the true hybridisation pattern for Stu I.

In support of the proposal that an intron lies between the Sac I site (found in the cDNA) and the 3'-end of the ALAS-1 gene, double digestion of pBK2.E11 DNA with Stu I/Sac I and Stu I/Eco RI resulted in three fragments cross-hybridising with the probe. The sizes of the fragments were 0.42, 0.6 and 0.75kb for the Sac I/Stu I digested pBK2.E11 DNA and 0.75, 4.5 and 6.11kb for the Eco RI/Stu I digested pBK2.E11 DNA. A single Stu I site, at +2011, is located between the Sac I site and the end of the ALAS-1 cDNA and therefore if the region of the ALAS-1 gene complementary to the 3'-probe was contained within a single exon, the digestion of pBK2.E11 with Sac I and Stu I should have resulted in only two fragments cross-hybridising with the probe.

Figure 5.4: **Restriction Mapping of the 3'-end of the Human ALAS-1 Gene.**

2μg of pBKE.11 DNA was cut with the following enzymes: lanes 1 and 7, EcoRI; lanes 2 and 8, Stu I; lanes 3 and 9, Sac I; lanes 4 and 10, Eco RI and Stu I; lanes 5 and 11, Eco RI and Sac I and lanes 6 and 12, Stu I and Sac I. Lanes M1 and M2 contained Eco RI cut SPP-1 and Hind III cut λ DNA respectively. The DNA fragments were resolved on a 1.0% agarose gel and were stained and photographed (A) prior to Southern blot analysis. The resultant filter was then probed with the Sac I to Eco RI 3'-end probe. Fragments which cross-hybridised with the probe were detected by exposing the filter to a PhosphorImager cassette. The image produced is shown in part B.


A
B
8.51
7.35
6.11
4.54
3.59
2.51
1.95
1.86
1.51
1.39
1.16
0.98
0.72
0.48
0.36
1 2 3 4 5 6 M1 7 8 9 10 11 12 M2 1 2 3 4 5 6 M1 7 8 9 10 11 12

Furthermore one of these fragments should have been 215bp in size. Digestion of pBK2.E11 with Eco RI and Stu I also produced three fragments which cross-hybridised with the probe suggesting that a second Stu I site is located in this region of the human ALAS-1 gene. This Stu I site must also be located within an intron as there is no corresponding Stu I site in the ALAS-1 cDNA.

The genomic organisation has been determined for the chicken (Maguire *et al.*, 1986) and the rat (Yamogida *et al.*, 1993) ALAS-1 gene and for the chicken (Lim *et al.*, 1994), mouse (Schoenhaut and Curtis, 1989) and human (Conboy *et al.*, 1992) ALAS-2 genes (fig 5.5 A). The exon-intron boundaries of all the genes are highly conserved, except for the chicken ALAS-1 gene which lacks the intron found in the 5'-UTR of all the other genes, including the human ALAS-1 gene (unpublished). Therefore, it is likely that the human ALAS-1 gene has a very similar genomic structure. Comparison of the position of the exon-intron boundaries with the amino-acid sequence of the protein, revealed that the last intron of the chicken and rat ALAS-1 and human and mouse ALAS-2 genes falls within a specific glutamic acid codon that forms part of a conserved region of the protein (fig 5.5 B). Although an aspartate residue is found at this position, instead of glutamic acid, in the chicken ALAS-2 protein, this codon is nevertheless still interrupted by intron 10 in the chicken ALAS-2 gene (fig 5.5 B). Therefore it was predicted that the terminal intron of the human ALAS-1 gene occurs at the glutamic acid residue found in this conserved region. This glutamic acid residue is located downstream of the Sac I site, which is in agreement with the Southern blot analysis data that suggested that an intron occurs between the Sac I site and the 3'-end of the gene. Furthermore it can be predicted that the 5'-end of the intron is located approximately 60bp away from the Sac I site, and the 3'-end of the intron is located approximately 155bp away from the Stu I site, based on the position of the glutamic acid codon at +1856. Comparison of the intron-exon structure of the other genes also predicts that the remaining introns are located upstream of the Sac I site.

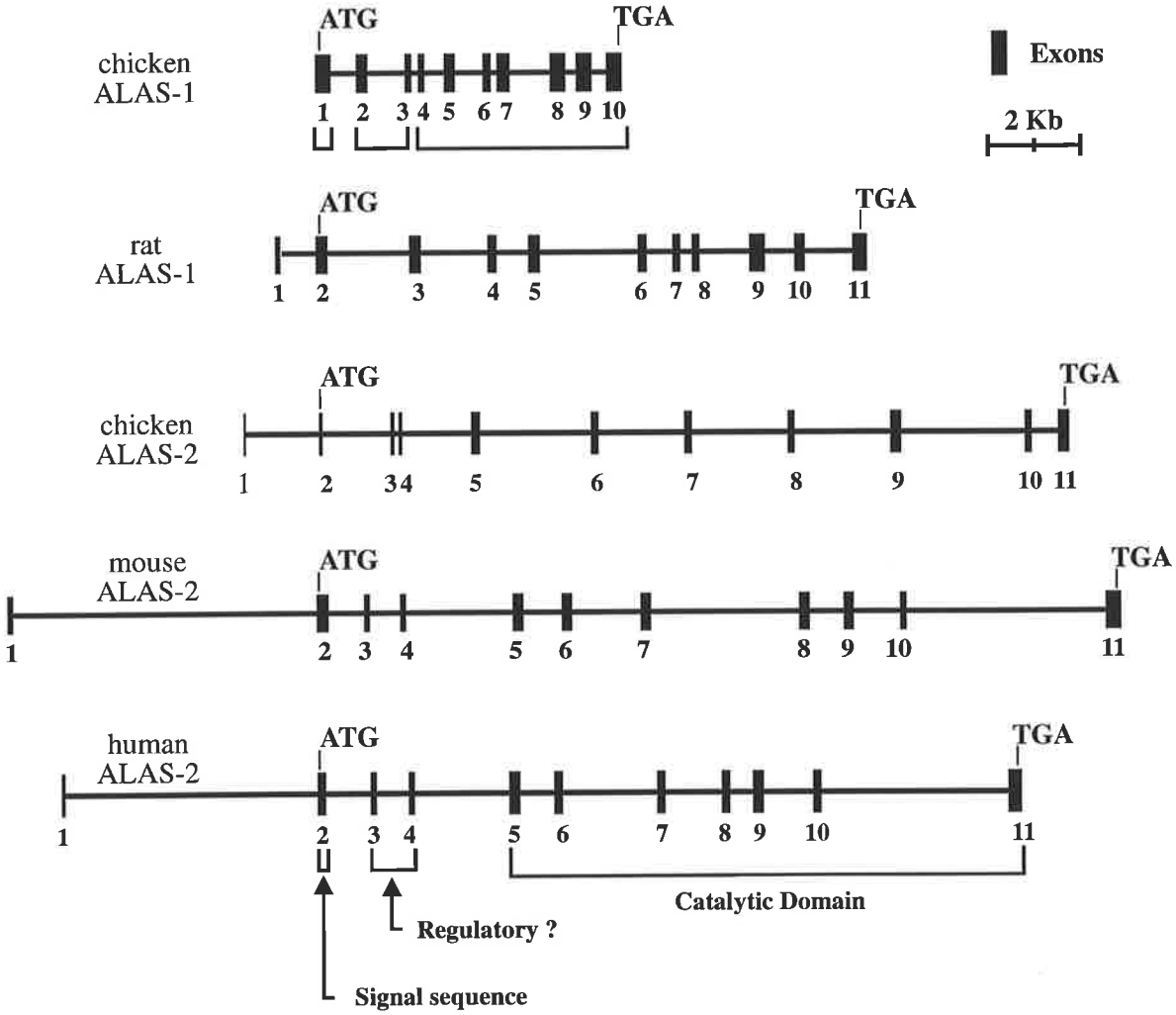
To orientate the Sac I fragments with respect to the intron, pBK2.E11 DNA was digested with the restriction endonucleases Pvu II or Pvu II and Sac I and Southern blot analysis performed, using the same 3'-probe (Fig 5.6). A unique Pvu II site at +1911 is

Figure 5.5: **Comparison of the structure of the ALAS genes.**

(A): The structural organisation of the genes for ALAS-1 and ALAS-2. Exons are numbered. The initiation and termination codons and the proposed functional domains are indicated.

(B): The comparison of the amino acid sequence surrounding the terminal intron of the genes for ALAS-1 and ALAS-2. The amino acid encoded for by the exon / intron boundary of the 3'-terminal intron is boxed. A similar sequence is also found in the human ALAS-1 enzyme where the glu residue predicted to be encoded by the exon / intron boundary is shown by an asterisk.

A:

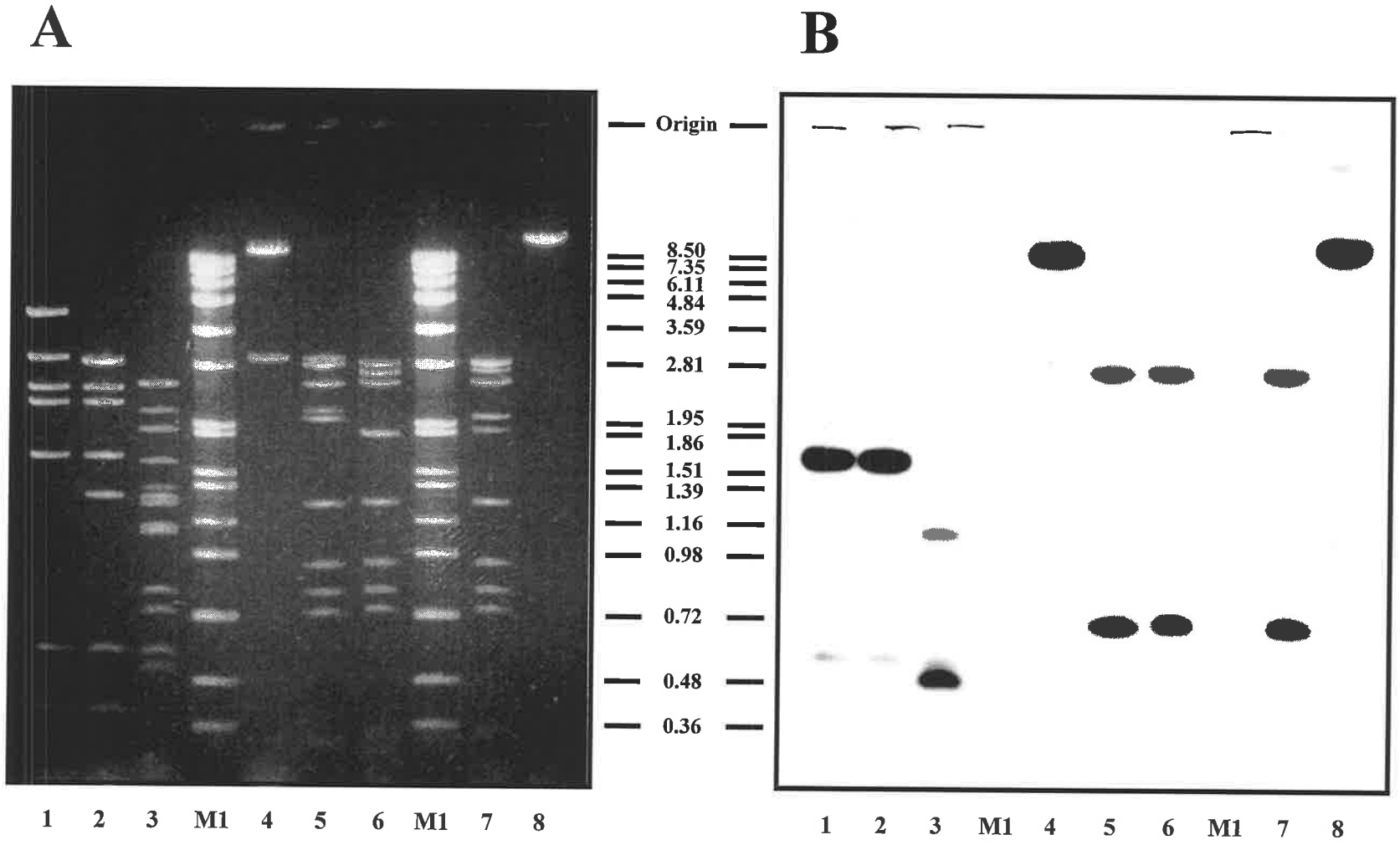


B:

					*						
Human ALAS-1	**Asn**	**Tyr**	**Phe**	**Leu**	**Glu**	**Asn**	**Leu**	**Leu**	**Leu**	**Thr**	**Trp**
Chick ALAS-1	**Ser**	**Tyr**	**Phe**	**Leu**	**Glu**	**Lys**	**Leu**	**Leu**	**Ala**	**Thr**	**Trp**
Rat ALAS-1	**Asp**	**Tyr**	**Phe**	**Leu**	**Glu**	**Lys**	**Leu**	**Leu**	**Leu**	**Thr**	**Trp**
Chick ALAS-2	**Glu**	**Asn**	**Leu**	**Ala**	**Asp**	**Lys**	**Leu**	**Ser**	**Glu**	**Cys**	**Trp**
Human ALAS-2	**Glu**	**Asp**	**Phe**	**Val**	**Glu**	**Lys**	**Leu**	**Leu**	**Leu**	**Ala**	**Trp**
Mouse ALAS-2	**Glu**	**Asp**	**Phe**	**Val**	**Glu**	**Lys**	**Leu**	**Leu**	**Leu**	**Ala**	**Trp**

Figure 5.6: **Localisation of the Human ALAS-1 Terminal Intron by Southern Blot Analysis.**

2μg of pBK2.E11 DNA was digested with the indicated restriction enzymes. The DNA was electrophoresed on a 1.0% agarose gel and photographed under UV light after the gel stained with ethidium bromide (A). Lane 1, Sac I; lane 2; Eco RI and Sac I; lane 3, Pvu II and Sac I; lane 4, Eco RI; lane 5, Pvu II: lane 6, Pvu II and Eco RI; lane 7 Pvu II and Xba I and lane 7, Xba I. Lanes M1 contain Eco RI cut SPP-1 DNA. The resultant filter was probed with the Sac I / Eco RI 3'-end probe. The restriction fragments that cross hybridised with the probe are shown in part B.


A
B
Origin
8.50
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72
0.48
0.36
1 2 3 M1 4 5 6 M1 7 8
1 2 3 M1 4 5 6 M1 7 8

found in the human ALAS-1 cDNA and should thus be located downstream of the predicted intron at +1857. Digestion of pBK2.E11 DNA with Pvu II resulted in two fragments cross-hybridising with the probe (lane 5), indicating that only one Pvu II site is found in the region spanned by the probe. Three bands of size 1.1, 0.6 and 0.5kb cross-hybridise to the 3'-probe when pBK2.E11 DNA is digested with Sac I and Pvu II (lane 3). This indicates that the 1.6kb Sac I fragment (lane 1) contained the Pvu II site and hence is cleaved by Pvu II to give the 1.1 and 0.5kb fragments observed when pBK2.E11 DNA is cleaved by Sac I and Pvu II (lane 3). The 0.6kb fragment must therefore be a result of the cleavage of Sac I at the Sac I site in exon 10 and a Sac I site 0.6kb away in intron 10. Based on the strength of the signal produced by the hybridisation of the probe to the 1.1kb and 0.5kb fragments it would appear that the smaller 0.5kb fragment contains more complementary sequence to the probe than the 1.1kb fragment. This suggests that the 0.5kb fragment contains sequences downstream of the Pvu II site as this region contains approximately 250bp of sequence complementary to the probe compared to approximately 57bp between the Pvu II site and the predicted 3'end of the intron. Even if the intron is located further upstream towards the Sac I site in exon 10, or further downstream towards the Pvu II site, the maximum amount of sequence complementary to the probe would be 117bp (the distance between the Sac I site and the Pvu II site in the cDNA). The Pvu II site in exon 11 is 250bp away from the end of the 3'-end of the mature ALAS-1 mRNA and thus the 0.5kb Sac I to Pvu II restriction fragment is estimated to contain about 250bp of 3'-flanking region (Bishop, 1990). This amount of 3'-flanking sequence should be sufficient to ensure the proper processing of the ALAS-1 mRNA 3'-end.

To confirm that the 1.6kb Sac I fragment contained the 3'UTR and to determine the precise position of intron 10, the 1.6kb Sac I fragment was first cloned into a modified pSP73 vector, termed pSP*Pv, which lacked the Pvu II site normally found in the pSP73 polylinker. The vector pSP*Pv was constructed by the religation of pSP73 following its digestion with Xho I and Sal I. This destroys the Sal I and Xho I sites and removes the sites for Pst I, Sph I, Hind III and Pvu II from the pSP73 polylinker. The vector was designed to allow upstream portions of the ALAS-1 cDNA to be added onto

the 3'-end of the ALAS-1 gene at the unique Pvu II site located in exon 11 of the 1.6kb Sac I fragment. The 1.6kb Sac I clones were orientated by digestion with Pvu II and Eco RI (present in the polylinker). To confirm the identity of the 1.6kb Sac I fragment, clones containing this fragment in both orientations were digested with Pvu II and Eco RV, religated and sequenced using a primer complementary to the SP6 RNA polymerase promoter (Section 2.1.7). The sequence obtained confirmed that the 1.6kb Sac I fragment contained the 3'UTR of the human ALAS-1 gene. The sequence data also suggested that intron 10 occurs between the codon for glutamic acid and asparagine in the human ALAS-1 gene rather than between the first and second nucleotides of the glutamic acid codon as found in the chicken and rat ALAS-1 and mouse and human ALAS-2 genes (fig 5.7 A). The intron-exon boundary was assigned to this position in the human ALAS-1 gene, rather than between the first (G) and second (A) nucleotide of the glutamic acid codon, as it provided the best match with the 3'-slice acceptor site consensus (Mount, 1982). The significance of this difference is unknown. However, to unequivocally assign the position of intron 10 the 0.6kb Sac I fragment, which contains the 5'-slice donor site, needs to be cloned and sequenced. A preliminary restriction map of the 3'-end of the human ALAS-1 gene is shown in fig 5.7 B.

ii) Construction of the Parental Human ALAS-1 gene (AAA).

The human ALAS-1 (AAA) gene was generated as outlined in figure 5.8. To isolate the ALAS-1 5'UTR a Kpn I site was first introduced 8bp upstream of the ALAS-1 CAP site. To achieve this an Eco RV to Hind III fragment was isolated from a 11kb Hind III clone containing the 5'-end of the ALAS-1 gene (Healy, 1990) and cloned into the vector pBluescript SK^+ to make pBSK-RV/H. The restriction enzyme Eco RV cuts within the ALAS-1 promoter 140bp upstream of the ALAS-1 CAP site, while Hind III cuts the DNA approximately 2kb downstream of the CAP site. This Hind III site must be located in another intron as there are no Hind III sites in the human ALAS-1 cDNA. The polymerase chain reaction was then used to introduce the Kpn I site upstream of the

Figure 5.7: **Structure of the 3'-end of the Human ALAS-1 Gene.**

(A) The intron / exon boundary sequence of the terminal intron of the chicken and rat ALAS-1, and the chicken, mouse and human ALAS-2 genes were compared to the proposed intron / exon boundary of the human ALAS-1 gene. The terminal intron of the human ALAS-1 gene was predicted to lie between the G nucleotide (+1859) and the A nucleotide (+1860). The number refers to the nucleotide position on the full length ALAS-1 cDNA. The human ALAS-1 genes 3'-terminal intron was predicted to lie at this position (between the glu and asn residues), rather than between the first and second nucleotide of the glu codon, to provide a better match with the proposed consensus intron / exon boundary sequence (Mount, 1982).

(B). Partial restriction map of the 3'-end of the human ALAS-1 gene. Hatched boxes represent coding portions, open boxes intronic sequences, grey boxes 3'UTR and the wavy line 3'-flanking sequences.

A

Exon 10		Intron 10	Exon 11
		5' Splice Site ▼	▼ 3' Splice Site
Chick ALAS-1	Leu CTC G	Glu gtgagtg - - - - -ccctgtttgtaacag	Lys AA AAG
Rat ALAS-1	Leu CTA G	Glu gtgagtcc - - - - - - - -ccccacag	Lys AG AAG
Chick ALAS-2	Ala GCC G	Asp gtaacc - - - - - - - - - -ccccag	Lys AT AAG
Mouse ALAS-1	Val GTG G	Glu gtaagt - - - - - - - tccattttcag	Lys AG AAG
Human ALAS-2	Val GTG G	Glu gtaagt - - - - - ccctaccaccttcag	Lys AG AAG
Human ALAS-1	Leu Glu CTT GAG	? - - - - - - -aactgtttctcctcag	Asn Leu AAT CTG
Consensus	C AAG	gtgat - - - - - - - - - $\binom{C}{T}_{11}$ N $^{C}_{T}$ag	GN

B

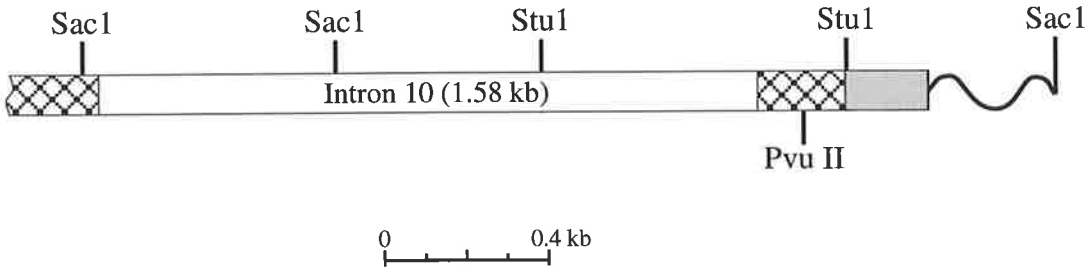
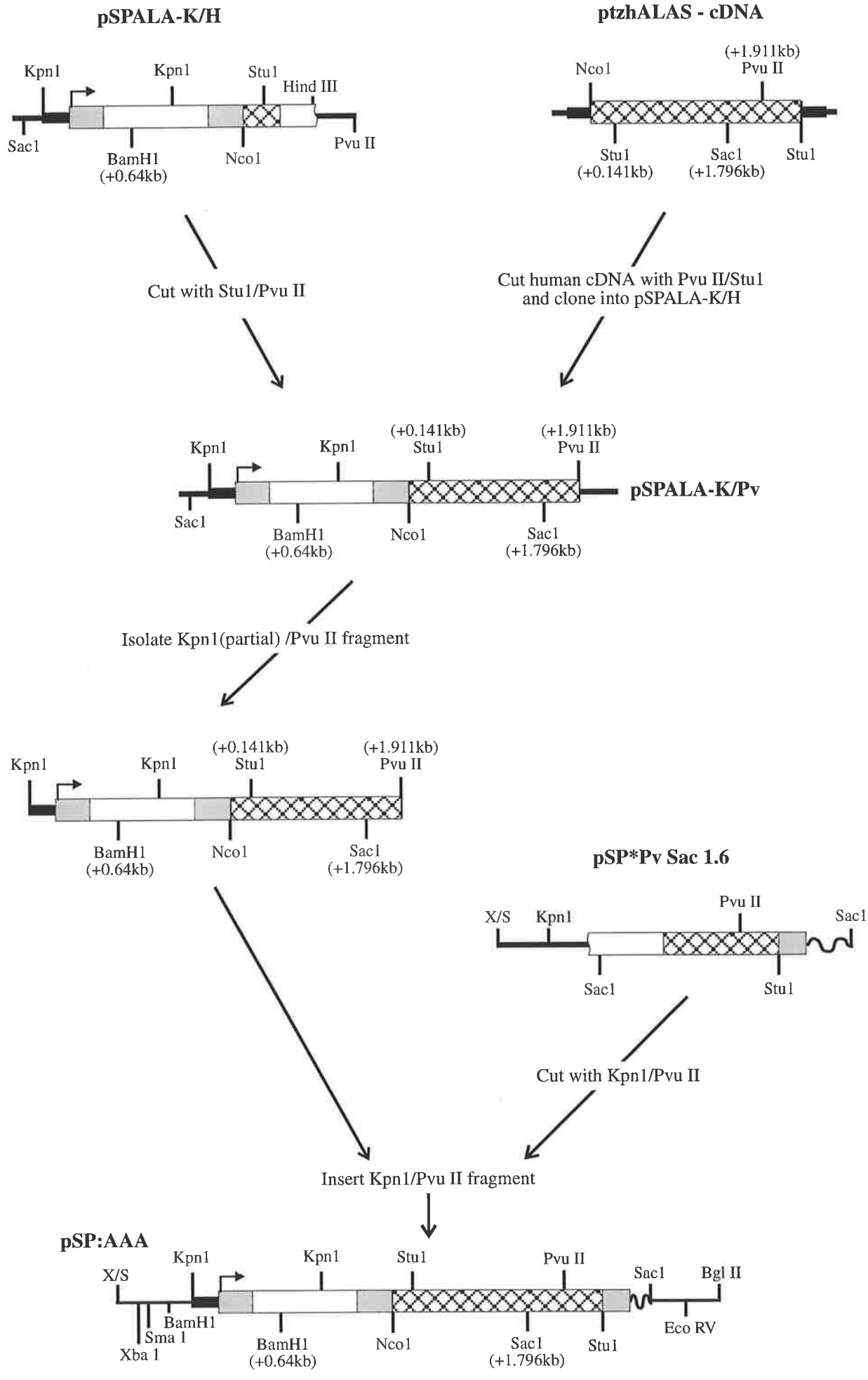
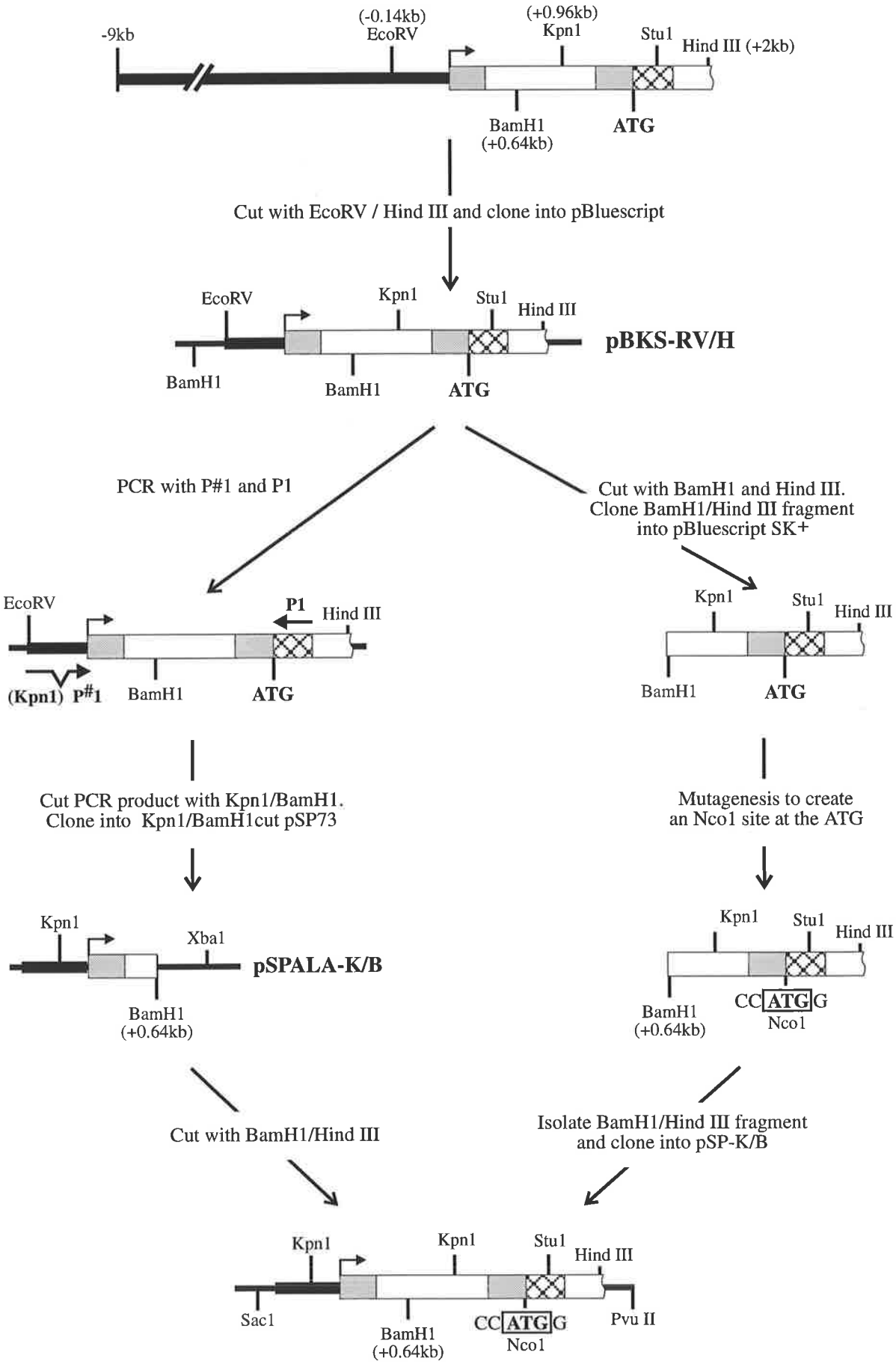


Figure 5.8: **Cloning strategy to synthesis the human ALAS-1 gene construct pSP AAA.**

Details of the cloning procedure are outlined in the text. The thick black line indicates the 5'-flanking region; hatched rectangles protein coding regions; open rectangles introns; grey rectangles untranslated regions; wavy lines 3'-flanking regions and thin lines vector sequences. X/S indicates the religation of the Xho I and Sal I sites in the pSP73 vector to remove the Pvu II site found in the pSP 73 polylinker. The CAP site is denoted by the open ended arrow and the PCR primers by close headed arrows.


pSPALA-K/H
Kpn1
Kpn1
Stu1
Hind III
Sac1
BamH1
(+0.64kb)
Nco1
Pvu II
ptzhALAS - cDNA
(+1.911kb)
Pvu II
Nco1
Stu1
(+0.141kb)
Sac1
(+1.796kb)
Stu1
Cut with Stu1/Pvu II
Cut human cDNA with Pvu II/Stu1
and clone into pSPALA-K/H
(+0.141kb)
Stu1
(+1.911kb)
Pvu II
pSPALA-K/Pv
Isolate Kpn1(partial) /Pvu II fragment
pSP*Pv Sac 1.6
X/S
Kpn1
Pvu II
Sac1
Sac1
Stu1
Cut with Kpn1/Pvu II
Insert Kpn1/Pvu II fragment
pSP:AAA
X/S
Kpn1
Kpn1
Stu1
Pvu II
Sac1
Bgl II
BamH1
Sma 1
Xba 1
BamH1
(+0.64kb)
Nco1
Sac1
(+1.796kb)
Stu1
Eco RV


-9kb
(-0.14kb)
EcoRV
(+0.96kb)
Kpn1
Stu1
Hind III (+2kb)
BamH1
(+0.64kb)
ATG
Cut with EcoRV / Hind III and clone into pBluescript
EcoRV
Kpn1
Stu1
Hind III
pBKS-RV/H
BamH1
BamH1
ATG
PCR with P#1 and P1
Cut with BamH1 and Hind III.
Clone BamH1/Hind III fragment
into pBluescript SK+
EcoRV
P1
Hind III
(Kpn1)
P#1
BamH1
ATG
Kpn1
Stu1
Hind III
BamH1
ATG
Cut PCR product with Kpn1/BamH1.
Clone into Kpn1/BamH1cut pSP73
Mutagenesis to create
an Nco1 site at the ATG
Kpn1
Xba1
pSPALA-K/B
BamH1
(+0.64kb)
Kpn1
Stu1
Hind III
BamH1
(+0.64kb)
CCATGG
Nco1
Cut with BamH1/Hind III
Isolate BamH1/Hind III fragment
and clone into pSP-K/B
Kpn1
Kpn1
Stu1
Hind III
Sac1
BamH1
(+0.64kb)
CCATGG
Nco1
Pvu II

CAP site. One Primer used (Primer 1 section 2.1.7) binds immediately upstream (-20 to -1) of the ALAS-1 CAP site. This primer contains several mismatches to the wild type ALAS-1 promoter sequence which results in the incorporation of a Kpn I site at the 5'-end of the PCR product. The second primer used (P1 section 2.1.7) is complementary to the 5'-end of the ALAS-1 coding region from +118 to + 139 and thus is located 3' to intron 1. The primer P1 was originally designed to the rat ALAS-1 cDNA sequence and as such carries a single base mismatch (A instead of G) to the human ALAS-1 cDNA sequence at the 5'-end of the primer. However due to the cloning procedure this mismatch is not introduced into the human ALAS-1 constructs. Following the PCR reaction the approximately 1.1 kb PCR product was digested with Kpn I and Bam HI (a single Bam HI site is located in intron 1, 640bp away from the CAP site) and cloned into Kpn I / Bam HI cut pSP73 to form pSPALA-K/B. To confirm the identity of this fragment and to check for any additional mutations inserted by the Taq DNA polymerase during the PCR reaction, the pSPALA-K/B clones were sequenced from both ends using primers, complementary to the T7 and SP6 RNA polymerase promoters which flank the pSP73 polylinker. Cloned next to this insert was the Bam HI to Hind III fragment of pBSK-RV/H, which had a Nco I site created at the ALAS-1 initiation codon by site directed mutagenesis, to form pSPALA-K/H.

To create the Nco I site at the ALAS-1 initiation codon, the Bam HI (+640) to Hind III (+2kb) fragment of pBSK-RV/H was isolated and cloned into pBluescript SK$^+$ to form pBSK-B/H (fig 5.8). Double stranded site directed mutagenesis was then performed, as described in section 2.2.11, on the pBSK-RV/H template using primer TS-1 (section 2.1.7) and the uracil incorporation selection procedure of Kunkel *et al.* (1987). Although, a lower mutation efficiency of about 10% was achieved using a double stranded template than reported with a single stranded template (>50%) the mutation rate was still high enough to conveniently isolate mutants. To ensure no other mutations were introduced into this fragment by the T4 DNA polymerase during the *in vitro* extension reaction the Bam HI to Stu I region of the insert was sequenced. To obtain the sequence near the 3'-end of intron 1 and into exon 2 several restriction fragments were subcloned prior to being sequenced.

Attempts were then made to cut pSPALA-K/H with Stu I and Pvu II in order to insert the Stu I (+141) to Pvu II (+1911) fragment of the human ALAS-1 cDNA into this clone to form pSPALA-K/Pv. A Stu I site is located in exon 2, 45bp downstream of the ALAS-1 initiation codon, thus cutting pSPALA-K/H with Stu I and Pvu II removes any downstream intronic sequences from the clone pSPALA-K/Pv. Several attempts, to cut pSPALA-K/H and the human ALAS-1 cDNA clones with Stu I resulted in very inefficient cutting at the Stu I site at +141 and this was attributed to an overlapping dcm methylase site at this position which inhibits Stu I digestion (Nelson and McClellend, 1991). The second Stu I site at +2011 of the human ALAS-1 cDNA is not overlapped by a dcm methylase site and is cut efficiently by Stu I. Therefore prior to digestion with Stu I the pSPALA-K/H and ALAS-1 cDNA clones were passaged through the dcm$^-$ dam$^-$ *E. coli* strain GM119 (Section 2.1.8). Lastly, the 5'-end ALAS-1 gene fragment was excised from the vector pSPALA-K/Pv as a Pvu II and Kpn I (partial) fragment, and inserted into the Kpn I and Pvu II sites of pSP*Pv-Sac 1.6 (section 5.2.2.i) to form pSP (AAA). The 5'-end of the ALAS-1 gene needed to be isolated by partial Kpn I digestion as a second Kpn I site is located in intron 1 at +960.

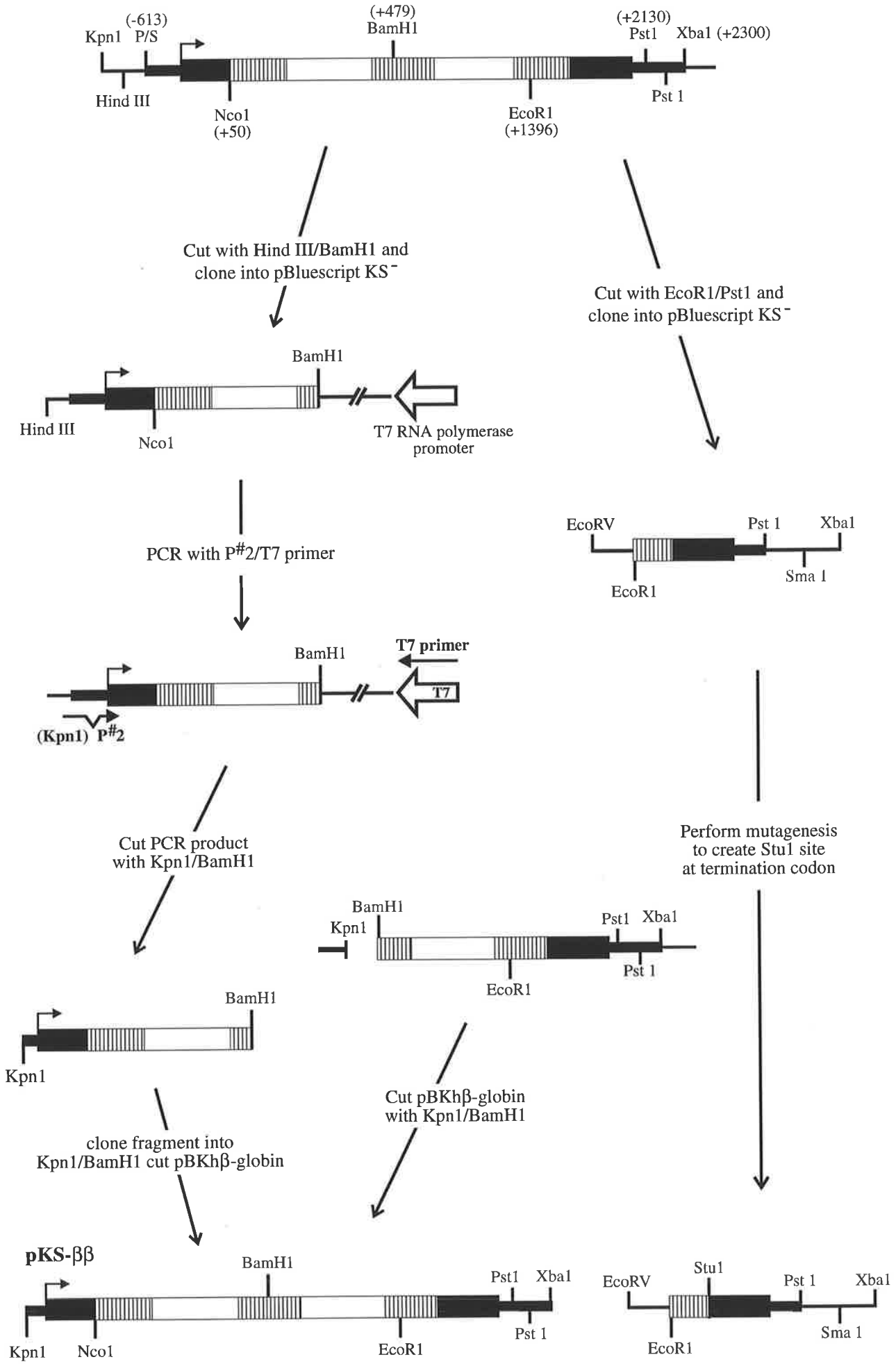
iii) Construction of the Parental Human β-globin gene (BBB).

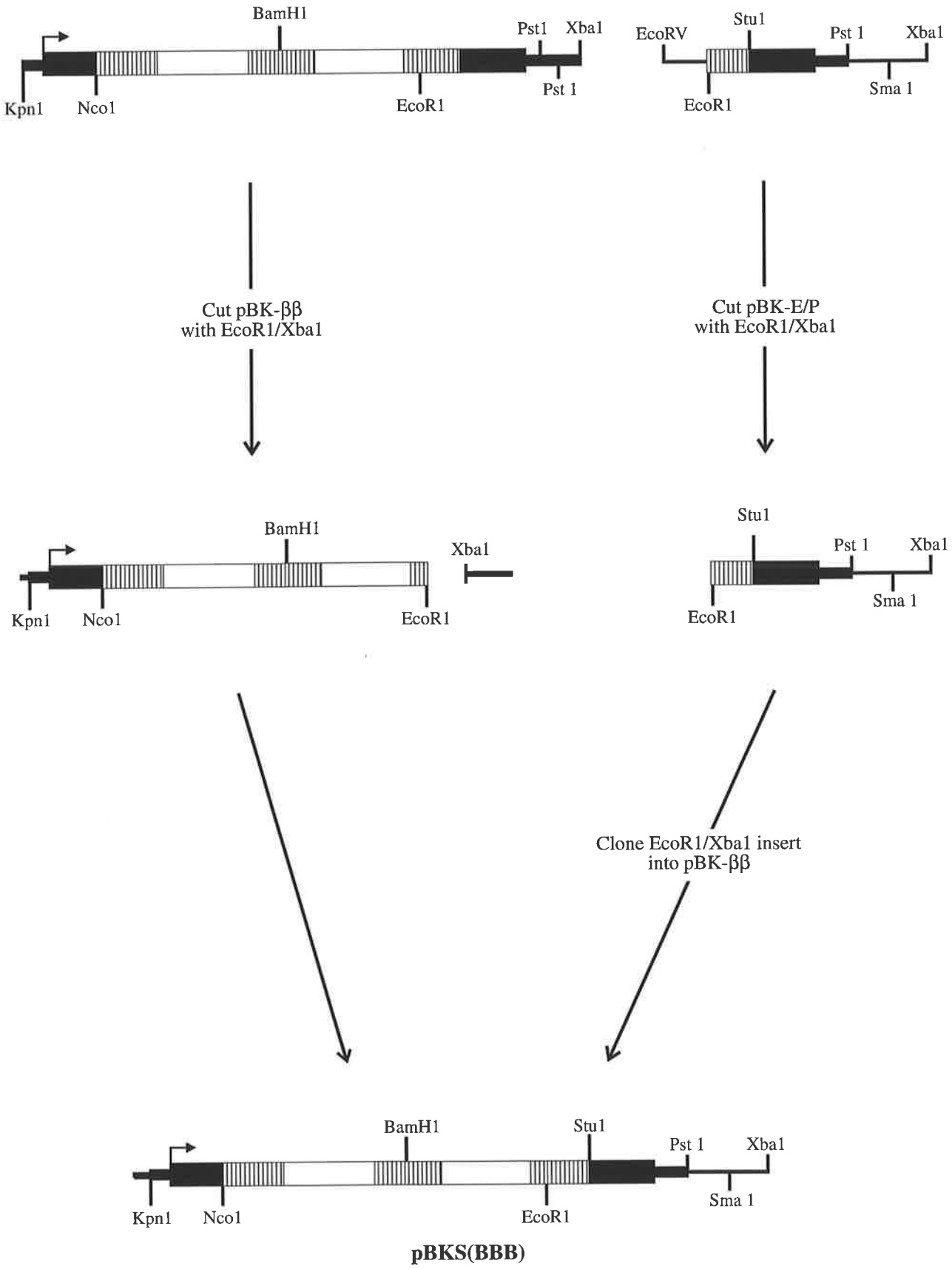
The entire β-globin gene (Lawn *et al.*, 1980) in pBluescript was a kind gift of Dr. Brooker (Section 2.1.6). The genomic clone contained β-globin sequences from the Sph I site at -613 to the Xba 1 site located approximately 1kb downstream of the β-globin gene. As discussed in section 5.2.1, the β-globin gene needed to be modified to allow the construction of the hybrid ALAS-1/β-globin constructs. An outline of the alterations is shown in figure 5.9.

Briefly, to place a Kpn I site adjacent to the β-globin CAP site, a Hind III (polylinker) to Bam HI (exon 2 β-globin) fragment containing the 5'-end of the β-globin gene was isolated and cloned into Hind III/Bam HI cut pBluescript KS$^-$. PCR was then performed using primer 2 (section 2.1.7) which binds adjacent (-22 to +2) to the β-

Figure 5.9: **The cloning strategy to synthesis the human β-globin gene construct pBKS BBB.**

Details of the cloning procedure outlined in the text. Black rectangles denote β-globin untranslated regions; stripped rectangles protein coding regions; open rectangles introns; thick lines 5' and 3' flanking regions and thin lines vector sequences. P/S indicates the 5' cloning site for the β-globin gene. The CAP site is denoted by the open ended arrow and the PCR primers by close headed arrows.


(-613)
Kpn1 P/S
(+479)
BamH1
(+2130)
Pst1 Xba1 (+2300)
Hind III
Nco1
(+50)
EcoR1
(+1396)
Pst 1
Cut with Hind III/BamH1 and
clone into pBluescript KS⁻
Cut with EcoR1/Pst1 and
clone into pBluescript KS⁻
BamH1
Hind III
Nco1
T7 RNA polymerase
promoter
EcoRV
Pst 1
Xba1
Sma 1
EcoR1
PCR with P#2/T7 primer
BamH1
T7 primer
T7
(Kpn1) P#2
Cut PCR product
with Kpn1/BamH1
Perform mutagenesis
to create Stu1 site
at termination codon
BamH1
Kpn1
Pst1 Xba1
Pst 1
EcoR1
BamH1
Kpn1
Cut pBKhβ-globin
with Kpn1/BamH1
clone fragment into
Kpn1/BamH1 cut pBKhβ-globin
pKS-ββ
BamH1
Pst1 Xba1
Kpn1
Nco1
EcoR1
Pst 1
EcoRV
Stu1
Pst 1
Xba1
Sma 1
EcoR1


BamH1
Pst1
Xba1
Kpn1
Nco1
EcoR1
Pst 1
EcoRV
Stu1
Pst 1
Xba1
EcoR1
Sma 1
Cut pBK-ββ
with EcoR1/Xba1
Cut pBK-E/P
with EcoR1/Xba1
BamH1
Xba1
Kpn1
Nco1
EcoR1
Stu1
Pst 1
Xba1
EcoR1
Sma 1
Clone EcoR1/Xba1 insert
into pBK-ββ
BamH1
Stu1
Pst 1
Xba1
Kpn1
Nco1
EcoR1
Sma 1

pBKS(BBB)

globin CAP site. This primer contains a Kpn I restriction site within it. The second primer used was complementary to the T7 RNA polymerase promoter of pBluescript. Following the PCR reaction the product was digested with Kpn I and Bam HI and inserted into pBluescript KS$^-$. The entire PCR product was then sequenced to ensure no additional changes were introduced during the amplification reaction. To obtain the entire sequence of this fragment several regions of this fragment were subcloned prior to being sequenced. The Kpn I to Bam HI fragment was then re-isolated from the appropriate clones and re-inserted into Kpn I/Bam HI cut pBluescript β-globin to form pBKS-ββ. This cloning procedure also caused the deletion of a region of the pBluescript polylinker from the Pst I to the Kpn I site, which includes the Eco RI site.

To introduce a Stu I site at the β-globin stop codon (fig 5.9) the Eco RI to Pst I fragment containing the 3'-end of the gene was cloned into pBluescript KS$^-$. Double stranded site directed mutagenesis, using primer 3 (section 2.1.7) was performed as described in section 2.2.11. After selection against wild type strands in dut$^+$ and ung$^+$ cells, DNA from 36 colonies was isolated and screened via restriction enzyme digestion with Stu I. Three positive clones were identified and the creation of a Stu I site at the β-globin stop codon confirmed by sequencing the insert. The sequence data also showed no additional mutations had been introduced into the β-globin gene at least for the region from the Eco RI site in exon 3 to 30bp downstream of the cytosine residue which forms the 3'-end of the mature mRNA. The mutated 3'-end fragment was then re-isolated from pBluescript KS$^-$ as an Eco RI/Xba I fragment (the Xba I site is found in the pBluescript polylinker 3' to the β-globin insert) and re-inserted into Eco RI / Xba I cut pBKS-ββ to form pBKS (BBB).

5.2.3 Construction of the Hybrid ALAS-1/β-globin Genes.

To transpose the 3'UTRs of the two genes to form the hybrid genes AAB and BBA (figure 5.1), the parental clones BBB and AAA were digested with either Stu I/Sma I or Stu I/Eco RV respectively and both the vector (plus 5'UTR and coding

region) and 3'UTR fragments isolated (figure 5.10). A unique Sma I site is located in the 3'-end of the BBB gene, while a unique Eco RV site is found at the 3'-end of the AAA gene. As mentioned in section 5.2.2.ii, the Stu I site at the 5'-end of the human ALAS-1 coding region cuts extremely poorly in wild type (dam^+ and dcm^+) *E. coli* strains and therefore the majority of pSP AAA vectors produced by Stu I digestion were only cut at the downstream Stu I site. Furthermore the partially Stu I cut Eco RV digested pSP AAA vector forms were easily separated from each other on an agarose gel. The 3'-end of each of the two genes was then inserted into the other vector. Clones were checked and orientated by extensive restriction enzyme digestions.

The creation of the Hybrid Genes BAA and ABB is outlined in figure 5.11. Briefly, the parental genes AAA and BBB were digested with either Nco I/Eco RV or Nco I/Sma I and the vector plus 5'UTR fragment isolated. In addition the restriction fragment containing the β-globin coding and 3'-untranslated regions was isolated from the Nco I/Sma I digested BBB gene construct. This insert was then cloned into the Nco I/Eco RV cut AAA gene construct to form pSP ABB. To isolate the ALAS-1 coding and 3'-untranslated regions on a single restriction fragment, the AAA gene construct was initially digested with Eco RV. A partial Nco I digestion was then performed (apart from the Nco I site introduced at the ALAS-1 initiation codon, Nco I cuts the ALAS-1 cDNA coding region at two other sites +784 and +995). The 2476bp fragment containing the full length ALAS-1 coding and 3'-flanking regions was separated from the 1796bp and 1596bp 5'-end truncated fragments and cloned into the Nco I / Sma I cut BBB gene construct to form pBKS BAA. The identity of these clones was checked by extensive restriction enzyme digestions.

To produce the hybrid gene BAB, the construct pBKS BAA was cut with Kpn I and Pvu II and the fragment, which contained the β-globin 5'UTR and the 5'-end of the ALAS-1 coding region up to the Pvu II site at +1811, isolated (fig 5.12). This fragment was then cloned into Kpn I / Pvu II cut pSP AAB vector (the Pvu II site of the pSP73 polylinker had been deleted in this vector) to form pSP BAB.

Initially, several attempts were made to insert the β-globin coding region as an Nco I to Stu I fragment into Nco I/Stu I digested pSP AAA to produce the hybrid gene

Figure 5.10: **Cloning strategy used to generate the chimeric gene constructs pSP AAB and pBKS BBA.**

Details of the cloning procedure to synthesis pSPAAB are outlined in the text. Grey rectangles denote ALAS-1 untranslated regions; black rectangles β-globin untranslated regions; hatched rectangles ALAS-1 protein coding regions; stripped rectangles β-globin protein coding regions; open rectangles introns; thick lines β-globin 5'- and 3'-flanking regions; wavy lines ALAS-1 3'-flanking regions and thin lines vector sequences. Sm/Rv and denotes the 3'-cloning site. Sm/RV and RV/Sm denotes the 3'-cloning sites. The CAP site is denoted by the open ended arrow

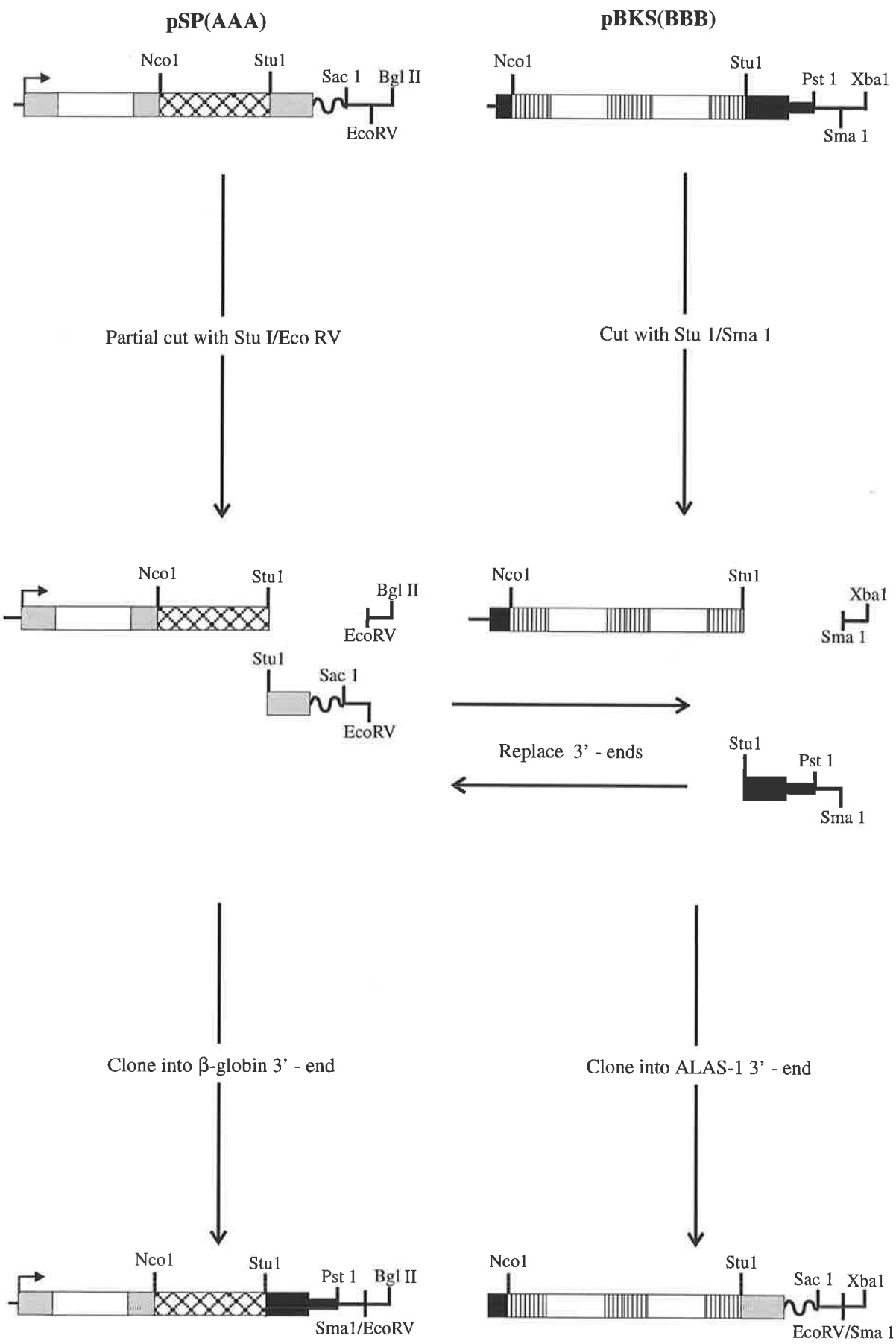

pSP(AAA)
Nco1
Stu1
Sac 1
Bgl II
EcoRV
pBKS(BBB)
Nco1
Stu1
Pst 1
Xba1
Sma 1
Partial cut with Stu I/Eco RV
Cut with Stu 1/Sma 1
Nco1
Stu1
Bgl II
EcoRV
Stu1
Sac 1
EcoRV
Nco1
Stu1
Xba1
Sma 1
Replace 3' - ends
Stu1
Pst 1
Sma 1
Clone into β-globin 3' - end
Clone into ALAS-1 3' - end
Nco1
Stu1
Pst 1
Bgl II
Sma1/EcoRV
Nco1
Stu1
Sac 1
Xba1
EcoRV/Sma 1
pSP(AAB)
pBKS(BBA)

Figure 5.11: **Cloning strategy used to generate the chimeric gene constructs pSP ABB and pBKS BAA.**

(A) Details of the cloning procedure to synthesis pSP ABB are outlined in the text. Grey rectangles denote ALAS-1 untranslated regions; black rectangles β-globin untranslated regions; hatched rectangles ALAS-1 protein coding regions; stripped rectangles β-globin protein coding regions; open rectangles introns; thick lines β-globin 5'-and 3'-flanking regions; wavy lines ALAS-1 3'-flanking regions and thin lines vector sequences. Sm/Rv denotes the 3'-cloning site. The CAP site is denoted by the open ended arrow

(B) Details of the cloning procedure to synthesis pSP BAA are outlined in the text. Grey rectangles denote ALAS-1 untranslated regions; black rectangles β-globin untranslated regions; hatched rectangles ALAS-1 protein coding regions; stripped rectangles β-globin protein coding regions; open rectangles introns; thick lines β-globin 5'-and 3'-flanking regions; wavy lines ALAS-1 3'-flanking regions and thin lines vector sequences. RV/Sm denotes the 3'-cloning site. The CAP site is denoted by the open ended arrow.

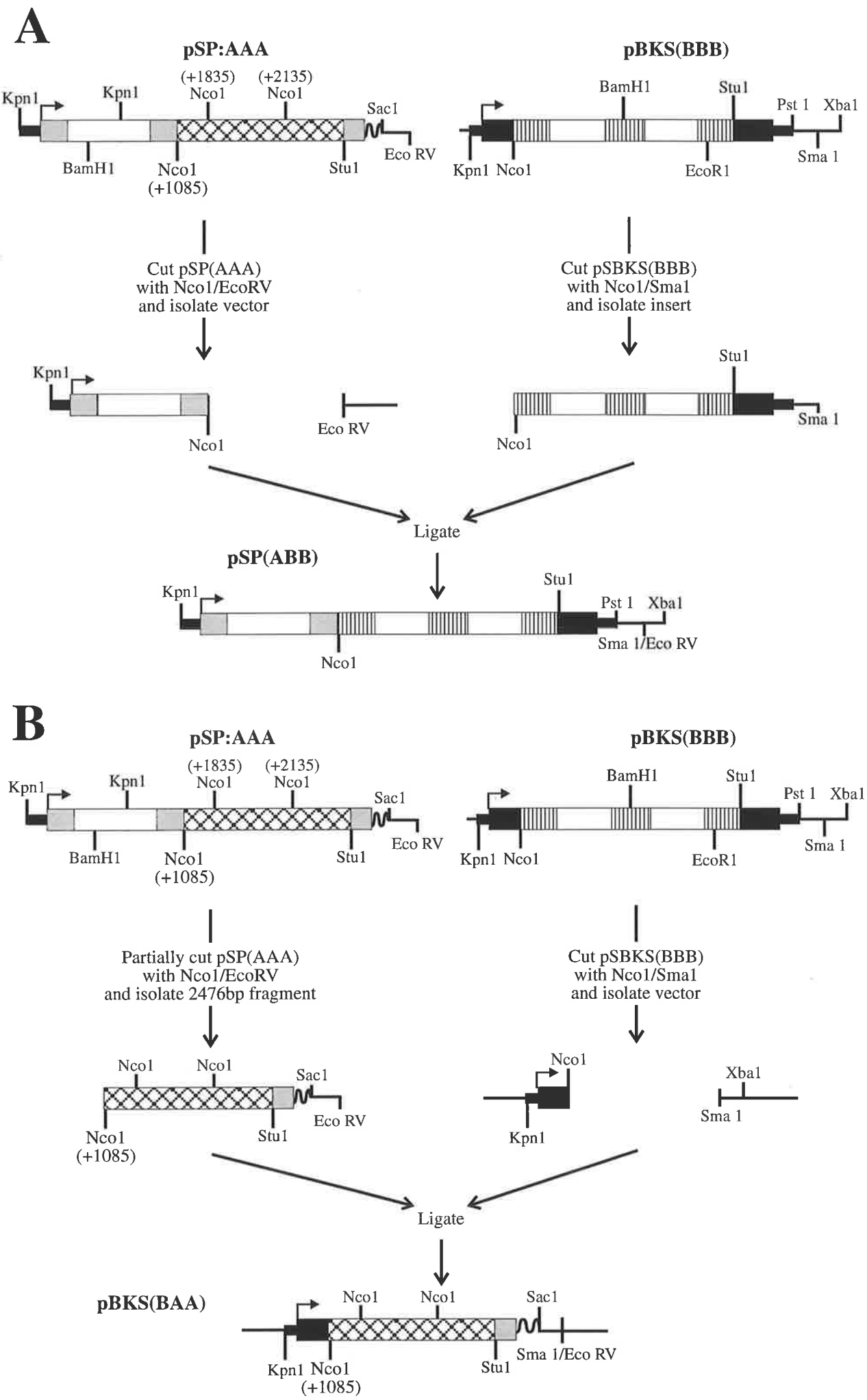
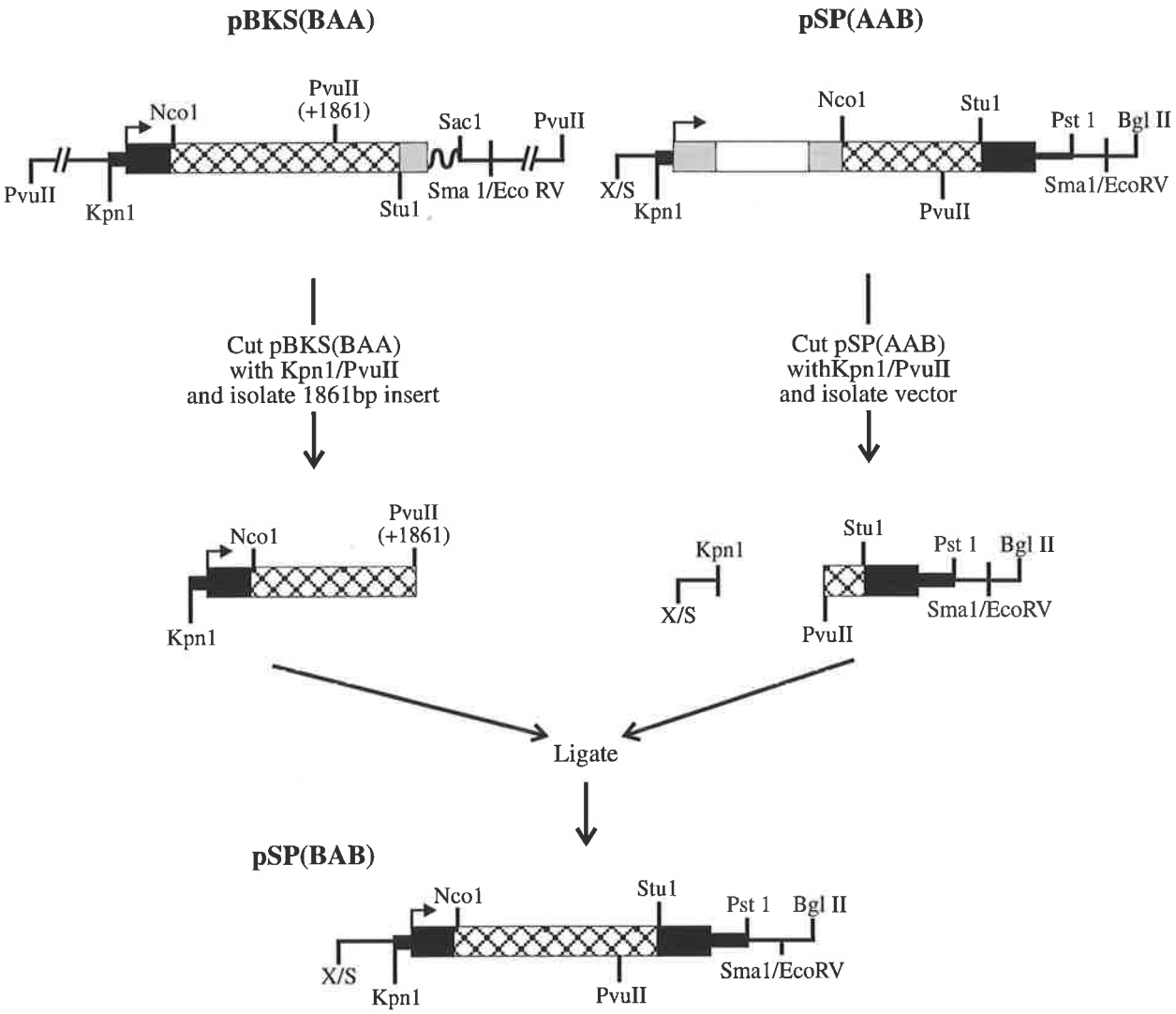

A
pSP:AAA
pBKS(BBB)
Kpn1
Kpn1
(+1835)
Nco1
(+2135)
Nco1
Sac1
Eco RV
BamH1
Nco1
(+1085)
Stu1
BamH1
Stu1
Pst 1
Xba1
Kpn1
Nco1
EcoR1
Sma 1
Cut pSP(AAA)
with Nco1/EcoRV
and isolate vector
Cut pSBKS(BBB)
with Nco1/Sma1
and isolate insert
Kpn1
Nco1
Eco RV
Stu1
Sma 1
Nco1
Ligate
pSP(ABB)
Kpn1
Stu1
Pst 1
Xba1
Sma 1/Eco RV
Nco1
B
pSP:AAA
pBKS(BBB)
Kpn1
Kpn1
(+1835)
Nco1
(+2135)
Nco1
Sac1
Eco RV
BamH1
Nco1
(+1085)
Stu1
BamH1
Stu1
Pst 1
Xba1
Kpn1
Nco1
EcoR1
Sma 1
Partially cut pSP(AAA)
with Nco1/EcoRV
and isolate 2476bp fragment
Cut pSBKS(BBB)
with Nco1/Sma1
and isolate vector
Nco1
Nco1
Sac1
Eco RV
Nco1
(+1085)
Stu1
Nco1
Kpn1
Xba1
Sma 1
Ligate
pBKS(BAA)
Nco1
Nco1
Sac1
Sma 1/Eco RV
Kpn1
Nco1
(+1085)
Stu1

Figure 5.12: **Cloning strategy used to generate the chimeric gene constructs pSP BAB.**

Details of the cloning procedure to synthesis pSP BAB are outlined in the text. Grey rectangles denote ALAS-1 untranslated regions; black rectangles β-globin untranslated regions; hatched rectangles ALAS-1 protein coding regions; stripped rectangles β-globin protein coding regions; open rectangles introns; thick lines β-globin 5'-and 3'-flanking regions; wavy lines ALAS-1 3'-flanking regions and thin lines vector sequences. Sm/Rv and denotes the 3'-cloning site. Sm/RV denotes the 3'-cloning site. The CAP site is denoted by the open ended arrow


pBKS(BAA)
pSP(AAB)
PvuII
(+1861)
Nco1
Sac1
PvuII
PvuII
Kpn1
Stu1
Sma 1/Eco RV
Nco1
Stu1
Pst 1
Bgl II
X/S
Kpn1
PvuII
Sma1/EcoRV
Cut pBKS(BAA)
with Kpn1/PvuII
and isolate 1861bp insert
Cut pSP(AAB)
withKpn1/PvuII
and isolate vector
PvuII
(+1861)
Nco1
Kpn1
Kpn1
X/S
Stu1
Pst 1
Bgl II
Sma1/EcoRV
PvuII
Ligate
pSP(BAB)
Nco1
Stu1
Pst 1
Bgl II
X/S
Kpn1
PvuII
Sma1/EcoRV

pSP ABA. However, these attempts proved to be unsuccessful. The few clones produced from the ligation reactions lacked the Nco I and Stu I sites at the cloning boundaries (as judged by their inability to be cut by Nco I or Stu I) and were too small to contain a full length copy of the ABA gene. The reason for this inability to insert the β-globin coding region into the pSP AAA vector is still unknown. The restriction pattern of the transformants produced suggested that the Nco I/Stu I cut pSP AAA vector had re-ligated without the insert (this was most likely due to a low level of exonuclease activity blunting the Nco I 5'-overhang). Therefore an alternative approach was used to form pBKS ABA (fig 5.13). Briefly, the ALAS-1 5'UTR attached to the β-globin coding region was isolated from the construct pSP ABB as a Stu I/Kpn I (partial) fragment and was then inserted into Kpn I/Stu I digested pBKS BAA to form pBKS ABA

To express these hybrid genes in tissue culture cells, the genes were cloned behind the Rous Sarcoma virus long terminal repeat promoter in the eukaryotic expression vector pRSVN.06. The vector pRSVN.06, as shown in figure 5.14, was a kind gift of Dr. A. Robbins (Bresatec Ltd.). The vector contains the M13mp18 polylinker inserted between the RSV promoter and the human growth hormone gene 3'-end to allow for the efficient expression of any recombinant gene in eukaryotic cells. To form the expression clones pRSV BBB, BBA and BAA, the hybrid genes BBB, BBA and BAA were excised from pBluescript KS^- with Kpn I/Xba I and inserted into Kpn I/Xba I cut pRSVN.06. To make the expression clones pRSV AAB, ABB and BAB the corresponding hybrid genes were excised from pSP*Pv 73 with Xba I/Sca I and inserted into Xba I/Sca I cut pRSVN.06. Sca I cuts both vectors in the ampicillin resistance gene which lies 3' to the hybrid gene and the human growth hormone 3'-end in pSP*Pv and pRSVN.06 respectively. It was impossible to make the expression clone pRSV AAA in a similar manner due to a Sca I site at the extreme 3'-end of the ALAS-1 3'-flanking region. To form pRSV AAA, the pSP AAA construct was cut with Xba I and Sca I and the Xba I site end-filled. The AAA gene was then inserted into the Not I site in pRSVN.06.which had also been end-filled. The correct orientation was then determined by restriction map analysis. Lastly, the expression clone pRSV ABA was formed in a two step procedure. Firstly, the large Kpn I to Xba I fragment containing the sequence

Figure 5.13: **Cloning strategy used to generate the chimeric gene constructs pBKS ABA.**

Details of the cloning procedure to synthesis pBKS ABA are outlined in the text. Grey rectangles denote ALAS-1 untranslated regions; black rectangles β-globin untranslated regions; hatched rectangles ALAS-1 protein coding regions; stripped rectangles β-globin protein coding regions; open rectangles introns; thick lines β-globin 5'-and 3'-flanking regions; wavy lines ALAS-1 3'-flanking regions and thin lines vector sequences. Sm/Rv and denotes the 3'-cloning site. RV/Sm denotes the 3'-cloning site. The CAP site is denoted by the open ended arrow .

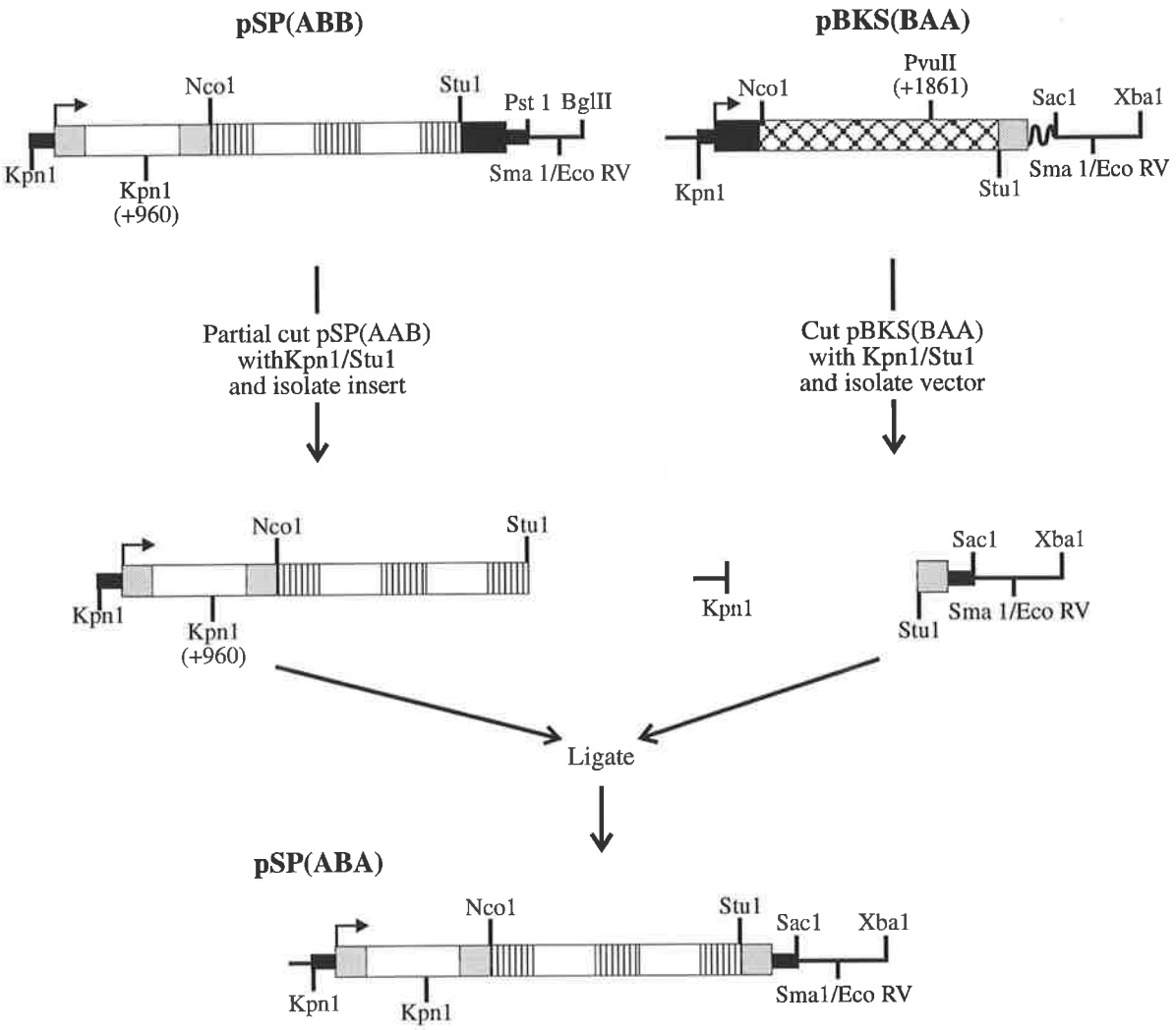
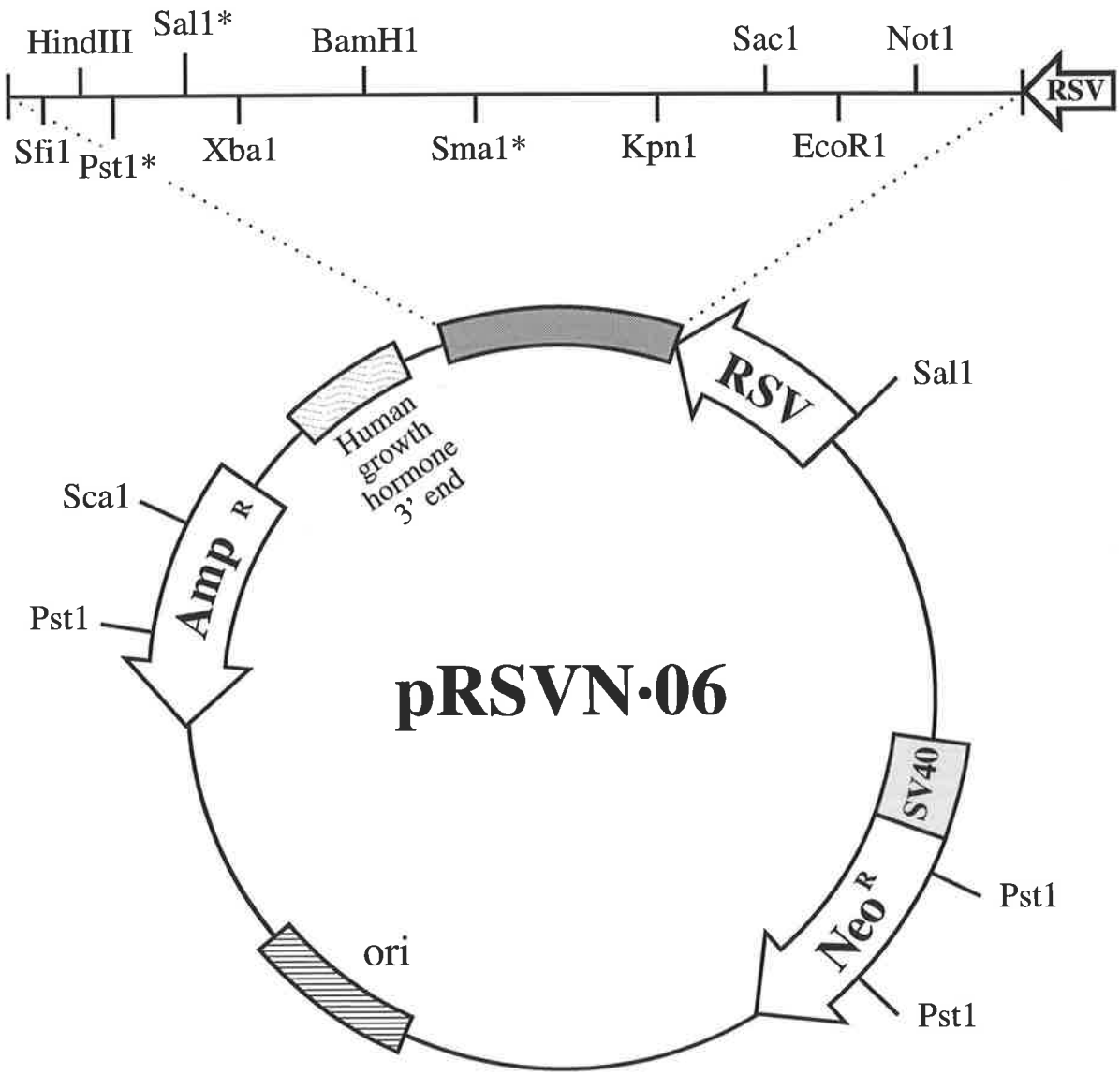

pSP(ABB)
Nco1
Stu1
Pst 1 BglII
Kpn1
Kpn1
(+960)
Sma 1/Eco RV
pBKS(BAA)
Nco1
PvuII
(+1861)
Sac1
Xba1
Kpn1
Stu1
Sma 1/Eco RV
Partial cut pSP(AAB)
withKpn1/Stu1
and isolate insert
Cut pBKS(BAA)
with Kpn1/Stu1
and isolate vector
Nco1
Stu1
Kpn1
Kpn1
(+960)
Kpn1
Sac1
Xba1
Stu1
Sma 1/Eco RV
Ligate
pSP(ABA)
Nco1
Stu1
Sac1
Xba1
Kpn1
Kpn1
Sma1/Eco RV

Figure 5.14: **Map of the Eukaryotic Expression Vector pRSVN.06.**

Sites denoted by an asterisk in the polylinker are aslo found elsewhere in the vector.


HindIII
Sal1*
BamH1
Sac1
Not1
RSV
Sfi1
Pst1*
Xba1
Sma1*
Kpn1
EcoR1
RSV
Sal1
Human growth hormone 3' end
Sca1
Amp R
Pst1
pRSVN·06
SV40
Neo R
Pst1
ori
Pst1

from +960 in intron I (Kpn I) to the 3'-end of the gene (Xba I) was isolated from pBKS ABA and cloned into Kpn I/Xba I cut pRSVN.06. to form pRSV BA. Subsequently, the small Kpn I fragment containing the 5'-end of the ALAS-1 5'UTR to +960 in intron 1 was inserted into pRSV-BA to form pRSV ABA. Clones were orientated with respect to the small Kpn I fragment by restriction digestion analysis.

To generate stable transfectants of the RSV driven hybrid gene constructs the vectors were first linearised to facilitate the incorporation of the constructs into the FRL 4.1 genome. The hybrid gene constructs pRSV ABB, AAB and BAB were linearised with Sca I, while the remaining gene constructs were linearised with Hind III. A Hind III site is located in the pRSVN.06 polylinker 3' to the hybrid genes. The restriction enzyme Hind III was chosen to linearise these constructs over Sca I in order to separate the hybrid genes from the human growth hormone gene 3'-flanking region found downstream of the polylinker. This was to ensure there was no interference by the growth hormone poly(A) signal with the ALAS-1 and β-globin poly(A) signals. Several studies have found that a strong downstream poly(A) signal can out-compete a weaker upstream poly-adenylation signal for poly-adenylation (Levitt *et al.*, 1989 ; Batt *et al.*, 1994). Although, the human β-globin poly (A) signal is thought to be a strong signal the relative strength of the human ALAS-1 signal is unknown, and therefore the presence of the growth hormone signal may have resulted in hybrid mRNAs containing human growth hormone 3'-ends. The cloning procedure used to synthesise the constructs pRSV ABB, AAB and BAB removed the growth hormone sequences from the vector (as well as the Hind III site). FRL 4.1 cells were transfected with 7.5μg of the linearised hybrid gene constructs using the electroporation conditions optimised in section 4.2.3. Stable transfectants were selected with G418 treatment for 10 days after which the surviving colonies (approximately 200) for each hybrid gene construct were pooled and stocks of low passage number cells frozen down.

The probes used to detect the transcripts produced from the hybrid genes in a RNase protection assay were as follows. To detect transcripts containing the coding region of the human ALAS-1 gene, a 361bp Bgl II (+1203) to Bam HI (+1564) fragment of the human ALAS-1 cDNA was cloned into the Bam HI and Bgl II sites of the vector

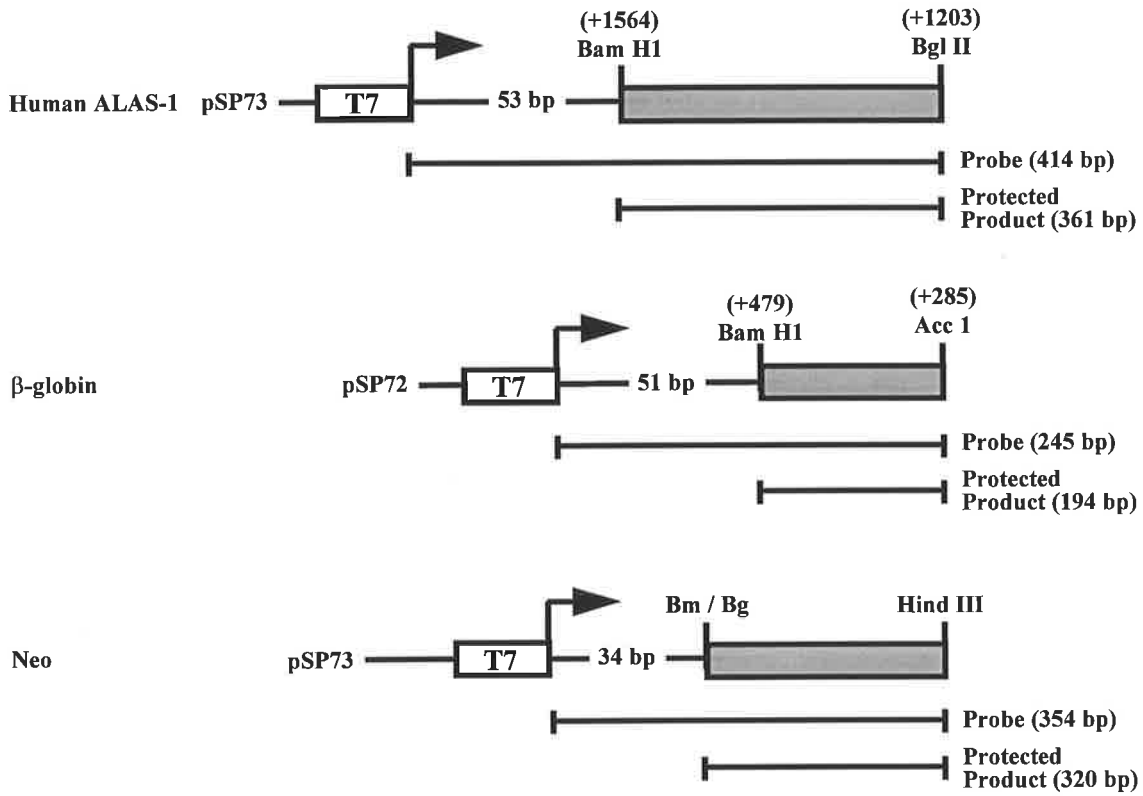
pSP73. Clones were orientated by the ability of the insert to be re-isolated with Bam HI and Bgl II. Sufficient differences exist in the sequence of the rat and human ALAS-1 mRNAs in this region to prevent the probe from protecting an equivalently sized portion of the endogenously expressed rat ALAS-1 mRNA. This is demonstrated in figure 5.15. Total RNA was prepared from FRL 4.1 cells stably transfected with the vector pRSVN.06, pRSV AAA or pRSV BBB and a RNase protection assay performed on 10μg of RNA from each sample. A single prominent protected product of about the expected 361 bases is detected with the human probe in FRL 4.1 cells stably transfected with pRSV AAA (lane 10). No protected product of a similar size is detected by the human ALAS-1 probe in FRL 4.1 cells transfected with either the vector pRSVN 0.6 or pRSV BBB (lane 8 and 11). The prominent 320 base band detected in lane 8 and 9 corresponds to the neo transcript produced from the vector pRSVN.06. The probe to detect the neo transcript was synthesised from a clone kindly provided by Dr. Vakaloupolou and consists of a 320bp Hind III to Bgl II fragment which spans the 5'-end of the neo transcript (Jones and Cole, 1987). To detect the transcript containing the β-globin coding region an Acc I to Bam HI fragment of exon 2 of the human β-globin gene was cloned into the Acc I and Bam HI sites of pSP72. The anti-sense β-globin probe generated from this vector protects an 194 base region of the β-globin coding region (lane 11), but does not cross-hybridise with anything in FRL 4.1 cells transfected with pRSVN 0.6 or pRSV AAA (lanes 8 and 10). To specifically detect the level of the endogenous rat ALAS-1 mRNA, a Hind III (+1554) to Sac I (+1732) fragment was isolated from the rat ALAS-1 cDNA and cloned into the Hind III and Sac I sites of pSP72. The anti-sense probe generated from this construct protects a 178 base region of the rat ALAS-1 mRNA (Data not shown).

Figure 5.15: **Expression of the parental AAA and BBB gene constructs in FRL 4.1 cells.**

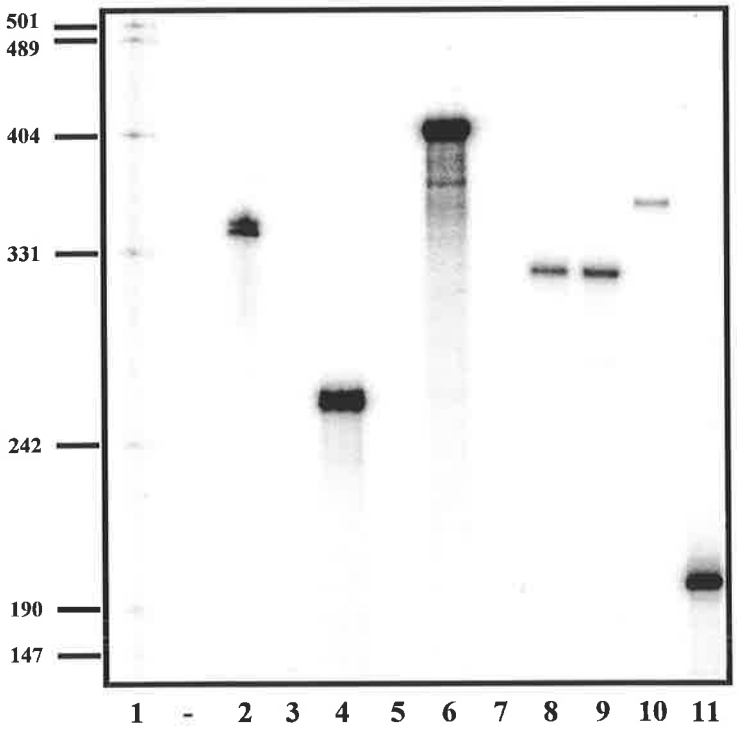
(A) Schematic of the anti-sense probes used to detect the human ALAS-1, human β-globin and Neo mRNA. Indicated below each construct is the size of the anti-sense transcript and the expected size of the protected product.

(B) RNase protection of total RNA (10μg) isolated from FRL 4.1 cells containing integrated copies of pRSVN AAA, pRSVN BBB and pRSVN.06. Lane 10, human ALAS-1 protected product from FRL 4.1 cells transfected with pRSVN AAA; lane 11, β-globin protected product from FRL 4.1 cells transfected with pRSVN BBB; lanes 8 and 9, Neo protected product from FRL 4.1 cells transfected with pRSVN.06. Also included in lanes 8 and 9 were the anti-sense probes for human ALAS-1 (lane 8) and β-globin (lane 9) mRNA. Lanes 2 and 3 contain the Neo antisense probes minus or plus RNase treatment; lanes 4 and 5 β-globin probe minus or plus RNase treatment and lanes 6 and 7 human ALAS-1 probe minus or plus RNase treatment.

A


Human ALAS-1
pSP73
T7
53 bp
(+1564)
Bam H1
(+1203)
Bgl II
Probe (414 bp)
Protected
Product (361 bp)
β-globin
pSP72
T7
51 bp
(+479)
Bam H1
(+285)
Acc 1
Probe (245 bp)
Protected
Product (194 bp)
Neo
pSP73
T7
34 bp
Bm / Bg
Hind III
Probe (354 bp)
Protected
Product (320 bp)

B


501
489
404
331
242
190
147
1 - 2 3 4 5 6 7 8 9 10 11

5.2.4 The Effect of Heme on the Decay of the Hybrid ALAS-1/β-globin mRNAs in FRL 4.1 cells.

i). The Effect of Heme on the Parental ALAS-1 and β-globin mRNAs AAA and BBB.

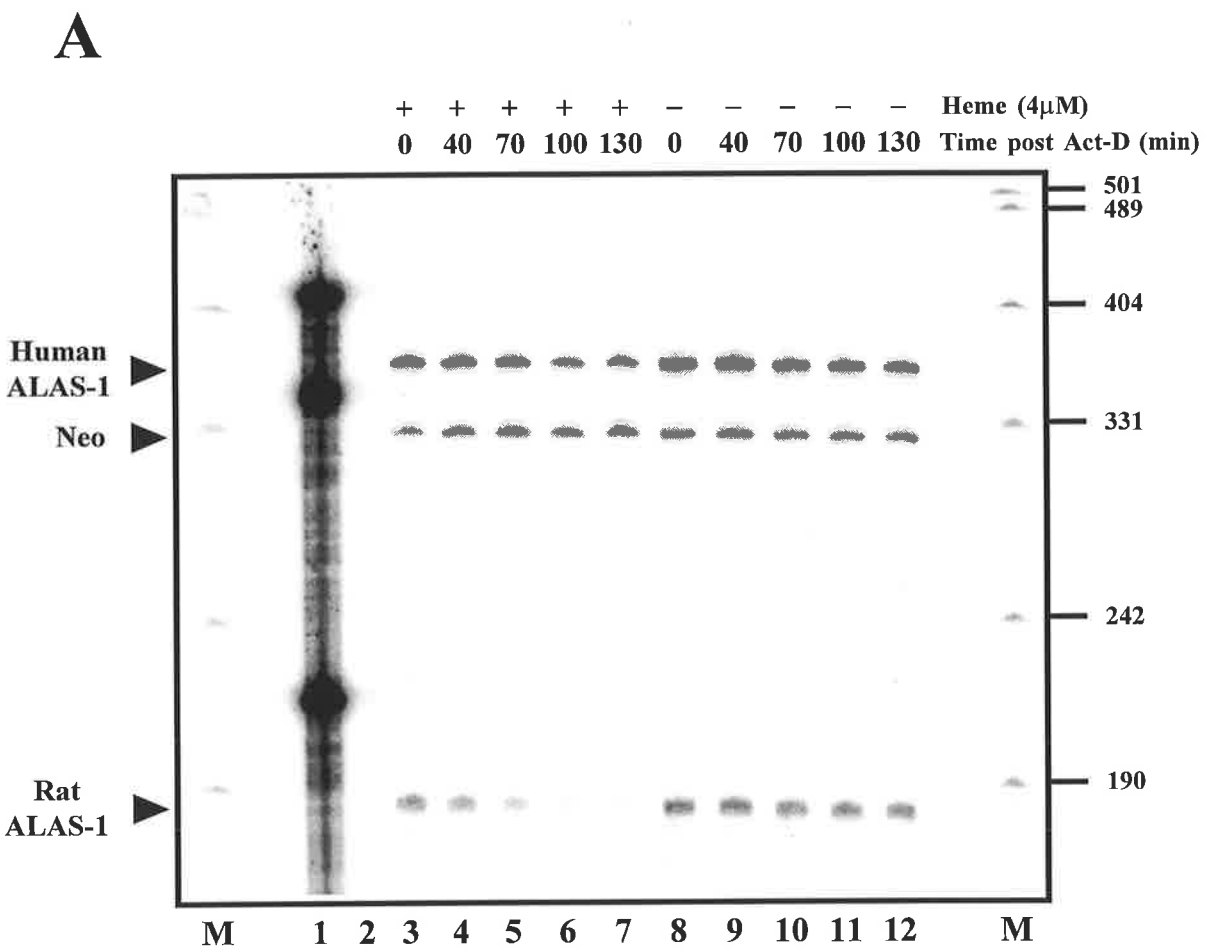
Initially, the effect of heme on the decay of the parental human ALAS-1 (AAA) mRNA was determined. This was to ascertain the feasibility of using the hybrid human ALAS-1/β-globin gene constructs to identify the determinants responsible for the heme-mediated destabilisation of the ALAS-1 mRNA. Total RNA was prepared from FRL 4.1 cells, stably transfected with pRSV AAA, at various times after the cells were treated with actinomycin D or actinomycin D plus 4μM heme. The relative concentrations of the human and rat ALAS-1 mRNA and the neo mRNA were then determined by RNase protection analysis on each RNA sample, using a mixture of uniformly radiolabeled α-sense RNA probes (figure 5.16, A). In the absence of any exogenously added heme only a small decline in the level of the human and rat ALAS-1 mRNA was observed over the 130 minutes assayed (lanes 8 to 12). The addition of heme, however, caused a significant drop in the level of both human and rat ALAS-1 mRNA in this time period (lanes 3 to 7). The level of the neo mRNA, which was synthesised from the SV40 early region promoter, does not decay detectably over this time period either in the presence or absence of heme and therefore served as an internal control to correct variations caused by sample handling.

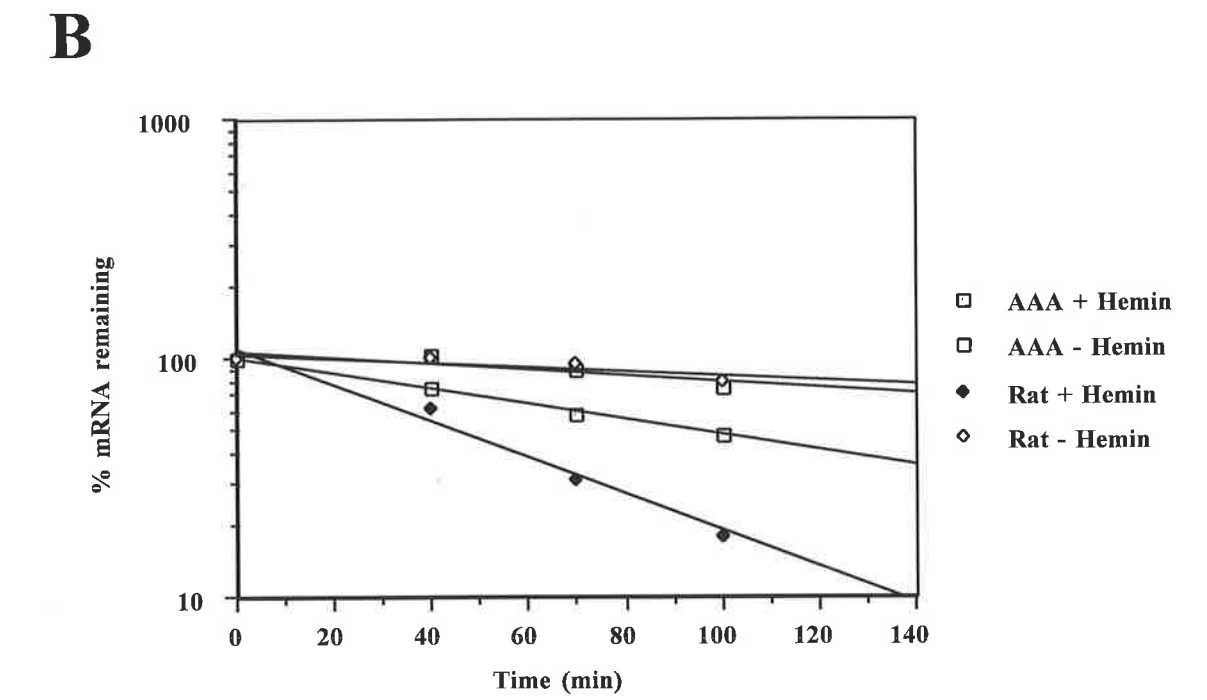
The relative levels of human and rat ALAS-1 mRNA were determined by quantitation of the PhosphorImage of the RNase protection assay and subsequently normalising the level of the ALAS-1 mRNAs to the level of neo transcript found in each sample. Data from two independent experiments were pooled and used to plot the percentage of ALAS-1 remaining versus time (fig 5.16, B). Calculation of the half-lives showed that heme caused a 2.8 fold decrease in the half-life of the human ALAS-1

Figure 5.16: **The Effect of Heme on the Decay of the Human ALAS-1 AAA mRNA.**

(A) Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pRSVN AAA. At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D plus 4μM heme (lanes 3 to 7) or actinomycin D plus Ca_2CO_3 vehicle (lanes 8 to 12). Final concentration Ca_2CO_3 ($1x10^{-4}$ % w/v). 10μg of total RNA was subsequently used in an RNase protection assay. Lane 1, antisense probes and lane 2 antisense probes following RNase treatment. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.

(B) The data from two independent experiments, as described above, was used to plot the % mRNA remaining versus time. Lines represent the best fit as determined by regression analysis, and were used to estimate the half-life of the human and rat ALAS-1 mRNAs under each treatment regiment.


A
+ + + + + − − − − − Heme (4μM)
0 40 70 100 130 0 40 70 100 130 Time post Act-D (min)
Human ALAS-1
Neo
Rat ALAS-1
501
489
404
331
242
190
M 1 2 3 4 5 6 7 8 9 10 11 12 M


B
1000
100
10
% mRNA remaining
0 20 40 60 80 100 120 140
Time (min)
AAA + Hemin
AAA - Hemin
Rat + Hemin
Rat - Hemin

mRNA from 253 minutes to 93 minutes. The difference in the half-life of the rat ALAS-1 mRNA in heme treated FRL 4.1 cells compared to non heme treated cells was 8 fold. This was considerably greater than that seen with the human ALAS-1 mRNA in the same cells. The half-life of the rat ALAS-1 mRNA was 43 and 345 minutes in heme treated and control cells respectively. Previously, the half-life of the rat ALAS-1 mRNA was calculated, following quantitation of ALAS-1 mRNA levels by Northern blot analysis, to be 42 and 131 minutes in heme treated and control FRL 4.1 cells respectively. The half-life of 43 minutes obtained for the rat ALAS-1 mRNA in heme treated FRL 4.1 cells, in this series of experiments is in good agreement with that obtained previously. However, a much longer half-life (2.6 times longer) was obtained for rat ALAS-1 mRNA in control FRL 4.1 cells, when mRNA levels were measured by RNase protection compared to that obtained previously, with Northern blot analysis. The reason for this difference in the half-life of the rat ALAS-1 mRNA in control cells when measured by the two techniques is unknown. However, it does not appear to be due to the over-expression of the human ALAS-1 mRNA in FRL 4.1 cells. The over-expression of some unstable mRNAs in cells has led to an increase in their half-lives, and this phenomena has been proposed to be due to the saturation of the cellular machinery involved in mRNA degradation (Shyu *et al.*, 1989). This does not appear to be the case in this situation, as a similar increase in the half-life of the rat ALAS-1 mRNA would have been expected to occur in heme treated cells, but this is not observed. An alternative explanation may lie in the size of the probe used in the RNase protection assay to detect the rat ALAS-1 mRNA. The rat probe only detects the presence of a small 178bp region in the middle of the rat ALAS-1 mRNA and therefore a decay intermediate which is substantially truncated in either the 5' or 3' end of the transcript will cross-hybridise with the probe. The signal generated from this truncated species will be identical to that produced by the intact ALAS-1 mRNA and this could account for the apparent increase in the half-life of the rat ALAS-1 mRNA. An example of this is seen in the decay of the histone H4 mRNA (Ross *et al.*, 1986). The mRNA for histone H4 mRNA appears to be degraded *in vitro* and *in vivo* in a 3' to 5' direction, initiated from an endonucleolytic cleavage event in a stem-loop found in the mRNA

3'UTR. A significant difference was observed in the decay rate of the H4 mRNA when probes specific to the 5' or 3' end of the mRNA were used in a S1 nuclease assay to determine the level of the mRNA. In this case the signal generated with the 3'-probe declined more rapidly than that detected by the 5'-probe. Northern blot analysis on the otherhand will only detect products of a defined size which in most cases corresponds to the full length mRNA. In certain situations if there is a relatively stable decay intermediate (which results in the build up of this species to detectable levels) a smaller truncated mRNAs may also be detected. An example of this is seen with the transferrin receptor mRNA where in the human plasma-cytoma cell line ARH-77 or in mouse fibroblasts cells a mRNA species truncated at its 3'-end is detectable (Binder, *et al.*, 1994). The addition of heme to the FRL 4.1 cells may increase the processivity of the machinery involved in the decay of the ALAS-1 mRNA or activate an alternative decay pathway and thus a build up of decay intermediates, which could cause an increase in the half-life of the mRNA in the RNase protection assay, does not occur.

The half-life of the human ALAS-1 mRNA was longer in heme treated FRL 4.1 cells than that of the rat mRNA. This difference may be accounted for in several ways. Firstly, this result may simply reflect an intrinsic difference in the half-lives of the two mRNAs. Alternatively, sequence differences between the human and rat ALAS-1 mRNAs such that the heme-responsive trans-acting factors found in FRL 4.1 cells have a higher affinity for the rat mRNA than that for the human mRNA may cause the longer half-life of the human AAA mRNA. The sequence difference could be natural variations or due to the changes introduced into the human gene during the cloning procedure used to synthesise pRSV AAA.

However, the finding that the half-life of the human ALAS-1 mRNA produced from the pRSV AAA construct is decreased by heme in FRL 4.1 cells is significant. This finding implicates heme in the turnover of the human ALAS-1 mRNA and thus the human ALAS-1 hybrid gene constructs can be used to identify determinants involved in the heme-mediated destabilisation of ALAS-1 mRNA.

The effect of heme on the parental β-globin (BBB) mRNA was also examined. Total RNA was prepared from FRL 4.1 cells, stably transfected with the construct pRSV

BBB, at various times after the cells had been treated with actinomycin D or actinomycin D plus heme. The level of β-globin mRNA was then determined in 10μg of total RNA from each time point by RNase protection (fig 5.17). As can be seen the mRNA for β-globin is very stable in FRL 4.1 cells, with no detectable decrease in the level of the mRNA over the 6 hours assayed. Heme treatment also did not have any effect on the amount of β-globin mRNA present (lane 1 to 4 versus 5 to 8).

ii) The Contribution of the ALAS-1 Coding and Untranslated Regions to the Instability of the ALAS-1 mRNA.

To assess the contribution of the ALAS-1 coding region sequences to the heme-mediated destabilisation effect, the stability of the hybrid mRNA produced from pRSV BAB was examined. In this construct the ALAS-1 coding region was tested for destabilising activity in the absence of any other ALAS-1 sequences. Quantitation of the initial RNase protection assays by PhosphorImaging found that the BAB mRNA gene appeared to be poorly expressed. The initial steady state level of the mRNA (at the start of the time course) appeared to be much lower than that for the neo mRNA when probes of approximately equal specific activity were used. This is in contrast to the expression of the AAA mRNA whose level appeared to be much higher, relative to the level of neo mRNA than BAB mRNA. This low level of the BAB mRNA is probably due to the lack of an intron in the BAB gene. As mentioned previously, there has been several reports describing the low expression of stably transfected intronless genes when compared to the corresponding intron containing gene (Korb *et al.*, 1993). In subsequent experiments the α-sense neo probe was synthesised at a lower specific activity to enable the human ALAS-1 and neo products to be clearly distinguishable. The result of a typical RNase protection assay is shown in figure 5.18, top. Treatment of FRL 4.1 cells with heme caused a more rapid disappearance of the BAB mRNA (lane 2 to 6) than in non-heme treated cells (lane 7 to 11). Data from two independent experiments was pooled and the results plotted as the percentage mRNA remaining versus time (fig 5.18, bottom). The half-life of the BAB mRNA in the presence or absence of added heme was 94 and 388

Figure 5.17: **The Effect of Heme on the Decay of the Parental β-globin BBB mRNA.**

(A) Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pRSVN BBB. At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D plus 4μM heme (lanes 1 to 4) or actinomycin D plus Ca_2CO_3 vehicle (lanes 5 to 8). 10ug of total RNA was subsequently used in an RNase protection assay. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.

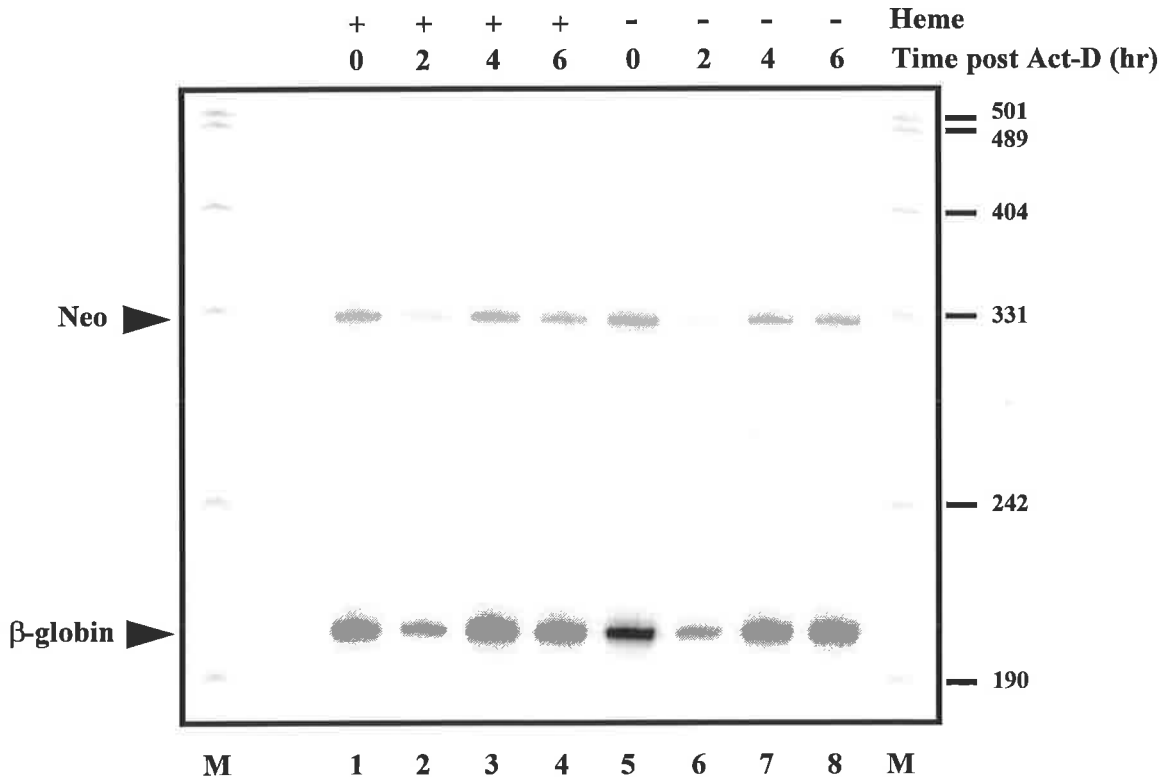
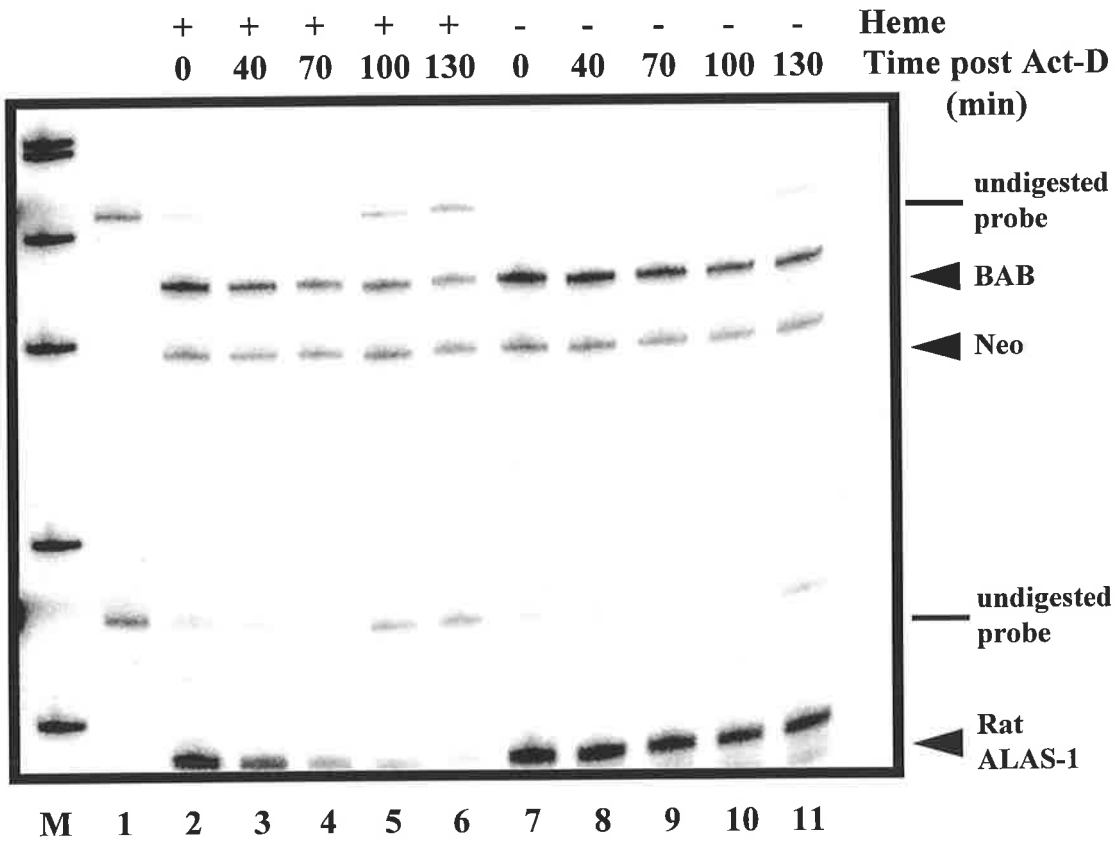
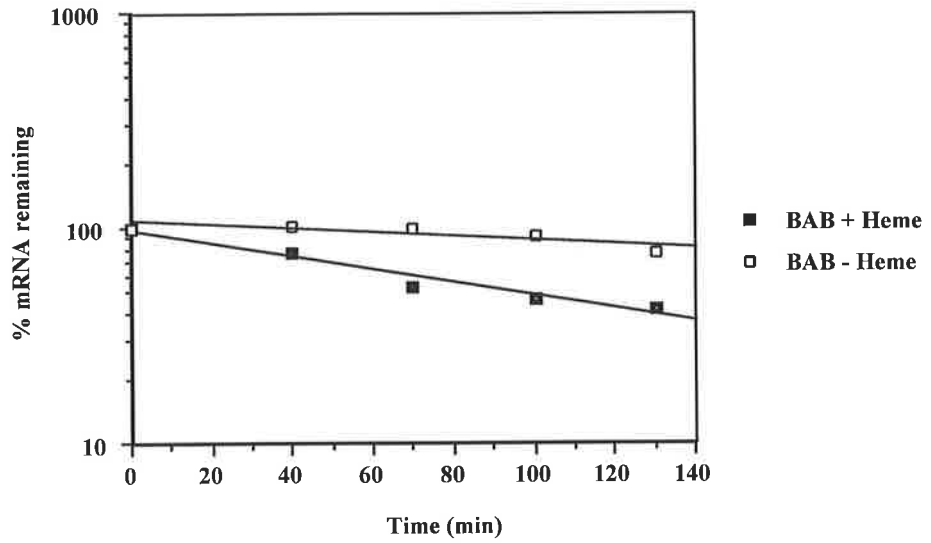

+ + + + - - - - Heme
0 2 4 6 0 2 4 6 Time post Act-D (hr)
501
489
404
331
242
190
Neo
β-globin
M 1 2 3 4 5 6 7 8 M

Figure 5.18: **The Effect of Heme on the Decay of the Hybrid BAB mRNA.**

(A) Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pRSVN BAB. At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D plus 4μM heme (lanes 2 to 6) or actinomycin D plus Ca_2CO_3 vehicle (lanes 7 to 11). 20μg of total RNA was subsequently used in an RNase protection assay. Lane 1, antisense probes following RNase treatment. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.

(B) The data from two independent experiments, as described above, was used to plot the % mRNA remaining versus time. Lines represent the best fit as determined by regression analysis, and were used to estimate the half-life of the hybrid BAB mRNA under each treatment regiment.


+ + + + + - - - - - Heme
0 40 70 100 130 0 40 70 100 130 Time post Act-D (min)
undigested probe
BAB
Neo
undigested probe
Rat ALAS-1
M 1 2 3 4 5 6 7 8 9 10 11


1000
100
10
% mRNA remaining
0 20 40 60 80 100 120 140
Time (min)
BAB + Heme
BAB - Heme

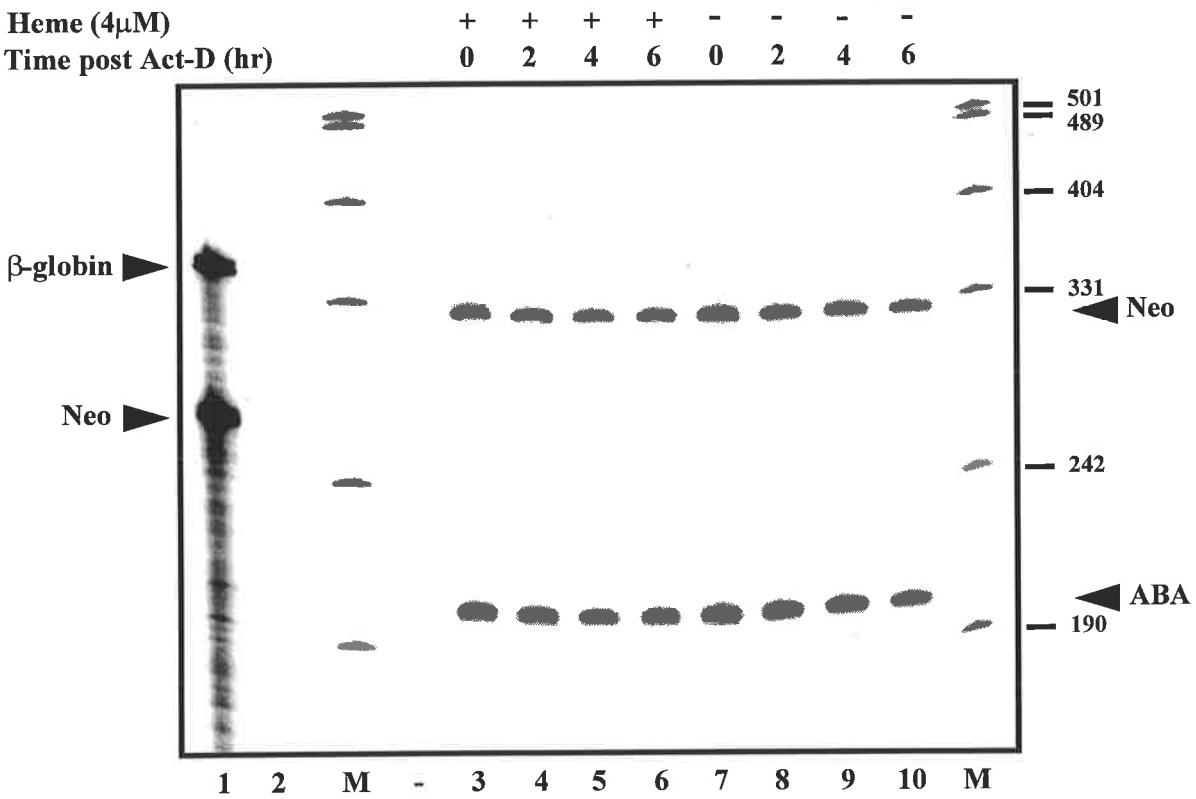
minutes respectively. The finding that the two mRNAs BAB and AAA decayed with similar kinetics (half-lives of 94 and 93 minutes, respectively) following heme treatment, suggested that all the sequences necessary for the heme-mediated destabilisation of the ALAS-1 mRNA occur in the mRNA coding region.

The half-life of the BAB mRNA in the absence of heme was longer (388 minutes) than that observed with the AAA mRNA (253 minutes). Whether this increase in the half-life is due to the absence of ALAS-1 untranslated region sequences or to some difference in the state of the FRL 4.1 cell population is unknown. For example, the half-life of the c-myc mRNA can vary from approximately 30 to 120 minutes depending on the cells growth conditions (Herrick and Ross, 1994).

The proposal that the ALAS-1 coding region is sufficient for the heme-mediated decay of the ALAS-1 mRNA was supported by experiments in FRL 4.1 cells that expressed hybrid ABA mRNA. The ABA mRNA was extremely stable in FRL 4.1 cells , with no detectable decline in its level up to 6 hours post actinomycin D treatment (fig 5.19). Furthermore heme had no effect on the decay of the hybrid mRNA in these cells (lanes 3 to 6 versus 7 to 10). As before, the level of ABA was corrected for sample handling variation by normalising neo mRNA levels. This experiment was repeated and a similar result obtained, implying that the untranslated regions of the ALAS-1 mRNA play no part in the heme-mediated destabilisation effect. As the ABA mRNA appears to be as stable as the full β-globin mRNA (BBB), the ALAS-1 untranslated regions also do not appear to play a role in the relatively instability of the human ALAS-1 mRNA (half-life 4 hours) in non-heme treated FRL 4.1 cells: most cytoplasmic mRNAs in mammalian cells turnover with apparent half-lives in excess of 12 hours (Greenberg, 1972 ; Singer and Penman, 1973). However to confirm this finding the time course needs to be extended to provide an accurate measurement of the half-lives of the BBB and ABA mRNAs.

Figure 5.19: **The Effect of Heme on the Decay of the Chimeric ABA mRNA.**

Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pRSVN ABA. At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D plus 4μM heme (lanes 3 to 6) or actinomycin D plus Ca_2CO_3 vehicle (lanes 7 to 10). 10μg of total RNA was subsequently used in an RNase protection assay. Lane 1, antisense probes and lane 2, antisense probes following RNase treatment. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.


Heme (4μM)
+ + + + - - - -
Time post Act-D (hr)
0 2 4 6 0 2 4 6
β-globin
Neo
501
489
404
331
Neo
242
ABA
190
1 2 M - 3 4 5 6 7 8 9 10 M

iii) The Effect of Heme on the Decay of the Hybrid mRNAs BAA, AAB, ABB and BBA.

If the ALAS-1 coding region is sufficient for the heme-mediated destabilisation effect we can predict that the hybrid mRNAs BAA and AAB should be unstable and heme-responsive, while the mRNAs for ABB and BBA should be stable and non-heme responsive.

Initially, FRL 4.1 cells expressing the mRNA for BAA were examined. Cells were treated with actinomycin D or actinomycin D plus heme and total RNA isolated from the cells at various times. Total RNA (20μg) from each time point was then used in an RNase protection assay to determine the levels of BAA and neo mRNA. Again due to the low steady state level of the BAA mRNA, the neo probe was re-synthesised as a low specific activity probe. A typical RNase protection is shown in figure 5.20. Data from two such experiments were used to calculate the half-life of the BAA mRNA (fig 5.20B). The half-life of the BAA mRNA was 146 and 512 minutes in the presence or absence of exogenously added heme respectively. As predicted the BAA mRNA was moderately unstable but more importantly was further destabilised (3.5-fold) by the addition of heme indicating that the BAA mRNA still responded to heme. However, the half-life of the BAA mRNA like the mRNA for BAB was longer in the absence of heme than that seen for the AAA mRNA perhaps suggesting a role for the ALAS-1 5'UTR in the instability of the ALAS-1 mRNA. Alternatively, the β-globin 5'UTR may be able to modulate the activity of the ALAS-1 coding region determinant in these hybrid mRNAs.

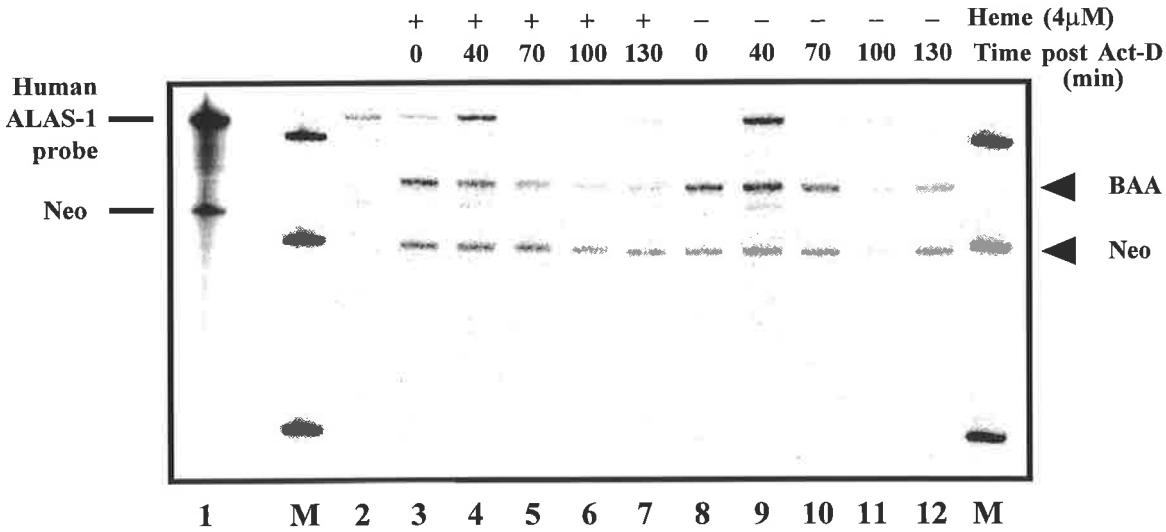
That the ALAS-1 5'UTR does not contain a true instability sequences is shown by the stability of the ABA mRNA. A similar finding was observed in the decay of the hybrid mRNA ABB. To assess the stability of the hybrid mRNA ABB, total RNA was isolated from FRL 4.1 cells expressing ABB at various times after the cells were treated with actinomycin D or actinomycin D plus heme. The level of ABB, neo and rat ALAS-1 mRNA was then determined in 10μg of total RNA from each time point by RNase protection analysis (fig 5.21). As can be seen in figure 5.21 the ABB mRNA is very stable in FRL 4.1 cells with little change in the level of ABB mRNA in either heme

Figure 5.20: **The Effect of Heme on the Decay of the Chimeric BAA mRNA.**

(A) Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pRSVN BAA. At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D plus 4μM heme (lanes 3 to 7) or actinomycin D plus Ca_2CO_3 vehicle (lanes 8 to 12). 20μg of total RNA was subsequently used in an RNase protection assay. Lane 1, antisense probes and lane 2, antisense probes following RNase treatment. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.

(B) The data from two independent experiments, as described above, was used to plot the % mRNA remaining versus time. Lines represent the best fit as determined by regression analysis, and were used to estimate the half-life of the hybrid BAA mRNA under each treatment regiment.

A


+ + + + + – – – – – Heme (4μM)
0 40 70 100 130 0 40 70 100 130 Time post Act-D (min)
Human ALAS-1 probe
Neo
BAA
Neo
1 M 2 3 4 5 6 7 8 9 10 11 12 M

B

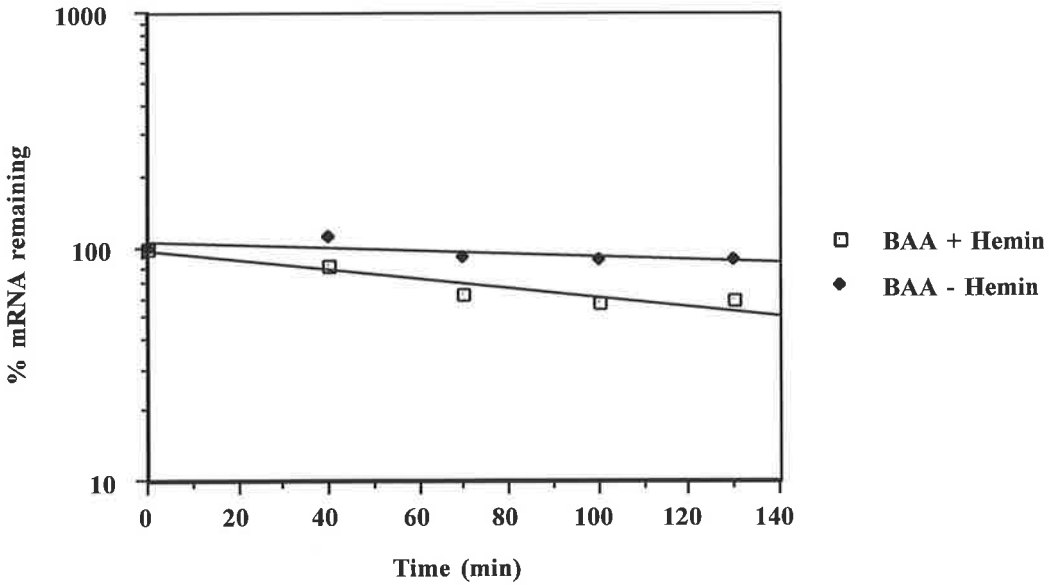
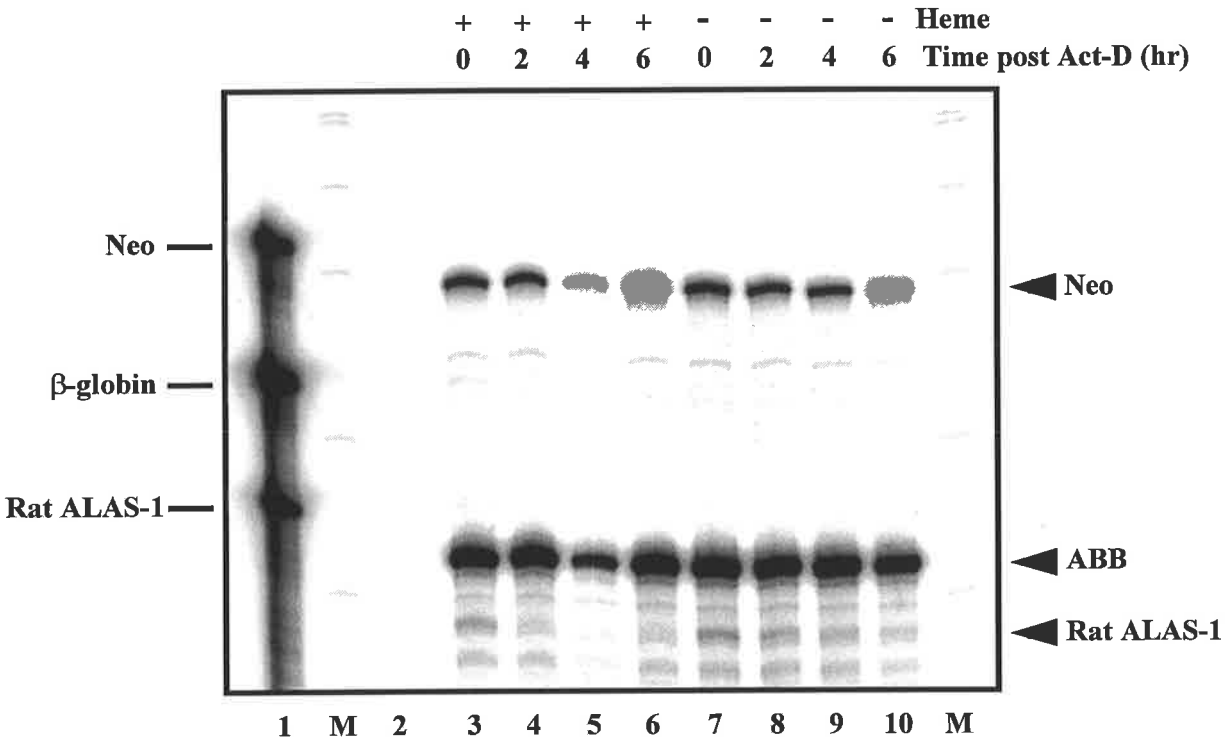

1000
100
10
% mRNA remaining
0 20 40 60 80 100 120 140
Time (min)
BAA + Hemin
BAA - Hemin

Figure 5.21: **The Effect of Heme on the Decay of the Chimeric ABB mRNA.**

(A) Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pRSVN ABB. At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D plus 4μM heme (lanes 3 to 6) or actinomycin D plus Ca_2CO_3 vehicle (lanes 7 to 10). 10μg of total RNA was subsequently used in an RNase protection assay. Lane 1, antisense probes and lane 2, antisense probes following RNase treatment. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.


+ + + + - - - - Heme
0 2 4 6 0 2 4 6 Time post Act-D (hr)
Neo
β-globin
Rat ALAS-1
Neo
ABB
Rat ALAS-1
1 M 2 3 4 5 6 7 8 9 10 M

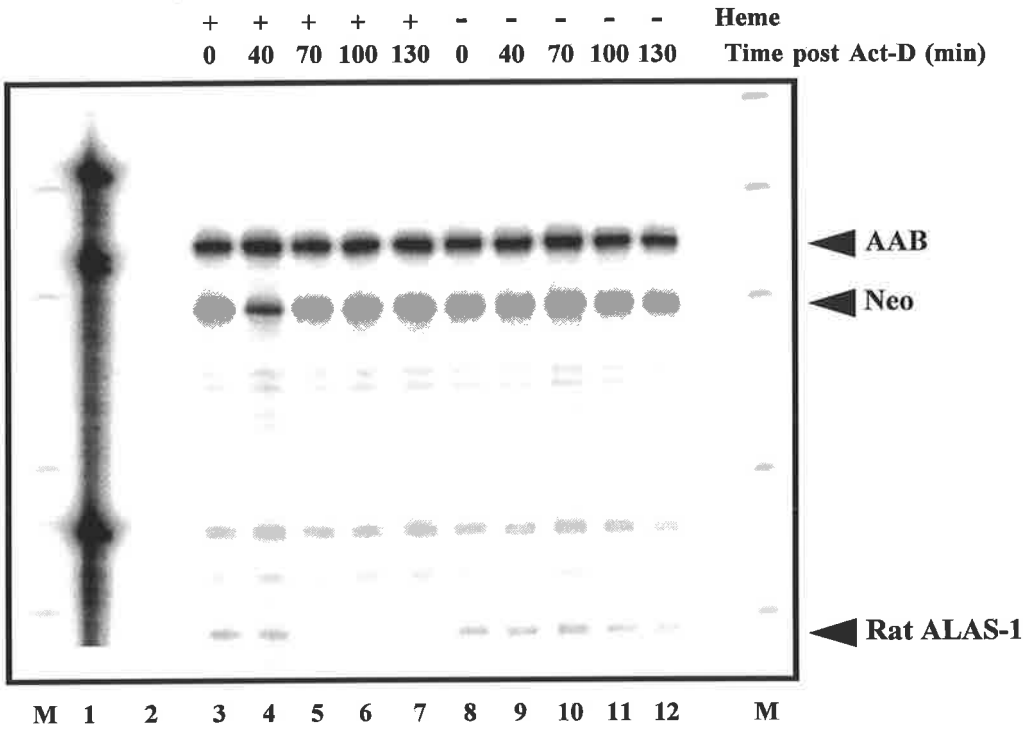
treated (lanes 3 to 6) or control (lanes 7 to 10) cells over the 6 hours assayed. The signal produced by the rat ALAS-1 mRNA is barely detectable over the background but it appears to rapidly drop in heme treated cells compared to control cells. This experiment was repeated and a similar result obtained, indicating that the ALAS-1 5'UTR probably does not contain any instability elements. However, for a more conclusive answer the time course needs to be extended to obtain an accurate measurement of the hybrid mRNA half-life.

Next the effect of heme on the decay of the AAB mRNA was investigated (fig 5.22). Surprisingly, the level of AAB mRNA remained unaltered in both heme treated (lanes 3 to 7) and untreated (lanes 8 to 12) FRL 4.1 cells during the 130 minutes assayed. Heme treatment appeared successful as a rapid decrease in the level of endogenous rat ALAS-1 in heme treated cells versus control cells is observed. This experiment was repeated and a similar result obtained. The time course was also extended (discussed later) but no detectable decline in the amount of AAB mRNA observed. Another indication of the apparently long half-life of the AAB mRNA was the high level of AAB mRNA (approximately 2-fold higher than that obtained by the SV40-driven neo gene) found in these cells at the start of the time course. The steady state level of a mRNA often reflects the half-life of the mRNA (Herrick and Ross, 1994). This result suggested that either the ALAS-1 coding region did not contain the heme-responsive instability element or alternatively, that the presence of the β-globin 3'UTR inhibited the function of ALAS-1 coding region instability determinant in this mRNA.

The second possibility appears to be unlikely as the half-life of the BAB mRNA, which also contains the β-globin 3'UTR, is very similar to that of the AAA mRNA. Therefore this result suggested that the heme-responsive instability sequence lies in the 3'UTR of the human ALAS-1 mRNA, as the replacement of this region of the ALAS-1 mRNA with the β-globin 3'UTR led to the stabilisation of the mRNA and the loss of heme-responsiveness. However, this conclusion is inconsistent with the results obtained when the effect of heme on the decay of the mRNAs for the hybrid genes BAB, BAA and ABA was determined. The decay profiles of these mRNAs suggested that the ALAS-1 coding region was sufficient for the heme-mediated destabilisation of the

Figure 5.22: **The Effect of Heme on the Decay of the Chimeric AAB mRNA.**

(A) Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pRSVN AAB. At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D plus 4μM heme (lanes 3 to 7) or actinomycin D plus Ca_2CO_3 vehicle (lanes 8 to 12). 10μg of total RNA was subsequently used in an RNase protection assay. Lane 1, antisense probes and lane 2, antisense probes following RNase treatment. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.


+ + + + + - - - - - Heme
0 40 70 100 130 0 40 70 100 130 Time post Act-D (min)
AAB
Neo
Rat ALAS-1
M 1 2 3 4 5 6 7 8 9 10 11 12 M

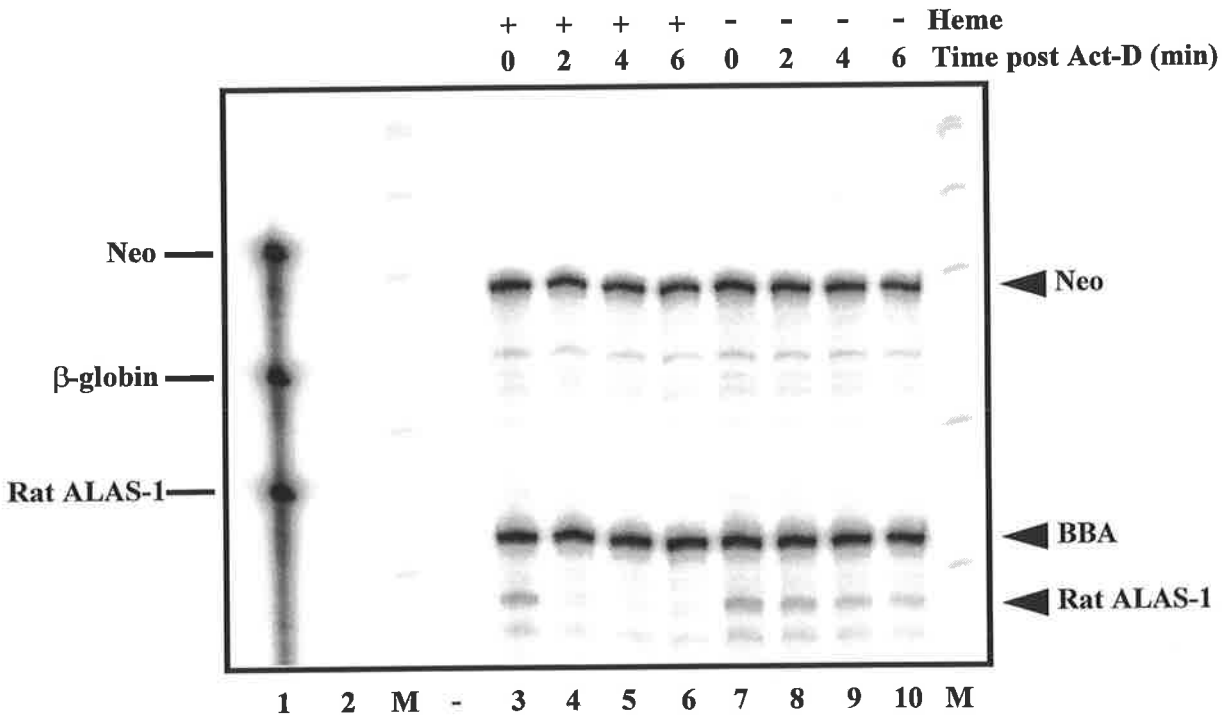
ALAS-1 mRNA. There are several possible reasons for these contradictory results. (i) A mutation was introduced into the AAB gene during the cloning procedure to make pRSV AAB or alternatively, part of the AAB construct was deleted or re-arranged during its integration into the FRL 4.1 cells genome. This mutation disrupts the function of the ALAS-1 coding region instability region. (ii) There was a problem with the export of the AAB mRNA from the nucleus to the cytoplasm (the proposed site of mRNA decay). (iii) The high level of the AAB mRNA produced in this cell population saturated the ALAS-1 specific decay machinery. (iv) The result obtained with the AAB mRNA is in fact the true result. These possibilities were then investigated.

The last possibility appears to be extremely unlikely, since not only do the results obtained with the decay of the mRNAs for BAB and BAA need to be the result of an artefact of the hybrid mRNAs, but also the decay profile of the ABA mRNA did not show any heme-responsive instability associated with the ALAS-1 3'UTR. To confirm that the 3'UTR of the human ALAS-1 mRNA does not contain an instability determinant the effect of heme on the decay of BBA mRNA was investigated by RNase protection analysis (fig 5.23). The hybrid BBA mRNA was found to be very stable in FRL 4.1 cells with no detectable decrease in the level of the β-globin product up to 6 hours post actinomycin D treatment. Heme, moreover did not have any effect on the level of BBA mRNA (lane 3 to 6). Heme did however, cause a substantial decline in the level of rat ALAS-1 mRNA in the same cells. Therefore it appears from the decay profiles of the mRNAs for ABA and BBA, that the human ALAS-1 3'UTR is incapable of conferring heme-mediated instability, at least, upon the β-globin coding region and hence is unlikely to contain the heme-responsive instability sequence.

To determine if there was a problem in the transport of the AAB mRNA from the nucleus to the cytoplasm, cytoplasmic RNA was isolated (section 2.3.2) from AAB expressing FRL 4.1 cells. For most mammalian pre-mRNAs a series of processing events must take place in the nucleus before the mature mRNA is exported to the cytoplasm, these events include the cleavage and poly-adenlyation reactions to form the 3'-end of the mRNA and the splicing reactions to remove the introns from the pre-mRNA. Several lines of evidence now indicate that these RNA-processing events are

Figure 5.23: **The Effect of Heme on the Decay of the Chimeric BBA mRNA.**

(A) Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pRSVN BBA. At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D plus 4μM heme (lanes 3 to 6) or actinomycin D plus Ca_2CO_3 vehicle (lanes 7 to 10). 10μg of total RNA was subsequently used in an RNase protection assay. Lane 1, antisense probes and lane 2, antisense probes following RNase treatment. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.


+ + + + - - - - Heme
0 2 4 6 0 2 4 6 Time post Act-D (min)
Neo
β-globin
Rat ALAS-1
Neo
BBA
Rat ALAS-1
1 2 M - 3 4 5 6 7 8 9 10 M

coupled to one another to ensure the appropriate and accurate expression of the gene. Further more it appears that only after the RNA is fully processed can the mature mRNA be transported to the cytoplasm (Eckner *et al.*, 1991 ; Reviewed Izaurralde and Mattaj, 1992 ; Davis, 1992). Several reports have suggested that the splicing reaction and the formation of the 3'-end of the mRNA are linked in intact cells. Firstly, the insertion of an α-globin intron into an intronless hybrid pre-mRNA which contained the 3'-end of the histone H2A mRNA, interfered with the formation of the histone 3'-end and instead favoured the use of a cryptic 3'-polyadenylation site found in the histone H2A genes 3'-flanking region. In the absence of introns the hybrid mRNA exclusively contained the histone 3'-end (Pandey *et al.*, 1990). Similarly, the insertion of an intron into an intronless CAT gene construct led to an increase in the amount of poly $(A)^+$ mRNA over poly $(A)^-$ mRNA in the nucleus. This was not seen with an intronless gene construct, suggesting that intronic sequences promote poly-adenylation (Haung and Gorman, 1990). Secondly, poly-adenylation can be stimulated *in vitro* by the presence of an upstream 3'-splice site (Niwa *et al.*, 1990).

This requirement for intron sequences for the efficient formation of the mRNAs 3'-end appears to be dependent on a functional 3'-terminal intron, at least for the human triosephosphate isomerase (TPI) gene (Nesic *et al.*, 1993). Conversely, a functional poly(A) signal appears to be involved in the removal of the 3'-terminal intron, at least *in vitro*, as mutations which disrupt the AAUAAA poly (A) signal also inhibit the splicing of an upstream 3'-terminal intron but not more distal introns (Niwa and Berget, 1991). However there does not appear to be any requirement for 3'-terminal-intron specific sequences in this enhancement process since a normally non-terminal intron can substitute for the 3'-terminal intron without inhibiting the formation of the 3'-end of the mRNA. Nevertheless a strong correlation was noted between the efficiency of 3'-terminal intron removal and the formation of the 3'-end of the TPI mRNA and that both processes were activated by the presence of upstream introns. This activation was most likely due to the apparent stimulatory influence of upstream introns on the removal of the 3'-terminal intron (Nesic and Maquat, 1994). Specifically, Nesic and Maquat (1994) observed that TPI gene constructs containing only a single intron produced a build up of

uncleaved and un-polyadenylated RNA in the nucleus with a resultant decrease in the correctly processed mRNA in the nucleus and cytoplasm. The addition of subsequent introns stimulated the removal of the 3'-terminal intron which in turn led to an increase in the amount of correctly processed mRNA in the nucleus (relative to unprocessed RNA) as well as an increase in the amount of TPI mRNA in the cytoplasm. Cooperativity between introns during the intron removal process has also been reported for tumor necrosis factor-β (TNF-β) pre-mRNA (Nell *et al.*, 1993).

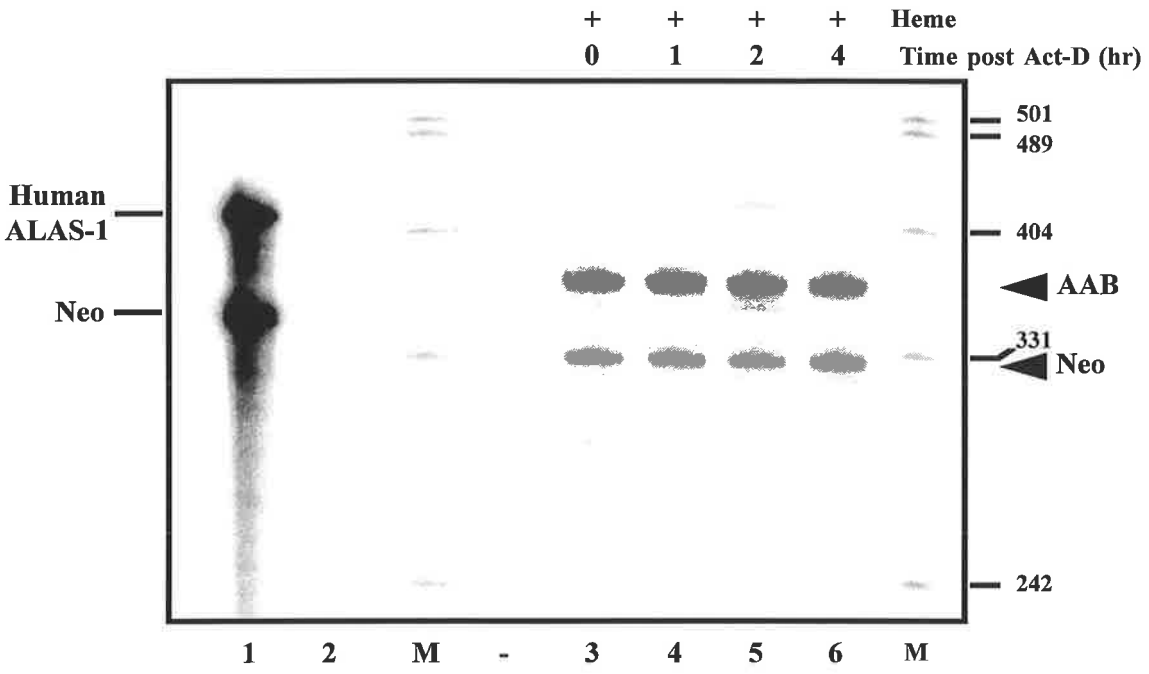
The build up of uncleaved and un-polyadenylated TPI RNA in the nucleus seen by Nesic and Maquat (1994) was most likely due to its inability to be exported from the nucleus in this state (Eckner *et al.*, 1991). Therefore the processing events that led to a mature mRNA in the cytoplasm appear to be highly interdependent on one-another so that the disturbance of one process leads to perturbation of the others.

The hybrid mRNA produced from the AAB gene only contained a single intron (intron 1) in its 5'UTR and therefore the splicing and formation of the AAB mRNAs 3'-end may be inefficient for the above reasons. This in turn could cause the majority of the AAB mRNA to be retained in the nucleus. As the heme-mediated destabilisation of the ALAS-1 mRNA is assumed to take place in the cytoplasm this could account for the apparent stability of the AAB mRNA. However, the parental human ALAS-1 gene construct AAA also only contains intron 1, but in this case the AAA mRNA still responds to heme indicating that the AAA mRNA could leave the nucleus. Therefore, it appeared that the human ALAS-1 intron 1 was sufficient to allow the processing and export of the hybrid pre-mRNAs from the nucleus. However, sequence differences between the AAB and AAA mRNAs which inhibit the export of the AAB mRNA from the nucleus cannot be ruled out.

Therefore, to examine the decay of the AAB mRNA in the cytoplasm, cytoplasmic RNA was isolated from FRl 4.1 cells at various times after the cells had been treated with actinomycin D and heme. The level of AAB and neo mRNA was then determined in 10μg of RNA from each time point by RNase protection (fig 5.24). The decay profile of cytoplasmic AAB mRNA was similar to that observed for total RNA. The level of AAB mRNA remained unchanged during the 4 hours assayed, indicating

Figure 5.24: **The Effect of Heme on the Decay of Cytoplasmic AAB mRNA.**

Cytoplasmic RNA was prepared from FRL 4.1 cells, stably transfected with pRSVN AAB as described in section 2.3.2. At time zero the cells were treated with 10μg/ml actinomycin D plus 4μM heme. 10μg of cytoplasmic RNA was then used in an RNase protection assay. Lane 1, antisense probes; lane 2, antisense probes following RNase treatment and lanes 3 to 6 cytoplasmic RNA. Cytoplasmic RNA was harvested at the indicated times above each lane. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.


+ + + + Heme
0 1 2 4 Time post Act-D (hr)
Human ALAS-1
Neo
501
489
404
AAB
331
Neo
242
1 2 M - 3 4 5 6 M

that the cytoplasmic form of the AAB mRNA was stable in the presence of heme. This result thus suggested that the AAB mRNA was not being trapped in the nucleus.

Another possible reason for the stability of the AAB mRNA may be its high expression level. Several studies have found that the over-expression of an unstable mRNA in a cell can saturate the cellular decay machinery, leading to an increase in the mRNAs half-life. This saturation effect can occur when the expression of the mRNA is being driven by a strong constitutive promoter, like RSV (Shyu *et al.*, 1989 ; Goodall personnel communication). This explanation does not appear to be valid in this case as the turnover of the endogenous rat ALAS-1 mRNA is unaffected by the high levels of AAB mRNA present. However, given that the level of AAB mRNA expression will vary from clone to clone, depending on the integration site of the construct in the FRL 4.1 genome, it is possible that a large proportion of the AAB mRNA signal detected came from a small percentage of clones which express very high levels of AAB mRNA. The level of endogenous rat ALAS-1 mRNA on the other hand is normally quite low in FRL 4.1 cells as a result of both a low transcription rate and a relatively unstable mRNA. Therefore, the contribution to the rat protected product by any one particular clonal cell line is likely to be small. So that even if the rat ALAS-1 mRNA is stabilised in cells massively over-expressing AAB mRNA this may be difficult to detect in a mixed cell population. This "dilution" effect may be compounded by the fact that the heme-responsive decay machinery in FRL 4.1 cells appears to have a higher affinity for the rat ALAS-1 mRNA than for the human mRNA, as judged by the difference in the half-lives of the two ALAS-1 mRNA species in heme treated cells. Therefore, the available heme-responsive factors are more likely to bind to the rat ALAS-1 mRNA than the AAB mRNA in these cells, making the rat ALAS-1 mRNA less sensitive to limiting amounts of the heme-responsive factor.

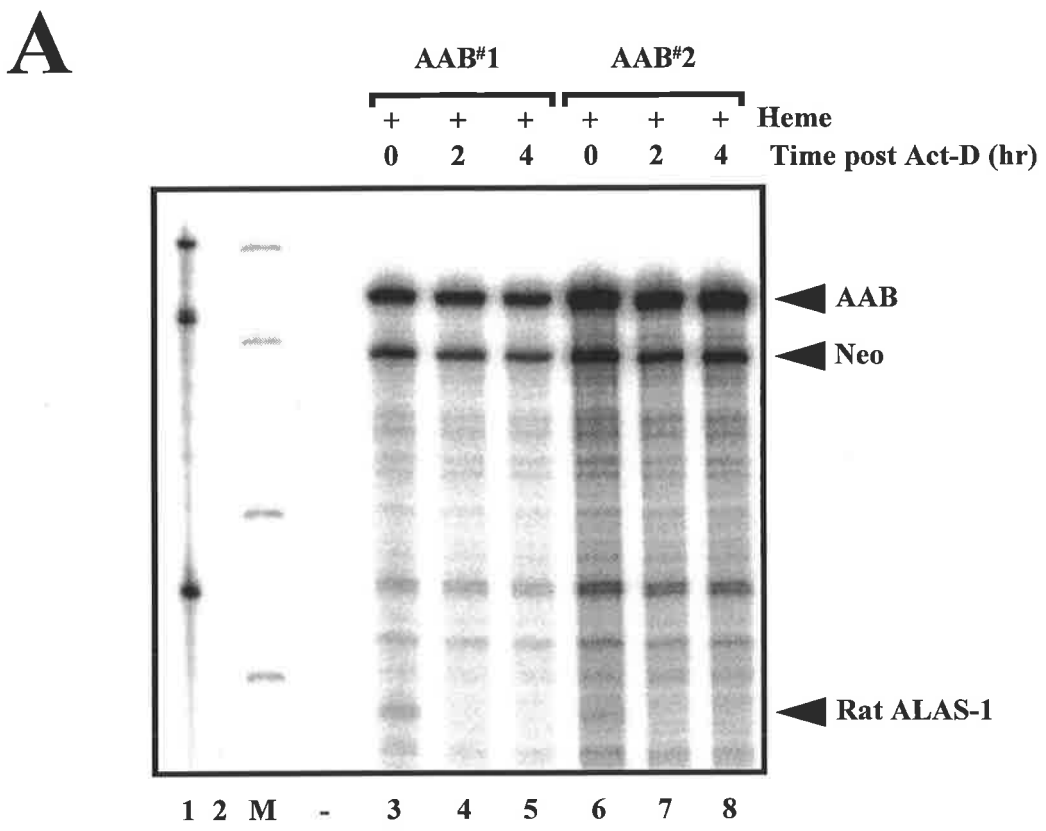
To assess if the level of AAB mRNA expression can affect the heme-mediated decay of the rat ALAS-1 and AAB mRNA, the effect of heme on the two mRNAs was examined in cell lines that expressed different levels of AAB mRNA. Briefly, pure clonal cell lines were isolated from the mixed cell population. To separate individual cells, aliquots of the AAB expressing cells were plated at a density of 10^3 cells/150mm

culture dish. These individual cells were allowed to grow up to a colony of between 100-200 cells, before five well separated colonies were picked, expanded and aliquots frozen down as stocks. The five clonal cell lines were termed AAB #1 to 5. Initial quantitation of the steady state level of AAB mRNA in these cell lines established that the expression of AAB mRNA varied considerably. The cell lines AAB# 2 and 5 expressed high levels of AAB mRNA, AAB# 4 low levels and AAB# 1 and 3 a moderate level of AAB mRNA (Data not shown). The effect of heme on the decay of the AAB mRNA was then investigated in the cell lines AAB #1,2,4 and 5. Total RNA was isolated from the different cell lines at various times after the cells were treated with actinomycin D plus heme and the level of AAB, neo and rat ALAS-1 mRNA in 10µg of total RNA determined by RNase protection analysis (fig 5.25). As can be seen in figure 5.25 the ABB mRNA was stable in the presence of heme in all four FRL 4.1 cell lines tested, irrespective of its initial steady state level (zero minutes ; lanes 3 and 6). The level of rat ALAS-1 mRNA however, dropped rapidly in the presence of heme to be virtually undetectable at 60 minutes in all four cell lines. This result indicated that the over-expression of the AAB mRNA was insufficient to stabilise the AAB mRNA by the saturation of the heme-responsive machinery.

Lastly, the apparent inconsistency between the decay of the AAB mRNA compared to the other hybrid mRNAs could be due to a mutation in the AAB mRNA such that the AAB mRNA is no longer recognised by the heme-responsive decay machinery. This mutation could either have been introduced during the synthesis of the pRSV AAB gene construct or during the integration of the construct into the FRL 4.1 genome. To determine if the AAB gene had undergone any deletions or re-arrangements, Southern blot analysis was carried out using specific probes for the 5', 3' and coding portion of the AAB gene. Genomic DNA was isolated from the five pure FRL 4.1 cell lines AAB#1 to 5 as described in section 2.2.13. Genomic DNA (20µg) from each cell line was digested with Pst I and Southern blot analysis performed in triplicate. Pst I digested rat liver genomic DNA and pRSV AAB vector was also included on the Southern blot. The resultant filters were then probed with the three different probes (figure 5.26). Figure 5.26B shows the result obtained when the filter

Figure 5.25: **Analysis of AAB mRNA Levels in Individual AAB Clones.**

Total RNA was isolated at the indicated times from FRL 4.1 cells, stably transfected with pRSVN AAB. At time zero the FRL 4.1 cells were treated with either 10μg/ml actinomycin D plus 4μM heme. 10μg of total RNA was subsequently used in an RNase protection assay. (A) RNase protection of RNA isolated from FRL AAB$^{\#}$1 (lane 3 to 5) and FRL AAB$^{\#}$2 (lanes 6 to 8). (B) RNase protection of RNA isolated from FRL AAB$^{\#}$4 (lane 3 to 5) and FRL AAB$^{\#}$5 (lanes 6 to 8). Lane 1, antisense probes and lane 2, antisense probes following RNase treatment. Markers (M) consisted of radiolabelled single stranded Hpa I cut pUC19 DNA. The position of the protected species is indicated by the arrow heads.


A
AAB#1
AAB#2
+ + + + + + Heme
0 2 4 0 2 4 Time post Act-D (hr)
AAB
Neo
Rat ALAS-1
1 2 M - 3 4 5 6 7 8

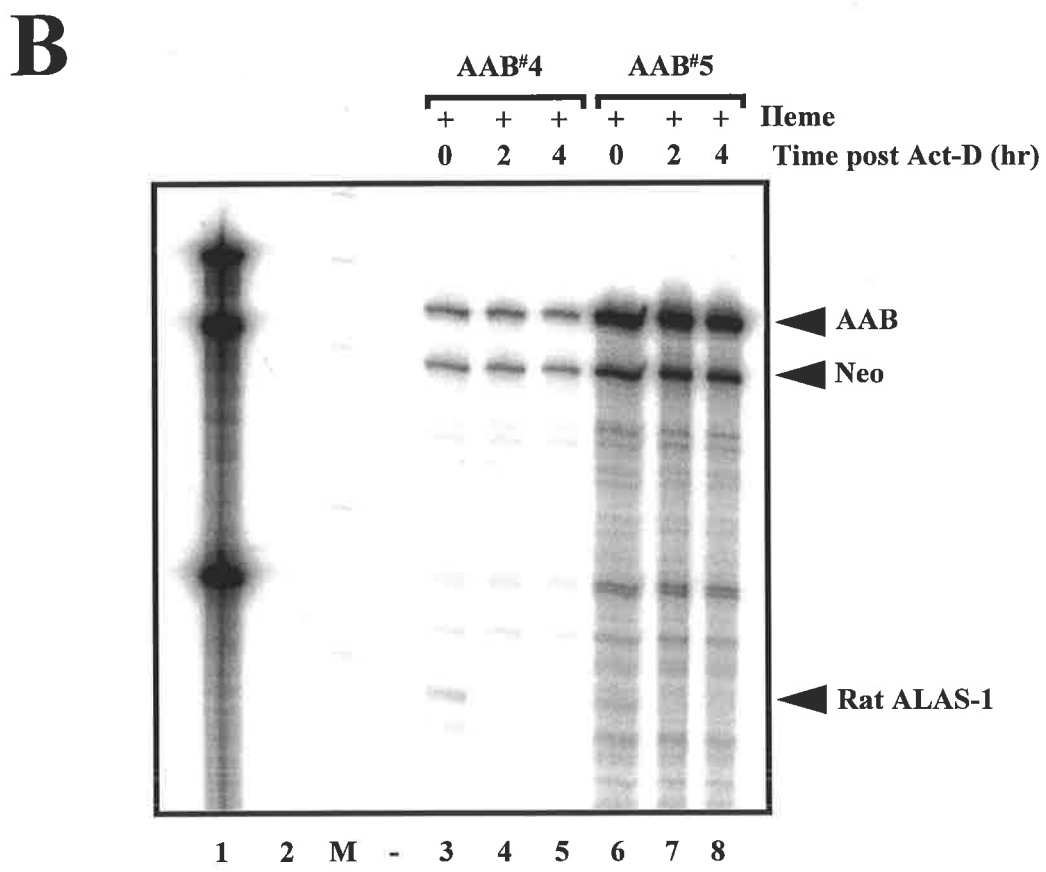
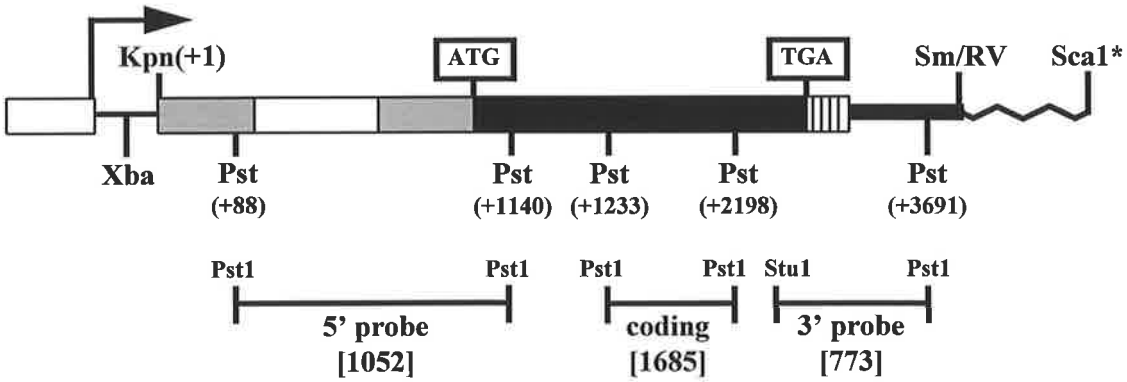

B
AAB#4
AAB#5
+ + + + + + Heme
0 2 4 0 2 4 Time post Act-D (hr)
AAB
Neo
Rat ALAS-1
1 2 M - 3 4 5 6 7 8

Figure 5.26: **Southern Blot Analysis of Pst I Digested AAB FRL 4.1 DNA.**

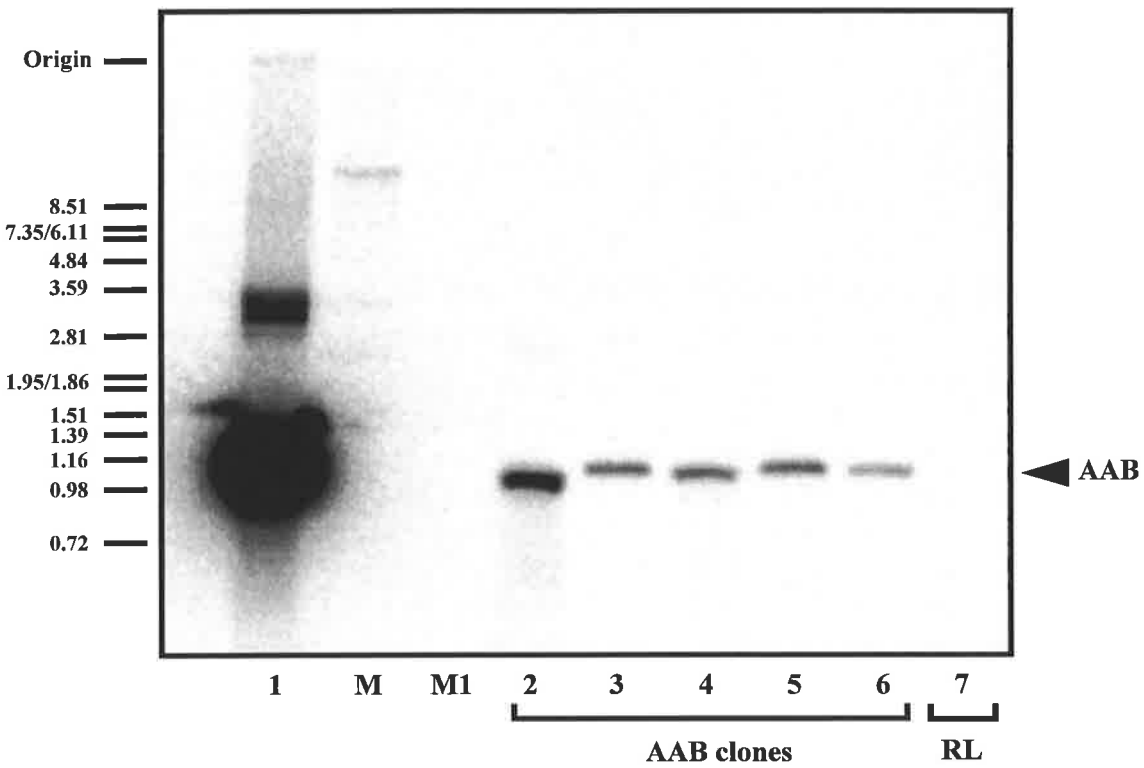
(A) Schematic of the AAB gene. Stripped rectangles, denote 5'-untranslated regions; open rectangles, introns; black rectangles, protein coding regions; vertical stripped rectangles, 3'-untranslated regions, thick lines, 3'-flanking sequences and wavy lines pRSVN.06 vector sequences. The position of the Pst I sites are given relative to the Kpn I site (+1) at the start of the ALAS-1 5'UTR sequences. The initiation and termination codons are boxed. Indicated below the gene are the three different probes used. The number in the square brackets is the expected size (bp) of the Pst I fragment detected by each probe.

(B) Genomic DNA (20μg) was digested with Pst I and electrophoresed on a 1.0% agarose TBE gel. The restriction fragments were then transferred to a Nytran^{TM} membrane and cross-linked to the filter by UV irradiation. The Southern blots were then probed with (B), the 5'-end probe; (C) the coding region probe and (D) the 3'-end probe. Lane 1, pRSVN AAB DNA; M, Hind III cut λ DNA; M1, Eco RI cut SPP-1 DNA; lane 2 AAB clone #5 DNA; lane 3, AAB clone #4 DNA; lane 4, AAB clone #3 DNA; lane 5, AAB clone #2 DNA, lane 6, AAB clone #1 DNA and lane 7, rat liver genomic DNA. AAB gene fragments that cross hybridise with the probe are indicated by arrow heads.

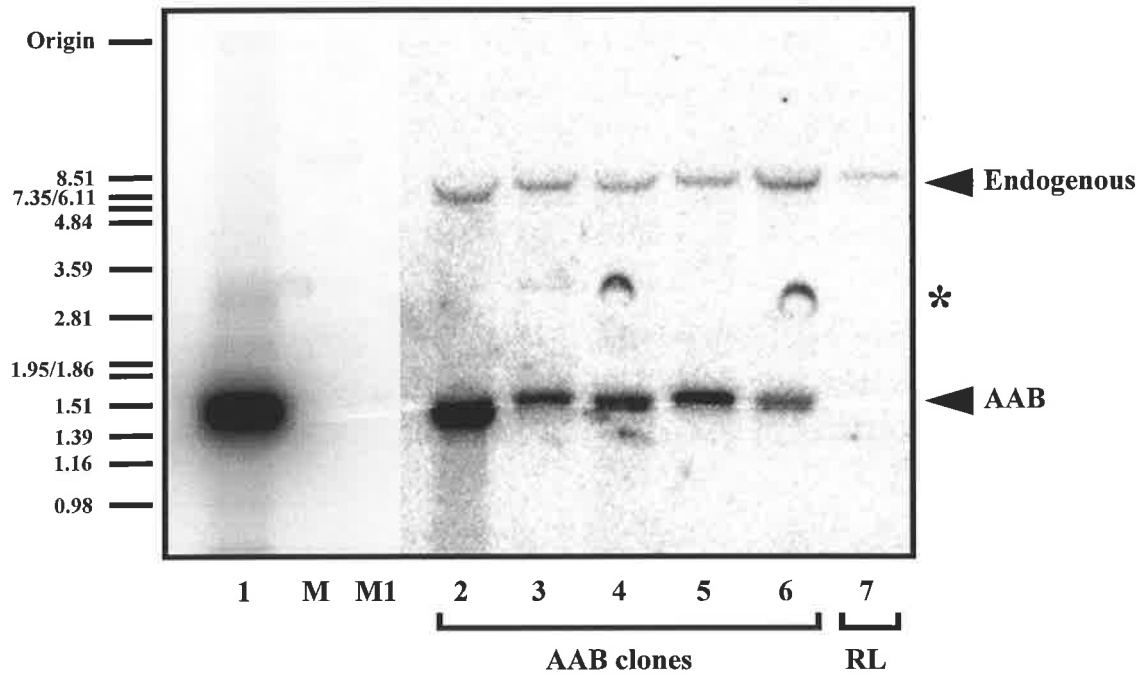
A


Kpn(+1)
ATG
TGA
Sm/RV
Sca1*
Xba
Pst
(+88)
Pst
(+1140)
Pst
(+1233)
Pst
(+2198)
Pst
(+3691)
Pst1
Pst1
Pst1
Pst1
Stu1
Pst1
5' probe
[1052]
coding
[1685]
3' probe
[773]

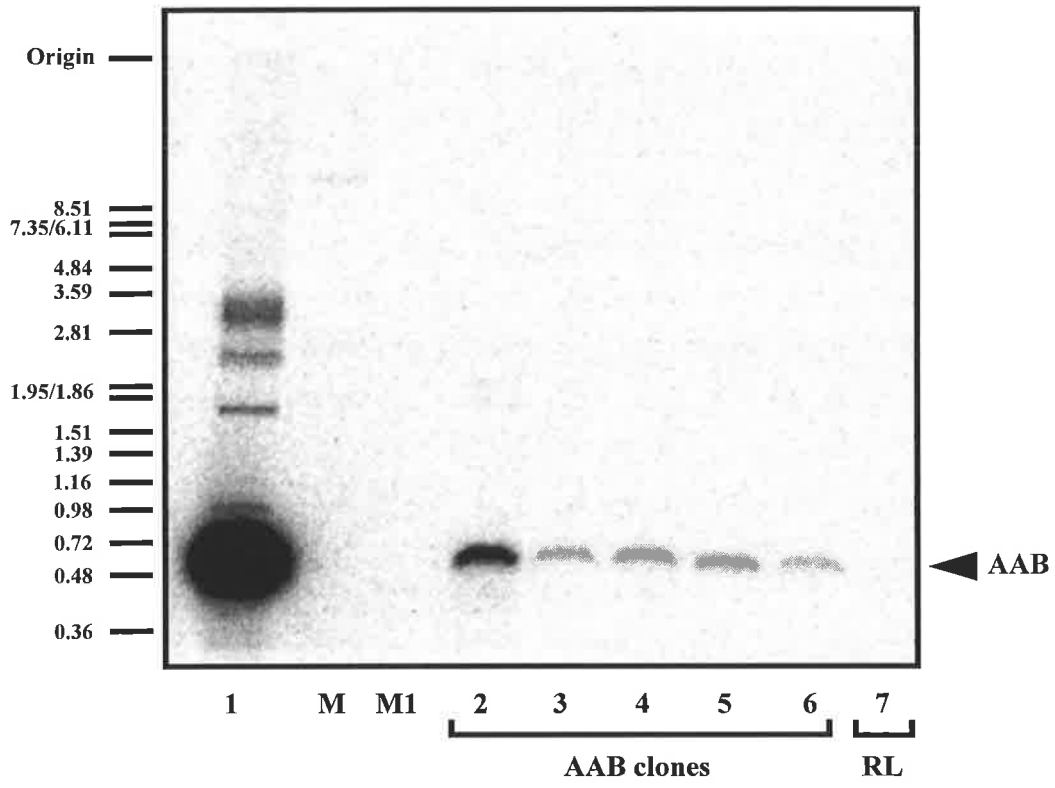
B


Origin
8.51
7.35/6.11
4.84
3.59
2.81
1.95/1.86
1.51
1.39
1.16
0.98
0.72
AAB
1
M
M1
2
3
4
5
6
7
AAB clones
RL

C


Origin
8.51
7.35/6.11
4.84
3.59
2.81
1.95/1.86
1.51
1.39
1.16
0.98
Endogenous
*
AAB
1
M
M1
2
3
4
5
6
7
AAB clones
RL

D


Origin
8.51
7.35/6.11
4.84
3.59
2.81
1.95/1.86
1.51
1.39
1.16
0.98
0.72
0.48
0.36
AAB
1
M
M1
2
3
4
5
6
7
AAB clones
RL

was probed with the 5'-end probe. The 5'-end probe consisted of the 1.044kb Pst I fragment from pBK-RV/Hd, that included sequences from the Pst I site at +86bp in the ALAS-1 genes 5'UTR to the Pst I site at 150bp in the human ALAS-1 cDNA. Figure 5.26C shows the result of probing the Pst I digested DNA with the ALAS-1 coding region probe. The ALAS-1 coding region probe consisted of the large 1684bp Pst I fragment of the human ALAS-1 cDNA. Figure 5.26D shows the result obtained when the filter was probed with the 3'-end probe. The 3'-end probe consisted of the 3'-end of the human β-globin gene and contained sequences from the Stu I site, introduced into the β-globin stop codon in pBK BBB, to the Pst I site approximately 700bp downstream.

A single band of approximately 1kb in size was detected with the 5'-probe in the Pst I digested genomic DNA from the AAB clones (part B). The size of this band was in good agreement with the expected size of the Pst I fragment (fig 5.26 A) indicating that the 5'-end of the AAB mRNA is intact. The 5'-probe does not cross-hybridise with the endogenous rat ALAS-1 gene as indicated by the lack of any bands in Pst I digested rat liver DNA (lane 7). The 5'-probe consists mainly of human ALAS-1 intron 1 sequences, which have limited homology to the corresponding rat intron 1. Two bands of size 8.5kb and 1.6kb cross-hybridised with the coding-region probe in Pst I digested FRL 4.1 AAB DNA. The larger of these two bands appears to be due to the cross-hybridisation of the probe to the rat ALAS-1 gene, as a band of the same size is detected in rat liver DNA (lane 7). The smaller band is only found in DNA from the AAB cell lines (lanes 2 to 6) and its size of 1.6 kb is in good agreement with the expected size of 1.684kb (fig 5.26 A). This result implied that the coding-region of the AAB gene had not undergone any major changes. A single band of approximately 700bp cross-hybridised with the 3'-specific probe in the AAB cell lines (lanes 2 to 6) but not in Pst I digested rat liver DNA (lane 7). The size of this band is also in good agreement with the expected size (fig 5.26 A) indicating that the 3'-end of the AAB mRNA is also intact.

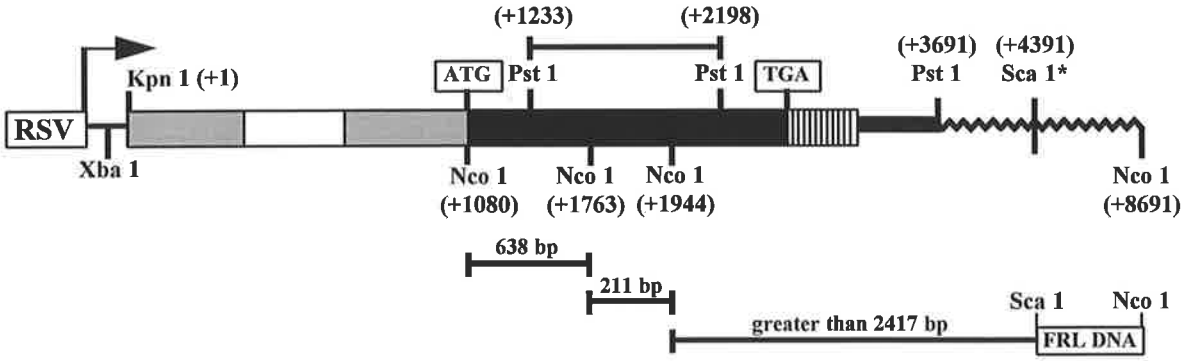
To further analyse the integrity of the human ALAS-1 coding region portion of the AAB gene, the genomic DNA samples were also cut with Nco I and Southern blot analysis performed, using the coding-region probe (fig 5.27B). There are three Nco I

Figure 5.27: **Southern Blot Analysis of Nco I Digested AAB FRL 4.1 DNA.**

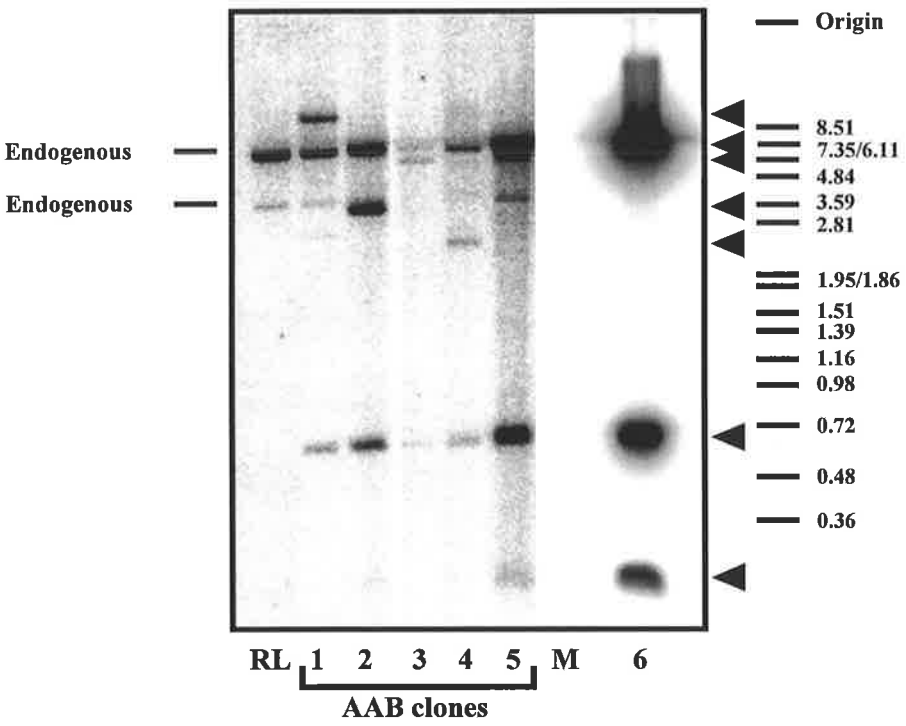
(A) Schematic of the AAB gene. Stripped rectangles, denote 5'-untranslated regions; open rectangles, introns; black rectangles, protein coding regions; vertical stripped rectangles, 3'-untranslated regions, thick lines, 3'-flanking sequences and wavy lines pRSVN.06 vector sequences. The position of the Pst I and Nco I sites are given relative to the Kpn I site (+1) at the start of the ALAS-1 5'UTR sequences. The initiation and termination codons are boxed. Indicated above the gene is the large Pst I fragment of the protein coding region used as a probe. Indicated below the gene are the expected size (bp) of the Nco I fragments detected by the probe. The Sca I site used to linearise the construct for transfection is denoted by an asterisk.

(B) Genomic DNA (20μg) was digested with Nco I and electrophoresed on a 1.0% agarose TBE gel. The restriction fragments were then transferred to a Nytran^{TM} membrane and cross-linked to the filter by UV irradiation. The Southern blots were then probed with the coding region probe. RL, rat liver genomic DNA; M, Eco RI cut SPP-1 DNA; lane 1 AAB clone $^{\#}1$ DNA; lane 2, AAB clone $^{\#}2$ DNA; lane 3, AAB clone $^{\#}3$ DNA; lane 4, AAB clone $^{\#}4$ DNA, lane 5, AAB clone $^{\#}5$ DNA and lane 6, pRSVN AAB DNA. AAB gene fragments that cross hybridise with the probe are indicated by arrow heads.

A


(+1233)
(+2198)
(+3691)
(+4391)
Kpn 1 (+1)
ATG
Pst 1
Pst 1
TGA
Pst 1
Sca 1*
RSV
Xba 1
Nco 1
(+1080)
Nco 1
(+1763)
Nco 1
(+1944)
Nco 1
(+8691)
638 bp
211 bp
Sca 1
Nco 1
greater than 2417 bp
FRL DNA

B


Origin
Endogenous
Endogenous
8.51
7.35/6.11
4.84
3.59
2.81
1.95/1.86
1.51
1.39
1.16
0.98
0.72
0.48
0.36
RL
1
2
3
4
5
M
6
AAB clones

sites in the human ALAS-1 coding-region spanned by the probe (fig 5.27A). The generation of the smaller restriction fragments by Nco I digestion, should allow for smaller deletions to be more readily identified in this region. Secondly, a small region at the 5'-end of the coding region (between the Pst I site at +1140 and +1223) does not hybridise to the different probes in Pst I digested DNA and therefore we wished to assay this region. Multiple bands cross-hybridised with the coding-region probe in FRL 4.1 cells, carrying the AAB gene (lane 1 to 5). Two of these bands of approximately 6kb and 3.5kb respectively appear to be a result of the cross-hybridisation of the probe to the rat ALAS-1 gene (lane RL). The band of similar size (6.5kb) in the Nco I digested pRSVN.06 vector (lane 6) is a result of the cleavage of the Nco I site at +1741 (fig 5.27 B) of the AAB gene and an Nco I site located approximately 4.3kb downstream of the AAB gene in the vector. Since the Sca I site used to generate the linear construct for transfection is located between these two sites, this fragment is not observed in the AAB cell lines. Rather, the probe should cross-hybridise to a different sized restriction fragment, in each of the AAB cell lines, due to the loss of the pRSVN.06 vector sequences (including the Nco I site) downstream of the Sca I site. The size of these fragments will be dependent on the constructs integration site. Also predicted from the map of the AAB gene construct to cross-hybridise with the probe were two small fragments of 211 and 664bp. Two bands of the predicted size were found in the AAB cell lines (lanes 2 to 5) indicating that the 5'-end of the coding region appears to be intact. Although the data obtained from the Southern blot analysis suggested that the AAB gene had not undergone any gross changes, it could not exclude the possibility of small changes such as point mutations or small deletions, causing the disruption of the heme-mediated effect. Therefore the reason for this discrepancy in the decay of the AAB mRNA relative to the decay of the other hybrid mRNAs is still unresolved. Currently, we assume that the problem lies in the AAB gene construct, and not in the other hybrid genes. Further experiments and reason for this discrepancy are considered below.

5.3 DISCUSSION.

The experiments performed in this chapter were designed to begin to localise the sequences responsible for the heme-mediated destabilisation of the ALAS-1 mRNA. However, attempts to define the region or regions involved were only partially successful. Several lines of evidence suggested that the coding region of the human ALAS-1 mRNA contained the sequences necessary for the heme-mediated destabilisation effect.

i) The hybrid mRNAs BAB and BAA were destabilised by heme and this effect appeared to be specific as neither the decay of the hybrid mRNAs ABA, ABB and BBA nor the mRNAs for β-globin and neo were similarly affected by heme. Since the ALAS-1 coding region was the only region common to both heme-destabilised mRNAs BAB and BAA and absent from the stable mRNAs ABA, ABB and BBA it was concluded that the coding region contained the sequences necessary for the heme-mediated decay of the mRNA.

ii) The magnitude of the heme-mediated destabilisation of the hybrid mRNA BAB was very similar (approximately 3-fold) to that observed for the effect of heme on the decay of the full length human ALAS-1 mRNA (AAA) and the hybrid mRNA BAA, implying that the ALAS-1 untranslated regions do not contribute to the heme-mediated destabilisation effect.

iii) The half-lives of the hybrid mRNAs BAB and BAA were close to that estimated for the human ALAS-1 (AAA) mRNA in heme treated cells, again suggesting that the heme-mediated decay of the hybrid mRNAs was unlikely to be non-specific. Normally, the insertion of a heterologous instability element into a stable reporter mRNA (such as β-globin) results in a hybrid mRNA with a half-life similar to that found for the unstable mRNA whose decay is normally mediated by this element (Shyu *et al.*, 1989 ; Schiavi *et al.*, 1994). This data is consistent with the proposal that the rate at which a mRNA is degraded is determined by the strength of its destabilising sequences (Sachs, 1993). Therefore, assuming the heme-responsive region was contained in a single region of the ALAS-1 mRNA, the hybrid mRNAs carrying this

sequence should have a half-life similar to that obtained for the full length ALAS-1 mRNA.

These results were also consistent with the work described in chapter 4, which suggested that the heme-responsive sequences reside in either the coding region or 5'UTR of the ALAS-1 mRNA. A coding region instability determinant is also consistent with the location of specific instability sequences in other unstable mRNAs, all of which have so far been localised to either the coding region or 3'UTR of the mRNA (Sachs, 1993).

However, heme had no detectable effect on the decay of the hybrid mRNA AAB. This result was unexpected given that the results with the other hybrid mRNAs were consistent with the heme-responsive instability sequences being contained in the coding region of the ALAS-1 mRNA. This lack of heme-responsiveness of the AAB mRNA could be interpreted in several ways, and these alternatives are discussed in detail below.

If the decay of the AAB mRNA is assumed not to be an artefact, how can this result be reconciled to the data on the decay of the other hybrid mRNAs? It seems unlikely that the human ALAS-1 3'UTR is critical for the heme-mediated destabilisation of the mRNA, as suggested by the decay of the AAB mRNA. Such an interpretation requires several assumptions. Firstly, that the heme-mediated destabilisation of the BAB mRNA was non-specific and secondly that the apparent inability of the ALAS-1 3'UTR to confer heme-mediated destabilisation onto the β-globin mRNA was either a temporal effect or due to β-globin coding region sequences inhibiting the function of the 3'UTR and not due to the lack of the heme-responsive instability determinants. However, as discussed, above the first assumption appears to be unlikely.

The apparent inability of the ALAS-1 3'UTR to confer heme-mediated destabilisation to the hybrid mRNAs BBA and ABA could potentially be explained by a temporal effect. In these experiments it was assumed that the presence of the ALAS-1 heme-responsive instability determinant in the hybrid mRNA would cause this mRNA to have a similar half-life, at least in heme-treated cells, to that for the human ALAS-1 mRNA (AAA). However, it is possible that that the heme-dependent instability element may simply cause a 3-fold decrease in the half-life of the hybrid mRNA. In this scenario

the half-life of the hybrid mRNA in the absence of added heme is close to that for the β-globin mRNA. Therefore, although the ABA and BBA mRNAs appeared to be as stable as the full length β-globin mRNA (BBB) in FRL 4.1 cells, the length of the experiment was too short to accurately determine this. Indeed both mRNAs did not show any significant decline in their respective levels during the course of the experiment, making an accurate measurement of their half-lives impossible. Subsequently, a role for sequences in either of the ALAS-1 untranslated regions in the heme-mediated instability of the ALAS-1 mRNA cannot be ruled out. However, a 3-fold destabilisation of the hybrid mRNAs BBA and ABA, caused by the heme-responsive instability determinant, should still have resulted in a measurable decrease in the level of β-globin protected product during the duration of the time course. The half-life of the β-globin mRNA in non-erythroid cells has been found to vary from 2.8 to 17.5 hours, depending on the cell type (Herrick and Ross, 1994 ; Kabnick and Housman, 1988). Assuming that the half-life of the hybrid mRNAs were similar to that of the full length β-globin mRNA, that is at most 18 hours, a 3-fold destabilisation of the mRNA would have resulted in a half-life of about 6 hours for the BBA and ABA mRNAs in heme-treated cells. This decline is clearly not seen, and therefore either the half-lives of the BBA and ABA mRNAs are substantially more in FRL 4.1 cells, so that the heme-mediated decline in their levels is missed, or that the human ALAS-1 3'UTR does not have an effect on the decay rate of the two mRNAs. Also the results obtained with the decay of the BAB and BAA mRNAs suggested that the ALAS-1 UTRs only play a minor, if any role, in the heme-mediated destabilisation effect. To address the role of the ALAS-1 untranslated region in the instability of the ALAS-1 mRNA, the actinomycin D time course will be repeated with time points taken over a much longer period. Time points will be taken up to 24 hours which is greater than the half-life of the human β-globin mRNA at least in fibroblasts (Kabnick and Housman, 1988)

The second possibility that sequences found in the β-globin coding region inhibit the function of an ALAS-1 3'UTR instability determinant also appears unlikely. The β-globin coding region was the only region common to both BBA and ABA mRNAs. The β-globin gene is the most widely used reporter gene in mammalian mRNA turnover

studies, and has been used successfully to measure the destabilising activity of a number heterologous instability determinants, without any apparent "inhibitory" effect of β-globin coding-region sequences on the activity of the various heterologous instability determinants. Instability determinants examined with β-globin hybrid mRNAs include the ARE element from a number of early response genes (Chen and Shyu, 1994), the coding region instability determinants from c-myc (Herrick and Ross, 1994) and c-fos (Schiavi *et al.*, 1994) and the 3'UTR instability region of the urokinase-type plasminogen activator (Nanbu *et al.*, 1994).

Taken together the data obtained for the decay of the hybrid mRNAs favour the proposal that the heme-responsive instability determinant is located in the human ALAS-1 protein-coding region. If the sequences in the ALAS-1 coding region are responsible for the heme-mediated decay, this raises the question as to why the decay of the AAB mRNA is not regulated by heme? Several possible reasons for this anomaly were investigated but no defect was found. These possibilities included, deletions or re-arrangements of the AAB gene, the retention of the AAB mRNA in the nucleus and the saturation of the heme-responsive decay machinery due to high levels of AAB mRNA.

This leaves several alternatives for the lack of heme-responsiveness on the part of the AAB mRNA. One possibility is that a point mutation or small deletion was introduced into the ALAS-1 coding region during the synthesis of the pRSV AAB construct, a small deletion, especially in the 3'-portion of the coding region may not have been picked up easily in the Southern analysis. This mutation would have to disrupt the function of the heme-responsive instability determinant perhaps by disrupting the sequence itself or by modulating the translation of the mRNA. Although, the heme-mediated decay of the rat ALAS-1 mRNA in FRL 4.1 cells appears independent of translation (chapter 3), this conclusion has not been fully proven due to a background of residual translation in the cycloheximide treated cells. Therefore it is possible that the AAB gene has been mutated in such a way to cause a change in the translation of the AAB mRNA which results in its stabilisation.

Several precedence's now exist for the coupling of translation to the function of protein-coding region instability determinants including the c-fos (Schiavi *et al.*, 1994),

c-myc (Herrick and Ross, 1994) and (GAP-43) neuronal growth associated protein-43 (Nishizawa, 1994) protein coding region instability determinants. In the case of c-myc and GAP-43 the creation of a pre-mature stop codon prior to the coding region instability determinant substantially stabilised the mRNA. The c-fos mRNA was also stabilised when translation was inhibited by the insertion of a large stem-loop structure into the 5'UTR of a mutant c-fos mRNA whose decay was solely dependent on the c-fos protein coding region instability determinant (Schiavi *et al.*, 1994).

Alternatively, the unnatural combination of the β-globin 3'UTR and ALAS-1 5'UTR on the same mRNA led to some sort of interaction between the two that inhibited the function of the protein coding region instability determinant. This interaction would need to be specific for the human ALAS-1 5'UTR and β-globin 3'UTR, as the ALAS-1 protein coding region instability determinant appears to function normally in the hybrid mRNAs BAB and BAA. Some precedents for such RNA-specific inhibition of destabilising activity exist. For instance a recent report on the function of the amino-terminal tetrapeptide instability element of β-tubulin found that although this element can confer autoregulated mRNA stability on β-tubulin mRNA and some heterologous mRNAs such as thymidine kinase, it cannot function in the amino-terminus of the α-tubulin mRNA (Bachurski *et al.*, 1994). Thus it seems that the function of some instability elements will be dependent on the RNA context into which they are placed.

In such a model the protein coding region instability determinant may normally interact with the 5' or 3'-end of the mRNA to promote exonucleolytic decay, but in the case of the AAB mRNA the ALAS-1 5'UTR and β-globin 3'UTR interaction prevents this. Evidence for the interaction of the 5' and 3' termini of a mRNA has been suggested in several experiments. Firstly, two proteins in yeast, poly(A) binding protein (PAB) and poly(A)-binding protein-dependent poly(A) nuclease (PAN), that are intimately associated with the poly(A) tail of the mRNA, have also been found to be required for the initiation of translation (Sachs and Davis, 1989 ; Sachs and Deardorff, 1992) suggesting a link between the poly(A) tail and the 5'-end of the mRNA. In the case of PAB, temperature-sensitive conditional mutants of PAB implicated PAB in the joining of the 60S ribosomal subunit to the 40S-mRNA complex (Manley and Proudfoot, 1994).

Also a linkage between the poly(A) tail and the initiation of translation has been suggested by the finding that the poly(A) tail binds several translation initiation factors *in vitro* (Gallie and Thanguay, 1994). Similarly, the initiation of translation in higher eukaryotes including Drosophilia and Xenopus appears to be coupled to the length of the mRNAs poly(A) tail (Sheets *et al.*, 1994). Physical evidence for the association of the 5' and 3' mRNA termini has also been obtained (Rubin *et al.*, 1994 ; Christensen *et al.*, 1987). Rubin *et al.* (1994) found that the radiolabelled poly(A) tail of the rabbit β-globin mRNA was co-captured, following ribonuclease V1 treatment, on a aminophenylboronate-agarose column, which specifically captures the CAP structure of a mRNA. This suggested that the 5' and 3' ends of the molecule must bind to each other (the captured fragments were sized to ensure that the captured products were not the native molecules). The interaction of the 5' and 3' termini of mRNA molecules has also been suggested by the finding of circular polysome structures in electron micrographs (Christensen *et al.*, 1987). Alternatively, the interaction of the ALAS-1 5'UTR with the β-globin 3'UTR may alter the global secondary and tertiary structure of the AAB mRNA which may in turn inhibit the recognition of the ALAS-1 protein coding region instability determinant.

Precedents, for the interaction between widely separated elements on a mRNA affecting its turnover, have been described and for instance, Saliocco *et al.* (1994) found that the formation of secondary structures between the 5' and 3' ends of a mRNA could influence the stability of the transcript. In this study the decay of hybrid mRNAs containing the CAT gene coding region linked to either the UTRs of the stable (PGK1) or unstable (CYP1) mRNAs were examined. The decay of these mRNAs appeared to be dependent on CAT sequences as the replacement of the unstable CYP1 UTRs with the UTRs from the stable PGK1 mRNA did not alter the half-life of the mRNA. However, when the 5'UTR and 3'UTR were predicted to form a secondary structure the half-life of the resultant mRNA increased about 2-fold, suggesting an interaction between a mRNAs termini can modulate the activity of an instability element.

A role for higher order structure has also been implicated in determining the endonucleolytic cleavage site of the mammalian insulin-like growth factor II (IGF II)

mRNA, as two widely separated elements are required to direct the appropriate cleavage reaction (Meinsma *et al.*, 1992). Therefore, it is possible that that the unique combination of the human ALAS-1 5'UTR and coding region with the human β-globin 3'UTR sequences in the AAB mRNA has led to a perturbation in the higher order structure surrounding the ALAS-1 coding region determinant. This change in the structure may then prevent the recognition of the determinant by a heme-responsive factor or prevent the cleavage of the site by an endonuclease. Whether, these explanations for the decay of the AAB mRNA are valid await a more detailed analysis of the coding-region instability determinant. This would allow any change in the secondary structure around this element to be determined.

Whether sequences in the ALAS-1 untranslated region have any role in the decay of the ALAS-1 mRNA has still not been conclusively established. That the ALAS-1 UTRs may play a role in the instability of the ALAS-1 mRNA is suggested by the increase in the half-life of the ALAS-1 mRNA in non-heme treated cells, when its UTRs were replaced by the corresponding β-globin sequences. However, any conclusion based on the comparison of the half-lives of the different hybrid mRNAs must be treated cautiously due to sequence heterogeneity at their 5'-ends. This heterogeneity was caused in several ways. Firstly, the RSV promoter used to drive the expression of the hybrid genes retained its own CAP site and therefore the mRNAs produced contained non-reporter sequences, that extended from the RSV CAP site to the restriction site used to insert the reporter gene into the vector. The length of this vector sequence included in the mRNA was dependent on the restriction sites used to clone in the hybrid genes. For example the AAA gene was inserted into the Not I site while the hybrid genes BAB and BAA began at the Xba I and Kpn I sites respectively (fig 5.14). Lastly, the 5'UTRs of ALAS-1 and β-globin carried 8bp and 15bp of their respective promoters due to the insertion of the Kpn I site (used in their isolation) upstream of their normal CAP site. Therefore all the hybrid mRNAs produced contained various lengths of non-reporter sequences at their 5'-ends, the effect of which on the stability of the respective mRNA is unknown.

Assuming, that the heme-responsive instability element is located in the ALAS-1 coding region how may this element function? To date eight examples of protein coding region instability determinants (CRDs) have been identified in yeast and higher eukaryotes. Surprisingly, each of these CRDs has unique properties indicating the existence of at least several different decay pathways in the cell. Whether these pathways converge at some point during the decay of a mRNA is still unclear. A theme common to many of these elements is their dependence on translation for activity. However, the specific requirement for translation varies markedly between the different elements. For example, the rapid degradation of β-tubulin mRNA, under conditions of tubulin monomer excess, appears to be dependent on the recognition of a specific N-terminal tetrapeptide (MREI) encoded by the first 12 nucleotides of the β-tubulin mRNA (Theodorakis and Cleveland, 1992), while the c-fos CRD is still functional when translated out of frame suggesting that it is recognised as an RNA sequence (Wellington *et al.*, 1993). The CRD of yeast MATα1 mRNA on the other hand appears to require the pausing of the ribosome at several rare codons located within this sequence for its activity (Caponigro *et al.*, 1993). The c-myc CRD also requires translation but in this case translation appears to stimulate the recognition of the CRD as an endonucleolytic cleavage site (Bernstein *et al.*, 1992). The endonuclease responsible for this cleavage has been proposed to be associated with the ribosome to explain this dependence (Bernstein *et al.*, 1992 ; Herrick and Ross, 1994). However, the heme-mediated decay of the rat ALAS-1 mRNA appears to be independent of translation, suggesting that the mechanism involved in targeting the ALAS-1 mRNA for rapid decay is likely to be different to those mentioned above.

Although the molecular events initiated by the recognition of cis-acting instability elements have only been established in a few cases, three different initial events have been observed. Several instability elements, including the c-fos CRD, appear to promote the rapid deadenylation of the mRNAs poly(A) tail and a strong correlation exists for this deadenylation to be a pre-requisite for the decay of the mRNA body (Laird-Offringa *et al.*, 1990 ; Shyu *et al.*, 1991 ; Decker and Parker, 1993).

A second mechanism by which mRNA turnover is initiated involves the sequence-specific cleavage of the mRNA within the instability element. To date the majority of these endonuclease sites have been found in the 3'UTR of the mRNAs (Stoeckle and Hanafusa, 1989 ; Meinma *et al.*, 1992 ; Brown *et al.*, 1993 ; Binder *et al.*, 1994), although at least one example of this type of instability element exists in the coding region of a mRNA. Since the characterisation of c-myc mRNA decay intermediates in both an *in vitro* decay system and in tissue culture cells suggested that the c-myc CRD undergoes endonuclease attack (Bernstein *et al.*, 1992).

The third type of instability element appears to directly promote the de-capping of the mRNA, leading to 5' to 3' exonucleolytic degradation. Currently, this mode of mRNA decay has only been observed for the rapid decay promoted by certain nonsense mutations in yeast (Muhlrad and Parker, 1994). Decay intermediates lacking a small portion of the 5' end have also been detected in transgenic mice expressing an unstable mutant β-globin mRNA that contain nonsense mutations (Lim *et al.*, 1992). These truncated β-globin mRNAs could indicate a similar de-capping in mammalian cells, alternatively these species could be generated by an endonucleolytic cleavage at the 5' end of the molecule.

The heme-mediated decay of the rat ALAS-1 mRNA, shares several mechanistic features with some mRNAs whose degradation is initiated by an endonucleolytic cleavage, namely its apparent insensitivity to translational inhibition and also its apparent independence on prior deadenylation. Several examples of mRNA turnover initiated by sequence specific endonucleolytic cleavage of the mRNA, independent of poly(A) tail removal have been reported in higher eukaryotes, including the chicken apolipoprotein II (Binder et al, 1989) and 9E3 (Stoeckle and Hanafusa, 1989), human insulin-like growth factor II (Nielsen and Christiansen, 1992 ; Meinsma *et al.*, 1992), Xenopus and Drosophila XIhbox 2B (Brown and Harland, 1990 ; Brown *et al.*, 1993) and human and mouse transferrin receptor (Binder *et al.*, 1994) mRNA. Moreover, the cleavage of the chicken 9E3 and Xenopus XIhbox 2B mRNAs also appear to occur in the absence of translation. Therefore, we currently favour a model in which the ALAS-1 heme-responsive coding region instability determinant (HRCRD) acts as an

endonucleolytic cleavage site. Presumably the products of this initial cleavage are then degraded by exonucleases. However, the type of nucleases (ie. 5' to 3' or 3' to 5') involved in the mRNA turnover in mammalian cells is still unknown although, as described later, in yeast many mRNAs appear to be degraded in a 5' to 3' direction. Alternatively, by analogy with the rapid decay mediated by certain nonsense codons in yeast, the ALAS-1 HRCRD may stimulate decapping of the ALAS-1 mRNA leading to 5' to 3' exonucleolytic decay. In the special case of nonsense codon-mediated rapid mRNA turnover in yeast, the instability signal stimulates the decapping of the mRNA independently of deadenylation (Muhlrad and Parker, 1994). Whether other instability elements promote mRNA degradation by a similar mechanism is unknown.

Interestingly, recent work in yeast found that the rapid deadenylation of the MATA2 mRNA, initiated by the instability determinant located in its 3'UTR, led to the removal of the 5'CAP and 5' to 3' exonucleolytic decay (Decker and Parker, 1993 ; Muhlrad *et al.*, 1994). To establish the order of decay events following deadenylation two strategies were used. Firstly, decay intermediates were trapped by the insertion of a strong secondary structure, known to slow the rate of exonucleolytic digestion (Vreken and Raue, 1992b) into either the 5'UTR or 3'UTR of the MATA2 mRNA. Secondly, the decay of the MATA2 mRNA was examined in yeast cells lacking the XRN1 gene product. The XRN1 gene is thought to encode the major 5' to 3' exonuclease in yeast (Larimer and Stevens, 1990). Insertion of the secondary structure into the unstable MATA2 mRNA was found to stabilise a truncated MATA2 mRNA which showed a correct precursor-product relationship with full length MATA2 mRNA in a transcriptional pulse-chase expression system. This truncated species lacked nucleotides 5' to the secondary structure, suggesting that the decay was initiated from the 5' end of the mRNA following deadenylation. Similar expression work in yeast cells lacking the XRN1 gene product found that the MATA2 mRNA accumulated as a decapped, full length species suggesting that removal of the 5' CAP preceded the degradation of the mRNA body by XRN1 nuclease. The XRN1 nuclease is normally blocked by the 5' CAP structure of mRNAs (Stevens, 1978). Decay intermediates consistent with de-capping followed by 5' to 3' exonucleolytic degradation have also been found for the stable yeast

mRNA PGK1 (Muhlrad *et al.*, 1995). Indeed, in a study by Hsu and Stevens (1993) in XRN1 mutant cells of five mRNAs examined, all showed an accumulation of full length species lacking the 5'CAP structure. This result suggested that the XRN1 nuclease forms part of an important mRNA degradation pathway in yeast and that decapping may be an early event in the turnover of a number of yeast mRNAs.

Therefore it appears that in yeast many of the mRNA degradation pathways specified by different instability elements converge at a common point, which involves the decapping and 5' to 3' exonucleolytic decay of the transcript. Decker and Parker (1994) proposed that deadenylation and endonucleolytic cleavage stimulated by the different types of instability elements both function to cause decapping by disrupting the normal interaction between the 5' and 3' termini of the mRNA. This disruption between the 5' and 3' ends of the mRNA could then indirectly or directly result in the exposure of the 5'CAP to the cells decapping activity. In the special case of rapid mRNA decay mediated by certain nonsense mutations, the nonsense signal leads to the decapping and 5' to 3' exonucleolytic decay directly (Muhlrad and Parker, 1994 ; Peltz *et al.*, 1993).

Whether, decapping and 5' to 3' exonucleolytic decay is a common pathway in mammalian cells is currently unknown. However, Decker and Parker (1994) have tentatively suggested that decapping may also occur in mammalian cells. Their proposal is based on several observations. Firstly, mRNAs lacking the 5'CAP structure are rapidly degraded in eukaryotic cells (Drummond *et al.*, 1985 ; Gallie, 1991). Moreover, an enzyme capable of removing the 5'CAP has been characterised from several mammalian sources (Nuss *et al.*, 1975 ; Nuss *et al.*, 1977). Therefore, it appears that all the enzymes required to degrade a mRNA by this pathway are present in mammalian cells. Secondly, many mammalian instability elements such as the ARE (Chen and Shyu, 1994) and c-fos CRD (Schiavi *et al.*, 1994) also promote deadenylation as the first step in the decay of their respective mRNAs. Since the 5' CAP and poly(A) tail are highly conserved between yeast and mammals, it is likely that many of the functions will also be conserved. A 5' to 3' exonuclease has recently been partially purified from mouse ascites cells, where this enzyme appears to be the major cytoplasmic nuclease (Coutts and Brawerman, 1993 ; Coutts *et al.*, 1993). Interestingly, the enzyme can

degrade protein-free RNA molecules containing 5'CAP structures but cannot degrade these same molecules in crude cytoplasmic extracts, implying that the action of the nuclease may be normally regulated by the binding of factors to the 5'-termini. Thus the potential exists in mammalian cells for alterations in the protein-RNA complex at or near the 5'-CAP, perhaps due to changes in the interaction of the 5' and 3' ends of the mRNA to lead to 5' to 3' exonucleolytic decay. However, in an *in vitro* decay system the c-myc mRNA is degraded in a 3' to 5' direction following deadenylation (Brewer and Ross, 1988) but whether this 3' to 5' decay is the major route for c-myc mRNA degradation in intact cells has not been established.

Clearly, the analysis of decay intermediates would be informative to the mechanism by which the ALAS-1 mRNA decays. Northern blots of FRL 4.1 mRNA however failed to detect any smaller ALAS-1 species that may have been decay intermediates, indicating that the intermediates produced may be extremely unstable. In addition the low level of ALAS-1 mRNA in FRL 4.1 cells means that any decay intermediate produced will also be extremely low, perhaps below the detection limit of Northern blots. To overcome these potential problems decay intermediates produced by a sequence-specific endonucleolytic cleavage could be searched for with the more sensitive RNase protection assay. Radiolabelled probes which span the ALAS-1 coding region would be used to detect specific truncated products in both control and heme treated cells. We predict that this truncated species should be more prominent in heme-treated cells than in control cells, due to the increased rate of ALAS-1 mRNA turnover in these cells. However, if the events following the endonucleolytic cleavage occur extremely rapidly, specific decay intermediates may still be difficult to detect. Also due to the constitutive expression of the endogenous rat ALAS-1 mRNA, a precursor-product relationship cannot be established making it impossible to formally demonstrate that the truncated species detected are the result of a specific endonuclease attack.

To attempt to stabilise any ALAS-1 decay intermediates, secondary structures, similar to those used to block exonucleases in yeast (Vreken and Raue, 1992b ; Muhlrad *et al.*, 1994 a and b), could be inserted into the 5' and 3' UTRs of the rat ALAS-1 gene. Presumably, mammalian exonucleases are also blocked by such structures, but this is

unknown. These hybrid genes would then be expressed in FRL 4.1 cells under the control of the transiently inducible c-fos promoter (Greenberg and Ziff, 1984). Serum treatment of serum starved cells should then led to a transient burst of transcription from the c-fos promoter. This method should stabilise ALAS-1 decay intermediates allowing their detection on Northern blots. RNA mapping using specific 5' and 3' probes would allow us to determine the direction of exonucleolytic shortening which would provide very useful information into not only the mechanism of ALAS-1 decay but also for the decay of mammalian mRNAs in general.

CHAPTER SIX

FINAL DISCUSSION AND SUMMARY.

CHAPTER SIX: FINAL DISCUSSION AND SUMMARY.

Since the early work of Granick (1966) established that ALAS levels in chick embryo hepatocytes could be increased by drugs and that heme could prevent this increase it has been proposed that heme biosynthesis in the liver is controlled by end product repression of ALAS, the first enzyme of the pathway. We are interested in studying this end product repression as it provides an interesting model into how the cell co-ordinates a metabolic process with its fluctuating demands for this process. Moreover, the deregulation of ALAS synthesis has been associated with a group of human disorders collectively termed the porphyrias and so it is of particular interest to determine how heme regulates ALAS levels as a model for these disorders.

As discussed, in chapter one, heme is thought to exert this control at several points. Experimental data indicates that heme negatively regulates the steady state levels of ALAS-1 mRNA and also the import of the precursor protein from the cytoplasm into the mitochondria. One of the aims of the laboratory is to determine how heme regulates the steady state level of ALAS-1 mRNA and the relationship of this regulation to the induction of the ALAS-1 gene in the liver by drugs that are inducers of the CYP system.

Recently, several reports have provided conflicting data on the regulation of ALAS-1 mRNA by heme. In rat liver, heme appears to negatively regulate the transcription of the ALAS-1 gene. However, a similar negative effect of heme on the transcription of the ALAS-1 gene is not observed in either chick embryo liver or transformed mammalian cell lines, such as the hepatoma cell lines HepG2 and H4-E-II/C3. Rather in these systems, heme appears to repress ALAS-1 mRNA levels post-transcriptionally by altering the half-life of the mRNA. In contrast, although the rat ALAS-1 mRNA turns over extremely rapidly in drug-induced rat liver, heme does not appear to increase this turnover. The reason for this observed difference in the regulation of the ALAS-1 gene by heme in the two species is still unknown.

However, one problem associated with the whole rat experiments was that the effects of heme on the transcription rate of the ALAS-1 gene were determined after several administrations of high heme doses (Srivastava *et al.*, 1988 ; Srivastava *et al.*,

1990). Also the effect of heme on the turnover of ALAS-1 mRNA was examined after the animals were given toxic substances such as actinomycin D and α-amanitin (Yamamoto *et al.*, 1988). Thus it is difficult to determine the physiological relevance of these studies since the actual dose of heme seen by the liver and the effect of such toxic substances as α-amanitin on general RNA metabolism was not adequately redressed. In contrast, the negative effect of heme on the stability of the ALAS-1 mRNA in chick embryo liver has been supported by studies in cultured chick embryo hepatocytes (Hamilton *et al.*, 1991 ; Drew and Ades, 1989b).

The work presented in this thesis was aimed at investigating the regulation of the rat ALAS-1 gene in the rat hepatoma cell line FRL 4.1. Heme was found to cause a rapid decrease in the steady state level of ALAS-1 mRNA in this cell line. However, unlike the situation in rat liver, we were unable to detect any effect of heme on the basal transcription rate of the ALAS-1 gene. Rather an investigation on the mechanism of heme repression in this cell line demonstrated that heme specifically increased the turnover of the ALAS-1 mRNA. Thus the regulation of ALAS-1 mRNA levels in this cell line appeared to be inconsistent with that reported previously in rat liver.

This finding raised two important questions. Firstly, was the data supporting heme repression of ALAS-1 transcription in rat liver an artefact of the treatment protocols used and secondly, if so, and heme regulates ALAS-1 mRNA turnover in the rat liver why wasn't an effect by heme on the stability of ALAS-1 mRNA detected in rat liver?

Re-examination of the transcription rate of the ALAS-1 gene in rat liver nuclei isolated from rats administered a single low dose of heme, supported the earlier data on the down-regulation of the ALAS-1 gene in response to heme. Thus in rat liver, heme does appear to be able to control the transcription rate of the ALAS-1 gene but not in transformed cell lines. One explanation for this apparent paradox is that a highly differentiated liver parenchymal cell phenotype is required to support some aspect of ALAS-1 gene transcription. Transformed cell lines have obviously lost some aspects of gene regulation as evidenced by their transformed phenotype. Furthermore, their adaptation to survival in tissue culture conditions often result in the down-regulation of

many features associated with a highly differentiated phenotype. In support of this proposal, it should be noted that another aspect of ALAS-1 gene transcription associated with liver parenchymal cells, namely the activation of gene transcription in response to drugs such as PB, has also been lost in all transformed cell lines examined to date. Indeed the ability of the liver to mount a response to the PB class of inducers is severely hampered in regenerating liver, thus it is tempting to speculate that some aspects of ALAS regulation are controlled by signals and transcription factors which are altered by dedifferentiation and proliferation. Therefore, although transformed cell lines have proven to be invaluable tools in determining the regulation of a number of tissue-specific genes, including liver-specific genes, it has become apparent that they are not suitable for investigating certain aspects of ALAS-1 transcriptional regulation. It would appear that these features of ALAS-1 regulation will need to be pursued in cells representing a more differentiated liver phenotype. Two possible systems for this include primary rat hepatocyte cultures and mouse transgenesis.

Recently a primary rat hepatocyte culture system has been developed that supports, albeit at a lower level, the phenobarbital mediated increase in ALAS-1 mRNA. Furthermore this induction was at least partly inhibited by ALA or heme addition; non-induced levels of ALAS-1 mRNA were also repressed by ALA or heme addition suggesting that primary rat hepatocytes may provide the best *in vitro* model to investigate the *in vivo* situation (Sinclair *et al.*, 1990). However the level at which heme is acting upon ALAS-1 mRNA levels in these cells remains to be determined. Mouse transgenesis has also recently been used successfully to investigate the control regions required for the liver-specific activation of the rat CYP2B2 gene by phenobarbital in mouse liver (Ramsden *et al.*, 1993) indicating that transgenesis could at least be used to investigate the activation of ALAS-1 gene transcription in response to phenobarbital.

The reason for the difference in the effect of heme on the turnover of the ALAS-1 gene in rat liver versus hepatoma cells is likewise not resolved. It seems unlikely that the effect seen in hepatoma cells is an artefact of the culture conditions, as the effect appears to be specific for the ALAS-1 mRNA. Specifically, heme administration was found not to affect the half-lives of three other mRNAs, namely the mRNAs for c-myc,

GAPDH and β-globin in the same cells. Of particular significance was the finding that the half-life of the c-myc mRNA was not altered by heme treatment. The turnover of c-myc mRNA is both extremely rapid in most cell types and can fluctuate in response to many changes in the cell including changes in the translation, growth and phosphorylation state of the cell. Therefore, any global changes in RNA metabolism caused by heme administration should have been reflected in a rapid change in the turnover of the c-myc mRNA. Moreover, since heme has been shown to affect the turnover of the ALAS-1 mRNA in chickens it seems likely that heme can also influence mRNA turnover in rats.

If this is the case, and ALAS-1 mRNA turnover is regulated by heme levels we are left with a paradox, since Yamamoto *et al.* (1988) could not detect a difference in the turnover of the ALAS-1 mRNA in the liver of heme treated versus control rats. We propose two possible explanations for this seeming paradox. In the first scenario, the level of heme in rat liver may have been still sufficiently high under the conditions used to cause the maximal destabilisation of the ALAS-1 mRNA thus the half-life for the ALAS-1 mRNA did not vary when more heme was added. In tissue culture cells, by contrast, the amount of free heme in the cell which contributes to the regulation of the ALAS-1 mRNA may be much lower, particularly if the gene has lost the ability to respond transcriptionally to a changes in heme levels. Therefore, heme levels may be limiting in this situation and the mRNA is stabilised. However when heme is administered, levels rapidly rise in the cytoplasm and this acts as signal to destabilise the mRNA.

Alternatively, the injection of the highly toxic transcriptional inhibitor α-amanitin into the rats to block transcription in the liver may have interfered in the turnover of the ALAS-1 mRNA. A precedent for such transcriptional blockers interfering in the turnover of a mRNA has been reported by Shyu *et al.* (1989). In this study, actinomycin D was found to inhibit the activity of the c-fos ARE element in mouse fibroblasts.

We therefore decided to continue to pursue the regulation of ALAS-1 mRNA stability in FRL 4.1 cells with two aims in mind. Firstly, we assumed that the elements

and the *trans*-acting factors that bind to them responsible for the rapid turnover of the ALAS-1 mRNA in FRL 4.1 cells would also be responsible for the extremely short half-life of the mRNA in rat liver. Since the regulation of mRNA stability has become increasingly apparent as an important process that determines the level of expression of a gene (Sachs, 1993), an understanding of the regulation of ALAS-1 mRNA turnover would contribute to the overall understanding of the control of heme synthesis in rat liver. Furthermore the mechanisms by which mammalian mRNAs turnover have only begun to be characterised, thus the turnover of ALAS-1 mRNA provided an interesting model for investigating mRNA decay in mammalian cells.

The second aim was to identify the mechanism for the heme-mediated instability of the ALAS-1 mRNA in hepatoma cells. On the basis of other mRNAs whose decay is regulated, such as the transferrin receptor mRNA, we predict that heme treatment will alter the binding pattern of specific *trans*-acting factors to these instability determinants. We further predict that the *cis*-elements involved in the heme-mediated turnover of ALAS-1 will be identical to the elements required for the rapid turnover of the mRNA in rat liver. These *trans*-acting factors can then be looked for in rat liver extracts, and the effect of heme on their binding activity determined. This data should allow an assesment of the likelihood of heme regulation of ALAS-1 mRNA turnover in rat liver.

Initially, important parameters for the instability of the endogenous ALAS-1 mRNA in hepatoma cells were investigated. The turnover of many eukaryotic mRNAs require translation. Interestingly, treatment of FRL 4.1 cells with the translational inhibitor cycloheximide did not stabilise the ALAS-1 mRNA. In contrast the half-life of the unstable c-myc mRNA was substantially increased. In this study translation in the cell was inhibited by 82% and therefore the possibility exits that the low level of translation persisting in the cells is sufficient to maintain the normal de-stabilisation of the ALAS-1 mRNA. Arguing against this interpretation is the fact that the turnover of the c-myc mRNA, which is known to be dependent on translation, was inhibited in these same cells.

To conclusively determine if translation is required for the destabilisation of the rat ALAS-1 mRNA these experiments need to be repeated under conditions where a

greater inhibition of protein synthesis is achieved. Alternatively the effect of specifically blocking the translation of the ALAS-1 mRNA could be examined by placing a stable secondary structure in the 5'UTR of the mRNA to inhibit either ribosome binding or movement.

Another feature common to the decay of many mammalian and yeast mRNAs is the de-adenylation of the poly(A) tail to a short oligo(A) tail prior to the onset of the decay of the transcribed portion of the mRNA. Indeed many of the instability elements characterised so far appear to promote this de-adenylation. Therefore we examined the de-adenylation of the ALAS-1 mRNA in FRL 4.1 cells, following transcriptional repression by actinomycin D, using the oligonucleotide directed cleavage procedure. Although, the ALAS-1 mRNA poly(A) tail became progressively shorter during the time assayed, heme did not appear to have an significant effect on this shortening. Also, the change in the length of the poly(A) tail of ALAS-1 mRNA in heme treated cells versus control cells did not appear to correlate with the rapid decrease in the main body of mRNA with heme. These results therefore suggest that de-adenylation of the poly(A) tail is not a prerequisite for the heme-mediated destabilisation of the ALAS-1 mRNA.

However, a major drawback in interpreting this experiment is that the population of ALAS-1 mRNA molecules at time zero was markedly heterogeneous in the length of their poly(A) tails, making it difficult to correlate changes in poly(A) tail length with the disappearance of the ALAS-1 mRNA. This heterogeneity is most likely due to the constitutive expression of the ALAS-1 gene, leading to a population of ALAS-1 mRNA molecules which have been in the cytoplasm for differing lengths of time. This fact makes it difficult to determine whether the decay of the main body of the mRNA occurs primarily on mRNA molecules containing no or very short poly(A) tails or whether the decay of the ALAS-1 mRNA can occur independently of prior poly(A) tail shortening.

This problem could be partially overcome by the use of an inducible/repressible promoter system driving the expression of the ALAS-1 gene. Transient induction of this promoter would lead to a pulse of newly synthesised transcripts which would be homogeneous in the length of their poly(A) tail. The discrete

starting size of the poly(A) tail would then allow a better determination of the relationship between the shortening of the poly(A) tail and the degradation of the ALAS-1 mRNA. The promoter of the c-fos gene which is transiently expressed following serum stimulation of serum-starved cells (Greenberg and Ziff, 1984) has been used extensively for this purpose (Shyu *et al.,* 1991 ; Wilson and Treisman, 1988 ; Wellington *et al.*, 1993). Future experiments using this promoter are planned which should hopefully allow a better characterisation of the role, if any, de-adenylation plays in the turnover of the ALAS-1 mRNA.

An examination was initiated to characterise the nature of the signal that causes the accelerated degradation of the ALAS-1 mRNA in conditions of high heme. Another metalloporphyrin (tin mesoporphyrin) was unable to decrease ALAS-1 mRNA levels at least when used at the same concentrations as heme. This suggests that the coordination chemistry of iron may be important in the interaction of heme with the presumptive heme responsive factor(s) since tin could not substitute for iron in the porphyrin ring. However, this study on the requirement for iron in the porphyrin ring was by no means exhaustive. A recent study by Cable *et al.* (1994) also found that tin-mesoporphyrin was ineffectual in destabilising ALAS-1 mRNA in cultured chick embryo hepatocytes; they did however show that another metalloporphyrin, zinc mesoporphyrin IX (ZnMeP), could promote the destabilisation of the ALAS-1 mRNA. Indeed, in their hands ZnMeP appeared to be a more effective promoter of ALAS-1 mRNA turnover than equivalent doses of heme, perhaps because ZnMeP did not induce HO-1 levels. Therefore it would be interesting to determine the effect of ZnMeP and a range of other metalloporphyrins on the turnover of rat ALAS-1 mRNA in FRL 4.1 cells.

The second aim of this thesis was to map the location of any *cis*-element(s) which target the ALAS-1 mRNA for rapid degradation. A common method to search for instability elements is to synthesise genes which express a hybrid mRNA containing portions of the unstable mRNA inserted into a normally stable mRNA. The half-life of the resulting hybrid mRNAs can then be measured to look for portions of the unstable mRNA which can destabilise this normally stable mRNA.

Initially the 3'UTR of the mRNA was examined for destabilising activity, since most unstable mRNAs contain instability determinants in this region (chapter 4). FRL 4.1 cells were stably transfected with a β-globin gene construct carrying the rat ALAS-1 3'UTR inserted into the 3'UTR of the β-globin gene. The decay of the β-globin mRNA was then compared to that of the parental β-globin mRNA, following heme administration. However, no significant destabilisation of the β-globin/ALAS-1 hybrid mRNA was observed. The most likely explanation for this is that the sequences responsible for the heme-mediated destabilisation of the ALAS-1 mRNA are located elsewhere in the mRNA.

A second series of constructs was then synthesised in which the untranslated and protein coding regions of the human ALAS-1 and β-globin genes were transposed. The data obtained for the decay of these hybrid mRNAs in FRL 4.1 cells was most consistent with the *cis*-acting instability determinants being located in the ALAS-1 coding region. This was also consistent with the findings of the earlier constructs. However, one construct containing the ALAS-1 coding region, and therefore expected to be unstable, was found to be stable.

Southern blot analysis indicated that the construct had not undergone any gross deletions or re-arrangements when incorporated into the FRL 4.1 cells genome. Also the over-expression of this mRNA could not account for its stability since it was equally stable in isolated clones that showed low levels of expression as well as in clones with high levels of expression. Furthermore, the regulation of the endogenous rat ALAS-1 mRNA was unaffected by the over-expression of this mRNA. Two plausible explanations for this difference are that the construct contains either a mutation or a mRNA-specific structure, that inhibits the activity of the ALAS-1 instability determinant. Investigating the first possibility may prove to be particular informative as the characterisation of any mutation in the mRNA immediately implicates this area in the function of the ALAS-1 instability determinant. This possibility could be tested relatively easily by PCR amplifying the hybrid mRNA from the stably transfected FRL 4.1 cell lines and sequencing the resultant product. Alternatively, this mutation may alter the translation of the mRNA and so implicate translation in the degradation process.

This however seems unlikely given the inability of cycloheximide to affect the turnover of the mRNA.

The second possibility is harder to test. However, some precedents for such RNA-specific inhibition of destabilising activity exist. For instance a recent report on the function of the amino-terminal tetrapeptide instability element of β-tubulin found that although this element can confer autoregulated mRNA stability on β-tubulin mRNA and some heterologous mRNAs such as thymidine kinase, it cannot function in the amino-terminus of the α-tubulin mRNA (Bachurski *et al.*, 1994). Thus it seems that the function of some instability elements will be dependent on the RNA context into which they are placed.

The next phase of this work will be to localise the elements in the ALAS-1 coding region responsible for the rapid turnover of the mRNA. To identify the regions(s) responsible we plan to insert fragments that span the rat ALAS-1 coding region into the coding region of the β-globin gene. These fragments will initially be cloned in frame to preserve both the normal termination of β-globin translation and the ALAS-1 reading frame. Transcription of these hybrid genes will be under the control of the c-fos promoter. As mentioned the c-fos promoter is normally silent in serum starved cells but is transiently activated by the addition of serum or phorbol esters.

The c-fos promoter provides two advantages over the use of a constitutive promoter as used in this study. Firstly it avoids any potential problems associated with the use of transcription inhibitors such as actinomycin D, which have been shown to interfere with the function of some instability elements (Shyu *et al.*, 1989) as well as with other cellular functions (Harrold *et al.*, 1991). Secondly, this promoter would be advantageous in the search for decay intermediates, since the starting population of molecules will be relatively homogeneous. This will be particularly useful in examining the requirement for de-adenylation of the poly(A) tail in the turnover of the ALAS-1 mRNA.

Following the identification of any putative *cis*-acting instability sequences, these could be tested by deleting the corresponding regions from the full length ALAS-1

mRNA. In addition, the binding of specific proteins to this region could be assayed for using *in vitro* RNA gel shift experiments.

The identification of decay intermediates would also provide information on the mechanism by which the ALAS-1 mRNA decays. Northern blots of FRL 4.1 mRNA however failed to detect any smaller ALAS-1 species that may have been decay intermediates, indicating that the intermediates produced may be extremely unstable. In addition the low level of ALAS-1 mRNA in FRL 4.1 cells means that any decay intermediate produced will also be extremely low, perhaps below the detection limit of Northern blots.

To attempt to stabilise any ALAS-1 decay intermediates, secondary structures, similar to those used to block exonucleases in yeast (Vreken and Raue, 1992b ; Muhlrad *et al.*, 1994 a/b), could be inserted into the 5' and 3' UTRs of the rat ALAS-1 gene. Presumably, mammalian exonucleases are also blocked by such structures, but this is unknown. These hybrid genes would then be expressed in FRL 4.1 cells under the control of the transiently inducible c-fos promoter (Greenberg and Ziff, 1984). Serum treatment of serum starved cells should then led to a transient burst of transcription from the c-fos promoter. This method should stabilise ALAS-1 decay intermediates allowing their detection on Northern blots. RNA mapping using specific 5'- and 3'-specific probes would allow the direction of exonucleolytic shortening to be determined which would provide very useful information into not only the mechanism of ALAS-1 decay but also for the decay of mammalian mRNAs in general.

In conclusion, we have begun to characterise the heme regulation of ALAS-1 mRNA levels in the rat hepatoma cell line FRL 4.1. In these cells, heme was found to specifically decrease the half-life of the ALAS-1 mRNA. Additionally in rat liver, heme also appears to negatively regulate the transcription of the ALAS-1 gene. Therefore, to date heme has been shown to control the production of (functional) mitochondrial ALAS-1 enzyme at three points. These points are the production and turnover of ALAS-1 mRNA and the import of the ALAS-1 precursor into the mitochondria. In addition to this tight control on the production of functional ALAS, the short half-life of the protein in mitochondria will ensure that any decrease in the synthesis of ALAS will be rapidly

reflected in a decrease in the amount of ALAS activity and hence a rapid decline in heme synthesis in the cell. Furthermore, an additional control on overall heme levels in the cell is provided by the heme inducibility of heme oxygenase-1, the rate-limiting enzyme of heme catabolism.

Therefore, a number of controls exist in the liver cell and presumably in every other cell type, that ensures the level of heme production closely matches the cells current requirement, thus preventing a build up of free heme in the cytoplasm. The reason for this extremely tight control on ALAS and hence heme synthesis are only now being understood. One likely, consequence of a build up of free heme in the cell may be to promote iron-catalysed oxidative stress. Indeed, the loading of endothelial cells with heme in culture renders these cells exquisitely sensitive to small, otherwise innocuous doses of hydrogen peroxide a byproduct generated in the cell by respiration (Muller-Eberhard and Fraig, 1993).

Finally, the turnover of mRNA is now recognised as an important process in regulating the expression of a variety of mammalian genes. Therefore, the destabilisation of the ALAS-1 mRNA by heme provides an interesting paradigm to study the differential regulation of mRNA turnover in response to changes in the metabolic status of the cell.

REFERENCES

REFERENCES.

Abraham, N. G., Nelson, J. C., Ahmed, T., Konwalinka, G. and Levere, R. D. (1989) *Exp. Hemat.* **17**, p908.

Adate, C., Baker, S. J., Lees-Miller, S. P., Anderson, C. W., Marshak, D. R. and Curran, T. (1993) *Proc. Natl. Acad. Sci. USA* **90**, p6766.

Ades, I. Z. (1983) *Biochem. Biophys. Res. Commun.* **110**, p42.

Ades, I. Z., Stevens, T. M. and Drew, P. D. (1987) *Arch. Biochem. Biophys.* **253**, p297.

Adrisani, O. M., Hayes, T. E. and Dixon, J. E. (1987) *Nucleic Acids Res.* **15**, p5715.

Affolter, M., Labbe, D., Jean, A., Raymond, M., Noel, D., Labelle, Y., Vablgeois, C. P., Lambert, M., Bojanowski, R. and Anderson, A. (1986) *DNA* **5**, p209.

Aharon, T. and Schneider, R. J. (1993) *Mol. Cell. Biol.* **13**, p1971.

Alam, J., Cai, J. and Smith, A. (1994) *J. Biol. Chem.* **269**, p1001.

Alam, J. and Den, Z. (1992) *J. Biol. Chem* **267**, p21894.

Alam, J., Shibahara, S. and Smith, A. (1989) *J. Biol. Chem.* **264**, p6371.

Alam, J. and Smith, A. (1992) *J. Biol. Chem.* **267**, p16379.

Alberta, J. A., Rundell, K. and Stiles, C. D. (1994) *J. Biol. Chem.* **269**, p4532.

Altardi, G. and Schatz, G. (1988) *Ann. Rev. Cell Biol.* **4**, p289.

Amarat, F. M., Chen, F. Y. and Wright, J. A. (1994) *J. Biol. Chem.* **269**, p6709.

Anderson, K. E., Drummond, G. S., Freddara, U., Sardana, M. K. and Sassa, S. (1981) *Biochim. Biophys. Acta* **676**, p289.

Andreason, G. L. and Evans, G. A. (1988) *Biotechniques* **6**, p650.

Andrews, N. C., Erdjunent-Bromage, H., Davidson, M. B., Tempst, P. and Orkin, S. H. (1993) *Nature* **362**, p722.

Angel, P., Imagawa, M., Chiu, B., Imbra, R. J., Rahmsdorf, H. J., Jonat, C., Herrlich, P. and Karin, M. (1987) *Cell* **49**, p729.

Applegate, L. A., Luscher, P. and Tyrrell, R. M. (1991) *Cancer Res.* **51**, p974.

Ashfield, R., Enriquez-Harris, P. and Proudfoot, N. J. (1991) *EMBO. J.* **10**, p4197.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1989) in "*Current protocols in molecular biology*" (Wiley).

Avissar, V. J. and Beale, S. I. (1989) *J. Bacteriol.* **171**, p2919.

Bachurski, C. J., Theodorakis, N. G. and Cleveland, D. W. (1994) *Mol. Cell. Biol.* **14**, p4076.

Badawy, A. A-B. and Evans, M. (1973) *Biochem. J.* **136**, p885.

Badawy, A. A-B. and Evans, M. (1975) *Biochem. J.* **150**, p511.

Balla, G., Ver*Cell*otti, G. M., Muller-Erberhard, U., Eaton, J. and Jacob, H. S. (1991) *Lab. Invest.* **64**, p648.

Barton, M. C., Madani, N and Emerson, B. A. (1993) *Genes & Dev.* **7**, p1796.

Batt, P. B., Luo, Y. and Carmichael, G. G. (1994) *Nucleic Acids Res.* **22**, p2811.

Battini, R., Ferrari, S., Kaczmarck, L., Calbretta, B., Chen, S. and Baserga, R. (1987) *J. Biol. Chem.* **262**, p4355.

Bawden, M. J., Borthwick, I. A., Healy, H. M., Morris, C. P., May, B. K. and Elliott, W. H. (1987) *Nucleic Acids Res.* **15**, p8563.

Beaumont, C., Deybach, J. C., Grandchamp, B., Silva, V. D., de Verneuil, H. D. and Nordmann, Y. (1984) *Exp. Cell Res.* **154**, p474.

Beinert, H. and Kennedy, M. C.(1993) *FASEB. J.* **7**, p1442.

Belgrader, P., Cheng, J. and Maquat, L. E. (1993) *Proc. Natl. Acad. Sci. USA* **90**, p482.

Belgrader, P., Cheng, J., Zhou, X., Stephenson, L. S. and Maquat, L. E. (1994) *Mol. Cell. Biol.* **14**, p8219.

Bernstein, P. L., Herrick, D. J., Prokipak, R. D. and Ross, J. (1992) *Genes & Dev.* **6**, p642.

Bhasker, C. R., Burgiel, G., Neupert, B., Emery-Goodman, A., Kuhn, L. C. and May, B. K. (1993) *J. Biol. Chem.* **268**, p12699.

Bickel, M., Iwai, Y., Pluznik, D. H. and Cohen, R. B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, p10001.

Binder, R., Horowitz, J. A., Basilion, J. P., Koeller, D. M., Klausner, R. D. and Harford, J. B. (1994) *EMBO. J.* **13**, p1969.

Binder, R., Hwang, S-P. L., Ratnasabapathy, R. and Williams, D. L. (1987) *J. Biol. Chem.* **264**, p16910.

Birnboim, H. C. and Doly, J. (1979) *Nucleic Acids Res.* **7**, p1513.

Bishop, D. F. (1990) *Nucleic Acids Res.* **18**, p7187.

Bishop, D. F., Henderson, A. S. and Astrin, K. H. (1990) *Genomics* **7**, p207.

Bishop, T. R., Miller, M. W., Frelin, L. P., Dierks, P.and Boyer, S. H. (1991*) Am. J. Hum. Genet.* (suppl) **49**, p425.

Blanchard, K. L., Acquaviva, A. M., Galson, D. L. and Bunn, H. F. (1992) *Mol. Cell. Biol.* **12**, p5373.

Blanchard, K. L., Fandrey, J., Goldberg, M. A. and Bunn, F. H. (1993) *Stem Cells* **11**, (Suppl. 1) p17.

Blumberg, P. M. (1988) *Cancer Research* **48**, p1.

Boggs, S. S., Gregg, R. G., Borenstein, N. and Smithies, O. (1986), *Exp. Hematol.* **14**, p988.

Bohjanen, P. R., Petryniak, B., June, C. H., Thompson, C. B. and Lindsten, T. (1991) *Mol. Cell. Biol.* **11**, p3288.

Bohjanen, P. R., Petryniak, B., June, C. H., Thompson, C. B. and Lindsten, T. (1992) *J. Biol. Chem.* **267**, p6302.

Borthwick, I. A., Srivastava, G., Day, A. R., Pirola, B. A., Snoswell, M. A., May, B. K. and Elliott, W. H. (1985) *Eur. J. Biochem.* **150**, p481.

Borthwick, I. A., Srivastava, G., Pirola, B. A., May, B. K. and Elliott, W. H. (1986) in "*Methods in Enzymology*" Chytil, F. and McCormick, D. B., eds., **123**, (Academic Press) p395.

Bottomley, S. S (1992) in "*Wintrobe's Clinical Hematology*" Lee, G. R., Bithell, T. C., Foerster, J., Athens, J. W. and Lukens, J. N., eds (Lea & Febiger) p852.

Bottomley, S. S. and Muller-Eberhard, U. (1988) *Semin. Hematol.* **25**, p282.

Bouvet, P., Paris, J., Philippe, M. and Osborne, H. B. (1991) *Mol. Cell. Biol.* **11**, p3115.

Boyer, T. D. and Olsen, E. (1991) *Biochem. Pharmacol.* **42**, p188.

Braidotti, G. (1992) *PhD thesis,* University of Adelaide.

Braidotti, G., Borthwick, I. A. and May, B. K. (1993) *J. Biol. Chem.* **268**, p1109.

Brewer, G. and Ross, J. (1988) *Mol. Cell. Biol.* **8**, p1697.

Brewer, G. and Ross, (1989) *J. Mol. Cell. Biol.* **9**, p1996.

Bridges, K., R. and Cudkowicz, A. (1984) *J. Biol. Chem.* **259,** p12970.

Brown, B. D. and Harland, R. M. (1990) *Genes & Dev.* **4**, p1925.

Brown, S. B., Houghton, J. D. and Wilks, A. in "*Biosynthesis of Heme and Chlorophylls*" (1990) Dailey, H. A. ed, (McGraw-Hill) p543.

Brown, B. D., Zipkin, I. D. and Harland, R. M. (1993) *Genes & Dev.* **7**, p1620.

Buchman, A. R. and Berg, P. (1988) *Mol. Cell. Biol.* **8**, p4395.

Buluwela, L., Forster, A., Buehm, T. and Rabbitts, T. H. (1989) *Nucleic Acids Res.* **17**, p452.

Burger, H-J., Schuetz, E. G., Schuetz, J. D. and Guzelian, P. S. (1990) *Arch. Biochem. Biophys.* **281**, p204.

Burger, H-J., Schuetz, J. D., Schuetz, E. G. and Guzelian, P. S. (1992) *Proc. Natl. Acad. Sci. USA* **89**, p2145.

Busfield, S. and Klinken, S. P. (1992) *Blood* **80**, p412.

Cable, E. E., Pepe, J. A., Karamitsios, N. C., Lambrecht, R. W. and Bonkovsky, H. L. (1994) *J. Clin. Invest.* **94**, p649.

Caponigro, G., Muhlrad, D. and Parker, R. (1993) *Mol. Cell. Biol.* **13**, p5141.

Casey, J. L., Koeller, D. M., Ramin, V., Klausner, R. D. and Harford, J. B. (1989) *EMBO. J.* **8**, p3693.

Carrier, F., Owens, R. A., Nebert, D. W. and Puga, A. (1992) *Mol. Cell. Biol.* **12**, p1856.

Caput, D., Beulter, B., Hartog, K., Thayer, R., Brown-Shimer, S. and Cerami, A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, p1670.

Caruccio, N. and Ross, J. (1994) *J. Biol. Chem.* **269**, p31814.

Chan, R. Y-Y., Seiser, C., Schulman, H. M., Kuhn, L. C. and Ponka, P. (1994) *Eur. J. Biochem.* **220**, p683.

Chandler, L. A., Ehretsmann, C. P. and Bourgeois, S. (1994) *Mol. Cell. Biol.* **14**, p3085.

Chau, C-M. A., Evans, M. J. and Scarpulla, R. C. (1992) *J. Biol. Chem.* **267**, p6999.

Chen, C-Y. A., Chen, T-M. and Shyu, A-B. (1994) *Mol. Cell. Biol.* **14**, p416.

Chen, C-Y. A. and Shyu, A-B. (1994) *Mol. Cell. Biol.***14**, p8471.

Chen J-J. and London I. M. (1995) *Trends Biochem. Sci.* **20**, p105.

Chen, J. J., Throop, M. S., Gehrke, L., Kuo, I., Pal, J. K., brodsky, M. and London, I. M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, p7729.

Cheng, J. and Maquat, L. E. (1993) *Mol. Cell. Biol.* **13**, p1892.

Chern, Y., Yonekura, S. and Sytkowski, A. J. (1990) *Blood* **76**, p2204.

Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, p156.

Choo, K. H., Filby, G., Greco, S., Lau, Y-F. and Kan Y. W. (1986) *Gene* **46**, p277.

Choy, H. A., McLaughlin, M. and Feigelson, P. (1989) *J. Biol. Chem.* **264**, p1754.

Chretien, S., Dubart, A., Beaupin, D., Raich, N., Granchamp, B., Rosa, J., Goossens, M. and Romeo, P-H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, p6.

Christensen, A. K., Kahn, L. E. and Bourne, C. M. (1987) *Am. J. Anat.* **178**, p1.

Chu, G., Hayakawa, H. and Berg, P. (1987) *Nucleic Acids Res.* **15**, p1311.

Chung, A. B., Stepien, G., Haraguchi, Y., Li, K and Wallace, D. C. (1992) *J. Biol. Chem.* **267**, p21154.

Ciechanover, A. (1994) *Cell* **79**, p13.

Conboy, J. G., Cox, T. C., Bottomley, S. S., Bawden, M. J. and May, B. K. (1992) *J. Biol. Chem.* **267**, p18753.

Connelly, S. and Manley J. L. (1988) *Genes & Dev.* **2**, p440.

Connelly, S. and Manley J. L. , (1989) *Cell* **57**, p561.

Cooper, D. N. (1993) *Ann. Med.* **25**, p11.

Cotter, P. D., Willard, H. F., Gorski, J. L. and Bishop, D. F. (1992) *Genomics* **13**, p211.

Coutts, M. and Brawerman, G. (1993) *Biochim. Biophys. Acta* **1173**, p57.

Coutts, M., Krowczynska, A. and Brawerman, G. (1993) *Biochim. Biophys. Acta* **1173**, p49.

Cox, T. C. (1993) *PhD thesis*, University of Adelaide.

Cox, T. C., Bawden, M. J., Abraham, N. G., Bottomley, S. S., May, B. K., Baker, E., Chen, L. Z. and Sutherland, G. R. (1990) *Am. J. Hum. Genet.* **46**, p107.

Cox, T. C., Bawden, M. J., Martin A. and May, B. K. (1991) *EMBO. J.* **10**, p1891.

Crosby, J. S., Lee, K., London, I. M. and Chen, J-J. (1994) *Mol. Cell. Biol.* **14**, p3906.

Cruse, I. and Maines, M. D. (1988) *J. Biol. Chem.* **263**, p3348.

Cui, Y., Hagan, K. W., Zhang, S. and Peltz, S. W. (1995) *Genes & Dev.* **9**, p423.

Cullen, B. R., Lemedico, P. T. and Ju, G. (1984) *Nature*, **307**, p241.

Culutta, V. C. and Hamer, D. H. (1989) *Mol. Cell. Biol.* **9**, p1376.

Dandekar, T., Stripecke, R., Grey, N. K., Goossens, B., Constable, A., Johansson, H. E. and Hentze, M. W. (1991) *EMBO. J.* **10**, p1903.

Dani, C., Blanchard, J. M., Piechaczyk, M., El Sabrouty, S., Martly, L. and Jeanteur, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, p7046.

Darwish, H. and Deluca, H. F. (1993) *Crit. Rev. Euk. Gene Exp.* **3**, p98.

Davis, L. I. (1992) *Curr. Opin. Cell Biol.* **4**, p424.

Davies, D. M., Smith, A., Muller-Eberhard, U. and Morgan, W. T. (1979) *Biochem. Biophys. Res. Commun.* **91**, p1504.

De Benedetti, A. and Baglioni, C. (1983) *J. Biol. Chem.* **258**, p14556.

De Benedetti, A. and Corrado, B. (1986) *J. Biol. Chem.* **261**, p338.

De Matteis, F. (1988) *Seminars in Hemat.* **25**, p321.

Decker, C. J. and Parker, R. (1993) *Genes & Dev.* **7**, p1632.

Decker, C. J. and Parker, R. (1994) *Trends Biochem. Sci.* **19**, p336.

Devereux, J. (1984) *Nucleic Acids Res.* **12**, p387.

Dewille, J. W., Jenh, C-H., Deng, T., Harendza, C. J. and Johnson, L. F. (1988) *J. Biol. Chem.* **263**, p84.

Dierks, P. in "*Biosynthesis of Heme and Chlorophylls*" (1990) Dailey, H. A. ed, (McGraw-Hill) p201.

Dodson, R. E. and Shapiro, D. J. (1994) *Mol. Cell. Biol.* **14**, p3130.

Dogra, S. C., Hahn, C. N. and May, B. K. (1993) *Arch. Biochem. Biophys.* **300**, p531.

Drew, P. D. and Ades, I. Z. (1989a) *Biochem. J.* **262**, p815.

Drew, P. D. and Ades, I. Z. (1989b) *Biochem. Biophys. Res. Commun.* **162**, p102.

Driscoll, J., Frydman, J. and Goldberg, A. L. (1992) *Proc. Natl. Acad. Sci. USA* **89**, p4986.

Drummond, D. R., Armstrong, J. and Colman, A. (1985) *Nucleic Acids Res.* **13**, p7375.

Drummond, G. S., Galbraith, R. A., Sardana, M. K. and Kappas, A. (1987) *Arch. Biochem. Biophys.* **255**, p67.

Duronio, V., Huber, B. F. and Jacobs, S. (1990) *J Cell Physiol.* **145**, p381.

Dzelzkalns, V, Foley, T. and Beale, S. I. (1982) *Arch. Biochem. Biophys.* **216**, p196.

Ebert, P. S. (1979) *Biochem. Biophys. Res. Commun.* **88**, p1382.

Eckner, R., Ellmeier, W. and Birnstiel, M. L. (1991) *EMBO. J.* **10**, p3513.

Efiok, B. J. S., Chiorini, J. A and Safer, B. (1994) *J. Biol. Chem.* **269**, p18921.

Eggermont, J. and Proudfoot, N. J. (1993) *EMBO. J.* **12**, p2539.

Ehrenman, K., Long, L., Wagner, B. J. and Brewer, G. (1994) *Gene* **149**, p315.

Eilers, M., Hwang, S and Schatz, G. (1988) *EMBO. J.* **7**, p1139.

Eisenstein, R. S., Garcia-Mayol, D., Pettingel, W. and Munro, H. N. (1991) *Proc. Natl. Acad. Sci. USA* **88**, p688.

Elferink, C. J., Sassa, S. and May, B. K. (1988) *J. Biol. Chem.* **263**, p13012.

Elferink, C. J., Srivastava, G., Maguire, D. J., Borthwick, I. A., May, B. K. and Elliott, W. H. (1987) *J. Biol. Chem.* **262**, p3988.

Elliott, W. H., May, B. K., Bawden, M. J. and Hanson, A. J. (1989) in "*Biochem. Soc. Symp. 55, Gene Expression: Regulation at the RNA and protein level*" Kay, J., Ballard, F. J. and Mayer, R. J. eds., The Biochem Soc. London p13.

Enriquez-Harris, P Levitt, N., Briggs, D. and Proudfoot, N. J., (1991) *EMBO. J.* **10**, p1833.

Essig, D. A., Kennedy, J. M. and McNabney, L. A. (1990) *Am. J. Physiol.* **259**, C310.

Essig, D. A. and McNabney, L. A. (1991) *Am. J. Physiol.* **261**, C691.

Evans, M. J. and Scarpulla, R. C. (1989) *J. Biol. Chem.* **264**, p14361.

Evans, M. J. and Scarpulla, R. C. (1990) *Genes & Dev.* **4**, p1023.

Farrell, P. F., Balkow, K., Hunt, T., Jackson, R. J. and Trachsel, H. (1977) *Cell* **11**, p187.

Ferreira, G. C., Andrews, T. L., Karr, S. W. and Dailey, H. A. (1988) *J. Biol. Chem.* **263**, p3835.

Ferreira, G. C., Neame, P. J. and Dailey, H. A. (1993) *Protein Sci.* **2**, p1959.

Fong, T. C. and Emerson, B. M. (1992) *Genes & Dev.* **6**, p521.

Fujita, H., Yamamoto, M., Yamagami, T., Hayashi, N., Bishop, T. R., de Verneuil, H., Yoshinaga, T., Shibahara, S., Morimoto, R. and Sassa, S. (1991a) *Biochim. Biophys. Acta.* **1090**, p311.

Fujita, H., Yamamoto, M., Yamagami, T., Hayashi, N. and Sassa, S. (1991b) *J. Biol. Chem.* **266**, p17494.

Fukuda, Y., Fujita, H., Garbaczewski, L. and Sassa, S. (1994) *Blood* **83**, p1662.

Fukuda, Y., Fujita, H., Taketani, S. and Sassa, S. (1993) *Br. J. Hemat.* **83**, p480.

(Funanage, V. L., Schroedl, N. A., Moses, P. A., Smith, S. M., Kirwin, J. J. and Hartzell, C. R. (1989) *J. Cell. Physiol.* **141**, p591.

Gallie, D. R. (1991) *Genes & Dev.* **5**, p2108.

Gallie, D. R. and Thanguay, R. (1994) *J. Biol. Chem.* **269**, p17166.

Galson, D. L., Tsuchiya, T., Tendler, D. S., Huang, L. E., Ren, Y., Ogura, T. and Bunn, F. H. (1995) *Mol. Cell. Biol.* **15**, p2135.

Garby, L. and Noyes, W. D. (1959) *J. Clin. Invest.* **38**, p1479.

Gardner, L. C. and Cox, T. M. (1988) *J. Biol. Chem.* **263**, p6676.

Gardner, L. C., Smith, S. J. and Cox, T. M. (1991) *J. Biol. Chem.* **266**, p22010.

Gibson, K. D., Laver, W. G. and Neuberger, A. (1958) *Biochem. J.* **70**, p71.

Gillies, S., Morrison, S., Oi, V. and Tonegawa, S. (1983) *Cell* **50**, p717.

Gilles-Gonzalez, M. A., Ditta, G. S. and Helinski, D. R. (1991) *Nature* **350**, p170.

Goldberg, M. A., Dunning, S. P. and Bunn, H. F. (1988) *Sci.* **242**, p1412.

Goldberg, M. A., Gaut, C. C. and Bunn, H. F. (1991) *Blood* **77**, p271.

Gonzalez, F. J. (1989) *Pharmacol. Rev.* **40**, p243.

Gorman, C. M., Moffat, L. F. and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, p1044.
Gough, N. M. (1988) *Anal. Biochem.* **173**, p93.

Grandchamp, B., Beaumont, C., de Verneuil, H. and Nordmann, Y. (1985) *J. Biol. Chem.* **260**, p9630.

Granick, S. (1966) *J. Biol. Chem.* **241**, p1359.

Greenberg, J. R. (1972) *Nature* **242**, p102.

Greenberg, M. E. and Ziff, E. B. (1984) *Nature* **311**, p433.

Grey, N. K. and Hentze, M. W (1994) *EMBO. J.* **13**, p3882.

Gross, D. S. and Garrard, W. T. (1988) *Ann. Rev. Biochem.* **57**, p159.

Gunning, P., Ponte, P., Okayama, H., Engel, J., Blau, H. and Kedes, L. (1983) *Mol. Cell. Biol.* **3**, p787.

Guo, G. G., Gu, M. and Etlinger, J. D. (1994) *J. Biol. Chem.* **269**, p12399.

Gutheridge, J. M. and Smith, A. (1988) *Biochem. J.* **256**, p861.

Guzelian, P. S., O'Connor, L., Fernandez, S., Chan, U., Giampietro, P. and Desnick, R. (1984) *Life Sci.* **31**, p1111.

Hagan, K. W., Ruiz-Echevarria, M. J., Quan, Y. and Peltz, S. W. (1995) *Mol. Cell. Biol.* **15**, p809.

Hahn, C. N. (1991) *PhD thesis,* University of Adelaide.

Hahn, C. N., Hansen, A. J. and May, B. K. (1991) *J. Biol. Chem.* **266**, p17031.

Haile, D. J., Rouault, T. A., Tang, C. K., Chin, J., Harford, J. B. and Klausner, R. D. (1992) *Proc. Natl. Acad. Sci. USA* **89**, p7536.

Hamilton, B. J., Nagy, E., Malter, J. S., Arrick, B. A. and Rigby, W. F. C. (1993) *J. Biol. Chem.* **268**, p8881.

Hamilton, J. W., Bement, W. J., Sinclair, P. R., Sinclair, J. F., Alcedo, J. A. and Wetterhahn, K. E. (1992) *Arch. Biochem. Biophys.* **298**, p96.

Hamilton, J. W., Bement, W. J., Sinclair, P. R., Sinclair, J. F., Alcedo, J. A. and Wetterhahn, K. E. (1991) *Arch. Biochem. Biophys.* **289**, p387.

Hamilton, J. W., Bement, W. J., Sinclair, P. R., Sinclair, J. F. and Wetterhahn, K. E. (1988) *Biochem. J.* **255**, p267.

Hansen, A. J., Elferink, L. A. and May, B. K. (1989) *DNA* **8**, p179.

Harbin, B. M. and Dailey, H. A. (1985) *Biochem* **24**, p366.

Hargrove, J. L. and Schmidt, F. H. (1989) *FASEB. J.* **3**, p2360.

Harrold, S., Genovese, C., Korbin, B., Morrison, S. L. and Milcarek, C. (1991) *Anal. Biochem.* **198**, p19.

Haung, M. T. F. and Gorman, C. M. (1990) *Nucleic Acids Res.* **18**, p937.

Hayashi, N., Kuashima, Y. and Kikuchi, G. (1972) *Arch. Biochem. Biophys.* **148**, p10.

Hayashi, N., Kurashima, Y. and Kikuchi, G. (1981) *Arch. Biochem. Biophys.* **209**, p451.

Hayashi, N., Terasawa, M. and Kikuchi, G. (1980) *Biochem. J.* **88**, p921.

Hayashi, N., Watanabe, N. and Kikuchi, G. (1983) *Biochem. Biophys. Res. Commun.* **115**, p700.

He, F. and Jacobson, A. (1995) *Genes & Dev.* **9**, p437.

Healy, H, M., (1990) *PhD Thesis*, University of Adelaide.

Helfman, T. and Falanga, V. (1993) *Am. J. Med. Sci.* **306**, p37.

Henics, T., Sanfridson, A., Hamilton, B. J., Nagy, E. and Rigby, W. F. C. (1994) *J. Biol. Chem.* **269**, p5377.

Herrick, D., Parker, R. and Jacobson, A. (1990) *Mol. Cell. Biol.* **10**, p2269.

Herrick, D. J. and Ross, J. (1994) *Mol. Cell. Biol.* **14**, p2119.

Hirling, H., Henderson, B. R. and Kuhn, L. C. (1994) *EMBO. J.* **13**, p453.

Hobbs, A. A., Mattschoss, L. A., May, B. K., Williams, K. E. and Elliott, W. H. (1986) *J. Biol. Chem.* **261**, p9444.

Hogan, C. N., Traverse, K. L., Sullivan, D. E. and Pardue, M-L. (1994) *J. Cell Biol.* **125**, p21.

Holloszy, J. O. and Winder, W. W. (1979) *Am. J. Physiol.* **236**, R180.

Houston, T., Moore, M. R., McColl, K. E. L and Fitzsimons, E. (1991) *Br. J. Hemat.* **78**, p561.

Hsu, C. L. and Stevens, A. (1993) *Mol. Cell. Biol.* **13**, p486.

Huang, L-Y., Tholanikunnel, B. G., Vakalopoulou, E. and Malbon, C. C. (1993) *J. Biol. Chem.* **268**, p25769.

Ibsen, K. H. and Fishman, W. H. (1979) *Biochim. Biophys. Acta.* **560**, p243.

Ikuta, T.and Tan, Y, W. (1991) *Proc. Natl. Acad. Sci. USA.* **88**, p10188.

Isseman, I., Prince, R. A., Tugwood, J. D. and Green, S. (1993) *J. Mol. Endocrinol.* **11**, p37.

Iwasa, F., Sassa, S. and Kappas, A. (1989) *Biochem. J.* **262**, p807.

Izaurralde, E. and Mattaj, I. W. (1992) *Sem. Cell Biol.* **3**, p279.

Jacob, W. F., Silverman, T. A., Cohen, R. B. and Safer, B. (1989) *J. Biol. Chem.* **264**, p20374.

Jakoby, W. B. and Ziegler, D. M. (1990) *J. Biol. Chem.* **265**, p20715.

Jackson, R. J. (1988) *Nature* **334**, p292.

Jackson, R. J. (1993) *Cell* **74**, p9.

Jackson, R. J. and Standart, N. (1990) *Cell* **62**, p15.

Jelkman, W. (1992) *Physiol. Rev.* **72**, p449.

Jones, T. R. and Cole, M. D. (1987) *Mol. Cell. Biol.* **7**, p4513.

Jordan, P. M. (1990) in "*Biosntheis of Heme and Chlorophylls*" Dailey, H. A. ed, (McGraw-Hill) p55.

Kabnick, K. S. and Housman, D. E. (1988) *Mol. Cell. Biol.* **8**, p3244.

Kappas, A., Sassa, S., Galbraith, R. A. and Nordmann, Y. (1989) *in "The Metabolic Basis of Inherited Disease"* Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D. eds. 6th Ed. (McGraw-Hill) p1305.

Karlsson, S. and Nienhuis, A. W. (1985) *Ann. Rev. Biochem.* **54**, p1071.

Kaufman, R. J. and Murtha, P. (1987) *Mol. Cell. Biol.* **7**, p1568.

Kaya, A. H., Plewinska, M., Wong, D. M., Desnick, R. J. and Wetmur, J. G. (1994) *Genomics* **19**, p242.

Kenna, M., Stevens, A., McCammon, M. and Douglas, M. G. (1993) *Mol. Cell. Biol.* **13**, p341.

Kikuchi, G. and Hayashi, N. (1981) *Mol. Cell. Biochem.* **37**, p734.

Kikuchi, G., Kumar, A., Talmage, P. and Shemin, D. (1958) *J. Biol. Chem.* **233**, p1214.

Klausner, R. D., Rouault, T. A. and Harford, J. B. (1993) *Cell* **72**, p19.

Klinken, S. P., Busfield, S., Keil, U., Farr, T., Colley, S., Callus, B., Chappel, D and Papadimitriou, J. (1993) *J. Compt. Asst. Microscopy* **5**, p81.

Klinken, S. P., Nicola, N. A. and Johnson, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, p8506.

Ko, L. J. and Engel, J. D. (1993) *Mol. Cell. Biol.* **13**, p4011.

Koeller, D. M., Casey, J. L., Hentze, M. W., Gerhardt, E. M., Chan, L-N. L., Klausner, R. D. and Harford, J. B. (1989) *Proc. Natl. Acad. Sci. USA* **86**, p3574.

Koeller, D. M., Horowitz, J. A., Casey, J. L., Klausner, R. D. and Harford, J. B. (1991) *Proc. Natl. Acad. Sci. USA* **88**, p7778.

Korb, M., Ke, Y. and Johnson, L. F. (1993) *Nucleic Acids Res.* **21**, p5901.

Koury, M. J. and Bondurant, M. C. (1990) *Sci.* **248**, p378.

Knutson, J. C. and Yee, D. (1987) *Anal. Biochem.* **164**, p44

Krieg, P. A. and Melton D. A. (1987) *Methods Enzymol.* **155**, p397.

Kugler, W., Enssle, J., Hentze, M. W. and Kulozik, A. E. (1995) *Nucleic Acids Res.* **23**, p413.

Kunkel, T. A., Roberts, J. D. and Zakour, R. A., (1987) *Methods Enzymol.* **154**, p367.

Lagnado, C. A., Brown, C. Y. and Goodall, G. J. (1994) *Mol. Cell. Biol.* **14**, p7984

Laird-Offringa, I. A. (1992) *BioEssay* **14**, p119.

Laird-Offringa, I. E., de Wit, C. L., Elfferich, P. and Van Der EB, A. J. (1990) *Mol. Cell. Biol.* **10**, p6132.

Landschulz, K. T., Noyes, A. N., Rogers, O and Boyer, S. H. (1989) *Blood* **73**, p1476.

Larimer, F. W. and Stevens, A. (1990) *Gene* **95**, p85.

Lathrop, J. T. and Timko, M. P. (1993) *Sci.* **259**, p522.

Lautier, D., Luscher, P. and Tyrrell, R. M. (1991) *Carcinogenesis* **13**, p227.

Lavrovsky, Y., Schwartzman, M. L., Levere, R. D., Kappas, A. and Abraham, N. G. (1994) *Proc. Natl. Acad. Sci. USA* **91**, p5987.

Lawn, R. M., Efstratidis, A., O'Connell, C. and Maniatis, T. (1980) *Cell* **21**, p647.

Lazar, J. L., Desvergne, B., Zimmerman, E. C., Zimmer, D. B., Magnuson, M. A. and Nikodem, V. M. (1994) *J. Biol. Chem.* **269**, p20352.

Lee, W., Mitchell, P. and Tijan, R. (1987) *Cell* **49**, p741.

Leong, S. A., Williams, P. H. and Ditta, G. S. (1985) *Nucleic Acids Res.* **13**, p5965.

Lewin, B. (1990) *Cell* **61**, p1161.

Levitt, N., Briggs, D., Gil, A. and Proudfoot, N. J. (1989) *Genes & Dev.* **3**, p1019.

Liang, H.and Jost, J-P. (1991) *Nucleic Acids Res.* **19**, p2289.

Li, Y., Li, D., Osborn, K. and Johnson, L. F. (1991) *Mol. Cell. Biol.* **11**, p1023.

Liem, H, H., Grasso, J. A., Vincent, S. H. and Muller-Eberhard, U.(1990) *Biochem. Biophys. Res. Commun.* **167**, p528.

Lim, G. B. and Wintour, M. E. (1993) *Todays Life Sci.* **5**, p16.

Lim, K. C., Ishihara, H., Riddle, R. D., Yang, Z., Andrews, N., Yamamoto, M. and Engel, J. D. (1994) *Nucleic Acids Res.* **22**, p1226.

Lim, S-K., Sigmund, C. D. Gross, K. W. and Maquat, L. E. (1992) *Mol. Cell. Biol.* **12**, p1149.

Liou, H-C. and Baltimore, D. (1993) *Curr. Opin. Cell Biol.* **5**, p477.

Logan, J., Falck-Pedersen, E., Darnell Jr. J. E. and Shenk, T. (1987) *Proc. Natl. Acad. Sci. USA* **84**, p8306.

Lowell, J. E., Rudner, D. Z. and Sachs, A. B. (1992) *Genes & Dev.* **6**, p2088.

Lozano, F., Maertzdorf, B., Pannell, R. and Milstein, C. (1994) *EMBO. J.* **13**, p4617.

Luscher, B. and Eisenman, R. N. (1990) *Genes & Dev.* **4**, p2025.

Maguire, D. J., Day, A. R., Borthwick, I. A., Srivastava, G., Wigley, P. L., May, B. K. and Elliot, W. H. (1986) *Nucleic Acids Res.* **14**, p1379.

Mahadevan, L. C., Wills, A. J., Hirst, E. A., Rathjen, P. D. and Heath, J. K. (1990) *Oncagene* **5**, p327.

Maines, M. D. (1992) in "*Heme Oxygenase: Clinical Applications and Functions*" (CRC press).

Maines, M. D. (1993) *Mol. Cell. Neuro.* **4**, p389.

Maity, A., McKenna, W. G. and Muschel, R. J. (1995) *EMBO. J.* **14**, p603.

Malter, J. S. (1989) *Sci.* **246**, p664.

Malter, J. S. and Hong, Y. (1991) *J. Biol. Chem.* **266**, p3167.

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982) "*Molecular Cloning: A Laboratory Manual*" Cold Spring Harbour Laboratory (Cold Spring Harbour, New York).

Manley, J. L. and Proudfoot, N. J. (1994) *Genes & Dev.* **8**, p259.

Martasek, P., Camadro, J. M., Delfau-Larue, M-H., Dumas, J. B., Montagne, J. J., de Verneuil, H., Labbe, P. and Grandchamp, B. (1994) *Proc. Natl. Acad. Sci. USA* **91**, p3024.

Marzluff, W. F. (1987) "*Methods in Cell Biology*" Stein, G., Stein, J. and Kleinsmith, L. J. Eds. **XIX**, (Acad. Press) p313.

Mattia, E., Josie, D., Ashwell, G., Klausner, R. D. and van Renswoude, J. (1986) *J. Biol. Chem.* **261**, p4587.

Max-Audit, I., Eleouet, J. F. and Romeo, P-H. (1993) *J. Biol. Chem.* **268**, p5431.

May, B. K., Bhasker, C. R., Bawden, M. J. and Cox, T. C.(1990) *Mol. Biol. Med.* **7**, p405.

May, B. K., Borthwick, I. A., Srivastava, G., Pirola, B. A. and Elliott, W. H. (1986) *Curr. Top. Cell Reg.* **28**, p233.

May, B. K., Dogra, S. C., Sadlon, T. J., Bhasker, C. R., Cox, T. C. and Bottomley, S. S. (1995) *Prog. Nucleic Acids Res. Mol. Biol.* in press.

Mayeda, A. and Krainer, A. R. (1992) *Cell* **68**, p365.

McClung, C. R., Somerville, J. E., Guerinot, M. L. and Chelm, B. K. (1987) *Gene* **54**, p133.

McCoubrey, W. K. and Maines, M. D. (1994) *Gene* **139**, p155.

Meinsma, D., Scheper, W., Holthuizen, P. E., Van den Brande, L. and Sussenbach, J. S. (1992) *Nucleic Acids Res.* **20**, p5003.

Melefors, O., Goossens, B., Johansson, H. E.,Stripecke, R., Grey, and Hentze, M. W (1993) *J. Biol. Chem.* **268**, p5974.

Mercer, J. F. B. and Wake, S. A. (1985) *Nucleic Acids Res.* **13**, p7929.

Merika, M. and Orkin, S. H. (1993) *Mol. Cell. Biol.* **13**, p3999.

Merika, M. and Orkin, S. H. (1995) *Mol. Cell. Biol.* **15**, p2437.

Metcalf, D. (1989) *Nature* **339**, p27.

Mignotte, V., Wall, L., de Boer, E., Grosveld, F. and Romeo, P-H. (1989) *Nucleic Acids Res.* **17**, p37.

Mihm, S., Galter, D. and Droge, W. (1995) *FASEB J.* **9**, p248.

Miller, I. J.and Bieker, J. J., (1993) *Mol. Cell. Biol.* **13**, p2776.

Miller, J. L., Walsh, C. E., Ney, P. A., Samulski, R. J. and Nienhuis, A. W. (1993) *Blood* **82**, p1900

Miura, O., Nakamura, N., Ihle, J. N. and Aoki, N. (1993) *J. Biol. Chem.* **269**, p614.

Monette, F. C. and Holden, S. A. (1982) *Blood* **60**, p527.

Mount, S. M. (1982) *Nucleic Acids Res.* **10**, p459.

Muerhoff, S. A., Griffin, K. J. and Johnson, E. F. (1992) *J. Biol. Chem.* **267**, p19051.

Muhlrad, D., Decker, C. J. and Parker, R. (1994) *Genes & Dev.* **8**, p855.

Muhlrad, D., Decker, C. J. and Parker, R (1995) *Mol. Cell. Biol.* **15**, p2145.

Muhlrad, D. and Parker, R. (1992) *Genes & Dev.* **6**, p2100.

Muhlrad, D. and Parker, R. (1994) *Nature* **370**, p578.

Muller-Erberhard, U. and Fraig, M. (1993) *Am. J. Hemat.* **42**, p59.

Mullner, E. W. and Kuhn, L. C. (1988) *Cell* **53**, p815.

Munakata,H., Yamagami, T., Nagai, T., Yamamoto, M. and Hayashi, N. (1993) *J. Biochem.* **114**, p103.

Murphy G. and Kavanagh, T. (1988) *Nucleic Acids Res.* **16**, p5198

Nagy, E. and Rigby, W. F. C. (1995) *J. Biol. Chem.* **270**, p2755.

Nanbu, R., Menoud, P-A. and Nagamine, Y. (1994) *Mol. Cell. Biol.* **14**, p4920.

Nandi, A. L. (1978) *Arch. Biochem. Biophys.* **188**, p266.

Nascimento, A. L., Luscher, P. and Tyrrell, R. M. (1993) *Nucleic Acids Res.* **21**, p1103.

Nath, K. A., Balla, G., Ver*Cell*otti, G. M., Balla, J., Jacob, H. S., Levitt, M. D. and Rosenberg, M. E. (1992) *J. Clin. Invest.* **90**, p267.

Nell, H., Weil, D., Giansante, C. and Dautry, F. (1993) *Genes & Dev.* **7**, p2194.

Nelson, D. R., Kamataki, T., Waxman, D., Guengerich, P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K. and Nebert, D. W. (1993) *DNA Cell Biol.* **12**, p1.

Nelson, M. and McClellend, M. (1991) *Nucleic Acids Res.* **19**, Suppl. p2045.

Nesic, D., Cheng, J. and Maquat, L. E. (1993) *Mol. Cell. Biol.* **13**, p3359.

Nesic, D. and Maquat, L. E. (1994) *Genes & Dev.* **8**, p363.

Nielsen, F. C. and Christiansen, J. (1992) *J. Biol. Chem.* **267**, p19404.

Nishizawa, K. (1994) *Biochem. Biophys. Res. Commun.* **200**, p789.

Niwa M. and Berget, S. M. (1991) *Genes & Dev.* **5**, p2086.

Niwa, M., Rose, S. D. and Berget, S. M. (1990) *Genes & Dev.* **4**, p1552.

Novogrodsky, A., Suthanthiran, M. and Stenzel, K. H. (1989) *J. Immunol.* **143**, p3981.

Nuss, D. L. and Furuichi, Y. (1977) *J. Biol. Chem.* **252**, p2815.

Nuss, D. L., Furuichi, Y., Koch, G. and Shatkin, A J. (1975) *Cell* **6**, p21.

O'Halloran, T. V. (1993) *Sci.* **261**, p715.

Okinaga, S. and Shibahara, S (1993) *Eur. J. Biochem.* **212**, p167.

Ortiz de Montellano, P. R. and Carreia, M. A. (1983) *Ann. Rev. Pharmacol. Toxicol.* **23**, p581.

Owen, D and Kuhn, L. C. (1987) *EMBO. J.* **6**, p1287.

Padmanadan, G., Venkateswar, V. and Rangarajan, P. N. (1989) *Trends Biochem. Sci.* **14**, p492.

Palma, J. F., Gao, X. Y., Lin, C-H., Wu, S. and Solomon, W. B. (1994) *Blood* **84**, p1288.

Palmiter, R. D., Sandgren, E. P., Avarbock, M. R., Alten, D. D. and Brinster, R. L. (1991) *Proc. Natl. Acad. Sci. USA* **88**, p478.

Pandey, N. B., Chodchoy, N., Liu, T-J. and Marzluff, W. F. (1990) *Nucleic Acids Res.* **18**, p3161.

Peltz, S. W., Brown, A. H. and Jacobson, A. (1993) *Genes & Dev.* **7**, p1737.

Peltz, S. W., He, F., Welch, E. and Jacobson, A (1994) *Prog. Nucl. Acids Res. Mol. Biol.* **47**, p271.

Pfiefer, K., Kim, K. S., Kogan, S. and Guarente, L. (1989) *Cell* **56**, p291.

Philpott, C. C., Haile, D., Rouault, T. A. and Klausner, R. D. (1993) *J. Biol. Chem.* **268**, p17655.

Piechaczyk, M., Blanchard, J. M., Martly, L., Dani, Ch., Panabieres, F., El Sabrouty, S., Fort, Ph. and Jeanteur, P. (1984) *Nucleic Acids Res.* **12**, p6951.

Pirola, B. A. (1986) *PhD thesis*, University of Adelaide.

Pirola, B. A., Mayer, F., Borthwick, I. A., Srivastava, G., My, B. K. and Elliott, W. H. (1984) *Eur. J. Biochem.* **144**, p577.

Ponka, P., Schulman, H. M. and Martinez-Medellin, J. (1988) *Biochem. J.* **251**, p105.

Porter, T. D.and Coon, M. J. (1991) *J. Biol. Chem.* **266**, p13469.

Prokipcak, R. D., Herrick, D. J. and Ross, J. (1994) *J. Biol. Chem.* **269**, p9261

Proudfoot, N. J., (1986) *Nature* **322**, p562.

Proudfoot, N. J. (1989) *Trends Biochem. Sci.* **14**, p105.

Proudfoof, N. J. (1991) *Cell* **64**, p671.

Rahmsdorf, H. J., Schonthal, A., Angel, P., Litfin, M., Ruther, U. and Herrlich, P. (1987) *Nucleic Acids Res.* **15**, p1643.

Rahuel, C., Vinit, M-A., Lemarchandel, V., Cartron, J-P. and Romeo, P-H. (1992) *EMBO. J.* **11**, p4095.

Rajagopalan, L. E. and Malter, J. S. (1994) *J. Biol. Chem.* **269**, p23882.

Raju, V. S. and Maines, M. D. (1994) *Biochim. Biophys. Acta.* **1217**, p273.

Ramsden, R., Sommer, K. M. and Omiecinski, C. J. (1993) *J. Biol. Chem.* **268**, p21722.

Rao, M. V., Rangaranjan, P. N. and Padmanaban, G. (1990) *J. Biol. Chem.* **265**, p5617.

Rhoads, R. (1993) *J. Biol. Chem.* **268**, p3017.

Rhode, M., Srivastava, G., Rylatt, D. B., Bundesen, P., Zamattia, J., Crane, D. I. and May B. K. (1990) *Biochem. Biophys. Res. Commun.* **280**, p331.

Riddle, R. D., Yamamoto, M. and Engel, J. D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, p792.

Roberts, K. P. and Griswold, M. D. (1990) *Mol. Endocrinol.* **4**, p531

Rosbash, M. and Singer, R. H. (1993) *Cell* **75**, p399.

Rogers, J. and Munro, H. N. (1987) *Proc. Natl. Acad. Sci. USA* **84**, p2277.

Romana, M., Dubart, A., Beaupain, D., Chabret, C., Goossens, M. and Romeo, P-H. (1987) *Nucleic Acids Res.***15**, p7343.

Ross, J., Peltz, S. W., Kobs, G. and Brewer, G. (1986) *Mol. Cell. Biol.* **6**, p4362.

Roy, N., LaFlamme, G. and Raymond, V. (1992) *Nucleic Acids Res.* **20**, p5753

Rubin, H. N., Stefanko, R. S. and Halim, M. N. (1994) *Int. J. Biochem.* **26**, p703.

Ryan, G. and Ades, I. Z. (1991) *Biochem. J.* **274**, p619.

Sachs, A. B. (1993) *Cell* **74**, p413.

Sachs, A. B. and Davis, R. W. (1989) *Cell* **58**, p857.

Sachs, A. B. and Deardorff J. A. (1992) *Cell* **70**, p961

Sachs, A. B. and Wahle, E. (1993) *J. Biol. Chem.* **268**, p22955.

Sadzadeh, S. M. H., Graf, E., Panter, S. S., Hallaway, P. E. and Eaton, J. W. (1984) *J. Biol. Chem.* **259**, p14354.

Saliocco, F. A., Zhu, D., Lasso, M. R., McCarthy, J. E. G., Tuite, M. F. and Brown, A. J. P. (1994) *J. Biol. Chem.* **269**, p18630.

Sanger, F., Niucklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, p5463.

Sardana, M. K. and Kappas, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, p2464.

Savant-Bhonsale, S. and Cleveland, D. W. (1992) *Genes & Dev.* **6**, p1927.

Scarpulla, R. C., Killar, M. C. and Scarpulla, K. M. (1986) *J. Biol. Chem.* **261**, p4660.

Schiavi, S. C., Belasco, J. G. and Greenberg, M. E. (1992) *Biochim. Biophys. Acta* **1114**, p95.

Schiavi, S. C., Wellington, C. L., Shyu, A-B., Chen, C-Y. A., Greenberg, M. E. and Belasco, J. G. (1994) *J. Biol. Chem.* **269**, p3441.

Schibler, U., Hagenbuchle, O., Wellauer, P. K. and Pittet, A. C. (1983) *Cell* **33**, p501.

Schoen, A., Krupps, G., Gough, S., Berry-Lowe, S., Kannangara, C. G. and Soll, D. (1986) *Nature* **322**, p281.

Schoenhaut, D. S. and Curtis, P. J. (1989) *Nucleic Acids Res.* **17**, p7013.

Schuetz, E. G., Hazelton, G. A., Hall, J., Watkins, P. B., Klaassen, C. D. and Guzelian, P. S. (1986) *J. Biol. Chem.* **261**, p8270.

Schuler, G. D. and Cole, M. D. (1988) *Cell* **55**, p1115.

Schuster, S. J., Badiavas, E. V., Costa-Giomi, P., Weinman, R., Erslev, A. J. and Caro, J. (1989) *Blood* **73**, p13.

Schwarz, E. and Neupert, W. (1994) *Biochim. Biophys. Acta* **1187**, p270.

Senjo, M., Ishibashi, T. and Imai, Y. (1985) *J. Biol. Chem.* **260**, p9191.

Shaw, G. and Kamen, R. (1986) *Cell* **46**, p659.

Sheets, M. D., Fox, C. A., Hunt, T., Woude, G. V. and Wickens, M. (1994) *Genes & Dev.* **8**, p926.

Sheiness, D. and Darnell, J. E. (1973) *Nature New Biol.* **241**, p265.

Shibahara, S. (1994) in "*Regulation of Heme Protein Synthesis*" Fujita, H. ed. (AplhaMed) p103.

Shibahara, S., Muller, R. M., Taguchi, H. and Yoshida, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, p7865.

Shyu, A-B., Belasco, J. G. and Greenberg, M. E. (1991) *Genes & Dev* **5**, p221.

Shyu, A-B., Greenberg, M. E. and Belasco, J. G. (1989) *Genes & Dev.* **3**, p60.

Sinclair, P. R., Schuetz, E. G., Bement, W. J., Haugen, S. A., Sinclair, J. F., May, B. K., Li, D. and Guzelian, P. S. (1990) *Arch. Biochem. Biophys.* **282**, p386.

Singer, R. H. and Penman, S. J. (1973) *Mol. Cell. Biol.* **78**, p321.

Smith, A. in "*Biosynthesis of Heme and Chlorophylls*" (1990a) Dailey, H. A. ed, (McGraw-Hill) p435.

Smith, A., Alam, J., Escriba, P. V. and Morgan, W. T. (1993) *J. Biol. Chem.* **268**, p7365.

Smith, A. and Hunt, R. C. (1990b) *Eur. J. Cell Biol.* **53**, p234.

Smith, A., Tatum, F. M., Muster, P., Burch, M. K. and Morgan, W. T. (1988) *J. Biol. Chem.* **263**, p5224.

Solanagi, K., Sacerdoti, D., Goodman, A. I., Schwartzman, M. L., Abraham, N. G., Levere, R. D. (1988) *Am. J. Med. Sci.* **296**, p387.

Solomon, W. B., Lin, C-H., Palma, J. F., Gao, X. Y. and Wu, S. (1993) *J. Biol. Chem.* **268**, p5089.

Srivastava, G., Bawden, M. J. Anderson, A. and May, B. K. (1989a) *Biochim. Biophys. Acta* **1007**, p192.

Srivastava, G., Bawden, M. J., Hansen, A. J. and May, B. K. (1989b) *Eur. J. Biochem.* **178**, p689.

Srivastava, G., Borthwick, I. A., Brooker, J. D., May, B. K. and Elliott, W. H. (1983) *Biochem. Biophys. Res. Commun.* **117**, p344.

Srivastava, G., Borthwick, I. A., Brooker, J. D., Wallace, J. C., May, B. K. and Elliott, W. H. (1982) *Biochem. Biophys. Res. Commun.* **109**, p305.

Srivastava, G., Borthwick, I. A., Maguire, D. J., Elferink, C. J., Bawden, M. J., Mercer, J., F. B. and May, B. K. (1988) *J. Biol. Chem.* **263**, p5202.

Srivastava, G., Hansen, A. J., Bawden, M. J. and May, B. K. (1990) *Mol. Pharmcol.* **38**, p486.

Srivastava, G. Kwong, S. K. Y., Lam, K. S. L. and May, B. K. (1992) *Eur. J. Biochem.* **203**, p59.

Staden, R. (1982) *Nucleic Acids Res.* **10**, p2951.

Stanton, L. W., Fahrlander, P. D., Tesser, P. M. and Marcu, K. B. (1984) *Nature* **310**, p423.

Stevens, A. (1978) *Biochem. Biophys. Res. Commun.* **81**, p656.

Stevens, C. F. and Wang, Y. (1993) *Nature* **364**, p147.

Stocker, R., (1990) *Free Radic. Res. Commun.* **9**, p101.

Stocker, R., Yamamoto, Y., McDoagh, A. F., Glazer, A. N. and Ames, B. N. (1987) *Sci.* **235**, p1043.

Stoeckle, M. Y. (1992) *Nucleic Acids Res.* **20**, p1123.

Stoeckle, M. Y. and Hanafusa, H. (1989) *Mol. Cell. Biol.* **9**, p4738.

Stoecklin, G., Hahn, S. and Muroni, C. (1994) *J. Biol. Chem.* **269**, p28591.

Surinya, K. E. (1995) *personel communication.*

Sutherland, G. R., Baker, E., Callen, D. F., Hyland, V. J., May, B. K., Bawden, M. J., Healy, H. M. and Borthwick, I. A. (1988) *Am. J. Hum. Genet.* **43**, p331.

Takahama, V. and Singer, A. (1992) *Sci.* **258**, p1456.

Takeda, K., Fujita, H. and Shibahara, S. (1995) *Biochem. Biophys. Res. Commun.* **207**, p160.

Takeda, K., Ishizawa, S., Sato, M., Yoshida, T. and Shibahara, S. (1994) *J. Biol. Chem.* **269**, p22858.

Taketani, S., Inazawa, J., Nakahashi, Y., Abe, T. and Tokunaga, R. (1992*) Eur. J. Biochem.* **205**, p217.

Taketani, S., Kahno, H., Okuda, M., Furukawa, T. and Tokunaga, R. (1994) *J. Biol. Chem.* **269**, p7527.

Theodorakis, N. G. and Cleveland, D. W. (1992) *Mol. Cell. Biol.* **12**, p791.

Urban-Grimal, D., Volland, C., Garnier, T., Dehoux, P. and Labbe-Bois, R. (1986) *Eur. J. Biochem.* **156**, p511.

Vakalopoulou, E., Schaack, J. and Shenk, T. (1991) *Mol. Cell. Biol.* **11**, p3355.

Valius, M. and Kazlauskas, A. (1993) *Cell* **73**, p321.

Vannice, J., Varticovski, L., Kushner, J. P. and Burnham, B. F. (1980) *J. Biol. Chem.* **255**, p3742.

Vassarotti, A., Stroud, R. and Douglas, M. (1987) *EMBO. J.* **6**, p705.

Verma, A., Hirsch, D. J., Glatt, C. E., Ronnett, G. V. and Snyder, S. H. (1993) *Sci.* **259**, p381.

Virbasius, C-M. A., Virbasius, J. V. and Scarpulla, R. C.(1993) *Genes & Dev.* **7**, p2431.

Virbasius, J V., Virbasius, C-M. A. and Scarpulla, R. C.(1994) *Proc. Natl. Acad. Sci. USA* **91**, p1309.

Volland, C. and Felix, F. (1984) *Eur. J. Biochem.* **142**, p551.

Volloch, V. and Housman, D. E. (1981) *Cell* **25**, p509.

Vreken, P. Buddelmeijer, N. and Raue, H. A. (1992a) *Nucleic Acids Res.* **20**, p2503.

Vreken, P. and Raue, H. A. (1992b) *Mol. Cell. Biol.* **12**, p2986.

Wahle, E. and Keller, W. (1992) *Ann. Rev. Biochem.* **61**, p419.

Wang, G. L. and Semenza, G. L. (1993a) *J. Biol. Chem.* **268**, p21513.

Wang, G. L. and Semenza, G. L. (1993b) *Proc. Natl. Acad. Sci. USA* **90**, p4304.

Wang, X. M., Kiledjian, M., Weiss I. M. and Liebhaber S. A. (1995) *Mol. Cell. Biol.* **15**, p1769.

Warnick, G. R.and Burnham, B. F. (1971) *J. Biol. Chem.* **246**, p6880.

Watowich, S. S., Hilton, D. J. and Lodish, H. F. (1994) *Mol. Cell. Biol.* **14**, p3535.

Weiss, I. M. and Liebhaber, S. A. (1992) *Blood* **80**, p145a.

Weiss, I. M. and Liebhaber, S. A. (1994) *Mol. Cell. Biol.* **14**, p8123.

Weiss, I. M. and Liebhaber, S. A. (1995) *Mol. Cell. Biol.* **15**, p2457.

Wellington, C. L., Greenberg, M. E. and Belasco, J. G. (1993) *Mol. Cell. Biol.* **13**, p5034.

Whitelaw, E. and Proudfoot, N. J. (1986) *EMBO. J.* **5**, p2915.

Whitelaw, M., Pongratz, I., Wilhelmsson, A., Gustafsson, J-A. and Poellinger, L. (1993) *Mol. Cell. Biol.* **13**, p2504.

Whittemore, L-A. and Maniatis, T. (1990) *Mol. Cell. Biol.* **10**, p1329.

Wickens, M. (1992) *Sem. Dev. Biol.* **3**, p399.

Williams, A. S., Ingledue III, T. C., Kay, B. K. and Marzluff, W. F. (1994) *Nucleic Acids Res.* **22**, p4660.

Williams, R. S., Garcia-Moll, M., Mellar, J., Salmons, S. and Harlan, W. (1987) *J. Biol. Chem.* **262**, p2764.

Wilson, T. and Treisman, R. (1988) *Nature* **336**, p396.

Wisdom, R. and Lee, W. (1991) *Genes & Dev.* **5**, p232.

Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O. and Ihle, J. N. (1993) *Cell* **74**, p227.

Wright, C. V. E., Cho, K. W. Y., Fritz, A., Burglin, T. R. and DeRobertis, E. M. (1987) *EMBO. J.* **6**, p4083.

Wu, W. H., Shemin, D., Richards, K. E. and Williams, R. C. (1974) *Proc. Natl. Acad. Sci. USA* **71**, p1767.

Yamamoto, M., Hayashi, N. and Kikuchi, G. (1981) *Arch. Biochem. Biophys.* **209**, p451.

Yamamoto, M., Kure, S., Engel, J. D. and Hiraga, K. (1988) *J. Biol. Chem.* **263**, p15973.

Yamogida, K., Yamamoto, M., Yamagami, T., Fujita, H. and Hayashi, N. (1993) *J. Biochem.* **113**, p364.

Yang, M. J., London, I. M. and Chen, J-J. (1992) *J. Biol. Chem.* **267**, p20519.

Yen, T. J., Machlin, P. S. and Clevand, D. W. (1988) *Nature* **334**, p580.

Yeoh, G. C. T., Hilliard, C., Fletcher, S. and Douglas, A. (1990) *Cancer Res.* **50**, p7593

You, Y., Chen, C-Y., A. and Shyu, A-B. (1992) *Mol. Cell. Biol.* **12**, p2931.

Zaidi, S. H. E., Denman, R. and Malter, J. S. (1994) *J. Biol. Chem.* **269**, p24000.

Zanger, U. M., Kagawa, N., Lund, J. and Waterman, M. R. (1992) *FASEB. J.* **6**, p719.

Zeevi, M., Nevins, J. R. and Darnell Jr., J. E. (1982) *Mol. Cell. Biol.* **2**, p517.

Zenke, M., Munoz, A., Sap, J., Vennstrom, B. and Beug, H. (1990) *Cell* **61**, p1035.

Zhang, L. and Guarente, L. (1995) *EMBO. J.* **14**, p313.

Zhang, S, Ruiz-Echevarria, M. J., Quan, Y. and Peltz, S. W. (1995) *Mol. Cell. Biol.* **15**, p2231.

Zhang, W., Wagner, B. J., Ehrenman, K., Schaefer, A. W., deMaria, C. T., Carter, D., Dehaven, K., Long, L. and Brewer, G. (1993) *Mol. Cell. Biol.* **13**, p7652.

Zinn, K., Keller, A., Whittemore, L-A. and Maniatis, T. (1988) *Sci.* **240**, p210.

Zubiaga, A. M., Belasco, J. G. and Greenberg, M. E. (1995) *Mol. Cell. Biol.* **15**, p2219.