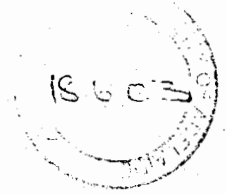


09PH
K439



**INVESTIGATING THE ROLE OF ARNT ISOFORMS AS
bHLH/PAS TRANSCRIPTIONAL REGULATORS**

This thesis is submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy in the Department of Molecular Biosciences
(Biochemistry), University of Adelaide

Robyn Jane Kewley, B. Sc. (Hons)

February 2003

TABLE OF CONTENTS

<u>TABLE OF CONTENTS</u>	II
<u>THESIS SUMMARY</u>	VII
<u>DECLARATION</u>	IX
<u>ACKNOWLEDGEMENTS</u>	X
<u>CHAPTER 1</u>	1
<u>INTRODUCTION</u>	1
<u>1.1 The bHLH superfamily of proteins</u>	1
<u>1.2 The bHLH/PAS sub-family of proteins</u>	2
1.2.1 The Dioxin Receptor.....	4
1.2.2 Hypoxia Inducible Factors.....	5
1.2.3 Single Minded proteins.....	7
1.2.4 Circadian Rhythm proteins.....	9
<u>1.3 The bHLH/PAS factor ARNT</u>	11
1.3.1 Expression of ARNT.....	12
1.3.2 <i>ARNT</i> ^{-/-} knockout mice.....	13
1.3.3 ARNT-like proteins.....	13
1.3.4 The role of ARNT proteins in non-mammalian species.....	16
1.3.5 A constitutive function for ARNT.....	17
1.3.6 Possible explanations for Dioxin Toxicity.....	19
<u>1.4 Structure of the mouse and human <i>ARNT</i> genes</u>	19
<u>1.5 Alternative splicing of ARNT</u>	20
<u>1.6 Alternative splicing of bHLH genes</u>	21
<u>1.7 A comparison between Max and ARNT</u>	24
<u>1.8 Phosphorylation of ARNT</u>	25
<u>1.9 Aims and approach</u>	26
<u>CHAPTER 2</u>	27
<u>MATERIALS AND METHODS</u>	27
<u>2.1 ABBREVIATIONS</u>	27
<u>2.2 MATERIALS</u>	28

2.2.1 Chemicals and reagents	28
2.2.2 Kits	29
2.2.3 Enzymes	29
2.2.4 Antibodies	30
2.2.5 Buffers and solutions	30
2.2.6 Plasmids	32
2.2.6.1 Cloning vectors	32
2.2.6.2 Expression vectors	33
2.2.6.3 Reporter plasmids	34
2.2.7 Oligonucleotides and primers	35
2.2.8 Bacterial strains	36
2.2.9 Bacterial growth media	37
2.2.10 Tissue culture cell lines	37
2.2.11 Tissue culture growth media	38
2.2.12 Miscellaneous	38
METHODS	38
2.3 DNA Manipulation Methods	38
2.3.1 Preparation of competent bacteria	38
2.3.2 Transformation of competent bacteria	39
2.3.2.1 Transformation of bacteria with intact plasmid	39
2.3.2.2 Transformation of bacteria with ligation mixes	39
2.3.3 Preparation of plasmid DNA	39
2.3.3.1 Alkaline lysis method of recombinant plasmid purification	39
2.3.3.2 Ammonium Acetate method of purification	40
2.3.4 Restriction digestion of plasmid DNA	41
2.3.5 Dephosphorylation of plasmid DNA	41
2.3.6 Generation of blunt-ended DNA using Klenow	41
2.3.7 Ligation of purified DNA fragments	41
2.3.8 DNA purification from agarose gels	41
2.3.9 Generation of new plasmids	41
2.3.10 Polymerase Chain Reaction	44
2.3.11 Labelling probes	45
2.3.11.1 Random labelling	45
2.3.11.2 End-fill reactions with Klenow	45
2.3.12 Southern blot analysis	45
2.3.12.1 Making dot blots	45
2.3.12.2 Pre-hybridisation	46
2.3.12.3 Hybridisation of probes to filter	46
2.3.13 Preparation of single stranded salmon sperm DNA	46
2.4 Gel Electrophoresis Methods	47
2.4.1 Agarose gel electrophoresis	47
2.4.2 Agarose/formaldehyde gel electrophoresis	47
2.4.3 Sequencing gel electrophoresis	47
2.4.4 Non-denaturing gel electrophoresis	48
2.4.5 Denaturing gel electrophoresis	48
2.4.6 Coomassie staining of gels	49
2.4.7 Western blotting	49
2.4.7.1 Protein transfer to nitrocellulose and antibody incubations	49
2.4.7.2 Chemiluminescent detection of proteins	50
2.5 Tissue Culture Methods	50
2.5.1 Maintenance of cell lines	50
2.5.2 Harvesting of cell lines	50
2.5.3 Making frozen stocks of cell lines	51
2.5.4 Analysis of reporter gene activity in transient transfected cell lines	51

2.5.4.1	Transient transfection of cell culture lines in 24-well format	51
2.5.4.2	Assaying luciferase activity	51
2.5.5	Whole cell Protein Extracts.....	52
2.5.6	Preparation of Nuclear and Cytosolic extracts	53
2.6	RNA manipulation Methods	53
2.6.1	Isolation of total RNA	53
2.6.2	Isolation of poly(A) RNA	54
2.6.3	Northern blotting of RNA	54
2.6.4	Generation of cDNA from RNA	55
2.6.5	Differential Display RT-PCR.....	56
2.6.6	Suppression Subtractive Hybridisation	57
2.7	Protein purification from bacteria	58
2.7.1	Protein expression from pET vectors	58
2.7.2	Nickel Affinity Purification.....	58
2.7.3	Size Exclusion Purification	59
2.7.4	Desalting of proteins	59
2.7.5	<i>In vitro</i> kinase assays	60
2.7.6	Electrophoretic mobility shift assays (EMSAs)	60
CHAPTER 3	62
	<u>AN EXPERIMENTAL SYSTEM TO SEARCH FOR ARNT TARGET GENES</u>	62
3.1	<u>Introduction</u>	62
3.2	<u>A dominant negative form of ARNT, ARNTΔb</u>	63
3.3	<u>The EF-IRES-puro expression system</u>	64
3.4	<u>Generation of ARNT and ARNTΔb overexpressing cell lines</u>	65
3.5	<u>ARNT RNA and protein expression in ARNT stable cell lines</u>	65
3.6.1	<u>Reporter gene analysis of ARNT stable cell lines</u>	66
3.6.1	XRE reporter gene activity.....	66
3.6.2	HRE reporter gene activity.....	67
3.6.3	E-box reporter gene activity.....	67
3.7	<u>Electrophoretic mobility shift analysis of E-box binding ability of the ARNT stable cell lines</u>	68
3.8	<u>Differential Display RT-PCR</u>	69
3.9	<u>Conclusion</u>	70
CHAPTER 4	72
	<u>SCREENING FOR DOWNSTREAM TARGET GENES OF ARNT</u>	72
4.1	<u>Introduction</u>	72
4.2	<u>Suppression subtractive hybridisation PCR</u>	73
4.3	<u>The generation of subtracted libraries by SSH PCR</u>	74

4.3.1 Assessment of subtraction efficiency	75
4.3.2 Screening the subtracted library for differentially expressed clones	76
4.4 Sequence analysis of differentially expressed clones.....	78
4.5 Expression of isolated clones in ARNT and ARNTΔb Hepa cell lines.....	79
4.6 Using microarray analysis to identify downstream targets of ARNT	80
4.7 Conclusion	82
CHAPTER 5	86
<u>DIFFERENTIAL REGULATION OF ARNT ISOFORMS</u>	86
5.1 Introduction	86
5.2 Expression of ARNT and Alt ARNT	86
5.3 Potential CKII phosphorylation sites in ARNT and Alt ARNT	87
5.4 Expression and purification of ARNT 142 and Alt ARNT 142.....	87
5.5 <i>In vitro</i> DNA-binding activity of ARNT and Alt ARNT 142.....	89
5.6 <i>In vitro</i> phosphorylation of ARNT and Alt ARNT 142 using cell extracts.....	89
5.7 Expression and purification of p22Max.....	90
5.8 <i>In vitro</i> DNA-binding activity of p22Max.....	90
5.9 The generation, expression and purification of Alt ARNT 142 mutants.....	90
5.9.1 Mutating potential CKII phosphorylation site in Alt ARNT 142	90
5.9.2 Expression and purification of Alt ARNT S77A, S82A and S77AS82A 142	91
5.9.3 Mutant Alt ARNT 142 proteins are functional DNA-binding homodimers	91
5.10 <i>In vitro</i> phosphorylation of ARNT and Alt ARNT using purified CKII.....	92
5.11 Theoretical implications of CKII phosphorylation of ARNT	92
5.12 The effects of CKII phosphorylation on ARNT DNA-binding activity.....	94
5.13 Studying differences between ARNT and Alt ARNT using larger sized proteins.....	95
5.13.1 Small scale expression of different sized ARNT proteins	95
5.13.2 Expression and purification of ARNT 358 proteins	96
5.13.3 ARNT 358, Alt ARNT 358 and mutant Alt ARNT 358 are functional.....	97
5.13.4 ARNT 358 proteins are differentially phosphorylated by CKII	97
5.13.5 The effects of CKII phosphorylation on DNA-binding.....	98
5.14 <i>In vivo</i> DNA-binding activity of ARNT and Alt ARNT.....	99
5.15 Conclusion	101

<u>CHAPTER 6</u>	105
<u>DISCUSSION</u>	105
<u>6.1 Introduction</u>	105
<u>6.2 Attempts to identify downstream ARNT target genes</u>	105
<u>6.3 Consequences of differential phosphorylation between ARNT and Alt ARNT</u>	108
<u>6.4 Final Conclusion</u>	110
<u>CHAPTER 7</u>	111
<u>REFERENCES</u>	111

THESIS SUMMARY

The basic helix-loop-helix PAS transcription factor ARNT functions as an obligate partner protein for signal regulated bHLH/PAS factors such as the Dioxin Receptor (DR) and Hypoxia Inducible Factors (HIF- α). The DR and HIF- α proteins respond to the respective environmental stimuli of xenobiotic exposure or low oxygen tension by forming active heterodimeric complexes with ARNT. Given the potential for ARNT to act as a general dimerisation partner for the emerging bHLH/PAS factor family, there has been some speculation as to the physiological role of ARNT in the absence of DR ligand or hypoxia.

The likelihood of further transcriptional regulatory roles is supported by evidence that ARNT can recognise the CACGTG E-box element as a homodimer *in vitro* and *in vivo*, the constitutive nuclear localisation of ARNT, the widespread expression of ARNT in mammalian tissues and the presence of a strong transactivation domain within the C-terminus of ARNT. The identification of an alternatively spliced form of the ARNT gene, known as Alt ARNT, provides additional complexity to the role of ARNT in the cell.

This thesis examines the possibility of alternative roles for ARNT in the cell. A search for downstream target genes of ARNT was performed utilising the suppression subtractive hybridisation-PCR method. A subtracted library was generated using cDNA from Hepa 1c1c7 cells stably overexpressing a dominant negative form of ARNT lacking the basic DNA-binding domain, known as ARNT Δ b. The subtracted library was screened to identify potential differentially expressed cDNAs. Subsequent Northern blot analysis revealed one of the clones identified during the screen was differentially expressed between the ARNT Δ b Hepa cells and Hepa 1c1c7 cells stably containing a blank expression vector. It is unlikely, however, to be a direct target of ARNT as it was expressed more highly in both ARNT and ARNT Δ b overexpressing cells compared to control Hepa 1c1c7 cells and contained no known ARNT binding sites within 712 bp of available promoter sequence. An additional differentially

expressed gene, CD47, was identified using a microarray data set obtained from another laboratory project, though the role of ARNT in regulation of CD47 expression remains unclear. These results suggest an alternative approach, perhaps detailed microarray analysis, is required to investigate novel roles for ARNT as a transcription factor.

This thesis also sought to investigate the function of Alt ARNT as a transcriptional regulator. Work herein describes the differential regulation of Alt ARNT and ARNT by Protein Kinase CKII. Phosphorylation was shown to have an inhibitory effect on DNA-binding to the E-box as homodimers. In contrast, DNA-binding by Alt ARNT/Dioxin Receptor heterodimers to the xenobiotic response element was not inhibited by phosphorylation. Together with the co-expression of ARNT and Alt ARNT found in all cell types tested, these results are supportive of alternative roles for these two ARNT isoforms existing within the cell.