Characterisation of the 5'-Flanking Region of the *CYP27B1* Gene in Osteoblast-Like Cells

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Abstract

The classical action of vitamin D hormone ($1,25(OH)_2D_3$) is to maintain calcium homeostasis by targeting such tissues as the intestine and bone. More recently, vitamin D has also been shown to be important in other processes such as the regulation of cellular proliferation and differentiation, immunity and reproduction. Production of $1,25(OH)_2D_3$ is catalysed by the enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (CYP27B1). Though highly expressed in the kidney, the *CYP27B1* gene is also expressed in many non-renal tissues including bone. As such, many of the non-classical actions of $1,25(OH)_2D_3$ are hypothesised to be autocrine or paracrine. At present, regulation of *CYP27B1* gene expression remains poorly understood. The aim of this study was to investigate the regulation of the 5'-flanking region of the human *CYP27B1* gene in osteoblast cells.

ROS 17/2.8 osteoblast-like cells were transiently transfected with reporter constructs containing deletions of the -1501 base pair 5'-flanking region of the human *CYP27B1* gene inserted upstream of the firefly *luciferase* gene. Deletion analysis showed that the 5'-flanking region of the *CYP27B1* gene in osteoblast cells comprised a proximal promoter, an enhancer region and an upstream repressive region. Site directed mutagenesis demonstrated that putative CCAAT and GC boxes within the proximal promoter were essential for expression, and two putative Ets-1 protein binding sites contributed to expression of the enhancer.

Interestingly, the upstream repressive region was stronger than that previously described in kidney cells and was located at a different region to that reported in

prostate cells. The repressive region in osteoblast cells lies almost exclusively between –1100 and –997. Within this region two adjacent Smad binding elements (SBEs) were identified. Inactivation of these sites within the 5'-flanking region of the *CYP27B1* gene caused derepression. Electrophoretic gel mobility shift assays showed that an oligonucleotide probe that contained the two SBEs bound recombinant GST-Smad4 protein, as well as nuclear proteins isolated from ROS 17/2.8 osteoblast cells.

The effect of exogenous hormones and growth factors on expression of the 5'-flanking region of the *CYP27B1* gene was also investigated. Interestingly, factors that regulate kidney *CYP27B1* gene expression, such as parathyroid hormone and $1,25(OH)_2D_3$, did not regulate expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. However insulin-like growth factor 1 (IGF-1) and transforming growth factor-beta (TGF-β1), which are secreted locally in bone, repressed expression of the CYP27B1 reporter gene by approximately 50%. The majority of inhibition by TGF-β1 was mediated by elements between -531 and -305.

This is the first study to investigate expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. Importantly, the results demonstrate that the *CYP27B1* gene is regulated differently within osteoblast cells to that in kidney and prostate cells. Basal expression of the 5'-flanking region of the *CYP27B1* gene involves a complex interaction of enhancer and repressor elements. Furthermore, rather than being regulated by calciotropic agents, *CYP27B1* gene expression is controlled by locally secreted growth factors such as TGF-β and IGF-1. These data are consistent with a model whereby synthesis of $1,25(OH)_2D_3$ by osteoblasts serves an autocrine or paracrine role.

Declaration

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Andrew Turner

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Publications Arising

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Presentations Arising

International

Regulation of the 25-hydroxyvitamin D_3-1α-hydroxylase Promoter in ROS 17/2.8 Osteoblast-like Cells

A Turner, PP Dwivedi, BK May and HA Morris

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National

Enhancer and Repressor Elements in the 25-hydroxyvitamin D_3-1α-hydroxylase Promoter

A Turner, PP Dwivedi, PH Anderson, BK May and HA Morris

ComBio 2005 (Combined ASBMB, ANZSCDB and ASPS Annual Meeting), Adelaide, South Australia, September, 2005.

25-hydroxyvitamin D_3-1α-hydroxylase Promoter Activity in the Rat Osteoblast-like Cell Line ROS 17/2.8

A Turner, PP Dwivedi, PH Anderson, BK May and HA Morris

15th Australian & New Zealand Bone and Mineral Society Conference, Perth, Western Australia, September, 2005.

25-hydroxyvitamin D_3-1α-hydroxylase Promoter Activity in the Rat Osteoblast-like Cell Line ROS 17/2.8

A Turner, PP Dwivedi, PH Anderson, BK May and HA Morris

14th Australian & New Zealand Bone and Mineral Society Conference, Hunter Valley, New South Wales, August, 2004.

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Chapter 1:

Introduction

1.1 Metabolic activation of vitamin D

1.1.1 Overview of endocrine vitamin D metabolism

The vitamin D endocrine system is critical to the maintenance of healthy bone owing to its central role in calcium homeostasis. Vitamin D deficiency in humans causes defects in bone mineralisation such as rickets and osteomalacia. Indeed, vitamin D was first identified in the 1920's as the agent behind the successes of cod-liver oil and sunlight as treatments for rickets (McCollum *et al.*, 1922). More recently, vitamin D has also been shown to be important in other processes such as the regulation of cellular proliferation and differentiation, immunity and reproduction.

The biologically active form of vitamin D is the steroid hormone 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). The first step in the production of $1,25(OH)_2D_3$ is the synthesis of vitamin D_3 from 7-dehydrocholesterol in the basal epidermal layer of the skin following exposure to ultra-violet light (Figure 1.1). Alternatively, vitamin D_3 can be derived from dietary sources. Vitamin D_3 is then transported in a complex with vitamin D_3 binding protein to the liver where it is hydroxylated by vitamin D_3-25-hydroxylase at carbon atom 25 to produce $25(OH)D_3$. This step is considered to be constitutive and $25(OH)D_3$ is the most abundant vitamin D metabolite in the circulation (Jones *et al.*, 1998). Conversion of $25(OH)D_3$ to its

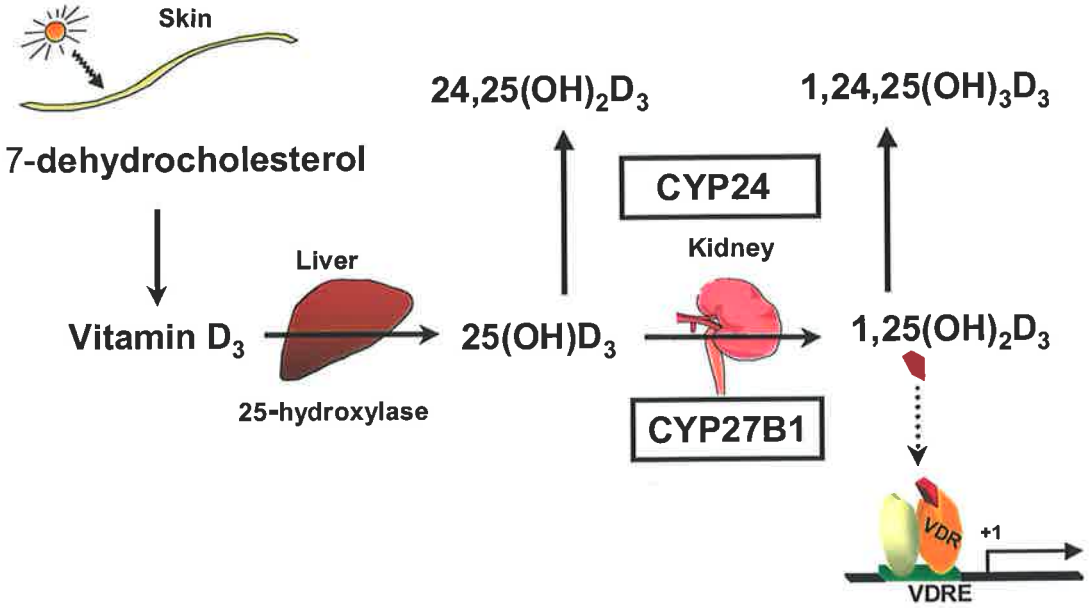

Figure 1.1: The endocrine pathway by which $1,25(OH)_2D_3$ is synthesised. Exposure to sunlight drives the conversion of 7-dehydrocholesterol in the skin to vitamin D_3. The liver and kidney are the principle sites of 25-hydroxylase and CYP27B1 activity respectively, supplying $1,25(OH)_2D_3$ to the circulation. Within target cells, $1,25(OH)_2D_3$ interacts with the vitamin D receptor which acts as a ligand induced transcription factor, binding to vitamin D responsive elements (VDREs) within the regulatory regions of target genes. Deactivation of $1,25(OH)_2D_3$ is catalysed by CYP24 within the kidney and other target tissues.

active form $1,25(OH)_2D_3$ is catalysed by 25-hydroxyvitamin D3 1α-hydroxylase (CYP27B1) which hydroxylates carbon atom 1.

The level of circulating $1,25(OH)_2D_3$ is primarily determined by CYP27B1 activity in the kidney and is tightly regulated by calciotropic hormones such as parathyroid hormone (PTH), calcitonin and $1,25(OH)_2D_3$ itself (Armbrecht et al., 2003b; Shinki et al., 1999). In order to exert a biological effect, $1,25(OH)_2D_3$ must first bind vitamin D receptor (VDR) which acts as a high affinity ligand activated transcription factor. Over sixty genes are thought to be regulated by $1,25(OH)_2D_3$ and major target tissues include the small intestine and bone. Inactivation of $1,25(OH)_2D_3$ is catalysed by the 25-hydroxyvitamin D_3 24-hydroxylase (CYP24). This is the first step in the degradation of $1,25(OH)_2D_3$ through the C-23/C-24 oxidation pathway. The *CYP24* gene is induced by $1,25(OH)_2D_3$ and is expressed in all of the hormone's target tissues. Therefore, endocrine vitamin D metabolism occurs in a step-wise manner involving processes within the skin, liver and kidney.

In recent years, synthesis of $1,25(OH)_2D_3$ has been identified within many of its target tissues, including bone. This extra-renal vitamin D metabolism is hypothesised to serve an as yet unidentified autocrine or paracrine role (Anderson *et al.*, 2003a). This chapter will introduce in greater depth our current understanding of vitamin D metabolism throughout the human body, as well as the physiological roles of $1,25(OH)_2D_3$ and its molecular action. An emphasis is placed on the physiological roles of *CYP27B1* expression and $1,25(OH)_2D_3$ in the context of the development and maintenance of bone tissue.

1.1.2 25-Hydroxylation of vitamin D_3

The principle site of 25-hydroxylation of vitamin D is considered to be the liver (Ponchon *et al.*, 1969) and several hepatic vitamin D_3-25-hydroxylases have been identified across species. The mitochondrial enzyme, CYP27A1, and several microsomal enzymes emerged as candidates for the critical 25-hydroxylase in vitamin D metabolism (reviewed by Prosser and Jones (2004)). However, mice with a disrupted *CYP27A1* gene show above normal serum $25(OH)D_3$ levels and normal $1,25(OH)_2D_3$ levels (Rosen *et al.*, 1998). Furthermore, the human *CYP27A1*-negative hepatic cell line Hep 3B shows 25-hydroxylase activity at low nanomolar concentrations (Byford *et al.*, 2002). Conversely, the microsomal *CYP2R1* gene was recently identified as being mutated in a Nigerian individual with rickets (Cheng *et al.*, 2004). The affected patient has low $25(OH)D_3$ levels despite having a normal *CYP27A1* gene. CYP2R1 has also been shown to hydroxylate both vitamin D_3 and vitamin D_2 and to be present mainly in the liver and testis (Cheng *et al.*, 2003). Therefore, although this area remains controversial, CYP2R1 is emerging as the most important hepatic 25-hydroxylase in humans.

1.1.3 Renal 1-hydroxylation

The final and rate-limiting step in the synthesis of $1,25(OH)_2D_3$ is the addition of a hydroxyl group to $25(OH)D_3$ at carbon position 1 (Figure 1.2). This step is catalysed by the 25-hydroxyvitamin D_3 1α-hydroxylase (CYP27B1) and unlike the 25-hydroxylase, there is little controversy as to its identity and physiological importance. The major source of circulating $1,25(OH)_2D_3$ was demonstrated using nephrectomised

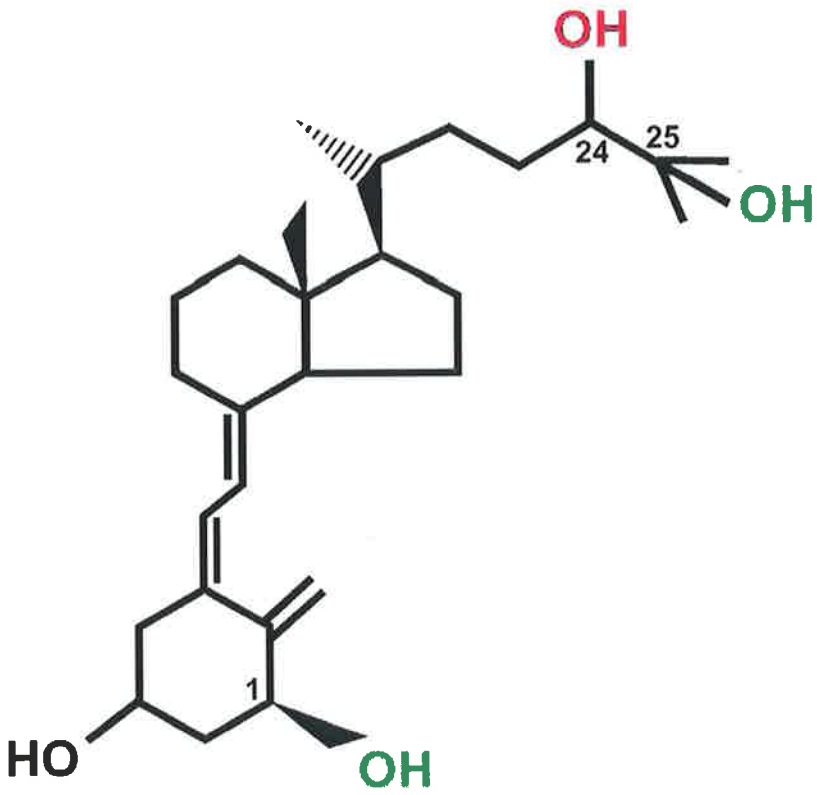

Figure 1.2: The structure of vitamin D showing hydroxyl groups that lead to either activation (green) or deactivation (red).

animals to be the renal CYP27B1 enzyme (Fraser and Kodicek, 1970) and further illustrated by patients suffering chronic renal failure (Mawer *et al.*, 1973). The renal CYP27B1 enzyme is located in the inner mitochondrial membrane of the proximal convoluted cells (Paulson and DeLuca, 1985) and its expression is strictly controlled (detailed in section 1.4.1). However CYP27B1 is also expressed in a variety of extra-renal tissues (Bland *et al.*, 2001) as described in section 1.3.

1.1.4 Inactivation by the 24-hydroxylase enzyme

The high potency of $1,25(OH)_2D_3$ to elevate circulating calcium and phosphorous levels necessitates a mechanism to limit its activity. This is performed within target cells by the vitamin D_3 24-hydroxylase (CYP24), which initiates the degradation of $1,25(OH)_2D_3$. CYP24 appears to be expressed in every $1,25(OH)_2D_3$ target tissue (Akeno *et al.*, 1997). PTH and $1,25(OH)_2D_3$ itself are the most important physiological regulators of *CYP24* gene expression in the kidney. Renal *CYP24* is induced by $1,25(OH)_2D_3$ whereas PTH suppresses expression (Shinki *et al.*, 1992). Depending on the species, CYP24 specifically catalyses the hydroxylation of either carbon 23 or 24. In humans, the C-23 pathway is preferred (Beckman *et al.*, 1996) and leads to more polar products which are eventually excreted via the kidney (Omdahl et al., 2002; Reddy and Tserng, 1989).

$1,25(OH)_2D_3$ can also undergo metabolism to the metabolite 1,25(OH)2-3-epi-D_3. This process is referred to as C-3 epimerization and has been identified in human keratinocytes (Reddy *et al.*, 2001), rat osteosarcoma cells (Siu-Caldera *et al.*, 1999) and human colon carcinoma cells (Messerlian *et al.*, 2000). 1,25(OH)2-3-epi-D_3

appears to be catabolised via the C-23/C-24 pathway more slowly whilst retaining significant biological activity. Moreover, its biological activity as compared to $1,25(OH)_2D_3$ varies between cell types. For example, 1,25(OH)2-3-epi-D_3 appears to be less potent at activating intestinal calcium transport (Norman *et al.*, 1993) but is equally capable of inhibiting keratinocyte proliferation (Norman *et al.*, 1993) and suppressing bovine PTH secretion (Brown *et al.*, 1999). Therefore, this pathway may contribute a degree of tissue specificity for the actions of $1,25(OH)_2D_3$.

cDNA clones for *CYP24* have been sequenced for the rat (Ohyama *et al.*, 1991), human (Chen *et al.*, 1993), chicken (Jehan *et al.*, 1998), mouse (Akeno *et al.*, 1997) and pig (Zierold *et al.*, 2000). The complete loss of 24-hydroxylase activity in *CYP24* knock-out mice results in impaired intramembranous bone mineralisation (St-Arnaud *et al.*, 2000). Importantly, this defective mineralisation during development was attributed to increased levels of circulating $1,25(OH)_2D_3$ and not the absence of $24,25(OH)_2D_3$. The human *CYP24* gene maps to chromosomal locus 20q13 (Hahn *et al.*, 1993) and has been identified as a putative oncogene in breast cancer (Albertson *et al.*, 2000).

1.1.5 Transport of vitamin D metabolites

Vitamin D metabolites are lipophilic molecules with a low aqueous solubility. Therefore transportation in the circulation requires binding to plasma proteins such as the vitamin D binding protein (DBP). This 58 kDa protein binds the major vitamin D metabolites with the following order of affinity: $25(OH)D_3$ =$24,25(OH)_2D_3$ >$1,25(OH)_2D_3$ >vitamin D_3 (Cooke and Haddad, 1989). The plasma level of DBP is

greatly in excess of all vitamin D metabolites and consequently >99% of vitamin D metabolites are protein bound in the circulation. DBP-bound metabolites have limited access to cells as compared with free metabolites (Cooke and Haddad, 1989). This is believed to buffer the biological activity of $1,25(OH)_2D_3$ and contribute to modulating vitamin D toxicity. The half-life of DBP-bound vitamin D metabolites is increased as they are less susceptible to hepatic metabolism and biliary excretion. In DBP-null mice, $25(OH)D_3$ and $1,25(OH)_2D_3$ levels are very low but the animals do not develop rickets (Safadi *et al.*, 1999), further suggesting that the biological activity of vitamin D is exerted only by the very small proportion of free $1,25(OH)_2D_3$.

1.2 Molecular cloning of the *CYP27B1* gene

The cloning of the *CYP27B1* gene in 1997 led to the identification of many extra-renal sites of expression, and allowed a greater understanding of the mechanisms involved in the regulation of its expression. The *CYP27B1* cDNA was first isolated from the kidney of mice lacking VDR where its synthesis is increased ten-fold (Takeyama *et al.*, 1997). At the same time, three other independent research groups reported the isolation of rat (Shinki *et al.*, 1997) and human *CYP27B1* cDNA clones (Fu et al., 1997; Monkawa et al., 1997; St-Arnaud et al., 1997). The present study focuses on the human *CYP27B1* gene that contains nine exons and eight introns spanning about 6.5 kb (Kong *et al.*, 1999).

1.3 CYP27B1 tissue distribution

Although the circulating level of $1,25(OH)_2D_3$ is determined by the kidney CYP27B1 enzyme, expression has also been reported in non-renal tissues. The first report that cells other than in the kidney possessed CYP27B1 activity came in 1980 when cultured chick calvarial cells were shown to convert $25(OH)_2D_3$ to $1,25(OH)_2D_3$ (Turner *et al.*, 1980b). This was followed by the identification of either CYP27B1 expression (ie mRNA or protein) or enzyme activity in numerous extra-renal sites (reviewed by Hewison and Adams (2005)). However the biological relevance of synthesising $1,25(OH)_2D_3$ at sites other than the kidney remains to be elucidated and is an area of ongoing interest.

Since the initial reports of CYP27B1 enzyme activity in bone, CYP27B1 activity or expression has been detected in cultures of chondrocytes (Schwartz *et al.*, 1992), macrophages (Adams *et al.*, 1983) keratinocytes (Bikle *et al.*, 1986b), colon cells (Bises *et al.*, 2004), prostate cells (Schwartz *et al.*, 1998), cervical cells (Friedrich *et al.*, 2002) and breast cells (Friedrich *et al.*, 2006) with the list continuing to grow. Zehnder *et al.* (2001) used immunohistochemical techniques to detect CYP27B1 protein in skin (basal keratinocytes, hair follicles), lymph nodes, colon, pancreas (islets), adrenal medulla, brain (cerebellum and cerebral cortex), and placenta. More recently, a transgenic mouse model was created in which the firefly luciferase gene was under the control of -1497 base pairs of the 5'-flanking region of the human *CYP27B1* gene together with 44 base pairs of the 5' untranslated region (Hendrix *et al.*, 2004). Results from these mice demonstrate that the *CYP27B1* gene promoter expresses strongly in a wide variety of tissues including the kidney, brain, testes, skin,

bone, bone marrow and spleen. A lower level of expression was also detected in other tissues such as muscle, distal small intestine and liver. In fact, the only tissue examined that did not express significant levels of luciferase, was the proximal small intestine. Importantly, expression of the luciferase reporter in the kidney occurred in the same cells as the endogenous CYP27B1 protein. Furthermore, regulation of the reporter in the context of dietary calcium and vitamin D status, was also comparable to the endogenous *CYP27B1* gene (Hendrix *et al.*, 2005). These final points are of particular relevance to investigations into the regulation of the *CYP27B1* promoter as they demonstrate that the major transcriptional regulatory elements for the *CYP27B1* gene lie within the first -1497 base pairs of the 5'-flanking region and +44 base pairs of the 5' untranslated region. These data provide credence for utilising this region of the gene in order to investigate the control of *CYP27B1* gene transcription, as has occurred in the present study and others.

1.4 Regulation of *CYP27B1* expression and activity

1.4.1 Regulation in the kidney

Since the kidney is the major source of circulating $1,25(OH)_2D_3$, it is not surprising that *CYP27B1* gene regulation has been most extensively studied in this organ. In keeping with the central role of $1,25(OH)_2D_3$ in calcium homeostasis, the renal *CYP27B1* is regulated by calciotropic factors that include PTH, calcitonin and $1,25(OH)_2D_3$ itself. The best documented of these actions is the positive regulation by PTH. Synthesis of PTH is up-regulated when serum calcium levels are low leading to an increase in CYP27B1 expression (Jones *et al.*, 1998) and enzyme

activity in kidney cell cultures (Kremer and Goltzman, 1982). As well, pharmacological doses of PTH infused into mice elevate *CYP27B1* gene expression two-fold (St-Arnaud *et al.*, 1997). This response is mediated, at least in part, through a cAMP signal transduction mechanism (Henry and Luntao, 1989). Furthermore, studies of the *CYP27B1* promoter suggest that PTH induction involves a CCAAT box located at –75 to –70 base pairs (Gao *et al.*, 2002).

In contrast to the induction of *CYP27B1* by PTH, $1,25(OH)_2D_3$ acts as a suppressor of *CYP27B1* gene expression. Studies with rodents show that the effect of $1,25(OH)_2D_3$ is to down-regulate *CYP27B1* mRNA levels in the kidney (Murayama *et al.*, 1999). This effect is also seen in kidney cell cultures (Henry, 1979; Turner et al., 1980a). In mice that express at least one copy of the *VDR* gene, expression of the *CYP27B1* gene is suppressed by $1,25(OH)_2D_3$, but not in *VDR*-null mice (Takeyama *et al.*, 1997). These data suggest that the negative feedback regulation of *CYP27B1* by $1,25(OH)_2D_3$ requires liganded VDR, however no vitamin D response element (VDRE) has been identified within the *CYP27B1* promoter. Murayama *et al.* (2004) have identified a region within the 5'-flanking region of the *CYP27B1* gene that is negatively regulated by $1,25(OH)_2D_3$ and requires the VDR for the inhibition to occur. Importantly though, VDR does not directly bind the regulatory element which resembles a pair of E-box motifs. However the precise molecular mechanism remains unclear at this time. It is important to note that the negative regulation by $1,25(OH)_2D_3$ by this mechanism has only been demonstrated in one cell line, that of MCT cells which are derived from mouse proximal kidney tubules.

There is substantial evidence that calcitonin regulates vitamin D metabolism within the intact animal (Galante et al., 1972; Nesbitt et al., 1987), but its action remains unclear. It is possible that *in vivo*, calcitonin induces hypocalcaemia which in turn induces PTH secretion. Early *in vitro* studies performed in isolated kidney cell cultures showed that calcitonin could either stimulate (Larkins *et al.*, 1974) or inhibit (Rasmussen *et al.*, 1972) the activity of the CYP27B1 enzyme. However *CYP27B1* mRNA levels were shown to be increased by calcitonin in normocalcaemic mice (Shinki *et al.*, 1999) suggesting a direct effect on the promoter of the *CYP27B1* gene. Furthermore, Murayama *et al.* (1999) were able to up-regulate the expression of the human *CYP27B1* gene fused to a CAT reporter in MCT kidney cells.

1.4.2 Regulation of extra-renal *CYP27B1* gene expression

CYP27B1 gene expression in non-renal cells is poorly understood both in terms of its regulating factors and molecular mechanisms of control. In general it appears that extra-renal *CYP27B1* gene expression is regulated differently to that in the kidney. For example in the kidney, there is an inverse relationship between *CYP27B1* and *CYP24* gene expression, but a positive relationship has been reported *in vivo* in rat bone (Anderson *et al.*, 2005). In the same study, no correlation was observed between circulating $1,25(OH)_2D_3$ and bone *CYP24* mRNA (Anderson *et al.*, 2005). However there is no information as to the molecular mechanisms controlling *CYP27B1* gene expression in bone.

Extra-renal *CYP27B1* gene expression has been best characterised in pulmonary alveolar macrophages isolated from patients with sarcoidosis, which have been shown

to synthesise $1,25(OH)_2D_3$ (Adams *et al.*, 1983). Both clinical and cell culture derived data suggest that traditional calciotropic factors do not regulate local CYP27B1 enzyme activity. For instance, while PTH is an important regulator of the renal *CYP27B1* gene, it has no effect on CYP27B1 enzyme activity in cultured macrophages (Reichel et al., 1987a). Likewise, there is no significant negative feedback response following treatment with $1,25(OH)_2D_3$ (Adams and Gacad, 1985). Further evidence that $1,25(OH)_2D_3$ synthesis by pulmonary alveolar macrophages is regulated independently to that in the kidney comes from blood analyses of sarcoidosis patients. In cases where this disease is associated with hypercalcemia, circulating $1,25(OH)_2D_3$ levels are high despite suppressed PTH levels (Sandler *et al.*, 1984).

Therefore, CYP27B1 activity within pulmonary alveolar macrophages is not regulated by the calciotropic factors that regulate renal *CYP27B1* gene expression. Rather, it appears to be responsive to immune cell regulators. A potent stimulator of CYP27B1 activity in macrophages is bacterial lipopolysaccharide (LPS) (Reichel et al., 1987b), a lipid found in the cell walls of infectious microorganisms. Macrophage CYP27B1 activity is also stimulated by cytokines such as interferon-γ (IFNγ) (Adams et al., 1989; Monkawa et al., 2000), tumour necrosis factor α (TNFα) (Reichel *et al.*, 1989) and interleukin-2 (IL-2) (Reichel et al., 1987a). These observations suggest that locally produced $1,25(OH)_2D_3$ may act in an autocrine or paracrine manner and serve a regulatory role in the immune system. The role of $1,25(OH)_2D_3$ in the immune system is discussed in 1.5.4.

1.5 Biological activity of $1,25(OH)_2D_3$

The list of biological actions of $1,25(OH)_2D_3$ is ever growing and too numerous for a complete description in this review. The classical action of $1,25(OH)_2D_3$ is to maintain calcium and phosphate homeostasis by targeting such tissues as the intestine and bone. However more recent studies have revealed new roles for this hormone such as the regulation of cellular proliferation and differentiation of numerous cell types. As such, $1,25(OH)_2D_3$ has become a focus in the areas of cancer biology and the immune system. Many of these new roles for $1,25(OH)_2D_3$ are hypothesised to be autocrine or paracrine functions.

1.5.1 Regulation of calcium homeostasis

Perhaps the most important role for $1,25(OH)_2D_3$ is to stimulate dietary calcium and phosphate absorption in the small intestine. Vitamin D deficiency in children can result in rickets, a disease characterised by defective bone mineralisation owing to an inadequate supply of mineral (Omdahl *et al.*, 2002). The rickets phenotype is also seen in VDR-null mice, but can be rescued with a high calcium and phosphate diet (Li et al., 1998a). Maintenance of calcium homeostasis requires the coordination of mechanisms involving the intestine, kidney, bone and parathyroid glands (Fukugawa and Kurokawa, 2002). Circulating $1,25(OH)_2D_3$ plays an integral role in these processes to ensure an adequate supply of calcium and phosphate is available for the maintenance of a mineralised skeleton as well as nerve and muscle functions (Anderson *et al.*, 2003a).

A fall in serum calcium concentration is detected by the calcium sensing receptor within the parathyroid gland and leads to PTH synthesis and secretion. PTH then acts to up-regulate renal *CYP27B1* gene expression. The resultant increase in serum $1,25(OH)_2D_3$ increases intestinal calcium absorption by activating transcription of specific genes involved in active calcium absorption. Upregulated genes include the cytosolic calcium binding protein, calbindin-D9k (Darwish and DeLuca, 1996), which is thought to be involved in the translocation of calcium across the enterocyte. Circulating $1,25(OH)_2D_3$ also acts to increase renal calcium reabsorption by increasing the expression of the renal calcium transport protein, calbindin-D28k (Varghese *et al.*, 1988). A third mechanism by which $1,25(OH)_2D_3$ maintains adequate serum calcium levels is by mobilising calcium from bone. It does so by increasing osteoclastogenesis leading to greater resorption of bone as is described in section 1.6.1.

The essential role that $1,25(OH)_2D_3$ plays in calcium homeostasis is demonstrated by examination of *VDR*-null and *CYP27B1*-null mice which lack all biological $1,25(OH)_2D_3$ activity. These mice develop hypocalcemia as a result of calcium malabsorption (Dardenne et al., 2001; Li et al., 1998b). The VDR-null mice develop a phenotype identical to patients suffering Vitamin D-Dependent Rickets, a disorder associated with mutations in the *VDR* gene. In addition to bone defects, these mice also display defects in the immune response, hair growth process, reproduction and the regulation of cell growth (Yoshizawa *et al.*, 1997). However, with the exception of hair growth processes and cell growth, these defects appear to be normalised when animals are fed a high calcium and phosphate 'rescue' diet which restores mineral ion balance. These data suggest that $1,25(OH)_2D_3$ activity may not be essential for bone

mineralisation when high dietary calcium is available, but appears to be necessary for normal hair cycling and cell growth processes.

CYP27B1-null mice develop the phenotype seen in patients suffering Pseudo-Vitamin D-Dependent Rickets (Panda *et al.*, 2001). Besides bone defects, these mice were infertile and displayed immune defects. However, the rescue diet was able to prevent most of these defects, such as osteomalacia and rickets, suggesting that the observed defects were due to hypocalcemia resulting from the loss of functional CYP27B1 enzyme. Importantly though, the femora of these animals were smaller than control animals despite being weaned on the rescue diet. Therefore, $1,25(OH)_2D_3$ itself appears to play an important role in normal bone growth. Direct actions of $1,25(OH)_2D_3$ on bone include the control osteoblast proliferation and differentiation *in vitro*, as described in 1.6.1.

1.5.2 Control of cell proliferation and differentiation

In addition to osteoblasts, $1,25(OH)_2D_3$ also regulates the proliferation and differentiation of many other cell types including keratinocytes. As described earlier, endocrine vitamin D metabolism begins in the skin with the production of vitamin D_3 from 7-dehdyrocholesterol. However keratinocytes also express the 25-hydroxylase and 1-hydroxylase and as such, are themselves capable of further metabolism to $1,25(OH)_2D_3$ (Bikle *et al.*, 1986a). It is likely that $1,25(OH)_2D_3$ synthesised by keratinocytes serves an autocrine or paracrine function, possibly related to control of differentiation. Numerous reports have described the control of keratinocyte proliferation and differentiation by $1,25(OH)_2D_3$ (Hosomi et al., 1983; Matsumoto et

al., 1990; Smith et al., 1986). The mechanism of action is poorly understood but may involve the inhibition of growth signals from the Transforming growth factor-α/Epidermal growth factor receptor (TGF-α/EGFR) growth loop (Cordero *et al.*, 2002). Specifically, $1,25(OH)_2D_3$ induces the sequestering of EGFR into early endosomes, thus reducing growth signals. Despite the mechanism of action not being fully elucidated, $1,25(OH)_2D_3$ (Perez *et al.*, 1996) and various analogues such as MC 903 and maxacalcitol (Barker et al., 1999; Kragballe et al., 1988) have been successfully used to treat psoriasis, a hyperproliferative disorder of the skin.

1.5.3 $1,25(OH)_2D_3$ and cancer

Given that $1,25(OH)_2D_3$ controls proliferation and differentiation of many cell types, it is not surprising that it has also become a focus in the field of cancer research. The potential for $1,25(OH)_2D_3$ to be used as an anti-cancer agent was first recognised in 1981 with the demonstration that it differentiated mouse myeloid leukemic cells into macrophages *in vitro* (Abe *et al.*, 1981).

Epidemiological studies suggest that $1,25(OH)_2D_3$ may play a protective role against the risk of several types of cancers. As early as 1941, it was reported that people who lived in the higher latitude states of the U.S.A had a greater risk of dying from cancer compared to people in the southern states (Apperly, 1941). The suggestion was that this relative immunity to cancer in the southern states may be due to increased sunlight exposure. More recently, Grant (2002) sought to determine how many types of cancer correlated with reduced exposure to the sun in another U.S. based study. Thirteen types of cancer showed inverse correlations between UV exposure and

cancer mortality rate including breast, prostate, colon and ovarian cancer. It was estimated that 23,600 people per year died between 1970 and 1994 from cancers associated with lower UV-B radiation. In addition to geographical location, race also influences an individual's vitamin D status. Skin pigmentation reduces vitamin D synthesis in the skin and as a result black Americans have lower mean $25(OH)_2D_3$ levels than white Americans (Looker et al., 2002; Nesby-O'Dell et al., 2002). Garland *et al.* (2006) suggest that vitamin D deficiency may contribute to the higher cancer mortality rates seen among the black American population.

The hypothesis that sunlight exposure is protective against many types of cancer via a vitamin D mediated action is supported by *in vitro* experiments on cancer cells. The proliferation of various cancer cells in culture can be inhibited by the addition of $1,25(OH)_2D_3$ to the media, such as prostate carcinoma (Zhao *et al.*, 2000b), breast cancer (James *et al.*, 1996) and leukemia cells (Abe *et al.*, 1981).

The antiproliferative effects of $1,25(OH)_2D_3$ are predominantly due to blocking the transition through the G1/G0 phases of the cell-cycle. Proliferation can be arrested by $1,25(OH)_2D_3$ through numerous mechanisms depending on the cellular context (reviewed by Dusso et al (2005)). For example, $1,25(OH)_2D_3$ induces the cyclin-dependent kinase inhibitor p21 which arrests the growth of myelomonocytic U937 cells (Liu *et al.*, 1996). The growth of some tumours is driven by TGF-α/EGFR. As described for keratinocytes, $1,25(OH)_2D_3$ reduces growth signals by causing EGFR to be sequestered into early endosomes (Cordero *et al.*, 2002).

More recently, $1,25(OH)_2D_3$ has been shown to have protective effects against DNA photodamage in skin cells. Exposure to ultra-violet radiation (UVR) causes DNA damage such as pre-mutagenic cyclobutane pyrimadine dimers (CPD). Dixon *et al.* (2005) have shown that $1,25(OH)_2D_3$, as well as two low calcemic analogues, reduces UVR-induced CPD and cell death in cultured human skin cells. Moreover, topical application of $1,25(OH)_2D_3$ reduced solar simulated-CPD damage in mice *in vivo* (Dixon *et al.*, 2005). These authors postulate that $1,25(OH)_2D_3$ improves skin cell survival post UV exposure by improving DNA repair mechanisms.

1.5.4 Modulation of immune responses

As has been described for many cancer states, links also exist between vitamin D deficiency and immune dysfunction. The effects of $1,25(OH)_2D_3$ on immune function are complex but appear largely to be immunosuppressant. This has been demonstrated in animal models of autoimmune diseases including encephalomyelitis, rheumatoid arthritis, systemic lupus erythematosus, type I diabetes and inflammatory bowel disease (Deluca and Cantorna, 2001). Alternatively, vitamin D deficiency has been linked to a weakened immune system. For instance, the incidence of tuberculosis is higher among subjects with relatively low serum vitamin D levels and tends to occur during the colder seasons when sun exposure is reduced and serum vitamin D levels are lower (Chan, 2000). This observation relates to the role of $1,25(OH)_2D_3$ as a modulator of macrophage function, though $1,25(OH)_2D_3$ has also been shown to affect monocytes, dendritic cells and lymphocytes (van Etten and Mathieu, 2005). The VDR is expressed by most cells of the immune system and interestingly, CYP27B1 is also expressed by activated macrophages (Overbergh *et al.*,

2000) and dendritic cells (Hewison *et al.*, 2003) suggesting that modulation of the immune system by $1,25(OH)_2D_3$ may occur through paracrine mechanisms.

The effect of $1,25(OH)_2D_3$ on immune response varies according to the cell type. The chemotactic and phagocytic capacity of macrophages and monocytes is enhanced by exposure to $1,25(OH)_2D_3$ (Penna and Adorini, 2000). However, dendritic cell maturation is inhibited by $1,25(OH)_2D_3$ (Penna and Adorini, 2000). Similarly interleukin-12 (IL-12), which is the major cytokine that determines the direction of immune system activation, is inhibited by $1,25(OH)_2D_3$ (D'Ambrosio *et al.*, 1998). Proliferation of, and cytokine secretion by T lymphocytes is also inhibited by $1,25(OH)_2D_3$ *in vitro*.

1.5.5 Control of the nervous system

As was previously described in the bone, immune system and skin, the brain is both a $1,25(OH)_2D_3$ target organ as well as a site of $1,25(OH)_2D_3$ synthesis implying that it may exert a paracrine action at this site. VDR is expressed throughout the central nervous system (Veenstra *et al.*, 1998) and $1,25(OH)_2D_3$ has been shown to modulate the synthesis of several factors including nerve growth factor (NGF) (Musiol and Feldman, 1997; Wion et al., 1991). Importantly, $1,25(OH)_2D_3$ has been shown to have neuroprotective effects, possibly through its inhibition of inducible nitric oxide synthase (iNOS) (Garcion *et al.*, 1997). Expression of iNOS is induced during disease states such as Alzheimer's disease, Parkinson's disease, AIDS and multiple sclerosis, and produces neuron damaging nitric oxide.

1.6 The physiological role of extra-renal *CYP27B1* gene expression

As described in the preceding paragraphs, $1,25(OH)_2D_3$ has many different roles throughout the body. If $1,25(OH)_2D_3$ was solely produced by the renal CYP27B1 enzyme, every other tissue would be at the mercy of its overriding need to maintain calcium homeostasis. An extra level of control over $1,25(OH)_2D_3$ activity between tissues is provided by differential expression of the *CYP24* and *VDR* genes. However it could be argued that it is more efficient to synthesise only the amount of $1,25(OH)_2D_3$ that is required, rather than limiting its effect. Therefore as alluded to in previous sections, it is possible that many of the biological activities of $1,25(OH)_2D_3$ at extra-renal sites could be performed by locally produced hormone. Given that extra-renal $1,25(OH)_2D_3$ does not appear to contribute to circulating levels under normal physiological conditions (Anderson et al., 2005; Shultz et al., 1983), this seems a reasonable hypothesis.

Furthermore, there is evidence that the *CYP27B1* gene is regulated differently in extra-renal tissues to that in the kidney (section 1.4.1). This observation is consistent with a model whereby $1,25(OH)_2D_3$ can be either an endocrine factor or an autocrine/ paracrine factor. In the endocrine system, $1,25(OH)_2D_3$ is produced by the renal CYP27B1 enzyme and has essential actions in calcium homeostasis. As such, expression of the renal enzyme is regulated by endocrine factors that are associated with calcium balance such as PTH. In autocrine/ paracrine systems, $1,25(OH)_2D_3$ is locally produced and its roles include the control of cell proliferation and differentiation. Accordingly, *CYP27B1* gene expression in extra-renal tissues appears to be regulated by local factors.

1.6.1 Functions within bone

As previously mentioned, vitamin D deficiency in humans causes defects in bone mineralisation such as rickets and osteomalacia. The data from *CYP27B1* and *VDR*-null mice suggest that the defective bone mineralisation of vitamin D deficiency is due largely to the decreased supply of calcium resulting from impaired intestinal absorption. However, $1,25(OH)_2D_3$ also has direct actions on bone and can modulate processes associated with bone remodelling such as bone cell differentiation and gene expression (Aubin and Heersche, 2005). Moreover, these effects may be autocrine or paracrine as osteoblasts are capable of producing their own $1,25(OH)_2D_3$ (Howard et al., 1981; Turner et al., 1980b). Bone proteins that are regulated by $1,25(OH)_2D_3$ include type I collagen, bone sialoprotein-I, osteocalcin, osteopontin and alkaline phosphatase (Atkins *et al.*, 2005).

The purpose of remodelling is to repair microfractures and facilitate the exchange of calcium and phosphate between the bone and the extracellular fluid. Remodelling is an ongoing process in the adult skeleton and begins with resorption of bone by osteoclasts. $1,25(OH)_2D_3$ indirectly modulates the differentiation and function of osteoclasts. By acting on the osteoblast, $1,25(OH)_2D_3$ stimulates the synthesis of receptor activator of nuclear factor-kB ligand (RANKL), which is able to interact with its receptor, receptor activator of nuclear factor-kB (RANK) on the surface of osteoclast cells (Atkins *et al.*, 2003). This interaction promotes osteoclast differentiation and increased osteoclast activity. *RANKL* expression has been shown to be upregulated by $1,25(OH)_2D_3$ and this occurs preferentially in immature osteoblasts. Resorption is tightly coupled to the formation of new bone by

osteoblasts. Matrix formation and mineral deposition by osteoblasts is dependent upon their progressive differentiation to a mature phenotype. The process of osteoblast development can be described in broad terms in three stages, with each identifiable by specific gene expression (Stein *et al.*, 1990) (see Figure 1.3). Initially it is thought that bone lining cells, which reside on bone surfaces that are not undergoing remodelling, detach from the bone and begin proliferating. These immature cells express collagen allowing an extracellular matrix to form. As osteoblasts develop, proliferation is down-regulated and the osteoblasts progressively differentiate and express *alkaline phosphatase*. Continued alterations in expression of genes such as *osteocalcin* result in a mature osteoblast phenotype that is able to mineralise the collagen matrix. The function of osteocalcin is poorly understood but it is a marker of a mature osteoblast phenotype.

The regulation of osteoblast development and osteoblast specific genes by $1,25(OH)_2D_3$ has largely been investigated through *in vitro* experiments. In general, $1,25(OH)_2D_3$ acts to inhibit osteoblast proliferation (van den Bemd *et al.*, 1995) and to drive osteoblast differentiation (Matsumoto *et al.*, 1991). Owen *et al.* (1991) examined the effects of both acute and chronic treatment of $1,25(OH)_2D_3$ over the course of rat osteoblast development (approximately 30 days). They found that acute exposure (48 hours) to $1,25(OH)_2D_3$ inhibited *collagen* and *alkaline phosphatase* gene expression in osteoblasts on day thirteen but on day twenty-five their expression could be stimulated. When chronic $1,25(OH)_2D_3$ treatment was initiated during osteoblast proliferation, *collagen* and *alkaline phosphatase* expression were suppressed, and development could not proceed through to mineralisation. In direct contrast, when treatment began on day twenty after a mineralised matrix had started to form,

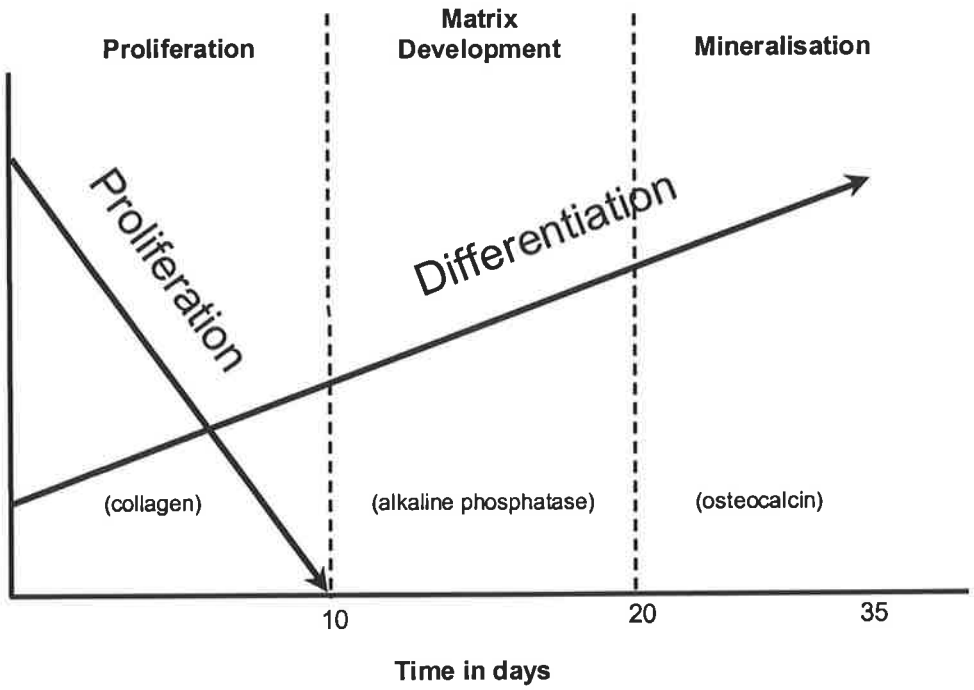

Figure 1.3: Schematic illustration of rat osteoblast cell development *in vitro*. Adapted from (Owen *et al.*, 1990). The osteoblast developmental sequence can be divided into three stages, that of proliferation, matrix development and mineralisation. As osteoblast proliferation decreases, gradual changes in gene expression see the formation of an extracellular, collagen matrix that is subsequently mineralised. Each stage can be identified by the expression of specific genes as shown in brackets.

osteocalcin gene expression was stimulated by $1,25(OH)_2D_3$ and development continued. In this manner, Matsumoto et al. (1991) have demonstrated that *in vitro* mineralisation can be stimulated by $1,25(OH)_2D_3$ in MC3T3-E1, mouse osteoblast-like cells. They found stimulation of calcium accumulation into the cell and matrix layer by $1,25(OH)_2D_3$ and that the effect was dose-dependent. Moreover, Gardiner *et al.* (2000) produced transgenic mice that overexpressed VDR under the control of the *osteocalcin* promoter, and therefore expressed higher levels of VDR in mature osteoblasts only. Analysis of the vertebrae from these mice revealed stronger cortical bone due to an increased mineral apposition rate as well as increased trabecular thickness due to decreased bone resorption.

Therefore not only is $1,25(OH)_2D_3$ crucial to bone through its central role in calcium homeostasis but it also has direct effects on bone. That is, it influences all stages of bone remodelling from osteoclastogenesis and resorption to osteoblast development and mineralisation. Regulation of osteoblast specific genes by $1,25(OH)_2D_3$ is dependent upon the maturity of the osteoblast.

1.6.2 Clinical evidence

Given that $1,25(OH)_2D_3$ appears to have pleiotropic functions in controlling osteoblast development (proliferating osteoblasts are inhibited by exposure to $1,25(OH)_2D_3$ whereas mineralisation is stimulated in mature cells), it may be beneficial for the osteoblast to control its own $1,25(OH)_2D_3$ levels. Indeed, bone *CYP27B1* gene expression has potential clinical significance given the association between decreased circulating $25(OH)_2D_3$ levels and increased risk of hip fracture

(Morris *et al.*, 1984). Interestingly, it has been observed that mature women (aged over 45 years) have similar circulating $1,25(OH)_2D_3$ levels to younger women but decreased bone levels of $1,25(OH)_2D_3$ (Sagiv *et al.*, 1992). This is an important point considering that age is a major contributing factor to fracture risk. Furthermore, a study by the same group reported that elderly women with sub-capital fractures of the femur have 5 times lower bone $1,25(OH)_2D_3$ levels than age matched women without a fracture (Lidor *et al.*, 1993). This was despite the fact that circulating $1,25(OH)_2D_3$ levels were only slightly lower in the women with fractures compared with the non-fracture group. These observations further suggest that there is additional control over the level of $1,25(OH)_2D_3$ in the bone compared with that in the kidney. Moreover, abnormal CYP27B1 activity in the bone may result in a decreased local supply of $1,25(OH)_2D_3$ which may lead to increased risk of fracture. This impaired bone strength may be due to the loss of stimulatory effects of $1,25(OH)_2D_3$ on osteogenesis or osteoclastogenesis.

1.6.3 Parallels with estrogen metabolism

In considering $1,25(OH)_2D_3$ as an autocrine/ paracrine factor, it is interesting to note the similarities to estrogen endocrinology. It is now apparent that estrogen, like $1,25(OH)_2D_3$, has wide ranging actions in addition to its traditional roles (reviewed by Simpson *et al.* (2002)). Estrogen biosynthesis is catalysed by the enzyme aromatase, which like CYP27B1, is a member of the cytochrome P450 gene superfamily. Aromatase is expressed in the ovaries and a number of other tissues including the bone (osteoblasts), adipose tissue, brain and breast. In the absence of estrogen production by the ovaries, for instance in men and postmenopausal women, estradiol

does not function as a circulating hormone. Rather it acts in an autocrine and paracrine manner in tissues other than the ovaries (Simpson *et al.*, 2002). Moreover, the expression of aromatase in bone is positively correlated with the stimulation of osteogenesis (Lea *et al.*, 1997).

1.7 Molecular action of $1,25(OH)_2D_3$

1.7.1 Vitamin D receptor

Most biological activities of $1,25(OH)_2D_3$ require binding to the VDR, which acts as a ligand activated transcription factor (Whitfield *et al.*, 2005). VDR was originally identified in chicken intestine (Haussler and Norman, 1969) and then other tissues involved in calcium homeostasis. Cloning of the VDR in avian (McDonnell *et al.*, 1987), human (Baker *et al.*, 1988), mouse (Kamei *et al.*, 1995) and rat (Burmester *et al.*, 1988) has led to its discovery in many other tissues. The human VDR is a member of the superfamily of nuclear receptors for steroid hormones with at least two variants existing, VDRA and VDRB1. VDRB1 varies from the original isoform by a 50 amino acid N-terminal extension (Crofts *et al.*, 1998). Both isoforms have been detected at similar levels in human kidney tissue as well as kidney, intestine and bone cell lines (Sunn *et al.*, 2001). Interestingly, the transactivation activity of VDRA and VDRB1 appears to vary (Gardiner *et al.*, 2004). Therefore the relative expression of each isoform in different tissues may represent a level of control over the vitamin D response (Esteban *et al.*, 2005).

The human VDR is composed of several functional domains including a DNA-binding domain and a ligand-binding domain (reviewed by Pike and Shevde (2005)). The DNA binding domain is highly conserved among nuclear steroid receptors and interacts specifically with vitamin D response elements (VDREs) within the promoter regions of target genes. DNA binding also requires the ligand binding domain which in addition to binding $1,25(OH)_2D_3$ with high affinity, is also required for dimerization with retinoid X receptor (RXR) (Jin et al., 1996; Whitfield et al., 1995). The formation of a VDR-RXR heterodimer is necessary for interaction with the two repeated half-sites of the VDRE (Nakajima *et al.*, 1994).

1.7.2 Binding to vitamin D responsive elements (VDREs)

The optimal VDRE as determined by random oligonucleotide selection procedures is a direct repeat of six base half sites separated by a three base pair spacer as follows: 5'-A/GGGTCAnngA/GGTTCA-3' (Colnot et al., 1995; Nishikawa et al., 1994). This is termed a DR3 type VDRE owing to its 3 base pair spacer, and is the most common VDRE. VDREs comprising direct repeats with a four or six nucleotide spacing, designated DR4- and DR6- type VDREs have also been described (Carlberg and Polly, 1998). The most responsive gene to $1,25(OH)_2D_3$ is *CYP24* which contains two separate VDREs within both the rat (Hahn et al., 1994; Zierold et al., 1995) and human gene promoters (Chen and DeLuca, 1995).

A number of studies have shown that in the absence of ligand, the VDR-RXR heterodimer binds the VDRE and recruits a co-repressor such as RIP13delta1 (Dwivedi *et al.*, 1998), Alien (Polly *et al.*, 2000), or Hairless (Skorija *et al.*, 2005).

Unliganded VDR/RXR heterodimers are thought to repress most, if not all, $1,25(OH)_2D_3$ target genes most likely through associated histone-deacetylase activity that facilitates a closed chromatin structure (Omdahl *et al.*, 2002).

Interestingly, alopecia is observed in *VDR*-null but not *CYP27B1*-null mice indicating that the underlying molecular mechanism that causes alopecia involves unliganded VDR and not $1,25(OH)_2D_3$. Furthermore targeted expression of the VDR in the keratinocytes of VDR-null mice prevents this phenotype by an unknown mechanism (Chen *et al.*, 2001). Therefore it is interesting to note that mutations in the *Hairless* gene in humans and mice cause alopecia (Ahmad et al., 1998; Mann, 1971). This points to a possible role of transcriptional repression by unliganded VDR and Hairless in hair follicle homeostasis (Skorija *et al.*, 2005).

Upon $1,25(OH)_2D_3$ binding its receptor, a conformational change occurs in the VDR-RXR heterodimer. This displaces the co-repressor facilitating the recruitment of co-activator complexes that are likely to act in a two step process. The first co-activator complex modifies chromatin structure and is replaced by a second complex that interacts with RNA polymerase II (reviewed by Omdahl *et al.* (2002)). Therefore, the VDR-RXR heterodimer can act as a repressor in the absence of $1,25(OH)_2D_3$ but also as a coactivator when $1,25(OH)_2D_3$ binds.

1.7.3 Regulation of VDR

As described earlier, the biological activity of $1,25(OH)_2D_3$ is determined largely by the activity of the CYP27B1 and CYP24 enzymes which regulate hormone levels in

the serum and target tissues. However alterations in the cellular content of VDR (Shao *et al.*, 2001) and post-translational modifications to the VDR, such as phosphorylation, also regulate $1,25(OH)_2D_3$ activity. The level of VDR protein has been shown to be modulated by hormones such as $1,25(OH)_2D_3$, PTH and estrogen, as well as growth factors (reviewed by Esteban *et al.* (2005)).

$1,25(OH)_2D_3$ has been shown to upregulate the abundance of VDR protein in kidney and intestinal cell lines and *in vivo* (Costa and Feldman, 1987; Issa et al., 1998; Sandgren and DeLuca, 1990; Strom et al., 1989). However, no increase in VDR levels following $1,25(OH)_2D_3$ treatment was reported *in vivo* in several non-calcemic rat tissues (Gensure *et al.*, 1998). Therefore $1,25(OH)_2D_3$ appears to increase the efficiency of its own action by increasing the level of its receptor, but only in specific tissues. The molecular mechanism by which $1,25(OH)_2D_3$ increases the cellular content of VDR is not clear but appears to involve stabilization of the VDR protein more so than an increase in expression of the *VDR* gene (Arbour et al., 1993; Wiese et al., 1992). No consensus VDRE has been identified within the 5'-flanking sequence of the mouse *VDR* gene (Jehan and DeLuca, 1997) which contains a TATA-less promoter and is driven by a cluster of Sp1 binding sites (Jehan and DeLuca, 2000).

1.7.4 Phosphorylation of VDR

Phosphorylation of the VDR is another mechanism by which VDR mediated transcription can be modulated. Upon ligand binding to the VDR, serine phosphorylation of VDR occurs at several sites and is mediated by kinases including casein kinase II (on serine 208) (Jurutka *et al.*, 1996), protein kinase C (on serine 51)

(Hsieh *et al.*, 1991) and protein kinase A (Jurutka *et al.*, 1993). Phosphorylation of the VDR is purported to increase VDR-mediated transcription by enhancing interaction between the VDR and coactivators. Such an effect has been reported between VDR and the coactivator DRIP205 (Barletta *et al.*, 2002).

1.7.5 Non-genomic actions of $1,25(OH)_2D_3$

$1,25(OH)_2D_3$ can also elicit more rapid responses that do not require changes in gene expression through the VDREs, and can occur in minutes. These non-genomic actions involve signalling pathways such as mitogen-activated protein kinases (MAP kinases) and protein kinase C (Dwivedi et al., 2002; Nemere et al., 1998; Song et al., 1998). For example, $1,25(OH)_2D_3$ opens Ca^{2+} channels via protein kinase C in 30 seconds in the rat osteoblast-like cell line ROS 17/2.8 (de Boland and Norman, 1990). A point of contention has been whether these non-genomic pathways are initiated via the classical nuclear VDR acting at the cell membrane or a novel membrane VDR. However, Capiati *et al.* (2002) have shown that $1,25(OH)_2D_3$ induces translocation of the nuclear VDR to the plasma membrane in chick myoblasts in less than 10 minutes. Moreover, non-genomic responses to $1,25(OH)_2D_3$ were abrogated in osteoblasts from VDR-null mice (Erben *et al.*, 2002), supporting the conclusion that the classical VDR mediates the nongenomic actions of $1,25(OH)_2D_3$.

1.8 Transforming growth factor–beta

As described in previous sections, $1,25(OH)_2D_3$ has many direct actions on bone and modulates the processes involved in bone remodelling. However, $1,25(OH)_2D_3$ does

not act alone in doing so. Bone homeostasis requires coordinated interactions between numerous agents such as transforming growth factor-beta (TGF-β), PTH, estradiol, and insulin-like growth factor (reviewed by Karsenty (1999)). This section will focus on the roles played by TGF-β in bone and what is known of its interactions with $1,25(OH)_2D_3$.

1.8.1 Functions of TGF-β

TGF-β is a polypeptide growth factor that belongs to a superfamily of more than twenty members that includes bone morphogenetic proteins (BMPs) and activins. Each member of this family plays significant roles in development and tissue homeostasis (Massague and Wotton, 2000). Within mammals, three isoforms of TGF-β exist - TGF-β1, TGF-β2 and TGF-β3. All three isoforms are present within bone, though TGF-β1 is the most abundant isoform (Hering *et al.*, 2001). In general, TGF-β regulates proliferation, differentiation and apoptosis of numerous cell types. Within bone, TGF-β1 plays a central role in remodeling by affecting both bone formation and bone resorption (reviewed by Janssens *et al.* (2005)).

1.8.2 TGF-β receptor signaling

Signaling of TGF-β family members occurs through type I and type II serine/ threonine kinase receptors (reviewed by ten Dijke and Hill (2004)). Ligand binding induces the two receptors to assemble, leading to the phosphorylation of the type I receptor by the type II receptor (Figure 1.4). The activated type I receptor then propagates the signal by phosphorylating Smad transcription factors, the nuclear

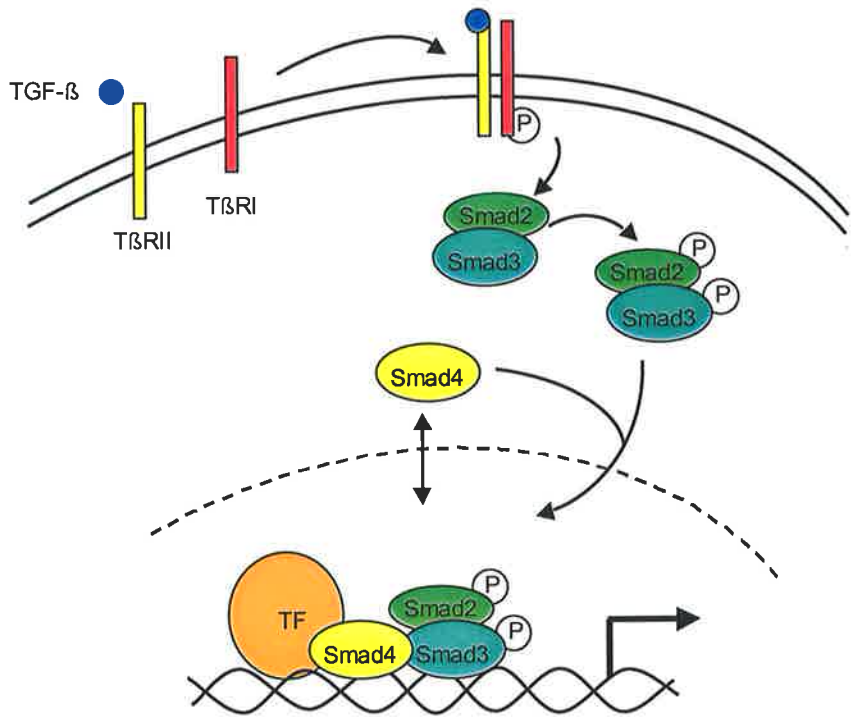

Figure 1.4: The TGF-β–Smad pathway. TGF-β binds to the TGF-β type II receptor (TβRII) which then forms a complex with, and phosphorylates, the TGF-β type I receptor (TβRI). The type I receptor in turn phosphorylates the receptor Smads (Smad2 and Smad3) which then accumulate within the nucleus in partnership with Smad4. Receptor Smad/ Smad4 complexes bind Smad Binding Elements (SBEs) within the promoters of TGF-β responsive genes in association with other transcription factors (TF). In the absence of TGF-β stimulus, Smad2 and Smad3 are predominantly cytoplasmic whereas Smad4 shuttles between the nucleus and the cytoplasm as indicated.

effectors of TGF-β signaling. However a number of responses to TGF-β occur too quickly (within minutes) to result from Smad-mediated changes in gene transcription and are therefore considered to occur by activating MAPK signaling (Janssens *et al.*, 2005).

1.8.3 Smad transcription factors

Smads were first identified as the products of the *Drosophilia Mad* and *C. elegans Sma* genes (Whitman, 1998). Eight Smad proteins have been identified in mammals and fall into three functional groups. Smad1, Smad2, Smad3, Smad5 and Smad8 are the receptor-regulated or R-Smads. Smad2 and Smad3 act as ligands for the TGF-β receptor whereas Smad1, Smad5 and Smad8 are ligands for BMP receptors. Following phosphorylation by the type I receptor, activated R-Smads form complexes with the common mediator Smad (Co-Smad), Smad4. These complexes accumulate in the nucleus where they can interact with other transcription factors on DNA to modulate gene expression. The third group of Smads is the inhibitory Smads (I-Smads), Smad6 and Smad7. These Smads inhibit TGF-β signalling by competing with the R-Smads for receptor interaction.

Rather than being static, Smads constantly shuttle between the cytoplasm and the nucleus. In the uninduced state, Smad2 and Smad3 are predominantly located in the cytoplasm whereas Smad4 is distributed in the nucleus and cytoplasm (ten Dijke and Hill, 2004). It is unclear whether activated R-Smads form complexes with Smad4 in the cytoplasm or the nucleus.

Structurally, Smads contain a Mad homology 1 (MH1) and a Mad homology 2 (MH2) domain (reviewed by Moustakas *et al.* (2001)). The MH1 domain regulates binding to DNA and other transcription factors and is highly conserved among R-Smads and Smad4, but not the I-Smads. The MH2 domain is highly conserved among all Smads and regulates receptor interaction, oligomerisation and interaction with other transcription factors.

1.8.4 Smad binding elements

Oligonucleotide selection experiments originally identified the palindrome 5'-GTCTAGAC-3' as the sequence specifically bound by recombinant Smad proteins (Zawel *et al.*, 1998). Shortly after, Shi *et al.* (1998) demonstrated that the Smad3 MH1 domain could interact with the palindrome's half-site 5'-GTCT-3', and this sequence, or its complement 5'-AGAC-3' were deemed Smad binding elements (SBEs). However in many Smad responsive promoters, SBEs contain an extra base, 5'-GTCTG-3' or 5'-CAGAC-3' (Lopez-Rovira et al., 2002; Massague et al., 2005; Zhao et al., 2000a).

The affinity of a Smad MH1 domain for DNA is low, in the order of the 10^{-7} M range (Shi *et al.*, 1998). Therefore, additional DNA contacts involving other transcription factors are necessary for high affinity binding of Smad complexes within the regulatory regions of genes. These additional interactions also add to target gene specificity, as the CAGAC sequence is predicted to be present, on average, once in the regulatory region of every average size gene (Massague and Wotton, 2000).

1.8.5 Smad transcriptional complexes

Stimulation of cells with TGF-β can lead to either positive or negative changes in gene expression. Microarray analysis of human epithelial cells revealed some 300 genes whose expression changed greater than two-fold following TGF-β treatment (Kang *et al.*, 2003). At present, the stoichiometry of active Smad complexes remains unclear, however all endogenous Smad complexes analysed so far have contained Smad4 (Massague *et al.*, 2005). It has been suggested that activated Smad heterodimers or heterotrimers may form depending on the promoter context and involvement of other transcription factors (Inman and Hill, 2002). For example, a Smad2-Smad2-Smad4 heterotrimer may bind the *Mix2* gene promoter in association with the co-factor FoxH1. On the other hand, evidence suggests that a Smad3-Smad4 heterodimer may target the *junB* gene promoter (Inman and Hill, 2002).

Whether a signal is positive or negative is determined largely by the recruitment of coactivators or corepressors to the Smad complex. R-Smads have been shown to recruit the general coactivators p300 and CBP (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998) via interactions with their MH2 domains. These coactivators possess histone acetyl transferase (HAT) activity and may therefore increase transcription by modifying nucleosome structure. Other coactivators include SMIF, which interacts specifically with Smad4 and p300 to potentiate transcriptional activation (Bai *et al.*, 2002).

1.8.6 Smad-mediated gene repression

There are also many examples of transcriptional repression by TGF-β. Interestingly though, the DNA elements involved in repression do not resemble canonical SBEs. For example, Smad3 represses c-Myc expression in association with the transcription factors E2F4 and E2F5, and the corepressor p107. Upon activation of Smad3, this complex translocates to the nucleus where it binds a Smad-E2F binding element (5'-GGCTTGGCGGGAAA-3') within the *c-Myc* promoter resulting in repression *of c-Myc* gene expression (Chen *et al.*, 2002). Other corepressors involved in Smad mediated transcriptional repression include TGIF (TG sequence-interacting factor) (Wotton *et al.*, 1999) and the proto-oncogene products Ski and SnoN (Luo *et al.*, 1999). The corepressors in turn recruit histone deacetylase (HDAC) leading to decreased transcription. Interestingly, Ski and SnoN interaction with Smads occurs under basal conditions and diminishes within the first hours of TGF-β treatment (Stroschein et al., 1999; Sun et al., 1999). TGF-β can also inhibit gene expression by interfering with transcriptional activators such as the myogenic differentiation factor MyoD. By binding MyoD, Smad3 prevents it from forming dimers with partner E-proteins and binding to E-boxes within target promoters (Liu *et al.*, 2001). Finally, TGF-β can induce the expression of the transcription factors TIEG1 and TIEG2 (TGF-β-inducible early-response gene). TIEG proteins bind to GC rich sequences (Chrisman and Tindall, 2003) within target genes and act as transcriptional repressors (Cook *et al.*, 1999).

1.8.7 Functions within osteoblasts

Like $1,25(OH)_2D_3$, TGF-β is both produced by osteoblasts and modulates osteoblast gene expression (Robey *et al.*, 1987). TGF-β is known to affect every stage of osteoblast development, from proliferation through to matrix production and mineralisation, though its role in each case remains controversial (Janssens *et al.*, 2005). In general, TGF-β appears to stimulate proliferation of cultured osteoblasts but inhibit their differentiation (Alliston et al., 2001; Bonewald and Dallas, 1994). TGF-β is highly expressed at fracture sites and as such its therapeutic use in fracture repair and osteoporosis is currently being investigated.

1.8.8 TGF-β and $1,25(OH)_2D_3$ interactions

It is clear that TGF-β and $1,25(OH)_2D_3$ each modulate osteoblast gene expression and development. Whereas $1,25(OH)_2D_3$ inhibits proliferation and stimulates differentiation of osteoblasts, the reverse is true for TGF-β. It is also apparent that in some cases, the two agents interact in modulating osteoblast gene expression. For example, synergistic activation of the osteocalcin promoter has been demonstrated in COS-1 cells and involves a VDRE and SBEs in close proximity (Subramaniam *et al.*, 2001). Similarly, $1,25(OH)_2D_3$ and TGF-β synergistically stimulate AP-1 protein activity in the mouse osteoblastic cell line MC3T3-E1 (Takeshita *et al.*, 1998). Furthermore, $1,25(OH)_2D_3$ increases TGF-β synthesis in several cell types including human osteoblasts (Wu *et al.*, 1997) and also increases TGF-β binding to the surface of normal human osteoblasts (Nagel and Kumar, 2002). However to the best of the

author's knowledge, there is no information as to the effect of TGF-β on $1,25(OH)_2D_3$ production.

1.9 Conclusion

Numerous *in vitro* studies have described pleiotropic actions of $1,25(OH)_2D_3$ in bone cells. An emerging view is that these effects may be performed by locally produced $1,25(OH)_2D_3$, acting in an autocrine or paracrine manner. However at present, the factors that regulate $1,25(OH)_2D_3$ production by osteoblasts are unknown. The aim of this work was to investigate the regulation of the *CYP27B1* gene 5'-flanking region in osteoblast cells. It was predicted that the pattern of expression would differ from that previously reported in kidney and prostate cells.

1.10 Aims of the thesis

1 To express plasmid constructs containing different lengths of the *CYP27B1* gene 5'-flanking region fused to a luciferase gene reporter in osteoblast-like cells to allow comparisons to be drawn between expression patterns in osteoblast, kidney and prostate cell lines.

2 To identify regulatory elements within the repressive region between –1100 and –997 that contribute to repression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells.

3 To identify the protein(s) that bind to the repressive elements by utilising electrophoretic mobility shift assays (EMSAs).

4 To determine the effect of exogenously added PTH, $1,25(OH)_2D_3$, TGF-β1 and IGF-1 on expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells.

5 To investigate the elements through which TGF-β1 represses the 5'-flanking region of the *CYP27B1* gene in osteoblast cells.

Chapter 2:

Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals and Reagents

The following reagents were used in the course of experimental procedures.

Sigma Chemical Company: Rat PTH (1-34), ampicillin, phenylmethylsulfonyl fluoride (PMSF), N, N, N, N-tetramethethylethenediamine (TEMED), ammonium persulphate (APS), lysozyme, sodium dodecyl sulphate (SDS), ethidium bromide, N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (Hepes).

Invitrogen (Carlsbad, CA): TRIzol reagent.

Tetrionics Inc (Madison, WI): 1,25 dihydroxyvitamin D_3.

R &D Systems (Minneapolis, MN): Recombinant human type 1 transforming growth factor beta (TGF-β1).

GroPep (Adelaide, Australia): Human Insulin-like growth factor-1 (IGF-1).

National Diagnostics (Atlanta, GA): Accugel (19:1).

Roche diagnostics Corp. (Indianapolis, IN): Caesium chloride, Fugene 6 transfection reagent, DOTAP liposomal transfection reagent.

Pharmacia (New York, NY): PolydI-dC.

Promega (Madison, WI): Dual luciferase reporter kit.

Qiagen Pty Ltd (Valencia, CA): Plasmid purification kits.

Calbiochem (Darmstadt, Germany): SB203580 pharmacological kinase inhibitor.

2.1.2 Radiochemical

[α-^{32}P]-dCTP (10μCi/μl) was purchased from Perkin Elmer (Boston, MA).

2.1.3 Antibodies

Smad4 (H-552): sc-7154 and Ski (H-329): sc-9140 were from Santa Cruz (Santa Cruz, CA). Immune serum raised against Smad4 was a kind gift from Assoc. Prof. Fang Liu, Rutgers University, New Jersey and has been reported previously (Denissova *et al.*, 2000).

2.1.4 Enzymes

All enzymes were from New England Biolabs (Ipswich, MA) with the exception of Pfu turbo polymerase (Pyrococcus furiosus) which was from Stratagene (La Jolla, Ca).

2.1.5 Buffers

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

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

Transformation buffer I (Tbf 1): 30mM KAc, 100mM RbCl, 10mM $CaCl_2(2H_2O)$, 50mM $MnCl_2(4H_20)$ and 15% Glycerol. pH 5.8.

Transformation buffer II (Tbf 2): MOPS (acid), 10mM RbCl, 10mM $CaCl_2(H_2O)$ and 15% Glycerol. pH 6.5.

Nuclear extract lysis buffer I: 10mM Hepes-KOH (pH 7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5mM Dithiothreitol and 0.2mM PMSF.

Nuclear extract lysis buffer II: 20mM Hepes-KOH (pH 7.9), 25% Glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM Dithiothreitol and 0.2mM PMSF.

GTE Buffer: 50mM Glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA.

Oligonucleotide annealing buffer (5X): 200mM Tris-HCl (pH 7.5), 100mM $MgCl_2$ and 250mM NaCl.

Dignam Buffer C: 20mM HEPES buffer (pH 7.6), 420 mM NaCl, 0.5 mM dithiothreitol, 5 mM MgCl2, 0.5 mM EDTA and 20% glycerol.

Buffers were sterilized by autoclaving where appropriate.

2.1.6 Vectors

pGL3-basic vector was purchased from Promega (Madison, WI).

pEFBos(Ets-1) is an expression clone for the Ets-1 protein and has been described previously (Thomas et al., 1995).

pcMV(Ets1T38A) is a dominant negative clone for the Ets-1 protein where T38A indicates a Thr^{38} to Ala substitution.

pcMV(Ets2T72A) is a dominant negative clone for the Ets-2 protein where T72A indicates the substitution of Thr^{72} to Ala. Both the Ets1 and Ets2 dominant negative clones were kindly provided by Professor David Hume, University of Queensland.

pact-c-Ski is a Ski protein expression clone and was a kind gift of Dr. Shunsuke Ishii, RIKEN Tsukuba Institute, Ibaraki, Japan.

pcMV5-Smad4 and pGEX-4T-1-Smad4(GST) are mammalian and bacterial expression clones for Smad4 respectively, and were kindly provided by Assoc. Prof. Fang Liu, Rutgers University, New Jersey.

pcMV5(ERK1K71R) is dominant negative clone for ERK1 where K71R stands for Lys^{71} to Arg. This clone has been described by our laboratory previously (Dwivedi *et al.*, 2002).

pLNCx(MKK4K116R) expresses a dominant negative MKK4 where K116R stands for Lys^{116} to Arg. This clone was donated by Dr D. Riches (National Jewish Medical and Research Centre, Denver, CO, USA).

Ras(17N) is a dominant negative clone for the Ras protein and has been used previously by our laboratory (Dwivedi *et al.*, 2002).

PRSα-HA1(MEK5A) expresses a dominant negative MEK5 in which Ser-311 and Thr-315 were replaced by Ala and Val, respectively (Kamakura *et al.*, 1999). The clone was kindly provided by Dr. E. Nishida (Kyoto University, Kyoto, Japan).

2.1.7 Synthetic oligonucleotides

Oligonucleotides for RT-PCR, site-directed mutagenesis and gel mobility shift assays were obtained from GeneWorks (Adelaide, Australia). The fluorogenic Taqman probe was synthesised by PE Applied Biosystems (Foster City, CA). Oligonucleotide sequences are listed below:

Primers used to amplify and detect *CYP27B1* mRNA by RT-PCR have been described previously (Anderson *et al.*, 2003b), and are as follows:

Forward primer: 5'-GAGATCACAGGCGCTGTGAAC-3'

Reverse primer: 5'-TCCAACATCAACACTTCTTTGATCA-3'

Taqman probe: 5'-6FAM-TGTCCCAGCTACCCCTGCTAAAGGCT-TAMRA-3'

Sequencing oligonucleotides:

300F: 5' AC GGT ACC TGA CTA GTG TAG CTT GGT C 3'

Hup8F: 5' ACG GTA CCA TGC CTA TTC TGC CAT 3'

Forward and reverse primers for site-directed mutagenesis with the core sequence underlined and mutated bases in bold:

GC box

Wild type 5'-GAAGTCAGCCCCAGCCCCGCCTACTGTTC-3' (-143/-115)

Forward 5'-GAAGTCAGCC**AA**AGC**TTT**GCCTACTGTTC-3'

Reverse 5'-GAACAGTAGGCAAAGCTTTGGCTGACTTC-3'

CAAT Box

Wild type 5'-CAGGAGGAGGGATTGGCTGAGGAGCTTG-3' (-85/-58)

Forward 5'-CAGGAGGAGGGA**GCTC**CTGAGGAGCTTG-3'

Reverse 5'-CAAGCTCCTCAGGAGCTCCCTCCTCCTG-3'

Ets binding site I (EBS I)

Wild type 5'-GAGCCACCACAGGATTCTGAGGC-3' (-812/-790)

Forward 5'-GAGCCACCACA**TTAT**TCTGAGGC-3'

Reverse 5'-GCCTCAGAATAATGTGGTGGCTC-3'

Ets binding site II (EBS II)

Wild type 5'-CCTCATGCCATCCTCCTGCCTCAG-3' (-864/-841)

Forward 5'-CCTCATGCC**AAAC**TCCTGCCTCAG-3'

Reverse 5'-CTGAGGCAGGAGTTTGGCATGAGG-3'

Ets binding site III (EBS III)

Wild type 5'-CCCAAAGTGTTGGGATTACAGTCATG-3' (-973/ -948)

Forward 5'-CCCAAAGTGTTG**TTAT**TACAGTCATG-3'

Reverse 5'-CATGACTGTAATAACAACACTTTGGG-3'

c-Myb

Wild type 5'-GTAGAGACAGTTCCACTATGTTG-3' (-1048/-1025)

Forward 5'-GTAGAGACAG**A**TCCACTATGTTG-3'

Reverse 5'-CAACATAGTGGATCTGTCTCTAC-3'

CRE

Wild type 5'-CCATGCCTGGCTAATTTTTG-3' (-1075/-1047)

Forward 5'-CCATGCCTG**TG**TAATTTTTG-3'

Reverse 5'-CAAAAATTACACAGGCATGG-3'

SBE1

Wild type 5'-AGGCTGGTCTCAGACTCCTG-3' (-1021/-1002)

Forward 5'-AGGCTGGTCTC**TTAC**TCCTG-3'

Reverse 5'-CAGGAGTAAGAGACCAGCCT-3'

SBE2

Wild type 5'-GCCCAGGCTGGTCTCAGACTCCTGAGC-3' (-1025/-999)

Forward 5'-GCCCAGGCTGGT**AA**CAGACTCCTGAGCT-3'

Reverse 5'-AGCTCAGGAGTCTGTTACCAGCCTCCCG-3'

SBE(1 and 2)

Wild type 5'-GCCCAGGCTGGTCTCAGACTCCTGAGC-3' (-1025/-999)

Forward 5'- GCCCAGGCTGGT**AA**C**TT**ACTCCTGAGC-3'

Reverse 5'-GCTCAGGAGTAAGTTACCAGCCTGGGC-3'

SBE(C)

Wild type 5'-CAGGCTGGTCTCAGACTCCTGAGC-3' (-1022/-999)

Forward	5'-CAGGCTGGTCTAAGACTCCTGAGC-3'
Reverse	5'-GCTCAGGAGTCTTAGACCAGCCTG-3'

TIEG

Wild type	5'-AGGAGGGGGAGGTGTGGCTAGTGCAGG-3' (-492/-466)
Forward	5'-AGGAGGGGGAGGCTTGGCTAGTGCAGG-3'
Reverse	5'-CCTGCACTAGCCAAGCCTCCCCCTCCT-3'

Fra-1

Wild type	5'-AATATCAGAGACTGACTAGTGTAGCTTG-3' (-315/-288)
Forward	5'- AATATCAGAGACTATCTAGTGTAGCTTG-3'
Reverse	5'-CAAGCTACACTAGATAGTCTCTGATATT-3'

SBE

Wild type	5'-TGGGAATATCAGAGACTGACTAGTG-3' (-318/-295)
Forward	5'-TGGGAATATCAGTTACTGACTAGTG-3'
Reverse	5'-CACTAGTCAGTAACTGATATTCCCA-3'

E box1+2

Wild type	5'-ATAGGTACCAACCCACCTGCCATCTGCCCAG-3' (-540/-510)
Forward	5'-ATAGGTACCAACCTGCCTGCTGTCTGCCCAG-3'
Reverse	5'- CTGGGCAGACAGCAGGCAGGTTGGTACCTAT-3'

Oligonucleotides used to create probes for electrophoretic mobility shift assays (S: sense, AS: antisense). Sequences of binding sites are underlined.

CYP27B1

S: 5'-GCTGGTCTCAGACTCCT-3'

AS: 5'-GAGGAGTCTGAGACCAG-3'

mSBE1

S: 5'-GCTGGTAACAGACTCCT-3'

AS: 5'-GAGGAGTCTGTTACCAG-3'

mSBE2

S: 5'-GCTGGTCTCTTACTCCT-3'

AS: 5'- GAGGAGTAAGAGACCAG -3'

CYP27B1-core

S: 5'-GGTCTCAGAC-3'

AS: 5'-GGTCTGAGAC-3'

Smad7

S: 5'-GGGTGTCTAGACGGC-3'

AS: 5'-CCACAGATCTGCCGG-3'

Consensus SBE

S: 5'-GACACGTAGTCTAGACGTCTAGACAATGT-3'

AS: 5'-GACATTGTCTAGACGTCTAGACTATGTGT-3'

2.1.8 Bacterial strains

The following *E.coli* strain was used as the host for recombinant plasmid DNA propagation and procedures:

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

The following strain was used as the host for expressing GST-Smad4 recombinant protein, and was a kind gift of the Molecular Regulation Lab, Division of Haematology, IMVS, South Australia:

E.coli BL21 Star™(DE3)pLysS: F-ompT hsdS_B (r_{B-mB}-) gal dcm rne131 (DE3) pLysS (Cam^R).

2.1.9 Bacterial growth media

All media were prepared with milliQ water and sterilised by autoclaving. Where required, ampicillin was added after the media had cooled to 50°C.

Luria Broth (LB): 1% (w/v) Bacto-tryptone (Difco, Franklin Lakes, NJ), 0.5% (w/v) yeast extract (Difco, Franklin Lakes, NJ), 1% (w/v) NaCl, pH 7.0.

Agar plates: plates were prepared by adding 1.5% (w/v) Bacto-agar (Difco, Franklin Lakes, NJ) to the LB. 100 ug/ml ampicillin was used where required.

2.1.10 Tissue culture cell lines

UMR 106.06: Rat osteosarcoma cell line was received from Professor TJ Martin, St. Vincent's Institute of Medical Research, University of Melbourne.

ROS 17/2.8: Rat osteosarcoma cell line obtained from the Garvan Institute, Sydney.

HEK-293T: Human primary embryonic kidney cell line was from Dr. Dan Peet, Discipline of Biochemistry, University of Adelaide.

MDA-MB-468: Human breast cancer cell line obtained from Assoc. Prof. David Callen, Hanson Institute, South Australia.

COS-1: Green African monkey kidney cell line was from the American Type Tissue Culture Collection (ATCC).

HOS: Human osteosarcoma cell line obtained from Dr. Gerald Atkins, Hanson Institute, South Australia.

2.1.11 Tissue culture media and supplies

a) Media and solutions

Dulbecco's Modified Eagle medium (DMEM), Hams F12 and RPMI 1640 were purchased from Gibco BRL (Carlsbad, CA).

Fetal Calf Serum (FCS) was from CSL (Parkville, Australia). FCS was heat inactivated prior to use.

Trypan Blue was from Sigma.

Trypsin/EDTA was from Gibco BRL (Carlsbad, CA).

Phosphate Buffered Saline (PBS): 136mM NaCl, 2.6mM KCl, 1.5mM KH_2PO_4 and 8mM Na_2HPO_4 (pH7.4). The solution was sterilized by autoclaving.

b) Flasks and plates for culture

Cell culture flasks: 25 cm^2, 75 cm^2 and 175 cm^2 flasks were purchased from BD Biosciences (Franklin Lakes, NJ).

Culture plates: 24-well plates, as well as Petri dishes (10mm, 60mm and 100mm) were from BD Biosciences (Franklin Lakes, NJ).

2.1.12 Miscellaneous

X-ray film: Agfa (Mortsel, Belgium).

2.2 ISOLATION AND ANALYSIS OF mRNA

2.2.1 Isolation of total RNA from cell cultures

Total RNA was extracted from UMR 106.06 and ROS 17/2.8 osteoblast cells using the commercially available TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, cultures were grown in 24 well plates to 80% confluency and 4 wells were pooled for each RNA extraction. To harvest the cell lyates, the media was carefully removed from each well and washed with PBS prior to cell lysis by the addition of 250μL of TRIzol per well. The Trizol solution was then collected and samples were incubated at room temperature for 5 minutes to allow dissociation of nucleoprotein complexes. 200μL of chloroform was added per ml of Trizol and shaken vigorously by hand. Following centrifugation for 15 minutes at 4°C, the aqueous phase was transferred to a clean tube, 500μL isopropanol and

20ug glycogen (Ultrapure, Invitrogen, Carlsbad, CA) was added to each tube and stored overnight at -80°C. RNA was then precipitated by centrifugation at 4°C, followed by washing with 75% ethanol, re-centrifugtion, and resuspension in 15µL dH_2O. The quantity and purity of RNA was determined by using a spectrophotometer (Model DU 530, Beckman, Fullerton, CA).

2.2.2 First strand cDNA synthesis

RNA was reverse-transcribed from 5ug of total RNA from each sample using a cDNA synthesis kit to generate first strand cDNA as per the manufacturer's instructions (Superscript III, Promega Corp, Madison, WI). A reaction mixture of 5µg of RNA, 200ng oligo-dT primer, 1µL dNTP mix (Geneworks, Australia) and deionised H_2O up to a total volume of 12µL was incubated at 65°C for 5 minutes, and then immediately cooled on ice. Reaction buffer (250mM Tris-HCL, pH 8.3, 375mM potassium chloride, 15mM $MgCl_2$), 10nM DTT and Superscript III reverse-transcriptase enzyme were then added to each sample and heated at 50°C for 60 minutes followed by 70°C for 5 minutes.

2.2.3 Real time polymerase chain reaction conditions

All PCR reactions were carried out in a final volume of 25 µl and were performed in duplicate for each cDNA standard in the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The reaction mix consisted of 12.5µL Universal Master Mix (1x TaqMan buffer, 5.5mM MgCl2, 200 µM dNTPs, 0.01 unit/µl AmpErase UNG, 0.05 unit/µl AmliTaq Gold) (PE Applied Biosystems,

Foster City, CA), 200nM probe) (PE Applied Biosystems, Foster City, CA) and 300nM each of the forward and reverse primers (Geneworks, Australia). The PCR condition was 50°C for 2 minutes and 95°C for 10 minutes, which was followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

2.3 RECOMBINANT DNA METHODS

2.3.1 General

The following methods were performed essentially as described in "Molecular Cloning: A laboratory manual" (Sambrook *et al.*, 1989): Growth, maintenance and preservation of bacteria; preparations and quantifications of DNA and RNA; restriction enzyme digestion and ligation; agarose and polyacrylamide gel electrophoresis; end-filled labelling of DNA fragments using the Klenow fragment of DNA polymerase and autoradiography. All manipulations involving viable organisms that contained recombinant DNA were carried out in accordance with the regulations of the Australian Academy of Science Committee on recombinant DNA, the University Council of the University of Adelaide and the Institute of Medical and Veterinary Science Institutional Biosafety Committee.

2.3.2 Synthesis of the pGL3 CYP27B1-luciferase reporter construct

The human *CYP27B1* gene 5'-flanking region deletion constructs have been previously described (Dwivedi et al., 2005; Gao et al., 2002). Briefly, -1501 base pairs (bp) of 5'-flanking sequence of the human *CYP27B1* gene together with the first

44 bp of the 5'-untranslated region of the *CYP27B1* gene were inserted upstream of the firefly luciferase cDNA coding sequence. This fragment was then cloned into the pGL3-basic vector (Promega, Madison, WI) using KpnI and XhoI restriction endonucleases (Figure 2.1). Deletions of the 5'-flanking region of length -1306, -1200, -1100, -997, -531 and -305 base pairs were then made using appropriate restriction enzymes. These constructs were designated pCYP27B1(-1501)-Luc, pCYP27B1(-1306)-Luc, pCYP27B1(-1200)-Luc, pCYP27B1(-1100)-Luc, pCYP27B1(-997)-Luc, pCYP27B1(-531)-Luc and pCYP27B1(-305)-Luc.

2.3.3 Small scale miniprep plasmid preparation

For analytical restriction digestion and sequencing, preparation of plasmid DNA was performed using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) to isolate DNA from 2ml of overnight bacterial culture.

2.3.4 Large scale plasmid preparation

Preparation of plasmid DNA for transfection of tissue culture cell lines, was obtained from either a plasmid preparation kit from Qiagen (Valencia, CA), or by cesium-chloride (CsCl) density gradient centrifugation.

Isolation of up to 100μg plasmid DNA was performed using the Qiagen midi-preparation kit according to the manufacturer's instructions. Larger amounts of plasmid DNA used for the transfection of tissue culture cell lines was routinely obtained using a modified version of (Sambrook *et al.*, 1989). 20ml of desired clone

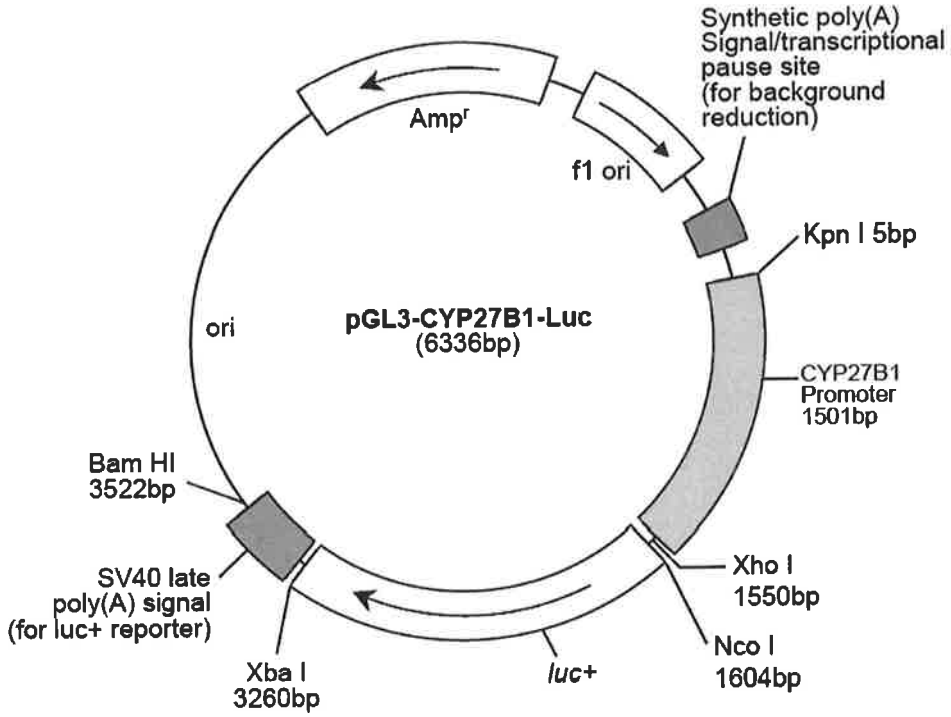

Figure 2.1: Schematic illustration of the pGL3-Basic-CYP27B1(-1501)-Luciferase (pCYP27B1(-1501)-Luc) reporter construct. -1501 base pairs (bp) of 5'-flanking sequence of the human *CYP27B1* gene together with the first 44 bp of the 5'-untranslated region of the *CYP27B1* gene were inserted upstream of the firefly luciferase cDNA coding sequence. This fragment was then cloned into the pGL3-basic vector. *luc*+: cDNA encoding the firefly luciferase; Amp^r:gene conferring ampicillin resistance in *E. coli*; f1 ori: origin of replication derived from filamentous phage; ori: origin of replication in *E. coli*. Arrows within *luc*+ and Amp^r indicate direction of transcription. The arrow within the f1 ori indicates the direction of single strand cDNA synthesis.

culture was inoculated into 250ml of LB medium with appropriate antibiotics (Ampicillin) and incubated at 37°C with shaking. The bacterial cells were pelleted at 4°C in a SS-34 rotor at 6000 rpm for 5 minutes. Plasmid DNA was extracted using the alkaline lysis procedure: 10ml of freshly prepared GTE buffer with lysozyme (2mg/ml) was used to lyse the cells on ice for 20 minutes; 20ml of freshly prepared Solution II (0.2M NaOH, 1% SDS) was added and mixed with the cell lysate by inverting the tubes and then 15ml of NaAc (3M, pH 4.6) was added, mixed and left on ice for 10 minutes. The supernatant was collected and filtered through a cloth lined funnel and the volume was recorded in a measuring cylinder. Plasmid DNA was precipitated by the addition of 0.6 volumes of isopropanol and incubated on ice for 10 minutes. The plasmid DNA was pelleted at 9000 rpm for 20 minutes and the supernatant discarded. The pelleted plasmid DNA was allowed to air dry and resuspended in 4ml of 1×TE. To separate plasmid DNA from RNA and genomic DNA, the plasmid was further purified by the CsCl/ethidium bromide density gradient procedure. 4.2g of CsCl was mixed with the dissolved plasmid DNA by vortexing thoroughly and 100µl of 10mg/ml ethidium bromide was added as the marker of plasmid DNA. The mixture was then centrifuged in a Beckman TL-100 benchtop ultracentrifuge and TLA-100.2 rotor at 80 000rpm at 20°C overnight. Plasmid DNA with ethidium bromide (the lower most central red band) was obtained using syringe and needles. The ethidium bromide was removed by butanol extraction. The plasmid DNA was then precipitated with 2.5 volumes of 100% ethanol, rinsed with 70% ethanol, resuspended in 1xTE buffer, and quantified by spectrophotometry and analysed by agarose gel electrophoresis to confirm concentration and supercoiling.

2.3.5 Quantification of DNA

The concentration of plasmid DNA was quantified by measuring the absorbance at 260nm on a DU530 spectrophotometer (Beckman Instruments, Fullerton, CA). DNA samples were diluted by adding 1μL of the plasmid DNA to 99μL of H_2O and absorbancy was then measured at a wavelength of 260nm. DNA concentration was calculated with the following formula:

DNA concentration (μg/μL) = Absorbance at 260nm x 50/1000 x 100 (dilution factor).

2.3.6 Restriction enzyme digestion of DNA

For analytical restriction digests, approximately 1μg of DNA was incubated with 1 unit each of the restriction enzyme(s) in the appropriate buffer suggested by the manufacturer for a minimum of 1 hour or overnight. The digestion was terminated by the addition of 1/6 volumes of urea DNA loading buffer. Separation of digested DNA was performed on a 2% Agarose gel in 1xTBE, then visualised by staining with 1ug/ml ethidium bromide and photographed.

2.3.7 Site-directed mutagenesis

All mutagenesis of reporter constructs was performed by the current investigator using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) with primer design and temperature cycling according to the manufacturer's instructions.

Primer design is shown in 2.1.7. Briefly, the Quickchange™ method employs two complementary oligonucleotide primers containing the desired mutation, that are complimentary to opposite strands of the vector to be mutated. The oligonucleotides are extended by the action of Pfu polymerase (2.5u/μl) in a cycling program (95°C for 30 seconds, 55°C for 1 minute and 68°C for 11 minutes for 18 cycles). Incorporation of the oligonucleotides results in a nicked vector that is unmethylated. The end product is then digested with DpnI (10U/μl), an endonuclease sensitive to methylated and hemi-methylated DNA, to remove the parental (non-mutated) DNA. Following digestion, the *CYP27B1* reporter constructs were transformed into competent DH5α cells. Small-scale plasmid purification was then performed on the recovered transformants and subsequently sequenced to confirm incorporation of the correct mutation.

2.3.8 Dye-terminator sequencing of PCR products

DNA sequencing was carried out using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Boston, MA). In PCR tubes, a 20μl mixture was set up containing 0.5μg of plasmid DNA, 1μl (100ng) of sequencing primer and 8μl of Big Dye reaction buffer. The mixture was then overlaid with 20μl of mineral oil to prevent evaporation during the following PCR: 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes in a PTC-100™ programmable thermal controller. To precipitate the extension products, 80μl of freshly prepared 75% isopropanol was added and left at room temperature for 1 hour. Samples were then pelleted by centrifugation at maximum speed for 20 minutes, and washed with 250μl of 75% isopropanol once. The samples were dried in a 37°C

heating block and delivered to the Division of Molecular Pathology, Institute of Medical And Veterinary Science for Big Dye sequencing analysis using an ABI Prism 3700 DNA Analyser.

2.3.9 Preparation of competent *E.coli* cells

E.coli cells were made competent by the calcium chloride method described in "Molecular Cloning" (Sambrook *et al.*, 1989). Briefly a single *E.coli* colony, obtained by streaking on the LB plate, was inoculated in 2ml of LB broth and grown at 37°C. The overnight culture was then subcultured into 200ml of warm LB broth and further grown with continuous shaking until an OD_{600} of 0.4-0.6. The cells were then placed on ice for 5 minutes and centrifuged in a HB-4 rotor at 4000rpm for 5 minutes at 4°C. The cells were resuspended in 80ml of Tfb1 (see 2.1.5) and kept on ice for a 5 minutes. Following centrifugation, the cells were resuspended in 8ml of Tfb2 (see 2.1.5) and placed on ice for a further 15 minutes. Aliquots of the competent cells were stored at -80°C until use.

2.3.10 Transformation of plasmids into competent bacteria

An aliquot of competent cells was thawed on ice. Approximately 100ng of plasmid DNA was added to 50µl of competent cells and incubated on ice for 30minutes. After heat shock at 42°C for 2 minutes, the cells were chilled on ice for 5 minutes, diluted with 500µl of LB medium and then grown at 37°C for 30 minutes. The cells were then pelleted by centrifugation for 30 seconds and resuspended in 150µl of LB and

then plated onto an L-agar plate containing Ampicillin (100μg/ml) and incubated at 37°C overnight.

2.4 METHODS FOR THE MAINTENANCE AND TRANSIENT TRANSFECTION OF CELL LINES

2.4.1 Maintenance of Cell lines

All cell lines were maintained in DMEM + 10% FCS, with the exception of ROS 17/2.8 which was maintained in 45% DMEM + 45% Hams F12 + 10% FCS. Cells were grown at 37°C in humidified air containing 5% CO_2. Cells were routinely passaged when they reached a confluency of 80-90%. Firstly, the attached cell layer was briefly washed with PBS to remove cell debris and old media. Cells were then dislodged by adding trypsin (1ml to a 75cm^2 flask) to the cell layer and incubating the flask at 37°C for 1-5 minutes depending on the cell line. The reaction was then stopped by the addition of 10ml of appropriate media and the cell suspension was centrifuged for 2 minutes at 1500 rpm. The supernatant was aspirated and the cell pellet was resuspended in media and aliquoted appropriately.

2.4.2 Transient transfection of cell lines

In preparation for transfection, cells were counted on a haemacytometer and seeded into 24 well plates at 5×10^5 cells/ well in a volume 400 ul of appropriate media. Cells were allowed to attach for 5-6 hours at which time the media was removed and replaced with RPMI 1640 that contained no FCS. Transfections were performed in

triplicate and each transfection was performed using 200ng of *CYP27B1* reporter construct and also 50ng of the thymidine kinase (TK) promoter-*Renilla* luciferase (Promega, Madison, WI) as a control for transfection efficiency. Over expression plasmids (eg. Ski and Smad4) and dominant negative mutants (eg. Ets-1 and Ets-2) were cotransfected in some experiments, in which case the total amount of DNA transfected was kept constant with the use of the empty vector. All cell lines were transfected using DOTAP liposomal reagent (Roche, Indianapolis, IN) with the exception of MDA-MB-468, which was transfected using Fugene 6 reagent (Roche, Indianapolis, IN). For one DOTAP transfection, all plasmid DNA was diluted in Hepes buffer (20mM, pH 7.4) to a final volume of 5ul and mixed with 1.5μg of DOTAP also diluted in 5ul of Hepes buffer. The DNA-DOTAP mixture was then incubated for 20 minutes at room temperature before addition to the appropriate well. After overnight transfection, the media was removed and dual luciferase activity was assayed (section 2.3.3). For those experiments involving treatment with exogenous factors such as TGF-β, the media was replaced with fresh serum free RPMI 1640 immediately following the overnight transfection. Cells were then treated with PTH (1-34), $1,25(OH)_2D_3$, TGF-β, IGF-1 or vehicles for 24 hours before measuring luciferase activity. Transfection of MDA-MB-468 cells with Fugene 6 was performed according to the manufacturer's instructions. The ratio of Fugene 6 (μl): DNA (μg) in the transfection mixture was 3:1 and as with DOTAP, cells were incubated overnight to permit transfection of the cells.

2.4.3 Dual luciferase assay

At the completion of the incubation period, dual luciferase activity was determined using the Dual Luciferase Reporter Kit from Promega (Madison, WI), according to the manufacturer's instructions. Briefly, the media was aspirated and the cells were lysed using 50µl of 1×passive lysis buffer for 20-30 minutes at 25°C with shaking. Dual luciferase activity was measured using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Luminescence of the firefly luciferase was assayed by adding 25µl of LAR II substrate to 10µl of cell lysate in a 1.5ml microcentrifuge tube. This was followed by measurement of the *Renilla* luciferase by the addition of 25µl of Stop and Glo reagent to quench the first reaction and simultaneously initiate the *Renilla* luciferase reaction. Data are presented as relative luciferase activity, calculated by dividing the first luminescence value by the second luminescence value.

2.5 ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSAs)

EMSA is a technique for determining protein: DNA interactions. The experiment is performed by incubating nuclear proteins with [α-^{32}P]-labelled oligonucleotides that contain putative protein binding sites. The reaction products are then analysed on a non-denaturing polyacrylamide gel. The assay is based on the principle that DNA oligonucleotides that form complexes with proteins migrate more slowly through non-denaturing polyacrylamide gel than free (unbound) oligonucleotides.

2.5.1 Isolation of nuclear proteins

Nuclear proteins were isolated from ROS 17/2.8 cells by hypotonic lysis followed by high salt extraction. The method is adapted from Andrews and Faller (1991). Briefly, cells were grown in a $75cm^2$ flask to 70-80% confluence and then washed and harvested by gentle scraping into 1ml cold PBS. The cells were then centrifuged for 15 seconds at room temperature and the pellet was resuspended with 400µl of cold Lysis Buffer I by gently flicking. The protease inhibitor phenylmethylsulphonylfluoride (PMSF) at 0.2mM was added to the suspension which was then incubated on ice for 15 minutes for hypotonic lysis. The cells were pelleted and then resuspended in 100µl of Lysis Buffer II and incubated on ice for 30 minutes for high salt extraction. Cellular debris was removed by centrifugation and the supernatant collected. The protein concentration of the supernatant was quantified by Bradford Assay (section 2.4.2). The nuclear proteins were then stored at –80°C in aliquots to avoid repeated freeze/ thaw events.

2.5.2 Preparation of recombinant Smad4 protein

The pGEX-4T-1-Smad4(GST) plasmid was transformed into the *E.coli* strain BL21 Star™(DE3)pLysS (Invitrogen, Carlsbad, CA) according to the supplier's instructions. A single colony was then used to inoculate 50ml of LB containing ampicillin and chloramphenicol and grown at 37°C overnight. The OD_{600} of the culture was measured and an aliquot was used to seed 500ml of LB plus ampicillin at an OD_{600} of 0.1. The culture was grown at 37°C with shaking until the OD_{600} reached 0.5. At this point the culture was induced using isopropyl-beta-D-

thiogalactopyranoside (IPTG) at 0.5mM and then grown at 30°C for a further 3 hours. To harvest, the culture was centrifuged at 6000 rpm for 10 minutes at 4°C. The pellet was then resuspended in 15ml 1 X PBS to which 150ul of 100mg/ml lysozyme had been added, and stored overnight at -80°C. The following day, the suspension was thawed on ice and 1% TritonX-100 and PMSF at 1mM were added. Cells were lysed by sonication (3 X 30 seconds, 30 second intervals) in a MiSonix XL2007 sonicator (Farmingdale, NY), and the solution was centrifuged at 12 000 rpm for 30 minutes at 4°C. The supernatant was then mixed with 1ml of glutathione Sepharose solution (Scientifix, Cheltenham, Australia) prewashed to remove ethanol. The protein-glutathione Sepharose solution was incubated on a rocking platform for 2 hours at 4°C to permit binding of the GST-tagged protein to the beads. The solution was applied to a column where it was washed with 100ml 1 X PBS. The protein was then eluted at room temperature using freshly prepared reduced glutathione (30mM in 50mM Tris, pH 8.0) in three 500ul fractions. Eluates were stored at 4°C and the presence of GST-Smad4 was confirmed by SDS-PAGE.

2.5.3 Protein analysis by SDS-PAGE

SDS-PAGE is a method whereby proteins are denatured with sodium dodecyl sulphate (SDS) and then separated by size by polyacrylamide gel electrophoresis (PAGE). During preparation of GST-Smad4, aliquots of solution were preserved in order to analyse the protein induction at specific stages. These samples, as well as an aliquot of the final eluate were added to loading buffer (10% SDS, 10mM diothiothreitol, 20% glycerol, 0.2M Tris-HCl, pH 6.8, 0.05% bromophenol blue) and denatured at 100°C for 5 minutes. A discontinuous buffer system was used whereby

the denatured samples were electrophoresed through a 4% stacking gel (60V for 30 minutes) and an 8% resolving gel (120V for 60 minutes) in order to achieve high resolution. The gel was then fixed at room temperature using an isopropanol fixing solution (25% isopropanol (v/v), 10% acetic acid (v/v) and 65% H_20) and transferred to a Coomassie blue staining solution (10% acetic acid (v/v), 0.006% (w/v) Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA) and 90% H_20). The gel was stained for 2 hours at room temperature before destaining in a solution of 10% acetic acid (v/v) until a clear background was seen. The gel was imaged using a PowerLook scanner (UMAX, Dallas, TX). Resolved proteins are shown in Figure 2.2.

2.5.4 Bradford Assay of protein concentration

The concentration of the nuclear proteins extracted from ROS 17/2.8 cells and recombinant Smad4 protein was estimated using 2ul of protein solution using the protein microassay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Bovine serum albumin (BSA) at 1μg/μl was used as the protein standard, and OD_{600} was measured in an E max microplate reader (Molecular Devices, Sunnyvale, CA).

2.5.5 Annealing single stranded oligonucleotides

Sense and antisense oligomers (2.5ug) (sequences shown in section 2.1.7) were annealed in a 20μl volume using 4ul of 5x annealing buffer. The oligomers were heated at 95°C for 2 minutes and transferred to a 65°C heating block. The block was then removed from its heat source and allowed to cool gradually to room temperature.

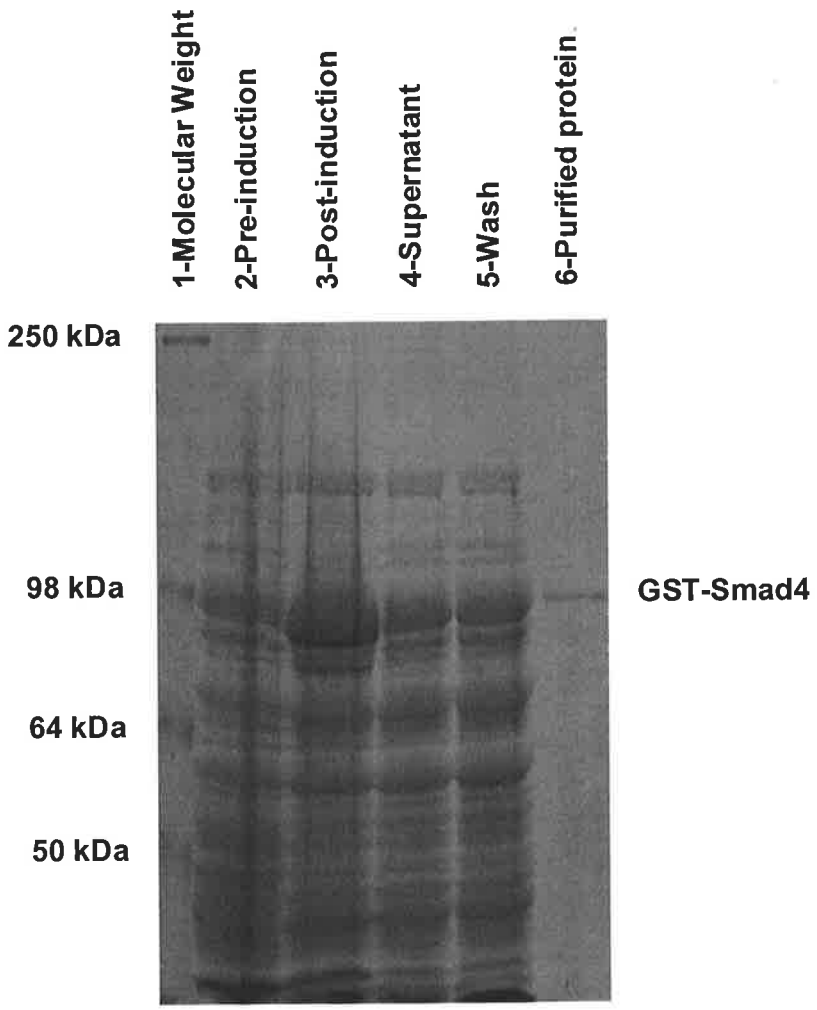

Figure 2.2: SDS-PAGE analysis of GST-Smad4 purification. Lane1: molecular weight marker; Lane 2: total bacterial proteins prior to induction; Lane 3: bacterial proteins following induction of pGEX-4T1-Smad4(GST) with isopropyl-beta-D-thiogalactopyranoside (IPTG); Lane 4: proteins in the supernatant applied to the Sepharose column; Lane 5: proteins that washed through the column without binding the Sepharose beads; Lane 6: purified GST-Smad4 eluted from the Sepharose beads has a similar mobility (size) to the 98 kDa molecular weight marker as expected.

2.5.6 End-filling of oligonucleotides

The annealed oligomers were end-fill labelled with [α-^{32}P] dCTP using Klenow fragment. The reaction contained 1.5μl of 10× Klenow buffer, 2 units of Klenow fragments (2U/ul), 9ul H_2O and 25uCi [α-^{32}P] dCTP. The reaction was incubated at room temperature for 30 minutes. Labelled probes were then precipitated with 1ug of poly (dI-dC), 2ul of 3M NaAc (pH 4.6) and 40ul of 100% on dry ice for 15 minutes. The probes were pelleted by spinning at maximum speed for 20 minutes and rinsed once with 50ul of 70% ethanol. Once air-dried, the probes were dissolved in 10ul of 1x TE buffer and 1ul was used to measure radioactivity in a 1214 Rackbeta Liquid Scintillation Counter (LKB Wallac, Turku, Finland).

2.5.7 Binding reaction and electrophoresis

Binding reactions contained 4ul (10ug) of protein and 10ul of Dignam Buffer C and were incubated on ice for 15 minutes. The appropriate labelled probe (100 000 cpm) was then added and the reaction further incubated on ice for 30 minutes. Retarded protein-DNA complexes were resolved on a 4% non-denaturing polyacrylamide gel in a low ionic strength running buffer (0.5× TBE) and electrophoresed for 80 minutes at 250 volts. The gel was subsequently dried using a GD2000 slab gel dryer (Hoefer, San Francisco, CA) and exposed to X-ray film in an intensifying screen at –80°C.

EMSA competition binding assays were performed using unlabelled oligonucleotide probe at 10X and 50X molar excess in the binding reaction prior to addition of the labelled probe. Similarly, 1-5ul of antibody was included in the binding reaction in

some experiments. In these experiments binding was tested both on ice and at room temperature, and longer incubation periods were tried.

2.5.8 Presentation of data and statistical analysis

The data obtained from reporter gene assays are presented as mean (n=3) luciferase ratios (firefly to *Renilla* luciferase). In each case the data shown are representative of at least 3 independent experiments. One-way analyses of variance were performed on the data presented in Figure 5.1 (Effect of exogenous factors on expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells). A Tukey's post-hoc test was then performed to determine significant differences between treatment groups and the control group.

Chapter 3:

Basal Expression of the Human CYP27B1 Gene 5'-Flanking Region in ROS 17/2.8 Cells

3.1 INTRODUCTION

Production of $1,25(OH)_2D_3$ is catalysed by the enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (CYP27B1), with the majority of circulating $1,25(OH)_2D_3$ arising from CYP27B1 activity in the kidney as described earlier in chapter 1. Renal *CYP27B1* expression is regulated by physiological factors, notably parathyroid hormone (PTH) and $1,25(OH)_2D_3$ (Chapter 1.4.1). PTH acts in a positive fashion through cAMP to increase transcription of the gene (Armbrecht *et al.*, 2003b). On the other hand $1,25(OH)_2D_3$ down regulates expression of the gene using VDR and a novel mechanism (Murayama *et al.*, 2004). There has been considerable interest in understanding the molecular aspects of these hormone actions and this has led to the identification of the transcriptional control elements involved. With PTH, there is evidence that in kidney cells, control elements in the early *CYP27B1* promoter contribute both to basal expression and to PTH induced expression (Gao *et al.*, 2002). These elements are a GC rich sequence which most likely binds Sp1 and a CCAAT box whose binding protein in kidney cells has not yet been established (Gao *et al.*, 2002). Other studies have identified CRE-like regulatory sequences that also contribute to PTH induction (Flanagan *et al.*, 2003).

CYP27B1 enzyme activity has also been identified in many non-renal cells, one of the first being osteoblasts some twenty-six years ago (Turner *et al.*, 1980b), and more recently prostate cells (Schwartz *et al.*, 1998). These non-renal activities are likely to play a role in the local production of $1,25(OH)_2D_3$ for cellular needs rather than contributing to circulating levels (Dusso *et al.*, 2005). What determines the level of expression of the *CYP27B1* gene in the different cell types is an important and interesting question.

There is evidence that basal regulation of the *CYP27B1* gene differs between cell types based on studies in transfected kidney and prostate cells. These studies used deletion constructs of the 5'-flanking region of the *CYP27B1* gene, as described in Chapter 1 (basal expression here is described as the expression in the cell lines in the absence of added hormones, growth factors etc). Particular differences identified in the transfected prostate cells but not in the kidney cells, were the presence of a stronger enhancer region beyond the proximal promoter and an upstream repressive region (Dwivedi et al., 2005; Gao et al., 2002). Of note were the presence of three functional Ets protein binding sites (EBS) in the enhancer region that contributed to the basal expression in the prostate cells (Dwivedi *et al.*, 2005). The findings strongly suggested that there is tissue specific regulation of the *CYP27B1* gene.

At present, there is little known about the regulation of the *CYP27B1* gene in osteoblast cells. The precise regions of the 5'-flanking sequence and the transcriptional control elements that are important for expression of the gene remain unknown. It seems reasonable to predict that basal regulation of the *CYP27B1* gene will differ in osteoblasts compared with kidney and prostate cells. The local

production of $1,25(OH)_2D_3$ may be critical for osteoblast and osteoclast development as described in Chapter 1.5.2. The overall aim of this chapter was to investigate basal expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells and initiate studies into the identification of control elements. The findings have then been compared with the previous data from transfected prostate and kidney cell lines.

3.2 SPECIFIC AIMS AND HYPOTHESES

3.2.1 Specific aims

1) The first aim was to identify a well characterised osteoblast cell line that is readily transfected and a significant level of endogenous mRNA for *CYP27B1* is expressed.

2) The second aim was to express plasmid constructs containing different lengths of the *CYP27B1* gene 5'-flanking region fused to a luciferase gene reporter in the selected osteoblast cell line, to allow comparisons to be drawn between expression patterns in osteoblast, kidney and prostate cell lines. Constructs would also be examined where the known transcription factor binding sites (GC, CCAAT and EBSs) are inactivated by mutagenesis to determine if they are important for basal expression in the osteoblast cell line.

3.2.2 Specific hypothesis

It is hypothesised that the expression pattern of the *CYP27B1* 5'-flanking region is tissue specific i.e. the expression pattern in osteoblasts will be different to that previously described in prostate and kidney cell lines.

3.3 OUTLINE OF METHODS USED IN THIS CHAPTER (Detailed descriptions are given in Chapter 2).

3.3.1 Measurement of endogenous *CYP27B1* mRNA in osteoblast cell lines

UMR 106.06 and ROS 17/2.8 osteoblast-like cells were cultured for 24 hours before total RNA was isolated using the commercially available TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was quantified by measuring absorbance at 260/280 nm and reverse transcribed to generate first strand cDNA. *CYP27B1* mRNA was quantified by Real-time RT-PCR using specific primers and Taqman fluorogenic probes (Perkin Elmer, CA, USA) (see Methods 2.2).

3.3.2 Preparation of human *CYP27B1* gene 5'-flanking region deletion constructs

The deletion constructs used in this study were prepared by Dr. Xiu-Hui Gao and Dr. Prem Dwivedi and have been previously described (Dwivedi et al., 2005; Gao et al., 2002). Briefly -1501 base pairs (bp) of 5'-flanking sequence of the human *CYP27B1* gene together with the first 44 bp of the 5'-untranslated region of the *CYP27B1* gene

were inserted upstream of the firefly luciferase cDNA coding sequence. This fragment was then cloned into the pGL3-basic vector. Deletions of the flanking region of length -1306, -1200, -1100, -997, -531 and -305 base pairs were then made using appropriate restriction enzymes. These constructs were designated pCYP27B1(-1501)-Luc, pCYP27B1(-1306)-Luc, pCYP27B1(-1200)-Luc, pCYP27B1(-1100)-Luc, pCYP27B1(-997)-Luc, pCYP27B1(-531)-Luc and pCYP27B1(-305)-Luc.

3.3.3 Mutagenesis of the GC box and CCAAT box

Site-directed mutagenesis of the GC box, CCAAT box, and Ets protein binding sites within the pCYP27B1(-997)-Luc construct was carried out using the QuikChange Site Directed Mutagenesis protocol (Stratagene, La Jolla, USA). Wild type and mutated sequences (shown in bold) were as follows, with the core binding sequence underlined:

GC box

Wild type 5' GAAGTCAGCCCCAGCCCCGCCTACTGTTC 3' (-143/-115)

Mutant 5' GAAGTCAGCC**AAAGCTTT**GCCTACTGTTC 3'

CCAAT box

Wild type 5' CAGGAGGAGGGATTGGCTGAGGAGCTTG 3' (-85/-58)

Mutant 5' CAGGAGGAGGGA**GCTC**CTGAGGAGCTTG 3'

Ets binding site I (EBS I)

Wild type 5'-GAGCCACCACAGGATTCTGAGGC-3' (-812/-790)

Mutant 5'-GAGCCACCACA**TTAT**TCTGAGGC-3'

Ets binding site II (EBS II)

Wild type 5'-CCTCATGCCATCCTCCTGCCTCAG-3' (-864/-841)

Mutant 5'-CCTCATGCC**AAAC**TCCTGCCTCAG-3'

Ets binding site III (EBS III)

Wild type 5'-CCCAAAGTGTTGGGATTACAGTCATG-3' (-973/ -948)

Mutant 5'-CCCAAAGTGTTG**TTAT**TACAGTCATG-3'

3.3.4 Transient expression of reporter constructs

Transfection of the osteoblast cells was performed with DOTAP transfection reagent according to the manufacturer's instructions. Briefly, osteoblast cell lines were seeded into 24 well plates and left 6 hours to attach. Cells were transfected overnight with a reporter plasmid in serum free media. A thymidine kinase (TK) promoter-*Renilla* luciferase was included to control for transfection efficiency. The following day, cells were lysed and dual luciferase activity was measured.

3.3.5 Ets protein expression clones

The expression clone for human Ets-1 (pEFBosEts-1) is based on the pEFBOS vector and has been described previously (Thomas *et al.*, 1995). The Ets-1 and Ets-2

dominant negative clones are detailed in Methods 2.1.6, and have been described previously (Yang *et al.*, 1996). These clones were co-expressed with the pCYP27B1(-997)-Luc reporter plasmid with the amount of DNA kept constant in all wells using the empty vector.

3.4 RESULTS

3.4.1 Choice of osteoblast cell line in which to study the regulation of the CYP27B1 gene

In order to investigate the mechanisms of *CYP27B1* gene regulation in osteoblasts, it was first necessary to choose an appropriate model cell line. The required cell line needed to have been previously characterised, to be readily transfected and to express endogenous *CYP27B1* mRNA. The rat osteoblast-like cell lines ROS 17/2.8 and UMR 106.06 were considered good candidates. The characteristics of these cell lines have been reviewed (Kartsogiannis and Ng, 2004). Each cell line is capable of forming a calcified matrix under specific culture conditions (Kartsogiannis and Ng, 2004) and as such are described as osteoblast-cell like. They are likely to represent different stages of osteoblast maturity and as such were of interest since *CYP27B1* gene expression may be regulated differently.in each. Both cell lines have been utilised extensively in our laboratory for high expression of gene promoters such as that for rat *CYP24* fused to the fire fly *luciferase* gene. The use of rodent cell lines to study a human promoter was considered a valid approach as a transgenic mouse model has previously been used to investigate *in vivo* regulation of the human *CYP27B1* promoter (Hendrix *et al.*, 2005). Endogenous *CYP27B1* mRNA was first

examined in ROS 17/2.8 and UMR 106.06 cells. Total mRNA was extracted from each cell line after culturing cells overnight in the absence of serum and the specific *CYP27B1* mRNA was quantified by real-time RT-PCR (Methods 3.3.1). Both ROS 17/2.8 and UMR 106.06 expressed similar levels of *CYP27B1* mRNA expressed as *CYP27B1* copy number/ug total RNA (Figure 3.1).

Therefore each cell line was transiently transfected with constructs containing various lengths of the -1501 base pair 5'-flanking region of the *CYP27B1* gene fused to the firefly *luciferase* gene as a reporter. A dual luciferase assay was employed that utilised a PRL-TK directed *Renilla* luciferase as an internal control to correct for transfection efficiency (Methods 3.3.4). The plasmids employed contained different lengths of this 5'-flanking region together with the first 44 base pairs of the 5'-untranslated region fused to the firefly *luciferase* gene (Methods 3.3.2).

Overnight transfection of the cells was performed with DOTAP transfectant (Methods 3.3.4). In preliminary experiments, the effect of 10% fetal calf serum was investigated on the expression of the pCYP27B1(-1501)-Luc construct. Surprisingly, serum was found to routinely inhibit expression by about 80%, not only of this plasmid but also of the internal control plasmid, indicating a general inhibitive effect. The reason for this was not pursued. In all of the transfection experiments to be now described, serum has been omitted from the media in which the cells were transfected (see Methods 3.3.4).

In repeated transfections, all of the deletion constructs expressed very weakly in UMR 106.06 cells and expression was not substantially greater than the promoter-less pGL3

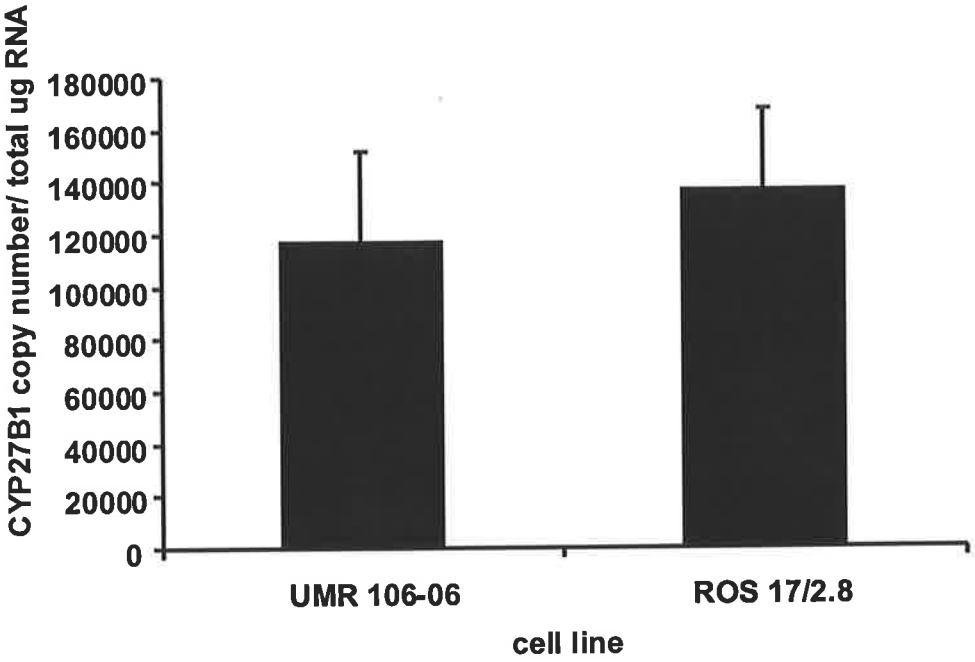

Figure 3.1: A comparison of *CYP27B1* mRNA expression in UMR 106-06 and ROS 17/2.8 cells. Cells were cultured overnight under basal conditions before total RNA was isolated from the cells. Specific *CYP27B1* mRNA was measured by real-time RT-PCR. Values are expressed as *CYP27B1* mRNA copy number/μg total RNA, and are the mean ± s.e.m. of triplicate samples.

vector (Figure 3.2). This result was unexpected because the first -1501 base pairs of flanking sequence is considered to contain all of the upstream elements necessary for *CYP27B1* gene expression (Hendrix *et al.*, 2005). By contrast, transfection of the deletion constructs into ROS 17/2.8 cells yielded markedly higher levels of expression (Figure 3.3). Therefore subsequent experiments to study the regulation of the deletion constructs were performed in ROS 17/2.8 cells.

3.4.2 Deletion analysis of the *CYP27B1* gene 5' flanking region

Figure 3.3 shows that the first -305 base pairs of the 5'-flanking region is sufficient to drive significant expression relative to the promoter-less pGL3 vector. This region is designated the proximal promoter. The level of expression was unaltered when the length of 5'-flanking sequence was increased to -531 but was highest when extended to -997. Interestingly, when the length of transfected 5'-flanking region was extended to -1100 there was a marked overall inhibition of expression with the level suppressed below that of the -305 promoter. Expression remained repressed in 5'-flanking sequence lengths of -1200 and -1306 and was lowest when the entire 5'-flanking length of -1501 was investigated. Overall these data indicate that the 5'-flanking region of the *CYP27B1* gene comprises an upstream inhibitory region located primarily between –997 and –1100, an enhancer region between -531 and –997 and a proximal promoter within the first –305 base pairs. These regions are depicted in Figure 3.4.

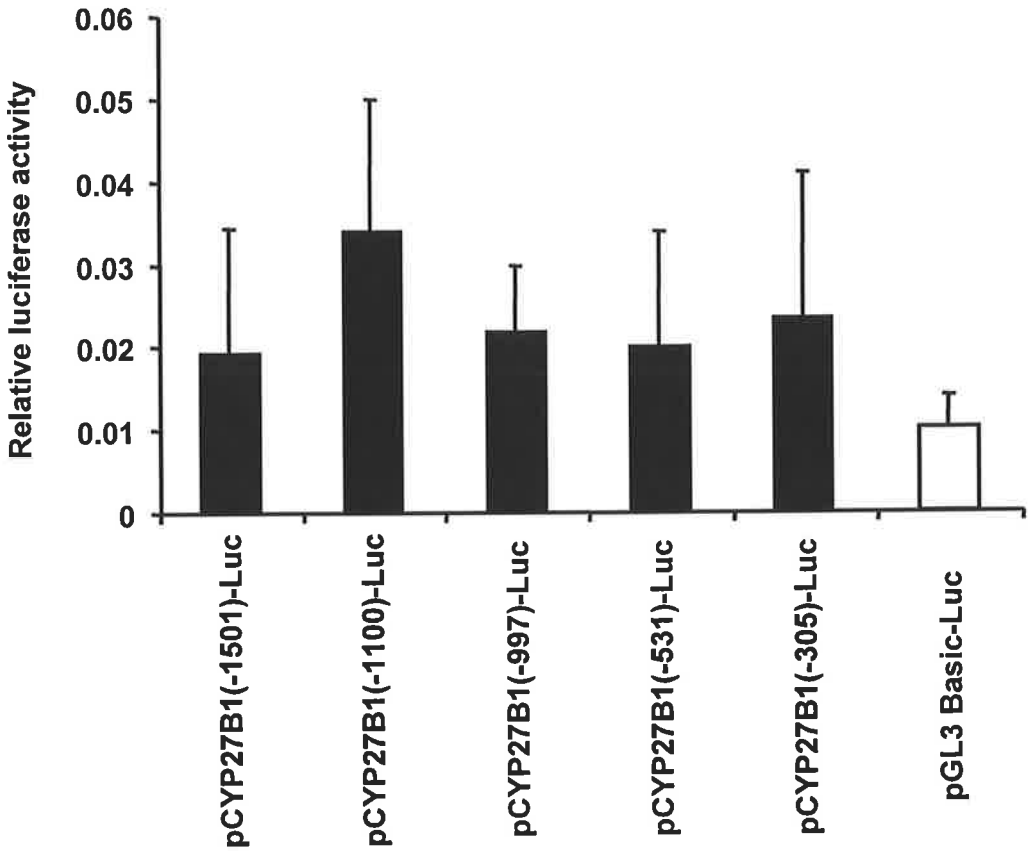

Figure 3.2: Expression of the 5'-flanking region of the human *CYP27B1* gene in UMR 106-06 cells. Deletion constructs containing -1501, -1100, -997, -531 and -305 bp of 5'-flanking region were transiently transfected into UMR 106-06 cells. For comparison the promoter-less pGL-3 Basic-Luc was also transfected. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

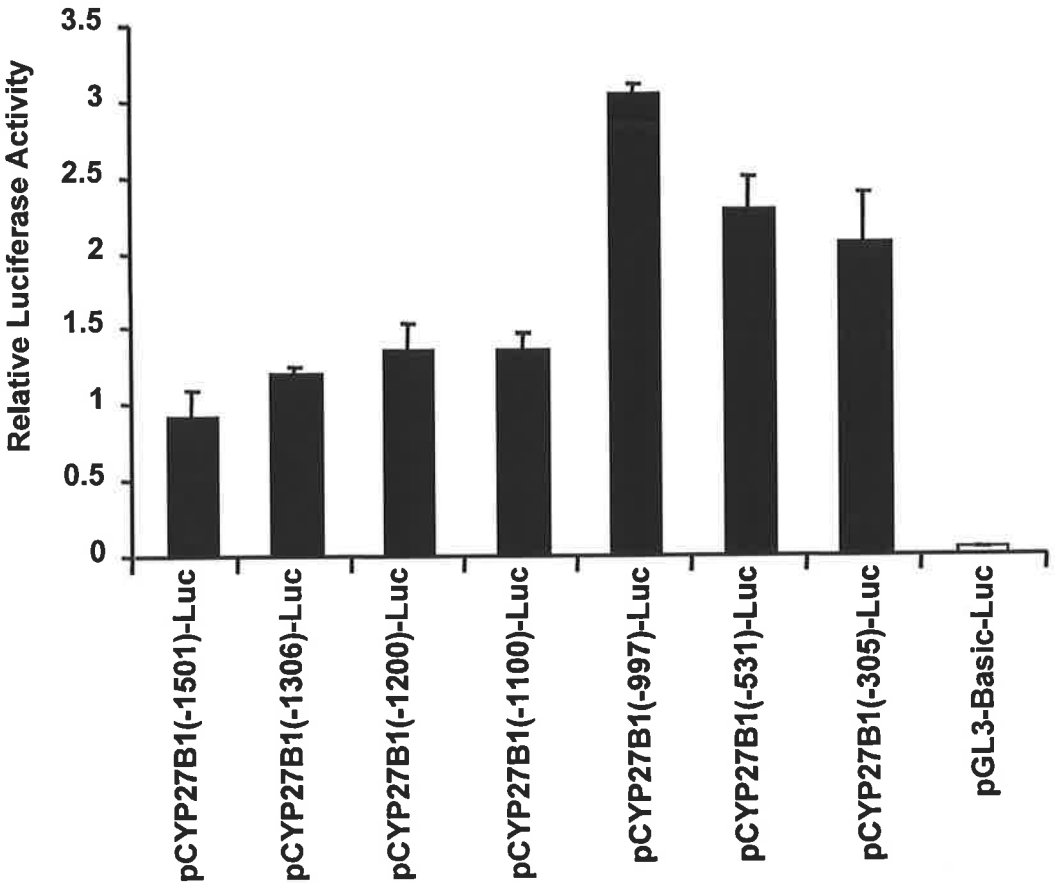

Figure 3.3: Expression of the 5'-flanking region of the human *CYP27B1* gene in ROS 17/2.8 cells. Deletion constructs containing -1501, -1100, -997, -531 and -305 bp of 5'-flanking region were transiently transfected into ROS 17/2.8 cells. For comparison the promoter-less pGL-3 Basic-Luc was also transfected. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

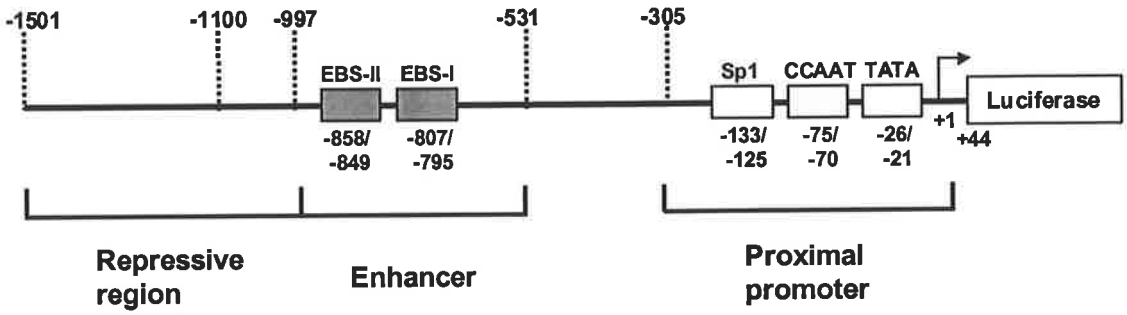

Figure 3.4: Illustration of the 5'-flanking region of the human *CYP27B1* gene (-1501 to +44). The location of transcription factor binding sites are indicated relative to the transcription initiation site of the gene.

3.4.3 Contribution of a putative Sp1 site (GC box) and CCAAT box within the proximal promoter

A number of possible transcriptional activator binding elements have been identified within the proximal promoter of the *CYP27B1* gene (Brenza et al., 1998; Ebert et al., 2004; Gao et al., 2002; Kong et al., 1999). In the kidney cell line AOK-B50, a GC-box that is an Sp1 binding site and a CCAAT box were demonstrated to be the most important contributors to expression of the proximal promoter (Gao *et al.*, 2002). To investigate whether these sites were functional in ROS 17/2.8 cells, the sites were inactivated by site directed mutagenesis within the -997 flanking sequence (which gave maximal basal expression) and tested following transient transfection. The specific mutations introduced are described in the Methods (3.3.3). The data show that the CCAAT box binding site and the GC box are critically important for expression of this construct in the osteoblast cells (Figure 3.5). Inactivation of the GC box lowered expression by approximately 80% and mutagenesis of the CCAAT box almost completely abolished expression.

The proteins that bind both the putative GC box and CCAAT box binding sites were not investigated in the present study. However earlier studies have shown that over expressed Sp1 acted on the promoter through the GC box in an insect cell line that lacks Sp proteins (Gao *et al.*, 2002). Therefore, it seems reasonable to assume that Sp1 present in bone cells will bind the GC rich sequence. There is no information from previous studies as to the possible identity of the functional protein that binds the CCAAT box in the present work.

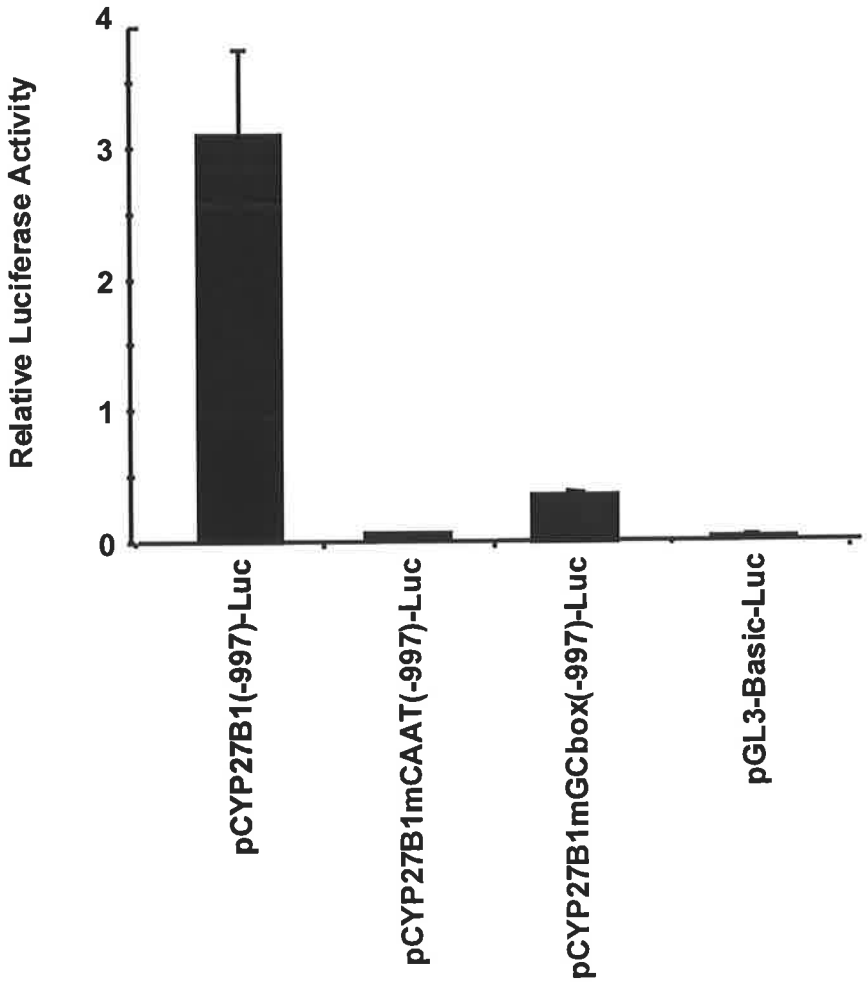

Figure 3.5: Mutational analysis of sites within the proximal promoter of the human *CYP27B1* gene in ROS 17/2.8 cells. The CAAT box and GC box were mutated in the pCYP27B1(-997)-Luc construct and transiently transfected into ROS 17/2.8 cells. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

3.4.4 The role Ets protein binding sites within the enhancer region

The enhancer region from –531 to –997 contains three possible Ets protein binding sites (EBSs) that contain a 5'-GGAT-3' core and have been previously identified (Dwivedi *et al.*, 2005). These sites designated as EBS-I (5'-CCACAGGATTCTGA-3'), EBS-II (5'-ATGCCATCCTCCTG-3') and EBS-III (5'-TGTTGGGATTACAGT-3') were inactivated by site directed mutagenesis within the pCYP27B1(-997)-Luc construct (see Methods 3.3.3). Mutagenesis of EBS-III was not observed to alter expression (data not shown) however EBS-I and EBS-II were shown to contribute. Figure 3.6 shows that EBS-I was most important with mutagenesis of this site lowering expression by approximately 60%. The expression seen following mutagenesis of this EBS was in fact less than that from the pCYP27B1(-305)-Luc construct. This was surprising and the reason is not clear. The mutation introduced does not generate any known inhibitory sequence.

The Ets family members Ets-1 and Ets-2 were considered possible transcription factors that may be acting through these EBSs as they have been reported as most highly expressed in a study of mouse osteoblasts. Ets-1 is highly expressed in proliferating osteoblasts whereas Ets-2 is only expressed in mature osteoblasts (Vary *et al.*, 2000). However over-expression of Ets-1 and Ets-2 dominant negative clones (see Methods 3.5.5) at 200, 500 and 1000ng had no effect upon expression of the pCYP27B1(-997)-Luc construct (Figure 3.7). Neither did over-expression of an Ets-1 expression clone at the same concentrations (Figure 3.8).

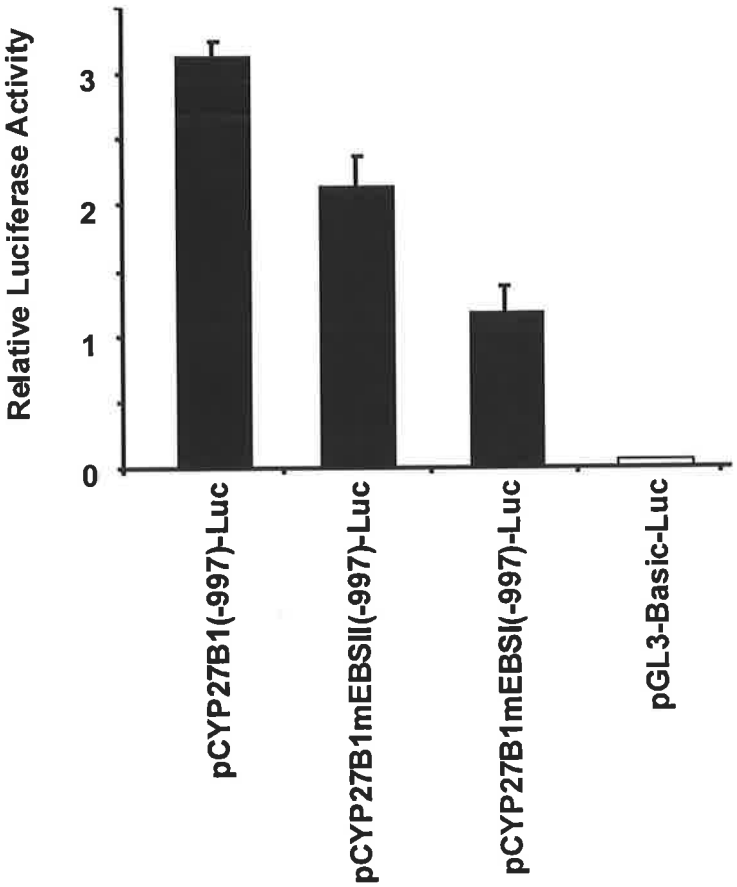

Figure 3.6: Mutational analysis of sites within the 5'-flanking region of the human *CYP27B1* gene in ROS 17/2.8 cells. Two Ets-1 protein binding sites (EBSI and EBSII) were mutated in the pCYP27B1(-997)-Luc construct and transiently transfected into ROS 17/2.8 cells. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

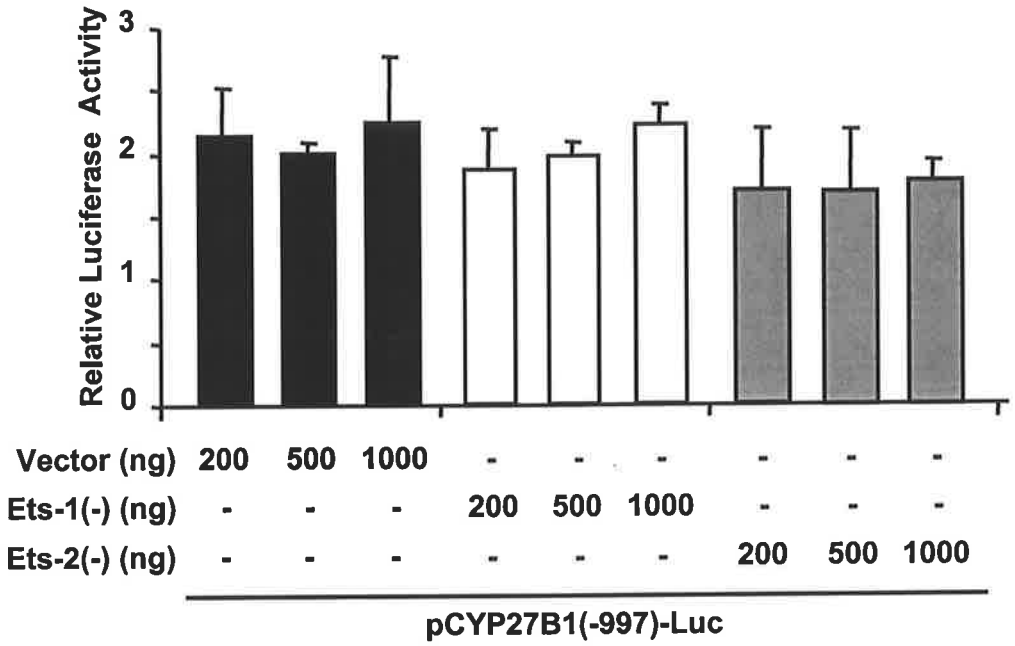

Figure 3.7: Effect of exogenously expressed Ets-1 and Ets-2 dominant negatives on the pCYP27B1(-997)-Luc in ROS 17/2.8 cells. The Ets-1 and Ets-2 dominant negative clones pcMV(Ets1T38A) and pcMV(Ets2T72A) (200, 500 and 1000ng), or empty vector were transiently transfected into ROS 17/2.8 cells along with the construct. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments experiments.

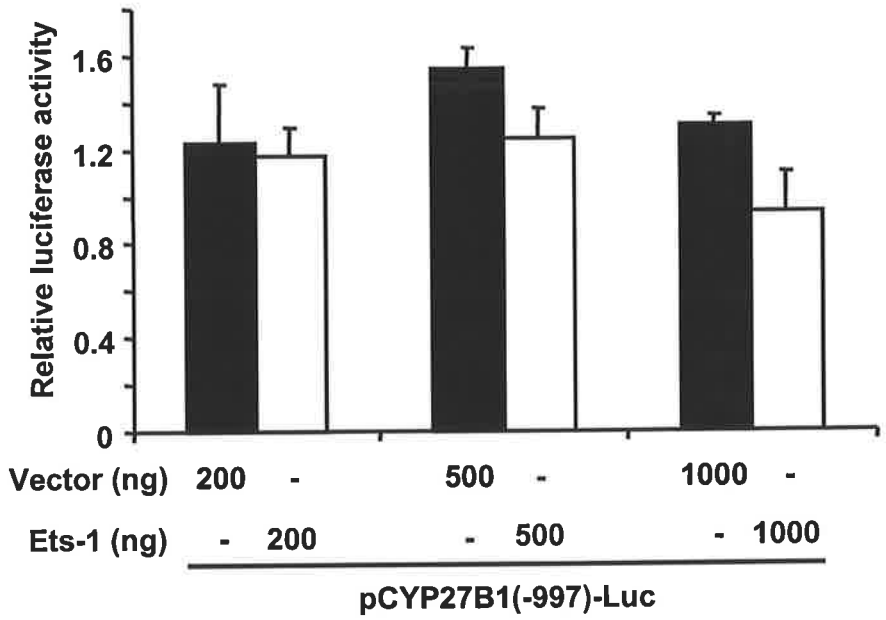

Figure 3.8: Effect of exogenously expressed Ets-1 in ROS 17/2.8 cells. The Ets-1 clone pEFBos(Ets-1) (200, 500 and 1000ng), or empty vector was transiently transfected into ROS 17/2.8 cells along with the pCYP27B1(-997)-Luc construct. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

3.5 DISCUSSION

Under the current paradigm the enzymes that control circulating levels of $1,25(OH)_2D_3$, notably CYP27B1, are localised in the kidney. However *CYP27B1* gene expression has been detected in numerous extra-renal tissues including the brain, prostate, skin and bone (Dusso et al., 1994; Hendrix et al., 2004; Zehnder et al., 2001) and is suggested to serve an autocrine or paracrine function in these tissues (as discussed in Chapter 1). Whilst the existence of the CYP27B1 enzyme in bone has been known for many years, its transcriptional control mechanisms remain unknown. The cloning of the human *CYP27B1* gene has allowed these mechanisms to be investigated but previous studies have focussed on expression in kidney (Gao et al., 2002; Murayama et al., 2004) and prostate cells (Dwivedi *et al.*, 2005). This is the first study of the *CYP27B1* 5'-flanking region in osteoblast cells.

In the current study, ROS 17/2.8 osteoblast-like cells were found to express endogenous *CYP27B1* mRNA and also to express transiently transfected plasmids comprising different lengths of human *CYP27B1* gene 5'-flanking region fused to the fire fly *luciferase* gene. The observation that UMR 106.06 cells expressed the endogenous *CYP27B1* gene but not the *luciferase* reporter gene constructs was unexpected and is difficult to explain. The first -1501 bp of flanking sequence is considered to contain all of the upstream elements necessary for *CYP27B1* gene expression (Hendrix *et al.*, 2005). This is because the sequence upstream from –1501 corresponds to sequence reported in the 3'-untranslated region of the gene for human methyltransferase-like protein (Bahr *et al.*, 1999). Moreover, a transgenic mouse model in which -1501 bp of 5'-flanking sequence of the *CYP27B1* gene was used to

direct luciferase expression, showed that the location and relative levels of luciferase activity correlated with endogenous *CYP27B1* gene expression (Hendrix *et al.*, 2004). On this basis it seemed likely that most, if not all of the upstream *CYP27B1* gene regulatory sites would lie within the first -1501 bp.

The UMR 106.06 cell line is routinely used for transfection studies in our laboratory, and transfections were successful in the present study as evidenced by the expression of the PRL-TK driven *Renilla* luciferase (data not shown). The reasons for the lack of expression of the deletion constructs in UMR 106.06 cells were not investigated since excellent expression was obtained with ROS 17/2.8 cells as described.

The deletion constructs transfected into ROS 17/2.8 cells showed that the *CYP27B1* 5'-flanking region comprises a proximal promoter that drives a modest level of expression, an enhancer region and an upstream repressive region. Importantly, this pattern of expression differs in one important aspect from that previously described in the kidney cell line AOK-B50 (Gao *et al.*, 2002). In kidney cells, only a very weak upstream repressive region was identified between -1501 and -997. However in the present study, a strong inhibitory region was detected between -1100 and -997. A strong inhibitory region was also reported for studies in DU145 prostate cells but this region was located between –1200 and –1100 (Dwivedi *et al.*, 2005), further upstream from the repressive region in ROS 17/2.8 cells. Detailed studies in our laboratory have shown that the majority of repression observed in DU145 cells is attributed to a Growth factor independent-1 (GFI-1) protein binding site at –1161 to –1138, that is within the –1200 to –1100 region. GFI-1 is not responsible for the marked repression observed in the ROS 17/2.8 cells since the GF-1 binding site lies outside the

repressive region. Specific binding elements within this repressive region are the subject of studies reported in Chapter 4.

As mentioned, the first -305 bp of the *CYP27B1* 5'- flanking region, designated as the proximal promoter, contains a GC box and a CCAAT box. Whilst these two sites have previously been shown to be essential for expression of the proximal promoter in both kidney and prostate cells, their functional relevance was unknown in osteoblasts. It has been shown here that these sites are again essential in osteoblasts for expression of the *CYP27B1* promoter. Based on the observation that Sp1 binds the GC box in an insect cell line (Gao *et al.*, 2002), it is predicted that Sp1 protein in ROS 17/2.8 cells would also bind this site. However the transcription factor that binds the CCAAT box is not known and has not been investigated in this study.

The finding that the GC and CCAAT box sites are important for expression of the proximal promoter in kidney, prostate and osteoblast cells is not surprising. These sites are commonly found in many gene promoters and bind transcription factors such as Sp1 that are found in most cell types. This study demonstrated that expression of a construct containing -997 bp of 5'-flanking region was greatly repressed when either of these sites were inactivated, but especially the CCAAT box. Therefore expression of the enhancer region requires intact GC box and CCAAT box binding sites. The fact that the enhancer is dependent on proximal promoter activity most likely reflects a cooperative interaction between transcription factors bound at these sites and a co-activator complex that contacts the RNA polymerase machinery (Jayaraman *et al.*, 1999).

Within the enhancer region, three putative Ets protein binding sites (EBS I, EBS II and EBS III) were investigated for their relative contributions to the *CYP27B1* enhancer activity in osteoblast cells. Each site has previously been shown to contribute to expression in prostate cells with EBS I being the most important and EBS III the least (Dwivedi *et al.*, 2005). In ROS 17/2.8 osteoblast cells, EBS I and EBS II were important but inactivation of EBS III did not alter expression. An attempt was made to identify the Ets family members that bound EBS I and EBS II. All Ets proteins bind the nucleotide sequence 5'-GGAT-3' (Oikawa and Yamada, 2003), therefore the sequences provided no further information as to the identity of the protein. However Ets-1 and Ets-2 are reported to be the Ets family members most highly expressed in osteoblasts (Vary *et al.*, 2000). Ets-1 is expressed mostly by proliferating osteoblasts whereas Ets-2 is expressed by more mature osteoblasts.

ROS 17/2.8 is considered to represent a mature osteoblast phenotype as it constitutively expresses osteocalcin (Price and Baukol, 1980) and is capable of producing a calcified matrix when implanted into diffusion chambers (Shteyer *et al.*, 1986). However, there is no specific information as to the relative expressions of Ets-1 and Ets-2 proteins in ROS 17/2.8.

In order to predict if either Ets-1 or Ets-2 associate with the *CYP27B1* enhancer, transfection experiments were performed that utilised the transient over expression of a native Ets-1 and dominant negative mutants of Ets-1 and Ets-2. Given the DNA sequences involved and the cellular context, it was predicted that one of these two transcription factors would affect *CYP27B1* gene expression. However, neither the over expression of Ets-1, nor an Ets-1 dominant negative had any effect upon the

expression of the pCYP27B1(-997)-Luc construct. Likewise, over expression of an Ets-2 dominant negative did not alter the expression of the enhancer either. These data suggest, but do not exclude, Ets-1 and Ets-2 from being the proteins that associate with EBS I and EBS II within the *CYP27B1* enhancer. It is likely that activation through these elements involves a complex interaction of transcription factors. Consequently, altering the level of one factor alone may not be sufficient to affect the level of *CYP27B1* gene expression. Therefore, although it was shown that as with prostate cells, the *CYP27B1* enhancer in osteoblasts requires intact Ets protein binding sites, no conclusions can be drawn as to the identity of the Ets family member that binds these sites. It may be that an Ets protein such as Elk1, that expresses weakly in osteoblasts but was not tested in this study, is involved. Accordingly, it would be useful to determine the relative expression of all Ets genes in ROS 17/2.8 by RT-PCR, in order to investigate this question further.

Overall, important observations can be made from the current work. There is a significant level of expression of the *CYP27B1* gene 5'-flanking region in ROS/172.8 cells and this will permit future studies aimed at identifying other transcription factor binding sites that contribute to the expression. Secondly, a strong inhibitory region has been identified upstream that lies between –1100 and -997 and appears to be a tissue specific since this region is not detected in kidney and prostate cells. Investigations into the identity of the transcription factors responsible for repression are described in the next chapter.

Hence the basal level of expression of the *CYP27B1* gene in the osteoblast cells reflects a complex balance between positive factors that bind to the promoter and

enhancer, and negative factors that bind to the repressive region. The local level of expression, and hence the local amount of $1,25(OH)_2D_3$ that may act in an autocrine/paracrine fashion to influence osteoblast development, will be critically dependent on the cellular concentration of these transcription factors.

3.6 CONCLUSIONS

3.6.1 The 5'-flanking region of the *CYP27B1* gene can be described in osteoblasts as comprising a proximal promoter, an enhancer region and an upstream repressive region. This pattern has similarities and differences to that previously reported in kidney and prostate cells.

3.6.2 Expression of the *CYP27B1* proximal promoter is dependent on a putative Sp1 binding site and a CCAAT box in osteoblasts. This mirrors the situation in kidney and prostate cells.

3.6.3 Maximal expression of the enhancer requires two intact Ets protein binding sites (EBS I and EBS II). The identities of the proteins that associate at these sites are yet to be elucidated.

Chapter 4:

Basal Repression of the Human CYP27B1 Gene 5'-Flanking Region in ROS 17/2.8 Cells

4.1 INTRODUCTION

It is apparent from *in vitro* studies that $1,25(OH)_2D_3$ has many direct actions on bone cells and can modulate processes associated with bone remodelling. For instance, $1,25(OH)_2D_3$ is able to modulate osteoblast development by inhibiting proliferation of immature osteoblasts and stimulating mineralisation in mature osteoblasts (Owen *et al.*, 1991). *In vivo*, the action of $1,25(OH)_2D_3$ may be autocrine or paracrine given the fact that osteoblasts are themselves capable of producing $1,25(OH)_2D_3$ (Anderson *et al.*, 2005; Turner *et al.*, 1980b). As described in Chapter 3, expression of the 5'-flanking region of the human *CYP27B1* gene in osteoblasts revealed an upstream repressive region. Repression of *CYP27B1* gene expression in osteoblasts may act to limit the local concentration of $1,25(OH)_2D_3$ allowing osteoblasts to proliferate. Therefore it is important to investigate the molecular basis of this repression.

A repressive region in the 5'-flanking region of the human *CYP27B1* gene has also been identified in transfection studies employing the prostate cancer cell line, DU145. Dwivedi *et al.* (2005) reported that a sequence from –1161 to –1138 was significantly involved in the repression of the 5'-flanking region in these cancer cells. They determined that the oncoprotein Growth Factor Independent-1 (GFI-1) bound this sequence and that exogenous expression of GFI-1 could directly further reduce

expression of the 5'-flanking region of the *CYP27B1* gene. Dwivedi *et al.* (2005) also noted the presence of a cAMP response element (CRE) at –1069 to –1062 which could also repress the 5'-flanking region of the *CYP27B1* gene in prostate cancer cells.

Basal expression of the 5'-flanking region of the human *CYP27B1* gene has also been studied in transfected kidney cells. In the porcine cell line AOK-B50, a weak repressive region was reported between –1501 and –997 (Gao *et al.*, 2002). Similarly in HEK 293 human embryonic kidney cells, expression increased about 1.5 fold when the length of *CYP27B1* 5'-flanking region was deleted from –1413 to –927 (Ebert *et al.*, 2004). This same study also reported down regulation of the *CYP27B1 gene* through NFκB signalling. A total of ten NFκB sites that bound NFκB in EMSA experiments were identified within the 5'-flanking region of the *CYP27B1* gene. However, these sites are located between –804 to –83, and are therefore not involved in the upstream repression phenomenon identified in the present study.

The aim of this chapter was to further characterise the repressive region between –1100 and –997 within the 5'-flanking region of the *CYP27B1* gene. A palindromic sequence was identified within this region that consisted of two putative Smad binding elements (SBEs). The possible role of these elements in the repressed expression of the 5'-flanking region of the *CYP27B1* gene has been investigated.

4.2 SPECIFIC AIMS AND HYPOTHESIS

4.2.1 Specific aims

1) To identify regulatory elements within the repressive region between –1100 and –997 that contributed to repression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells.

2) To investigate whether the repressive elements identified in osteoblast cells also caused repression in other cell types.

3) To identify the protein(s) that bind to the repressive elements utilising electrophoretic mobility shift assays (EMSA).

4.2.2 Specific hypothesis

It is hypothesised that repression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells involves Smad proteins binding the palindromic sequence 5'-GTCTCAGAC-3' (–1015/–1007).

4.3 OUTLINE OF METHODS USED IN THIS CHAPTER (Detailed descriptions are given in Chapter 2).

4.3.1 Mutagenesis of a putative c-Myb binding site, a CRE and SBEs

Site-directed mutagenesis of a putative c-Myb binding site, a CRE and the SBEs within the pCYP27B1(-1100)-Luc construct was carried out using the QuikChange site directed mutagenesis protocol (Stratagene, La Jolla, CA). Wild type and mutated sequences were as follows, with the core binding sequence underlined and the mutated nucleotides shown in bold:

c-Myb

Wild type	5'-TGTAGAGACAGTTCCACTATGTTG-3' (-1048/-1025)
Mutant	5'-TGTAGAGACAGATCCACTATGTTG-3'

CRE

Wild type	5'-CCATGCCTGGCTAATTTTTG-3' (-1075/-1047)
Mutant	5'-CCATGCCTGTGTAATTTTTG-3'

SBEs

Wild type	5'-AGGCTGGTCTCAGACTCCTG-3' (-1021/-1002)
Mutant SBE1	5'-AGGCTGGTAACAGACTCCTG-3'
Mutant SBE2	5'-AGGCTGGTCTCTTACTCCTG-3'
Mutant SBE(1 and 2)	5'-AGGCTGGTAACTTACTCCTG-3'
Mutant SBE(C)	5'-AGGCTGGTCTAAGACTCCTG-3'

4.3.2 Transient expression of reporter constructs

Transfection of the cells was performed using the appropriate transfection reagent according to the manufacturer's instructions. Briefly, cells were seeded into 24 well plates and left 6 hours to attach. Cells were transfected overnight with a reporter plasmid in serum free media. A thymidine kinase (TK) promoter-*Renilla* luciferase plasmid was included to control for transfection efficiency. The following day, cells were lysed and dual luciferase activity was measured. Experiments involving transforming growth factor-beta type 1 (TGF-β1) included a 24 hour treatment period immediately following the overnight transfection.

4.3.3 Over expression of the Ski oncoprotein and Smad4

The expression clones for human Ski (Ichikawa *et al.*, 1997) and Smad4 (Denissova *et al.*, 2000) have been described previously. These clones were co-expressed with specific pCYP27B1-Luc reporter plasmids with the total amount of DNA kept constant in all wells using the empty vector.

4.3.4 Purification of GST-Smad4

The clone for production of GST-Smad4 fusion proteins was obtained from Associate Professor Fang Liu (Rutgers University, New Jersey) and was based on the pGEX 4T-1 plasmid. The plasmid was transformed into the BL21* strain of *E. coli*. Bacterial cultures were induced with 0.5mM IPTG at 30°C for 3 hours and then purified by affinity chromatography using a glutathione Sepharose column (see Methods 2.5.2).

The purity of the eluted fusion protein was confirmed by SDS-PAGE analysis (see Methods 2.5.3) and its concentration was estimated by the Bradford assay (see Methods 2.5.4).

4.3.5 Electrophoretic mobility shift assays (EMSA) and analysis of the SBEs

The oligonucleotide sequences used to create the probes were as follows with SBEs underlined.

CYP27B1	5'-GCTGGTCTCAGACTCCT-3'
	5'-GAGGAGTCTGAGACCAG-3'
mSBE1	5'-GCTGGTAACAGACTCCT-3'
	5'-GAGGAGTCTGTTACCAG-3'
mSBE2	5'-GCTGGTCTCTTACTCCT-3'
	5'-GAGGAGTAAGAGACCAG -3'
CYP27B1-core	5'-GGTCTCAGAC-3'
	5'-GGTCTGAGAC-3'
Smad7	5'-GGGTGTCTAGACGGC-3'
	5'-CCACAGATCTGCCGG-3'

Consensus 5'-GACACGTAGTCTAGACGTCTAGACAATGT-3'

5'-GACATTGTCTAGACGTCTAGACTATGTGT-3'

Oligonucleotides were annealed and the resultant 5'-overhangs were labelled with [^{32}P]dCTP during an end filling reaction (Methods 2.5.6). The CYP27B1 probe contained SBE1 and SBE2 (underlined) and flanking sequence. The mSBE1 and mSBE2 probes contained mutations (shown in bold) that were identical to those used in transient transfections. Binding was also compared to the CYP27B1-core probe that contained SBE1 and SBE2, but lacked any additional flanking sequence except for the guanine overhang necessary for the end filling reaction. The Smad7 probe was of interest because it contains an almost identical pair of SBEs whose repressive function has been studied previously (Denissova and Liu, 2004). The sequence of the consensus probe contains two consecutive copies of the 5'-GTCTAGAC-3' palindrome and has been reported previously (Nicol and Stavnezer, 1998). Binding reactions contained either 10ug of total nuclear protein extracted from ROS 17/2.8 cells, or approximately 0.1ug-0.4ug of recombinant GST-Smad4 protein. All reactions contained 100 000 c.p.m of [^{32}P]-labelled oligonucleotide probe and were loaded directly on to a 4% polyacrylamide gel (Methods 2.5.7). Supershift experiments used commercial polyclonal antibodies against Smad4 (H-552, Santa Cruz) and Ski (H-329, Santa Cruz), as well as the immune serum against Smad4 that was used in studies of the *Smad7* promoter (Denissova *et al.*, 2000) (see Methods 2.1.3).

4.4 RESULTS

4.4.1 Identification of regulatory elements within the repressive region

In order to identify the regulatory elements that contribute to repression of the 5'-flanking region of the *CYP27B1* gene, possible transcription factor binding sites were identified between -1100 and -997 base pairs (Figure 4.1.a). Dwivedi *et al.* (2005) have described a CRE site (–1069/-1063) which partially contributed to repression of a pCYP27B1(-1200)-Luc reporter construct in transiently transfected prostate cancer cells. In addition, the web based software 'TESS: Transcription Element Search System' predicted that the transcription factor c-Myb could bind the core sequence 5'-CAGTTC-3' (-1040/-1035). Importantly, the presence of a palindromic sequence (-1015/-1007) comprising two adjacent consensus SBEs, designated SBE1 and SBE2, was also identified within the repressive region. This sequence differs by one base pair from a repressive sequence previously identified within the promoter of the *Smad7* gene (Nagarajan *et al.*, 1999) (Figure 4.1.b) and this is described later.

The putative SBEs, CRE and c-Myb sequences were changed by site-directed mutagenesis within the -1100 flanking sequence and tested following transient transfection. The specific mutations introduced are described in the Methods (4.3.1). As can be seen in Figure 4.2, expression of the pCYP27B1(-1100)-Luc construct was lower than that of the pCYP27B1(-997)-Luc construct as expected. However mutagenesis of the CRE within the pCYP27B1(-1100)-Luc construct did not alleviate the repression.

a)

CRE

(-1100) CTCAAGGCACTACAGGCATACGCCACCATGCCTGGCTAATTTTTGTATT

SBE1 SBE2

c-MYB

(-1051) TTTTGTAGAGACAGTTCCACTATGTTGCCCAGGCTGGTCTCAGACTCCTG

AGCTC (-997)

b)

SBE1 SBE2

CYP27B1: GTCTCAGAC

SBE1 SBE2

Smad7: GTCTAGAC

Figure 4.1:

a) The DNA sequence of the repressive region of the 5'-flanking region of the *CYP27B1* gene identified in osteoblast cells. Putative transcription factor binding sites are underlined.

b) Comparison of the palindromic sequence that mediates basal repression of the 5'-flanking region of the *CYP27B1* gene with a similar repressive sequence within the *Smad7* promoter. The *CYP27B1* sequence differs by the addition of a central cytosine.

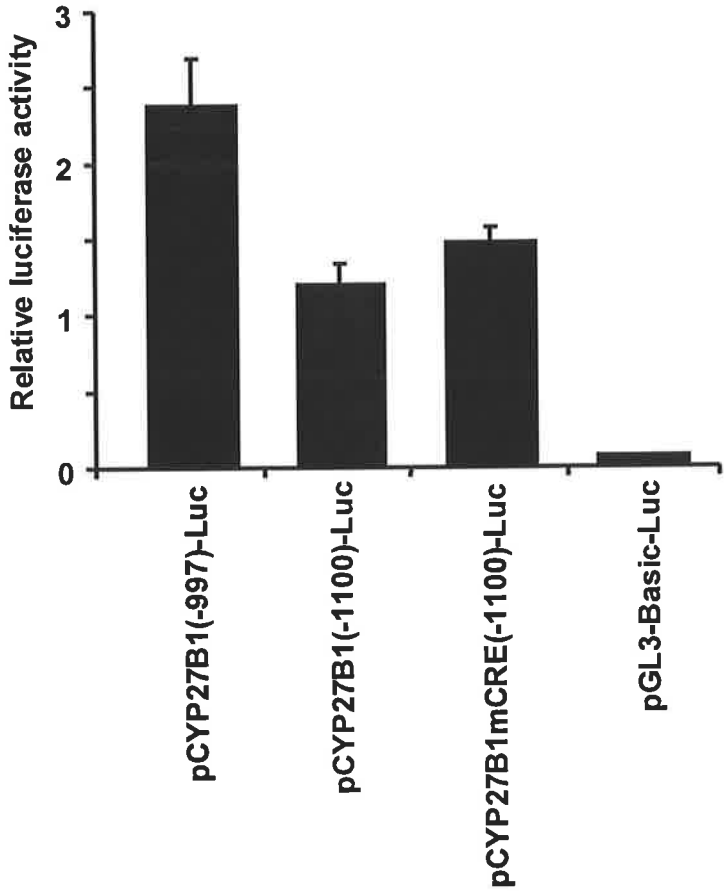

Figure 4.2: Mutational analysis of a CRE site within the repressive region of the 5'-flanking region of the human *CYP27B1* gene in ROS 17/2.8 cells. The CRE was mutated in the pCYP27B1(-1100)-Luc construct and transiently transfected into ROS 17/2.8 cells. The pCYP27B1(-1100)-Luc and pCYP27B1(-997)-Luc constructs were also transfected for comparison. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

Similarly in Figure 4.3, repression is again observed with the –1100 construct compared with the -997 construct and is not reversed by mutagenesis of the putative c-Myb site. However, mutagenesis of SBE1 and SBE2, either individually or in combination, increased expression of the -1100 construct to the level of the -997 construct (Figure 4.4). Interestingly, changing the central cytosine base that separates SBE1 and SBE2 to an adenine also reversed the observed repression of the 5'-flanking region (Figure 4.4).

4.4.2 Investigation of the repressive region in other cell lines

The pCYP27B1(-997)-Luc, pCYP27B1(-1100)-Luc and pCYP27B1mSBE(1and2)(-1100)-Luc reporter constructs were then transfected into another osteoblast cell line and also into three different cell types to determine whether the SBEs were functional (Figure 4.5). The data show that the repressive region between -1100 and -997 that was detected in ROS 17/2.8 osteoblast cells was also observed in the human osteoblast cell line HOS, as well as COS-1 cells. However, whilst repression was significantly reversed by inactivation of SBE1 and SBE2 together in the HOS cells, expression was not significantly restored in COS-1 cells. A much weaker repressive region was detected between –1100 and –997 in HEK-293T human kidney cells and MDA-MB-468 human breast cancer cells. In each case, inactivation of both SBEs together did not alter the expression of the 5'-flanking region of the *CYP27B1* gene (Figure 4.5).

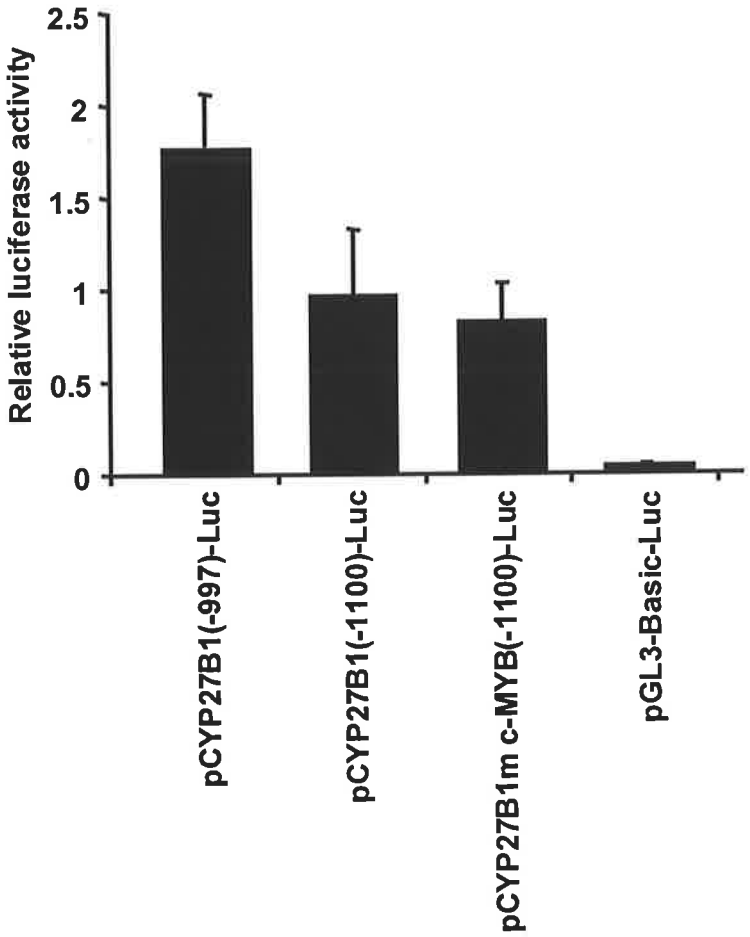

Figure 4.3: Mutational analysis of a putative c-Myb binding site within the repressive region of the 5'-flanking region of the human *CYP27B1* gene in ROS 17/2.8 cells. The c-Myb site was mutated in the pCYP27B1(-1100)-Luc construct and transiently transfected into ROS 17/2.8 cells. The pCYP27B1(-1100)-Luc and pCYP27B1(-997)-Luc constructs were also transfected for comparison. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

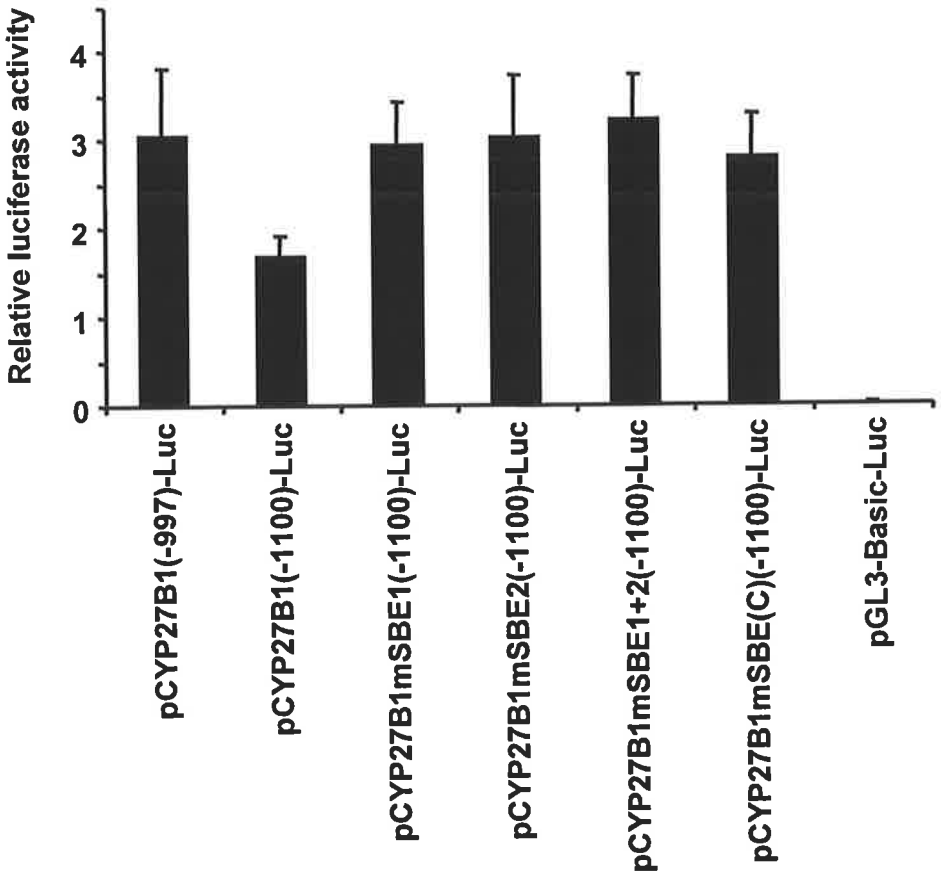

Figure 4.4: Mutational analysis of two putative SBEs within the repressive region of the 5'-flanking region of the human *CYP27B1* gene in ROS 17/2.8 cells. Site-directed mutagenesis was used to inactivate SBE1, SBE2, SBE1 and SBE2 together, and the central cytosine within the pCYP27B1(-1100)-Luc construct. Constructs were then transiently transfected into ROS 17/2.8 cells. The pCYP27B1(-1100)-Luc and pCYP27B1(-997)-Luc constructs were also transfected for comparison. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

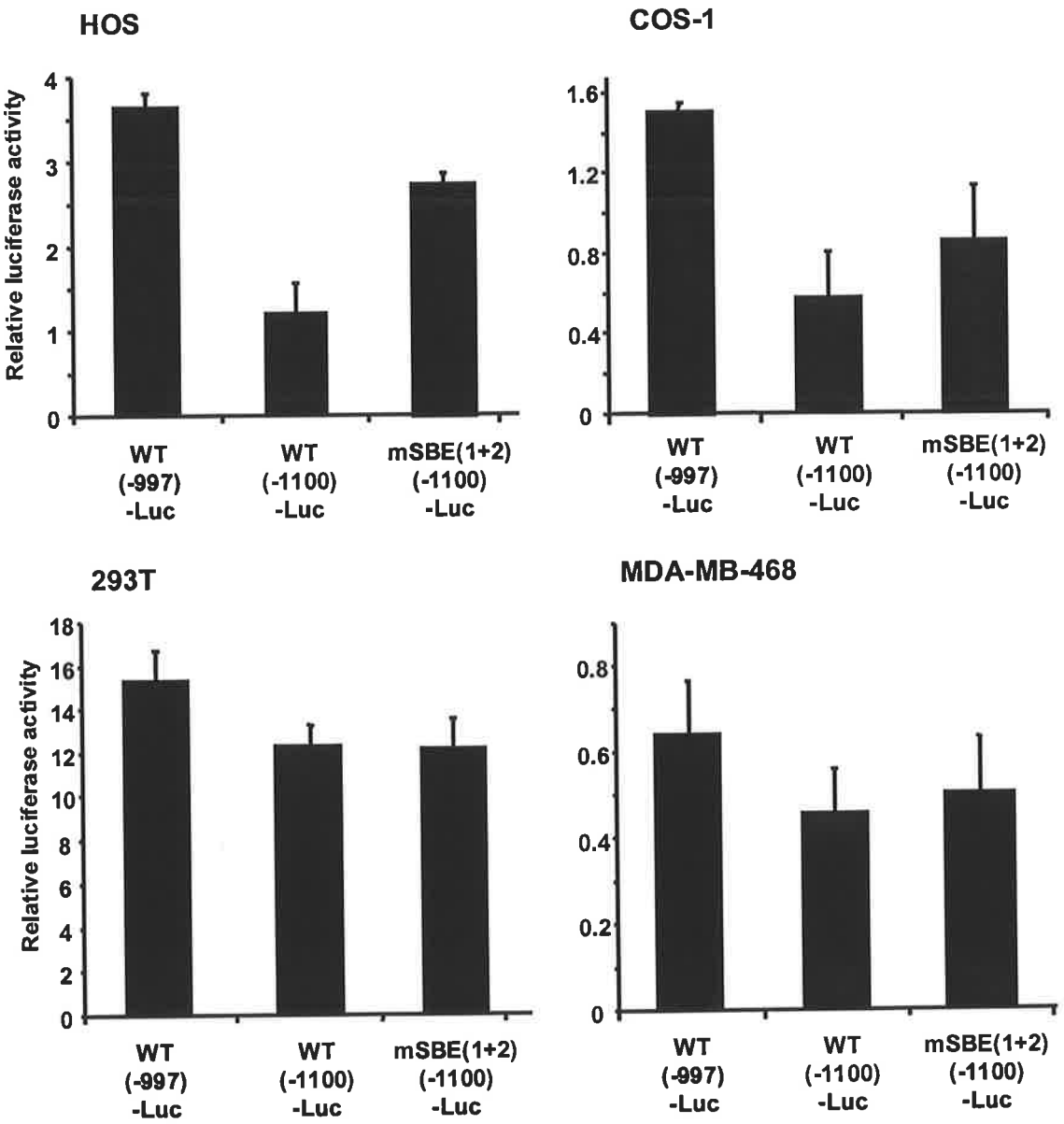

Figure 4.5: Investigation of repression in a variety of cell lines. The pCYP27B1(-997)-Luc, pCYP27B1(-1100)-Luc and pCYP27B1mSBE(1+2)(-1100)-Luc constructs were transiently transfected into HOS, COS-1, 293T and MDA-MB-468 cells as indicated. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

4.4.3 Involvement of Smad4 and Ski in the repression

As shown in Figure 4.1.b), the repressive element closely resembles a palindromic sequence that has been previously shown to mediate basal repression of the *Smad7* gene promoter. CHIP assays showed that a protein complex containing Smad4 and the oncoprotein Ski bound the *Smad7* gene promoter (Denissova and Liu, 2004). Denissova *et al.* (2004) also demonstrated that over expression of Ski inhibited expression of the *Smad7* gene promoter in HepG2 cells in a dose dependent manner. Hence it was concluded that Smad4 and Ski are important for basal repression of this promoter through interaction with the palindromic sequence. It was therefore important to determine what effect over expression of Ski would have on the *CYP27B1* reporter constructs in ROS 17/2.8 cells.

Co-transfection of up to 500ng of Ski expression clone failed to alter expression of the pCYP27B1(-1100)-Luc construct (Figure 4.6). It was thought that repression may require both Ski and Smad4 to be co-expressed, and therefore the experiment was performed again using 200ng each of Ski and Smad4 expression plasmids. Figure 4.7 shows that co-transfection of both the Ski and Smad4 plasmids partially lowered expression of the pCYP27B1(-1100)-Luc construct. However expression of the pCYP27B1mSBE(1and2)(-1100)-Luc construct, where both putative SBEs were mutated, was similarly lowered by over expression of Ski and Smad4 plasmids indicating that the observed effect was not dependent on the putative SBEs.

It was possible however that over expression of Ski and Smad4 failed to show a function through the putative SBEs because of high endogenous levels of one or both

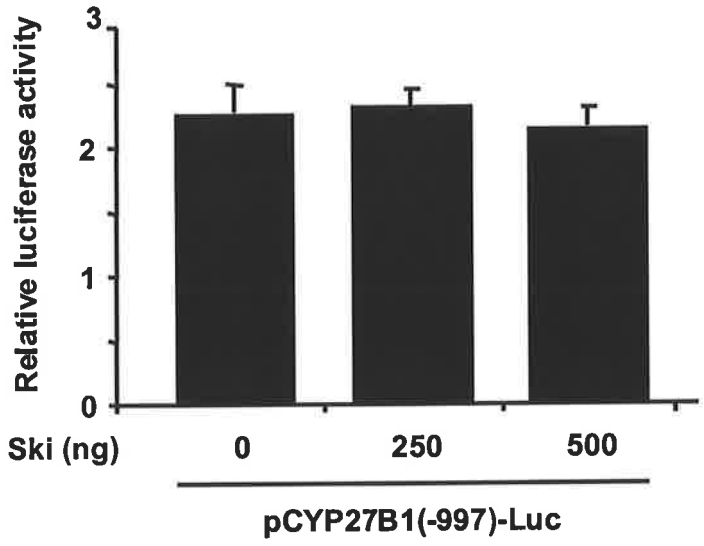

Figure 4.6: Effect of exogenously expressed Ski in ROS 17/2.8 cells. The effect of over expressing the Ski clone pACT-Ski (0, 250 and 500ng) on expression of the pCYP27B1(-997)-Luc construct in ROS 17/2.8 cells was tested. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

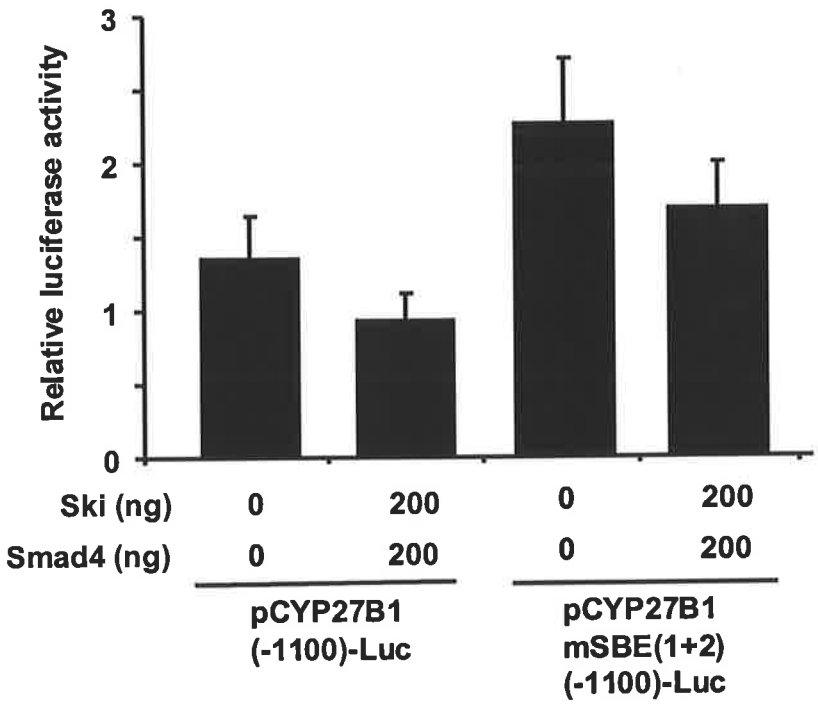

Figure 4.7: Effect of exogenously expressed Ski and Smad4 in ROS 17/2.8 cells. The effect of over expressing the Ski clone pACT-Ski and the Smad4 clone pCMV-Smad4 (200ng each) together on expression of the pCYP27B1(-997)-Luc construct in ROS 17/2.8 cells was tested. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

of these proteins in ROS 17/2.8 cells. In an attempt to circumvent this problem the Ski and Smad4 plasmids were individually over expressed in HEK-293T cells. HEK-293T cells were chosen because reporter constructs expressed in these cells showed the least amount of repression between -1100 and -997, and there was no indication that the putative SBEs were transcriptionally functional (Figure 4.5). The data in Figure 4.8 show that expression of the Smad4 plasmid in HEK-293T cells lowered expression of the pCYP27B1(-1100)-Luc construct in a dose dependent manner. However, the Smad4 expression clone was similarly capable of lowering expression of the pCYP27B1mSBE(1and2)(-1100)-Luc construct once again indicating that the effect was independent of the putative SBEs. Over expression of 200ng of the Ski plasmid in HEK-293T cells caused a marked increase in expression of the pCYP27B1(-1100)-Luc construct (Figure 4.9). Once again this effect was still observed with the pCYP27B1mSBE(1and2)(-1100)-Luc construct.

4.4.4 Effect of TGF-β1 on repression in ROS 17/2.8 cells

As described in chapter 1.8.2, Smads are downstream mediators of TGF-β signalling. Binding of TGF-β to its cell surface receptor leads to the phosphorylation of Smad2 and Smad3 which can then accumulate in the nucleus and complex with Smad4 to modulate gene expression. Treatment of HepG2 cells with TGF-β1 leads to an 8-fold activation of the *Smad7* gene promoter (Denissova and Liu, 2004). Therefore, it was of interest to know what effect TGF-β1 had on expression of the pCYP27B1(-1100)-Luc construct. Surprisingly, the addition of TGF-β1 (1ng/ml) to cultured ROS 17/2.8 cells for 24 hours post-transfection further lowered expression of the wild-type pCYP27B1(-1100)-Luc construct by approximately 50% relative to vehicle treated

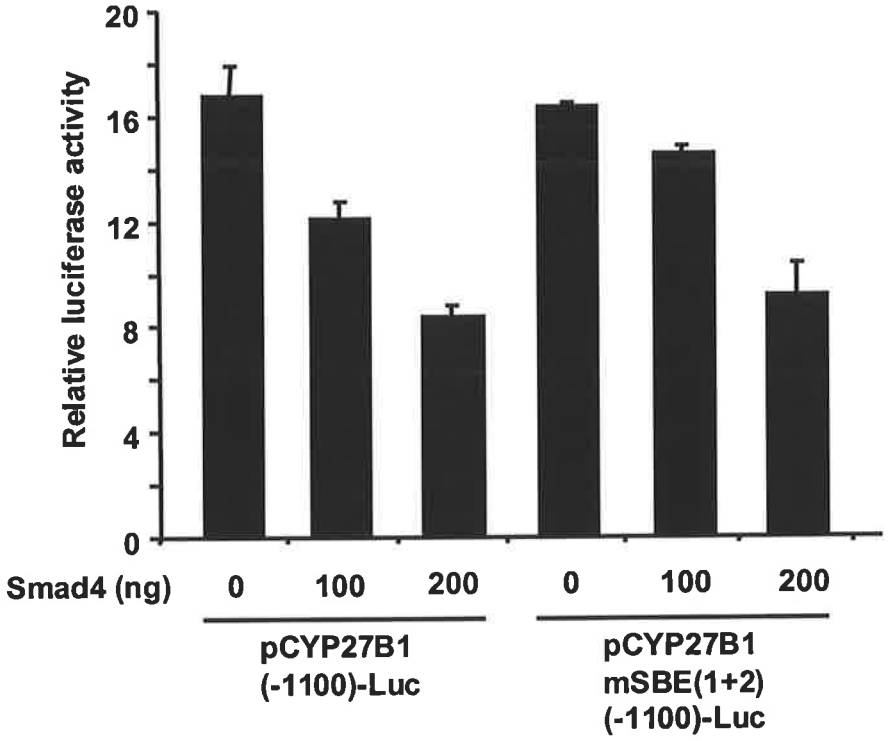

Figure 4.8: Effect of exogenously expressed Smad4 in HEK-293T cells. The effect of over expressing the Smad4 clone pCMV-Smad4 (0, 100 and 200ng) on expression of the pCYP27B1(-997)-Luc construct in HEK-293T cells was tested. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

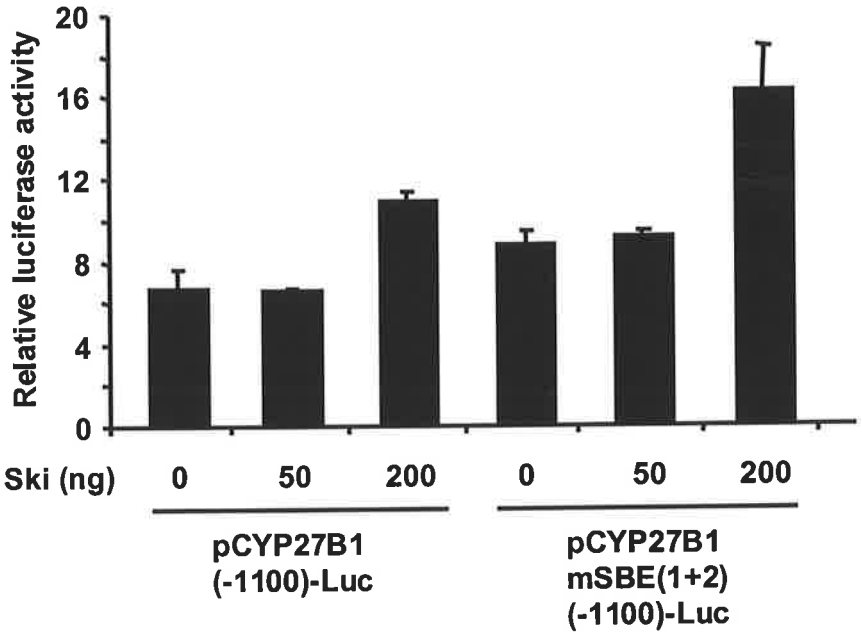

Figure 4.9: Effect of exogenously expressed Ski in HEK-293T cells. The effect of over expressing the Ski clone pACT-Ski (0, 50 and 200ng) on expression of the pCYP27B1(-997)-Luc construct in HEK-293T cells was tested. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

cultures (Figure 4.10). When the putative SBEs were inactivated, either alone or in combination, this additional repression by TGF-β1 remained.

4.4.5 EMSA analysis of the putative SBEs

EMSA experiments were performed in an attempt to identify the proteins binding the repressive sequences designated SBE1 and SBE2. The sequences of the oligonucleotide probes used are given in the Methods (4.3.5). As earlier stated, CHIP assays have previously shown Smad4 binds an almost identical palindrome comprising two SBE sequences within the *Smad7* promoter (Denissova and Liu, 2004). Therefore it was important to see whether Smad4 could also bind the repressive sequence under investigation here. The results showed that the probe designated CYP27B1, whose design includes SBE1 and SBE2 plus additional native flanking sequence successfully bound GST-Smad4 (Figure 4.11). The strength of the detected band was proportional to the amount of recombinant protein added to the binding reaction. Binding was also studied using probes that contained mutations identical to those introduced for transfection experiments. GST-Smad4 bound the mSBE2 probe, but not the mSBE1 probe establishing that SBE1 is important for binding. The mobility of the protein complex that bound the mSBE2 probe was the same as the complex that bound the native CYP27B1 probe, indicating that the two complexes are of the same size.

The binding of nuclear proteins isolated from ROS 17/2.8 cells was also studied. For these experiments 10ug of total nuclear proteins were included in a binding reaction with each of 3 oligonucleotide probes (Figure 4.12). Binding to the Smad7 probe

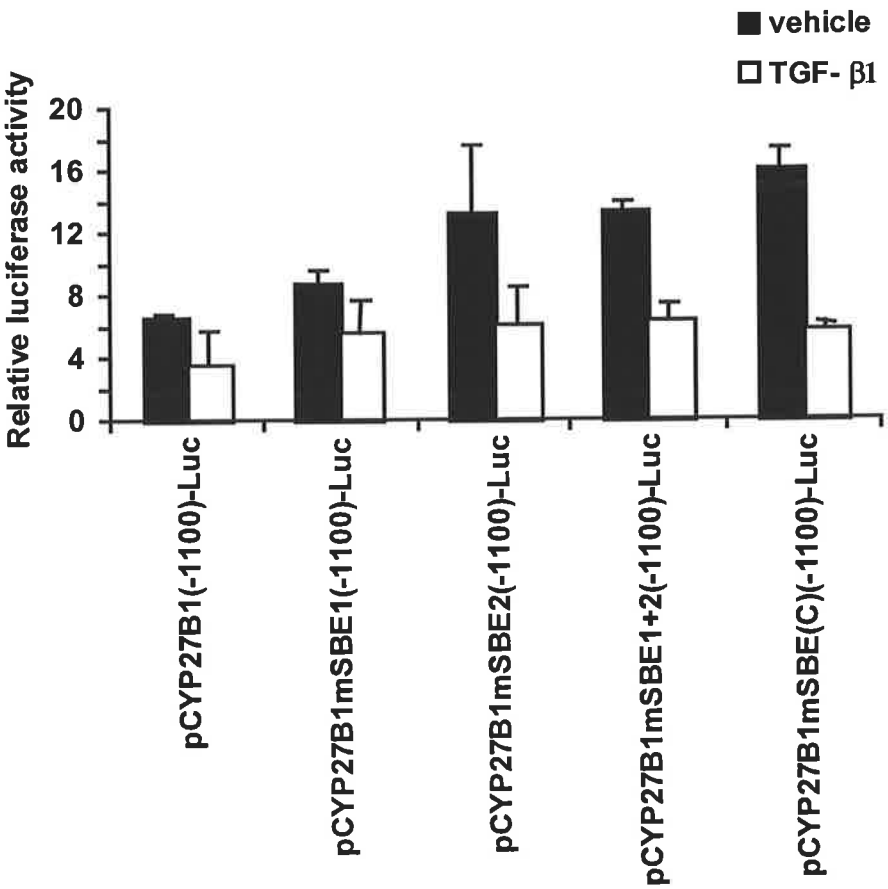

Figure 4.10: The effect of TGF-β1 on repression through the SBEs. pCYP27B1(-1100)-Luc constructs containing intact and inactivated SBEs were transiently transfected into ROS 17/2.8 cells and then treated with TGF- β1 or vehicle for 24 hours. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

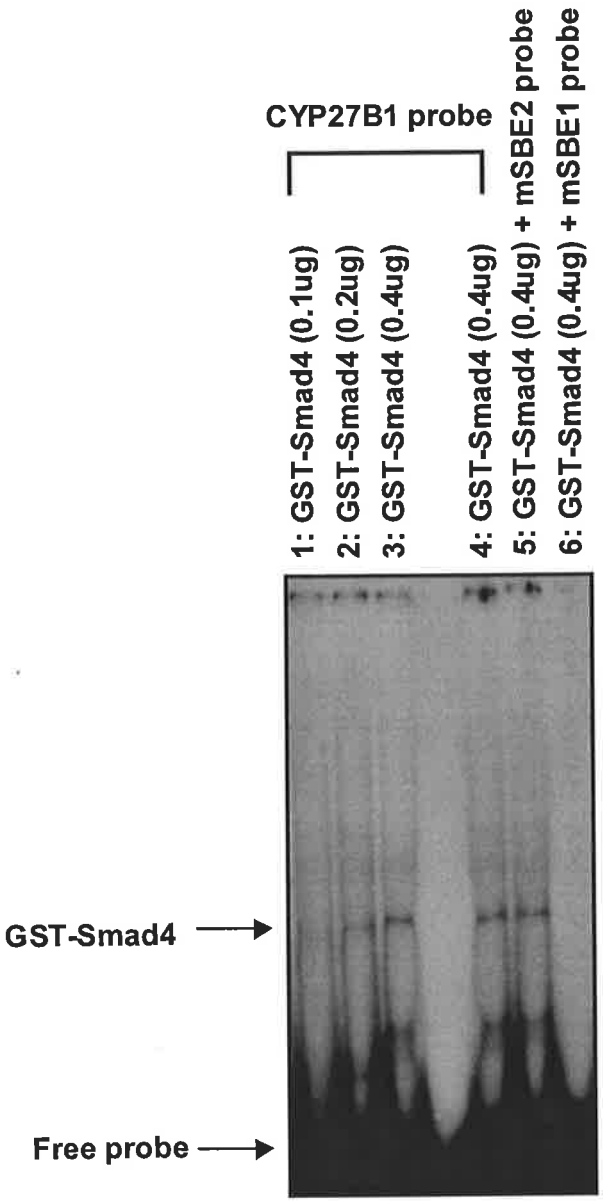

Figure 4.11: EMSA analysis of the binding of GST-Smad4 to the SBEs within the repressive region. Oligonucleotide probes were labelled with [α-^{32}P]dCTP and incubated with GST-Smad4. Retarded GST-Smad4 complexes and free probe are indicated with arrows. Lanes 1-3: Increasing amounts of GST-Smad4 protein bound the wild type CYP27B1 probe with increasing intensity. Lanes 4-6: 0.4ug of GST-Smad4 bound the wild type CYP27B1 and mSBE2 probes, but did not bind the mSBE1 probe.

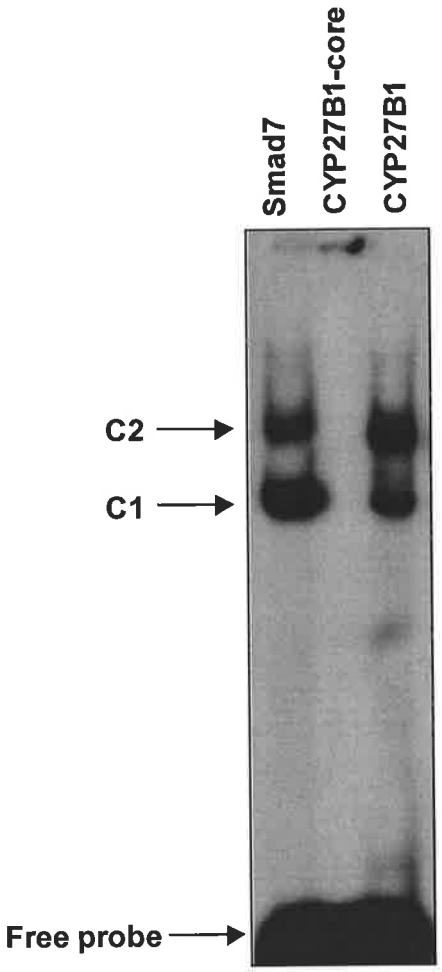

Figure 4.12: EMSA analysis of the binding of ROS 17/2.8 nuclear proteins to the repressive SBEs. The Smad7 probe contains the SBEs that mediate basal repression of the *Smad7* gene promoter. The CYP27B1-core probe comprises SBE1 and SBE2 within the regulatory region of the *CYP27B1* gene with no additional flanking sequence. The CYP27B1 probe is identical except it contains additional native flanking sequence. All probes were labelled with [α-^{32}P]dCTP and incubated with 10ug of nuclear proteins. Two retarded complexes (C1 and C2) and free probe are indicated with arrows.

yielded two distinct bands (C1 and C2), which were also seen when the CYP27B1 probe was tested. However the CYP27B1-core probe that contained only SBE1 and SBE2 without flanking sequence did not give rise to any bands.

In order to establish the specificity of binding of the bands observed with the nuclear proteins, competition analyses were performed using unlabelled probe. The results show that the bands produced by the CYP27B1 probe were effectively competed out by unlabelled CYP27B1 probe and by a previously reported 'consensus' SBE probe which contained two consecutive repeats of the sequence 5'-GTCTAGAC-3' (Nicol and Stavnezer, 1998) (Figure 4.13). However the CYP27B1-core probe, that lacked additional flanking sequence, was unable to compete for binding with the CYP27B1 probe.

Finally, supershift experiments were attempted using antibodies against Smad4 and Ski, and nuclear proteins extracted from ROS 17/2.8 cells. Neither commercial Smad4 nor Ski antibodies, nor the Smad4 antiserum (Methods 4.3.5) were able to shift any protein-DNA complex. A variety of binding conditions were tested but yielded no band shift, and this approach was not further investigated.

4.5 DISCUSSION

In Chapter 3, analysis of deletion constructs expressed in ROS 17.2.8 cells permitted the delineation of the 5'-flanking region of the *CYP27B1* gene into three regulatory regions. The first -305 base pairs of flanking comprises a proximal promoter that drives a modest level of expression. Between -997 and -531 lies an enhancer region

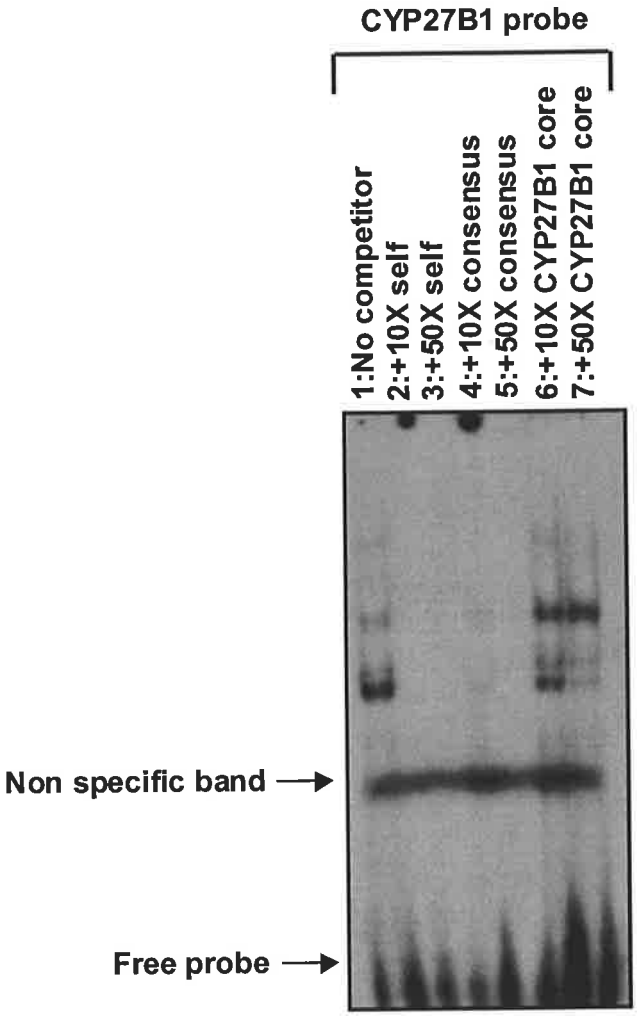

Figure 4.13: Unlabelled oligonucleotide probes containing SBEs compete for binding to the CYP27B1 probe. Binding of ROS 17/2.8 nuclear proteins to the CYP27B1 probe, containing SBE1 and SBE2 plus additional flanking sequence, (lane 1) was competed by unlabelled CYP27B1 probe (lanes 2 and 3) and by an unlabelled consensus SBE probe (lanes 4 and 5). Binding was competed for weakly by the unlabelled CYP27B1-core probe. The CYP27B1 probe was labelled with [α-^{32}P]dCTP and incubated with 10ug of nuclear proteins. A non specific band (complexes not competed by unlabelled probes) and free probe are indicated with arrows. Probe sequences are shown in 4.3.5.

that is required for maximal expression and beyond -997 an upstream repressive region was identified. This repressive region lay from -1501 to -997 with the majority of repression being attributed to a sequence from –1100 to –997. Interestingly, this repressive region is more proximal than that previously identified in prostate cancer cells (-1200 to –1100). The major aim of the present chapter was to identify the specific repressive elements within the region that lead to inhibition of expression in osteoblast cells. Inspection of the 5'-flanking sequence from –1100 to –997 revealed three putative transcription factor binding sites that were considered likely mediators of the repressive activity. These candidates were a cAMP response element (CRE), a binding site for the transcription factor c-Myb and a 9 base pair palindrome that contained two putative SBEs. Dwivedi *et al.* (2005) demonstrated that in prostate cancer cells, the majority of repression of the 5'-flanking region of the *CYP27B1* gene was attributed to a GFI-1 binding element at –1161 to –1138. As this GFI-1 binding site lies outside the repressive region under investigation in osteoblast cells, it was not relevant and not tested here. Dwivedi *et al.* (2005) also identified a CRE in this region that contributed to repression in prostate cancer cells. However it was not known if this site also functioned in osteoblast cells. The present data showed that the pCYP27B1mCRE(-1100)-Luc construct expressed at a similar level to the wild type pCYP27B1(-1100)-Luc construct. Therefore the CRE is not a functional component of the repressive region in osteoblast cells. Previous studies have shown that c-Myb can repress promoters such as c-*erbB*-2 (Mizuguchi *et al.*, 1995) and the mouse *plasma membrane Ca(2+)-ATPase-1* (*PMCA1*) promoter (Afroze and Husain, 2000). However, inactivation of the putative c-Myb binding site did not alter the level of expression relative to the wild type. Therefore it was also not considered to

contribute to repression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells.

The two adjacent putative SBEs that form the palindromic sequence 5'-GTCTCAGAC-3', within the *CYP27B1* gene repressive region, were the next focus. This sequence differs by only one base pair, at its central cytosine, from the previously characterised eight base pair repressive palindromic sequence 5'-GTCTAGAC-3' identified within the native promoter of the *Smad7* gene. A detailed discussion of this characterised eight base pair palindrome is relevant here before dealing with the data relating to the palindrome in the *CYP27B1* gene. Oligonucleotide selection experiments identified 5'-GTCTAGAC-3', the sequence present within the *Smad7* gene promoter, to be the sequence specifically bound by recombinant Smad3 and Smad4 proteins (Zawel *et al.*, 1998). Using a CHIP assay it was established that Smad4, but not Smad3 or Smad2 bound in the repressed state together with Ski, that is during basal expression (Denissova and Liu, 2004). While these authors have not presented a detailed model for the repression mechanism on the *Smad7* gene promoter, the model shown in Figure 4.14.a. attempts to bring together the known facts. It is envisaged that Smad4 binds to one half site of the palindrome (either through the 5'-GTCT-3' half site, or its reverse complement 5'-AGAC-3') although the possibility that Smad4 molecules bind to each half site cannot be eliminated (see Figure 4.14.a). Smad4 then recruits Ski which can inhibit gene expression by forming a complex with histone deacetylase (HDAC) through direct binding to the nuclear hormone receptor co-repressor (NCoR) (Nomura *et al.*, 1999).

In support of this model mutagenesis of the palindrome increases the basal level of expression indicating that the *Smad7* promoter is repressed by factors that bind to this site (Denissova and Liu, 2004) (Figure 4.14.a). As mentioned, Denissova *et al.* (2004) used CHIP assays to demonstrate that Ski and Smad4, but not Smad2 or Smad3, bound the endogenous *Smad7* promoter under basal conditions. Moreover, over expression of Ski repressed promoter expression, and RNAi knockdown of *ski* mRNA demonstrated that Ski could act as a co-repressor of Smad4 to inhibit basal expression of the *Smad7* gene (Denissova and Liu, 2004). This basal repression is over come in the presence of TGF-β. When cells transfected with the *Smad7* promoter were treated with TGF-β, expression was induced and this was found to be dependent on an intact palindrome (Denissova et al., 2000) (Figure 4.14.b). In the induced state, CHIP assays show increased binding of Smad4 to the *Smad7* promoter and an antibody against Smad2/3 also detected binding. Interestingly Ski remained associated with the promoter in the presence of TGF-β as shown by the CHIP assay. Hence the following model for induction of the gene can be proposed. Treatment of the cells with TGF-β causes phosphorylated Smad2 and Smad3 to accumulate in the nucleus where they form a complex with Smad4. This Smad complex then displaces the co-repressor from the palindrome allowing a coactivator complex to bind in its place. The coactivator complex then induces gene expression through increased RNA polymerase activity perhaps by direct association as shown (Figure 4.14.b).

Since the palindromic sequence was critical for repression of the *Smad7* gene promoter it seemed highly likely that a similar sequence in the *CYP27B1* gene repressive region would underlie the inhibitory action. When each putative SBE was individually mutated within the pCYP27B1(-1100)-Luc construct, the results clearly

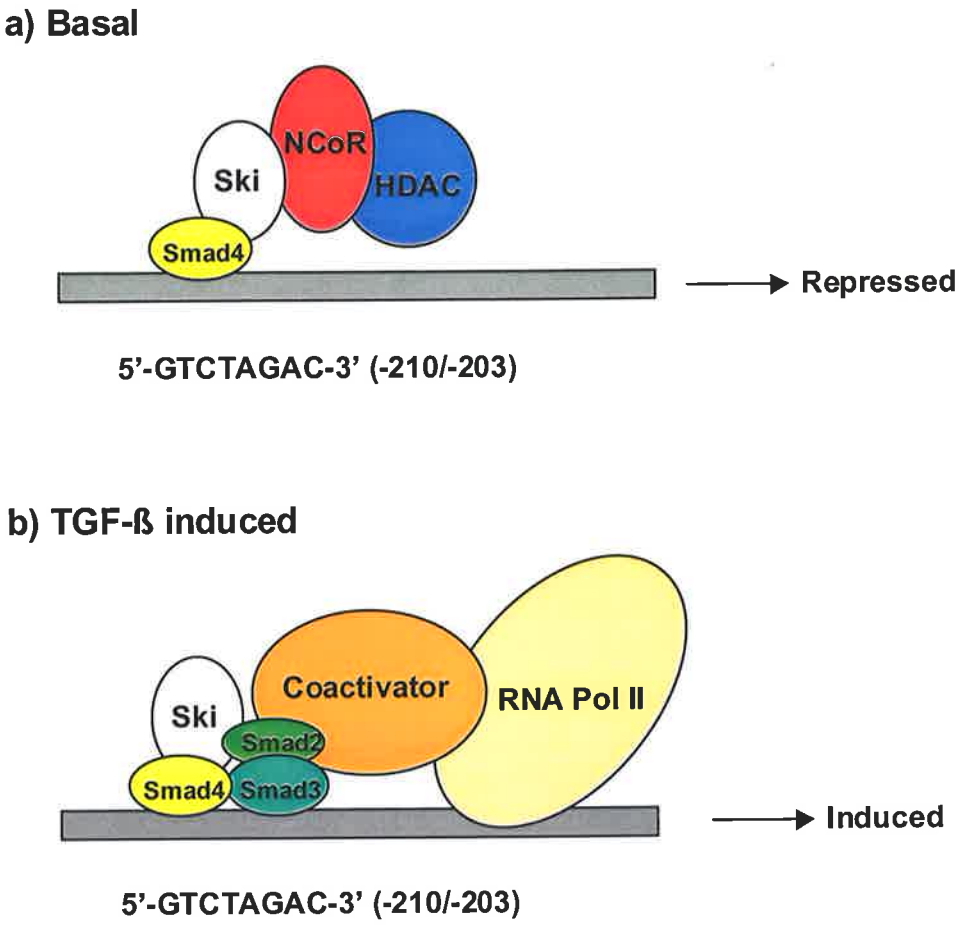

Figure 4.14. The proposed model whereby expression of the human *Smad7* gene is regulated. a) In the absence of TGF-ß, Smad4 binds the palindrome 5'-GTCTAGAC-3' recruiting Ski and a co-repressor complex that may involve NCoR and HDAC to repress transcription. b) TGF signalling causes Smad2 and Smad3 to associate with Smad4 in the nucleus. The Smad complex displaces the co-repressor and a coactivator binds in its place. The coactivator may induce gene expression by direct interaction with RNA polymerase II. Abbreviations: NCoR, nuclear receptor co-repressor; HDAC, histone deacetylase; RNA Pol II, RNA polymerase II.

demonstrated reversal of repression of *CYP27B1* reporter activity. Not surprisingly, inactivation of both SBEs within a double mutant also abolished repression.

It was also of interest to investigate the significance of the cytosine base that lies at the centre of this palindrome. Whilst flanking sequences vary, the inclusion of this extra base represents the only difference between the core of this sequence and that of the eight base palindrome that mediates repression of the *Smad7* gene promoter. When this cytosine was replaced with an adenine in the current study, derepression was also observed that was comparable with mutagenesis of either SBE. This result shows that the central cytosine is necessary for a multi-protein repressive complex to function through this nine base palindrome. This suggests that the complex may be different to that which functions through the eight base pair equivalent on the *Smad7* gene promoter or alternatively, it is possible that the central cytosine could be a part of the SBE2 sequence. Although the consensus SBE is normally described as 5'-GTCT-3' or 5'-AGAC-3', the sequence 5'-CAGAC-3' is found within many Smad responsive promoters (Massague *et al.*, 2005) (see Chapter 1.8.4). Therefore it is possible that the central cytosine, rather than separating the two SBEs, forms part of SBE2. In which case, the 9 base palindrome comprises exactly two SBEs, much like its 8 base pair equivalent within the *Smad7* gene promoter.

Having established that SBE1 and SBE2 were functional in ROS 17/2.8 cells, the question arose as to whether these sites mediate repression in other cell types. Of the four other cell lines tested, SBE1 and SBE2 were only found to function in HOS human osteoblast cells where their inactivation substantially reversed repression. The fact that no repressive region was detected between –997 and –1100 in HEK-293T

human kidney cells and MDA-MB-468 human breast cancer cells rules out SBE1 and SBE2 as having a repressive action in these cells. Weaker repression was observed in COS-1 cells, however inactivation of these sites did not restore expression to the extent that was observed in ROS 17/2.8 and HOS cells. Taken together, these results suggest that SBE1 and SBE2 may function as repressive elements specifically within osteoblast-like cancer cells.

This observation is consistent with cell specific repression of the 5'-flanking region of the *CYP27B1* gene in transfected prostate and kidney cells. Moreover, it suggests that repression of the *CYP27B1* gene may be a common mechanism of control in specific cell types, whereby inhibition is effected through different regions of the 5'-flanking region. Whereas in osteoblast cells, it appears that repression can be attributed to the palindromic sequence at -1015 to -1007, repression of the 5'-flanking region in prostate cells is mediated by GFI-1 through a sequence at -1161 to -1138. In HEK-293 kidney cells, a much weaker repressive region exists between -1413 and -927 (Ebert *et al.*, 2004). In addition, expression of the 5'-flanking region in HEK 293 cells can be down regulated through NFκB signalling, and ten NFκB binding sites have been identified between -804 and -83. Therefore each cell type in which expression of the 5'-flanking region has been studied has revealed differences in the level of repression, the location the repressive region, and perhaps the molecular mechanisms that mediate repression. This raises the interesting questions as to why repression occurs in each cell type and why the mechanism varies.

With regard to osteoblasts, repression of the 5'-flanking region of the *CYP27B1* gene may have physiological relevance. Repression of *CYP27B1* gene expression could be

necessary to limit the local production of $1,25(OH)_2D_3$ by proliferating osteoblasts. As described in Chapter 1.6.1, formation of bone by osteoblasts is dependent upon their ordered development. Before the production and subsequent mineralisation of an extracellular matrix, immature osteoblasts first undergo a period of proliferation (Owen *et al.*, 1990). When immature osteoblasts in culture are treated with $1,25(OH)_2D_3$, their development is interrupted and an extracellular matrix is not produced (Owen *et al.*, 1991). However, at later stages of osteoblast development, *in vitro* mineralisation can be stimulated by $1,25(OH)_2D_3$ (Matsumoto *et al.*, 1991). Therefore, it may be advantageous for osteoblasts to restrict *CYP27B1* gene expression during early stages of development.

Alternatively, it should be noted that ROS 17/2.8 and HOS are both cancer cell lines, and so too is the DU145 cell line used to investigate repression in prostate cells. Therefore, it is possible that repression of the 5'-flanking region of the *CYP27B1* gene may be a feature of the cancer phenotype. As described in Chapter 1.5.4, $1,25(OH)_2D_3$ has been shown to inhibit the proliferation of various cancer cell cultures including leukemia (Abe *et al.*, 1981), breast (James *et al.*, 1996) and prostate (Zhao *et al.*, 2000b). Moreover, CYP27B1 activity has been reported to be lower in prostate cancer cells compared to normal prostate cells (Hsu *et al.*, 2001). The molecular mechanism of repression of *CYP27B1* gene expression in osteoblast cells is clearly an important question.

Previous studies of the *Smad7* gene promoter suggested that Smad4 and Ski play a role in repression through the 5'-GTCTAGAC-3' palindrome (Denissova and Liu, 2004). Therefore expression plasmids for Smad4 and Ski were over expressed in

ROS 17/2.8 cells in an attempt to further repress expression of the pCYP27B1(-1100)-Luc construct. The observation that Ski alone could not cause further repression was in contrast to its effect on the *Smad7* gene promoter in HepG2 cells (Denissova and Liu, 2004). Since Ski requires a binding partner such as Smad4 in order to bind DNA, expression plasmids for these two proteins were then over expressed simultaneously. The combined over expression of Ski and Smad4 marginally lowered expression of the pCYP27B1(-1100)-Luc construct, although the effect was independent of the putative SBEs. However these results do not preclude Ski and Smad4 from involvement in the repression of the pCYP27B1(-1100)-Luc construct. It is possible that endogenous levels of one or both of these proteins are high in ROS 17/2.8 cells with repression already at a maximum level. This idea could be tested by Western blot analysis to measure the endogenous level of Smad4 and Ski proteins within ROS 17/2.8 cells.

However in an attempt to overcome this potential problem in the current study, Smad4 and Ski were independently over expressed in HEK-293T cells. The repressive action of the region between -1100 and -997 was not detected in earlier experiments in these cells, and no effect when the putative SBEs were mutated was detected (see 4.4.2). Interestingly, over expression of the Smad4 plasmid lowered expression of both the pCYP27B1(-1100)-Luc and pCYP27B1mSBE(1and2)(-1100)-Luc constructs confirming that Smad4 can act at another site within the 5'-flanking region of the *CYP27B1* gene. A search of the 5'-flanking region revealed eight other putative SBEs within the pCYP27B1(-1100)-Luc construct at -1043/-1040, -879/-876, -747/-744, -687/-684, -619/-616, -307/-304, -223/-226 and -160/-157. Once again, these results do not exclude the interaction of these proteins at the -1015/-1007 site.

Over expression of the Ski plasmid in HEK-293T cells increased expression of both the pCYP27B1(-1100)-Luc construct and also the pCYP27B1mSBE(1/2)(-1100)-Luc constructs. This result indicated that Ski can act via an SBE independent mechanism. The pCYP27B1(-997)-Luc construct was also activated by Ski (data not shown). These data suggest that Ski activates the 5'-flanking region of the *CYP27B1* gene by acting through a site that is proximal to –997. It is interesting to note that a search revealed the presence of a near perfect binding site for the nuclear factor I (NFI) family of transcription factors at –867/-855 (5'-TGGCCTCATGCCA-3'; core sequence is underlined). Ski has previously been shown to bind NFI and activate transcription of promoters containing NFI binding sites (Tarapore *et al.*, 1997). However the possibility of Ski acting through the NFI binding site within the 5'-flanking region of the *CYP27B1* gene was not further investigated here.

As explained earlier, SBEs commonly mediate promoter activation by TGF-β. For example, the promoter of the *Smad7* gene is activated by TGF-β1 in HepG2 cells (see Figure 4.14). Therefore it was relevant to know what effect, if any, TGF-β1 would have on the 5'-flanking region of the *CYP27B1* gene. Treatment of ROS 17/2.8 cells with TGF-β1 cells lowered expression of the pCYP27B1(-1100)-Luc construct by approximately 50% relative to untreated cultures. This result was surprising given that TGF-β1 activates the *Smad7* promoter via the palindrome 5'-GTCTAGAC-3'. The fact that TGF-β1 also repressed the pCYP27B1mSBE(1and2)(-1100)-Luc construct by approximately 50% suggested that the effect occurred independently of SBE1 and SBE2. Indeed subsequent investigation of the 5'-flanking region of the *CYP27B1* gene indicated that a more proximal TGF-β inhibitory region exists

between –531 and –305. This TGF-β1 inhibitory region is the subject of studies described in Chapter 5.

Having established that the sequences designated SBE1 and SBE2 contribute to repression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells, the identity of the proteins that bound these elements was then investigated. Based on their sequences, it was hypothesised that Smad proteins would bind SBE1 and SBE2. As described in Chapter 1.8.3, in the absence of TGF-β signalling, Smad2 and Smad3 are predominantly located in the cytoplasm whereas Smad4 is distributed in the nucleus and cytoplasm (ten Dijke and Hill, 2004). Given that repression had been detected under basal conditions in the present study (that is, osteoblasts were cultured in the absence of exogenous TGF-β), it was assumed that Smad4 would be the only Smad protein involved. This assumption is supported by previous work on the *Smad7* promoter, whereby CHIP assays using HepG2 cells showed that in the basal state, Smad4 but not Smad2 or Smad3, bound the *Smad7* promoter (Denissova and Liu, 2004). Therefore, recombinant GST-Smad4 was prepared and EMSAs were performed in order to test whether the protein could bind the CYP27B1 probe that contained SBE1, SBE2 and additional native flanking sequence. Indeed GST-Smad4 was found to bind this probe in a concentration dependent manner, supporting the view that these sites are SBEs. This result is consistent with a model whereby Smad4 binds the SBEs under basal conditions and recruits a co-repressor, such as Ski, to inhibit expression of the *CYP27B1* gene in a manner similar to the *Smad7* promoter.

As the palindrome contained two putative SBEs, it was of interest to know if Smad4 bound to one or both elements. Studies of the *Smad7* gene promoter demonstrated

that the palindrome conferred basal repression, and also mediated induction of promoter expression by TGF-β. However the palindrome's half-sites were not examined as individual SBEs in these studies (Denissova and Liu, 2004), and therefore there is no information as to the function of each SBE within the palindrome located in the *Smad 7* gene promoter. The result of EMSAs in the present study showed that GST-Smad4 bound the mSBE2 probe indicating that SBE2 was not required for binding to the palindrome. However mutagenesis of SBE1 abolished binding, showing that SBE1 is the site of GST-Smad4 binding (Figure 4.11). The protein bands produced by the mSBE2 and CYP27B1 probes were of the same mobility indicating that the size of the protein-probe complex is the same in each case. On this basis, it follows that GST-Smad4 binds to SBE1 (and not SBE2) on the wild type CYP27B1 probe (Figure 4.15.a). In addition there are no reports in the literature of Smad4 forming homo-dimers, therefore it is predicted that a Smad4 monomer binds SBE1. Interestingly, transfection experiments described in this chapter showed that inactivation of SBE2 abolished repression suggesting in fact it is involved in the repression phenomenon. Since SBE2 does not bind GST-Smad4, it could be predicted to bind an unknown protein that could interact with the co-repressor complex (Figure 4.15.a).

In order to investigate further the identity of the transcription factors that functioned through the SBEs, EMSAs were performed using nuclear proteins isolated from ROS 17/2.8 cells. To the author's knowledge, binding in EMSA experiments of Smad proteins present in nuclear extracts to DNA has not been reported in the literature without prior treatment of the cells with TGF-β to enhance the nuclear location of the protein. The Smad7 probe produced two molecular complexes (C1 and C2) and these

were also seen when the CYP27B1 probe was tested. This indicated that yet to be identified nuclear proteins within ROS 17/2.8 cells, may be capable of binding the SBEs within the 5'-flanking region of the *CYP27B1* gene. It also indicated that the proteins that bind each probe may be similar, which could be expected considering their sequence similarity. A second CYP27B1-based probe was tested that lacked the native sequence that flanks SBE1 and SBE2. The fact that in repeated EMSAs, no bands were detected with this probe indicated the importance of flanking sequence to the binding.

Competition analyses were then performed in order to establish the specificity with which the nuclear proteins were binding to the oligonucleotide sequence of the CYP27B1 probe. The result that unlabelled CYP27B1 oligomer successfully competed for binding suggests that the observed bands were specifically binding to this sequence of the CYP27B1 probe. The fact that the unlabelled consensus SBE probe (which contains two copies of the sequence 5'-GTCTAGAC-3') also competed for binding confirmed that the proteins that bound the CYP27B1 probe could also bind the SBE palindrome. However the CYP27B1-core probe that lacked flanking competed very weakly for binding to the nuclear proteins, again demonstrating the importance of flanking sequence in the protein-DNA interaction.

A final set of experiments was performed with the aim of confirming the identity of the nuclear proteins that bound the CYP27B1 probe. Antibodies against Smad4 and Ski were used in binding reactions in an attempt to 'shift' the protein complexes. However no shift was seen and the experiments yielded no evidence that either Smad4 or Ski bound the CYP27B1 probe. It should be noted that while these

antibodies have been reported to be functional in the literature, they were not tested for their ability to 'shift' protein complexes in control experiments in the present work.

In summary, transient transfections have been used to identify a palindromic sequence, 5'-GTCTCAGAC-3' (–1015/–1007), that mediates repression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. This sequence is almost identical to the original consensus SBE (5'-GTCTAGAC-3') identified *in vitro* by oligonucleotide selection experiments. However, the latter sequence has only been identified in one promoter, that of the *Smad7* gene where it mediates induction by TGF-β, as well as basal repression of the *Smad7* gene by association with Smad4 and Ski. Therefore the negative factors that repress the 5'-flanking region of the *CYP27B1* gene were hypothesised to include Smad4 and Ski. The binding of recombinant Smad4 protein to a probe that contained the repressive element(s) provides evidence of the involvement of Smad4. Based on the available data, the following model can be proposed for the repression of the *CYP27B1* gene promoter (Figure 4.15). Smad4 binds to SBE1 and then recruits an unknown factor (protein X) that binds SBE2. Smad4 and protein X then together recruit a co-repressor protein complex which results in repression of *CYP27B1* gene expression (Figure 4.15.a). Mutagenesis of SBE1 prevents Smad4 binding, interfering with the recruitment of the co-repressor complex with concomitant derepression of the gene (Figure 4.15.b). When SBE2 is mutated, Smad4 binds to SBE1 but the absence of protein X interferes with the recruitment of the co-repressor complex (Figure 4.15.c).

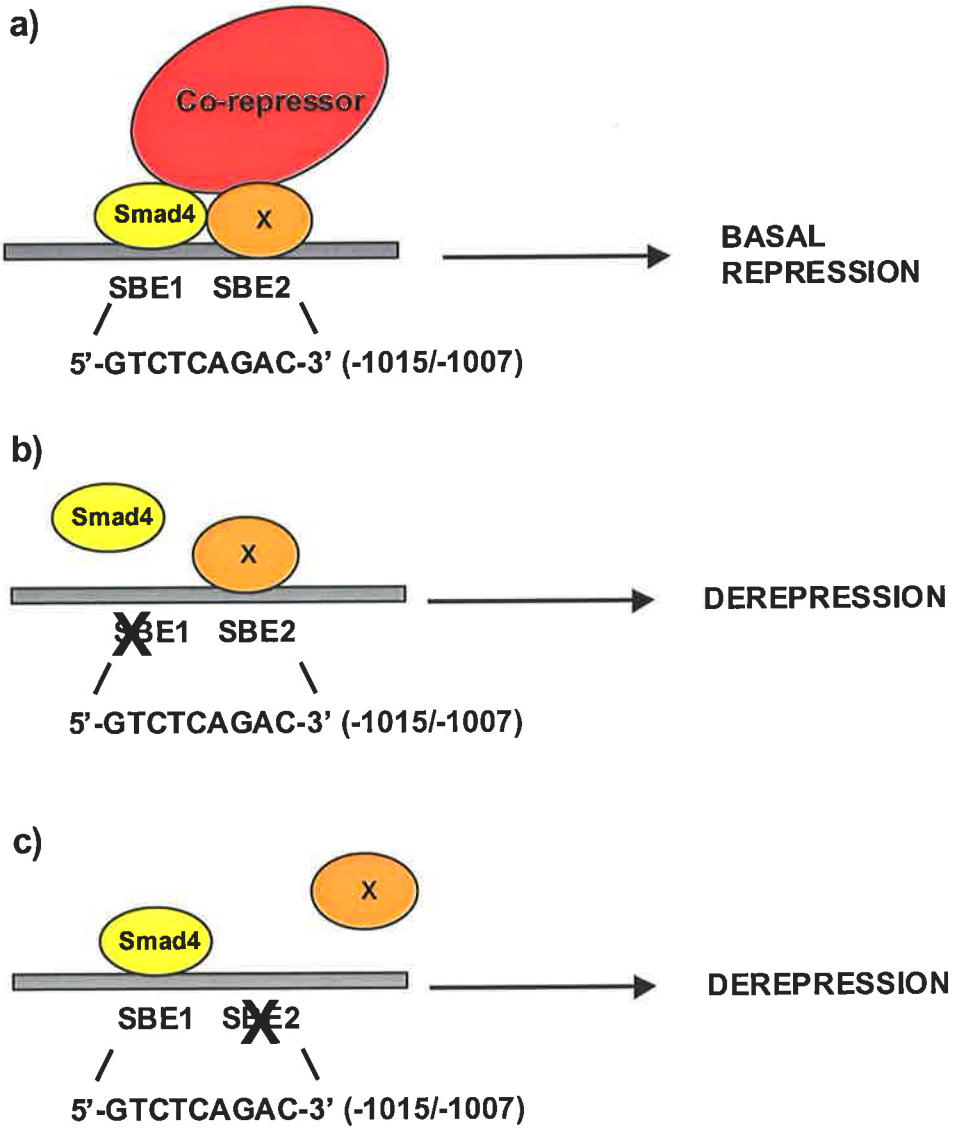

Figure 4.15. The proposed model of basal repression of *CYP27B1* gene expression in ROS 17/2.8 cells. a) Smad4 binds SBE1 and an unknown protein (protein X) associates with SBE2. Both proteins are required to recruit a co-repressor that represses transcription. Inactivation of SBE1 abolishes Smad4 binding and the co-repressor is not recruited. Similarly, inactivation of SBE2 prevents protein X binding and the co-repressor is not recruited to the palindrome.

Importantly, this newly identified repressive element, 5'-GTCTCAGAC-3' (-1015/-1007) within the 5'-flanking region of the *CYP27B1* gene is not responsible for the repression of the gene previously determined in kidney and prostate cancer cells. The present study is important because it further strengthens the concept that regulation of *CYP27B1* gene expression is tissue specific.

4.6 CONCLUSIONS

4.6.1 Repression of the 5'-flanking region of the *CYP27B1* gene in osteoblasts primarily involves the palindromic sequence 5'-GTCTCAGAC-3' (–1015/–1007). A nearby CRE that partially contributes to repression in prostate cancer cells appears not to function in osteoblast cells.

4.6.2 The palindrome was found to function as a repressive element in both ROS 17/2.8 and HOS osteoblast cells, and may be specific to cells of this lineage.

4.6.3 The repressive sequence comprises two consensus SBEs and was shown to bind GST-Smad4.

Chapter 5:

Investigations into the Effects of Growth Factors and Hormones on the Regulation of the CYP27B1 5'-Flanking Region in ROS 17/2.8 cells

5.1 INTRODUCTION

For more than two decades, expression of the *CYP27B1* gene has been observed in various extra-renal tissues, including cultured macrophages, keratinocytes and bone cells (Anderson *et al.*, 2003a; Zehnder *et al.*, 2001) (Chapter 1.3). During this time, regulation of the renal *CYP27B1* gene by physiological factors such as parathyroid hormone (PTH) and $1,25(OH)_2D_3$ have been well documented (Dusso *et al.*, 2005; Omdahl *et al.*, 2002) (Chapter 1.4.1). Briefly, PTH acts in a positive fashion through cAMP to increase transcription of the gene (Armbrecht *et al.*, 2003b), whereas $1,25(OH)_2D_3$ down regulates expression of the gene using VDR and a novel mechanism (Murayama *et al.*, 2004). However the role of these endocrine factors in regulating the *CYP27B1* gene within bone cells is unknown.

There is an emerging view that the local production of $1,25(OH)_2D_3$ is controlled differently at non-renal sites compared to that in the kidney. Chapters 3 and 4 have described differences in the basal expression of the 5'-flanking region of the *CYP27B1* gene between osteoblast, kidney and prostate cells. There is also evidence that the *CYP27B1* gene responds differently to circulating factors in non-renal tissues compared to the kidney. Interestingly, CYP27B1 activity within pulmonary alveolar macrophages is not regulated by the calciotropic factors that regulate renal CYP27B1

activity. Rather, it appears to be responsive to immune cell regulators. Macrophage CYP27B1 activity is stimulated by bacterial lipopolysaccharide (LPS) (Reichel *et al.*, 1987b), and cytokines such as interferon-γ (IFNγ) (Adams *et al.*, 1989; Monkawa *et al.*, 2000), tumour necrosis factor α (TNFα) (Reichel *et al.*, 1989) and interleukin-2 (IL-2) (Reichel *et al.*, 1987a) (Chapter 1.4.2).

It was therefore reasoned that *CYP27B1* gene expression in bone might be regulated by the same factors that modulate osteoblast development. The proliferation and differentiation of osteoblasts *in vitro* can be regulated by the locally secreted growth factors transforming growth factor-beta (TGF-β) and insulin-like growth factor-1 (IGF-1). As such, it was hypothesised that these factors might regulate *CYP27B1* gene expression in osteoblasts. Indeed, TGF-β was shown to repress the p*CYP27B1*(-1100)-Luc construct in experiments described in Chapter 4.

Accordingly, the present study asked whether TGF-β, IGF-1, PTH and $1,25(OH)_2D_3$ could regulate expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. The inhibitory effect of TGF-β is further discussed, and the overall results are compared to data previously reported in kidney cells.

5.2 SPECIFIC AIMS AND HYPOTHESES

5.2.1 Specific aims

1) To determine the effect of exogenously added PTH, $1,25(OH)_2D_3$, TGF-β1 and IGF-1 on expression of a reporter gene controlled by the 5'-flanking region of the *CYP27B1* gene in osteoblast cells.

2) To investigate the mechanism by which TGF-β1 represses the 5'-flanking region of the *CYP27B1* gene in osteoblast cells.

5.2.2 Specific hypothesis

It was hypothesised that the regulation of the 5'-flanking region of the *CYP27B1* gene by exogenous factors in osteoblast cells will be different to that previously described in kidney cells.

5.3 OUTLINE OF METHODS USED IN THIS CHAPTER (Detailed descriptions are given in Chapter 2).

5.3.1 Inactivation of putative transcription factor binding sites

Site-directed mutagenesis of a putative Fra-1 binding site, SBE and TIEG binding site within the pCYP27B1(-531)-Luc construct was carried out using the QuikChange Site

Directed Mutagenesis protocol (Stratagene, La Jolla, USA). Wild type and mutated sequences were as follows, with the core binding sequence underlined:

Fra-1

Wild type 5'-AATATCAGAGACTGACTAGTGTAGCTTG-3' (-315/-288)

Mutant 5'- AATATCAGAGACT**AT**CTAGTGTAGCTTG-3'

SBE

Wild type 5'-TGGGAATATCAGAGACTGACTAGTG-3' (-318/-295)

Mutant 5'-TGGGAATATCAG**TTAC**TGACTAGTG-3'

TIEG

Wild type 5'-AGGAGGGGGAGGTGTGGCTAGTGCAGG-3' (-492/-466)

Mutant 5'-AGGAGGGGGAGG**CTT**GGCTAGTGCAGG-3'

E-box(1+2)

Wild type 5'-ATAGGTACCAACCCACCTGCCATCTGCCCAG-3'(-540/-510)

Mutant 5'-ATAGGTACCAACC**TG**CCTGC**TGT**CTGCCCAG-3'

5.3.2 Transient expression of reporter constructs

Transfection of the cells was performed using DOTAP transfection reagent according to the manufacturer's instructions. Briefly, cells were seeded into 24 well plates and left 6 hours to attach. Cells were transfected overnight with a reporter plasmid in serum free media. A thymidine kinase (TK) promoter-*Renilla* luciferase was included

to control for transfection efficiency. Immediately following the overnight transfection, the media were replaced and cells were treated with rat PTH (1-34) (10^{-7}M), $1,25(OH)_2D_3$ (10^{-7}M), human recombinant TGF-β1 (1ng/ml), human IGF-1 (10^{-9}M) or vehicles for 24 hours before measuring luciferase activity.

5.3.3 Over expression of dominant negative plasmids for MAPK pathway enzymes

The dominant-negative expression plasmids for ERK1 (ERK1K71R), Ras (Ras 17N), MKK4 (MKK4K116R) and MEK5 (MEK5A) have been described in the Methods (2.1.6.). These clones were co-expressed with the pCYP27B1(-531)-Luc reporter construct with the total amount of DNA kept constant in all wells using the empty vector. The kinase inhibitor SB203580 was also used to disrupt p38 signalling.

5.4 RESULTS

5.4.1 Regulation of the *CYP27B1* gene 5'-flanking region by exogenous factors

As discussed earlier (Chapter 1.3), the first -1501 base pairs of flanking sequence is considered to contain all of the upstream elements that regulate *CYP27B1* gene expression (Hendrix *et al.*, 2005). Therefore, in order to investigate regulation of the 5'-flanking region of the *CYP27B1* gene by exogenous treatments, the pCYP27B1(-1501)-Luc construct was used in transfections. The endocrine factors PTH and $1,25(OH)_2D_3$ were tested because they have previously been shown to modulate expression of the 5'-flanking region of the *CYP27B1* gene in kidney cells (Gao et al.,

2002; Murayama et al., 1999). The effect of the growth factors TGF-β and IGF-1 (known to be locally secreted) on expression of the pCYP27B1(-1501)-Luc construct was also investigated as these factors modulate osteoblast development *in vitro*. The effect of these factors was investigated in a serum-free environment.

The results show that expression of the pCYP27B1(-1501)-Luc was unaltered by treatment with $1,25(OH)_2D_3$ (10^{-7}M) whereas treatment with PTH (10^{-7}M) was led to a small but non-significant ($p>0.05$) reduction in expression (Figure 5.1). Treatment of the cells with TGF-β1 (1ng/ml) and IGF-1 (10^{-9}M) substantially decreased pCYP27B1(-1501)-Luc expression by 58% and 53% respectively ($p<0.01$) (Figure 5.1). It was decided to investigate further the mechanism by which TGF-β1 inhibited expression of the pCYP27B1(-1501)-Luc construct. The mechanism by which IGF-1 lowered expression was not investigated further.

5.4.2 Dose dependency of repression by TGF-β1

In order to establish the most appropriate concentration to use in future transfections, a dose response experiment was performed. The most highly expressing construct, pCYP27B1(-997)-Luc, was chosen for this experiment in order to maximise the sensitivity of the repressive effect. Figure 5.2 illustrates firstly that this construct could be repressed by TGF-β1 establishing that responsive elements lie within the first –997 base pairs. Secondly, it can be seen that the greatest level of repression was observed when TGF-β1 was used at a concentration of 1ng/ml. Less repression was observed when the dose was decreased to 0.1ng/ml, but increasing the dose to

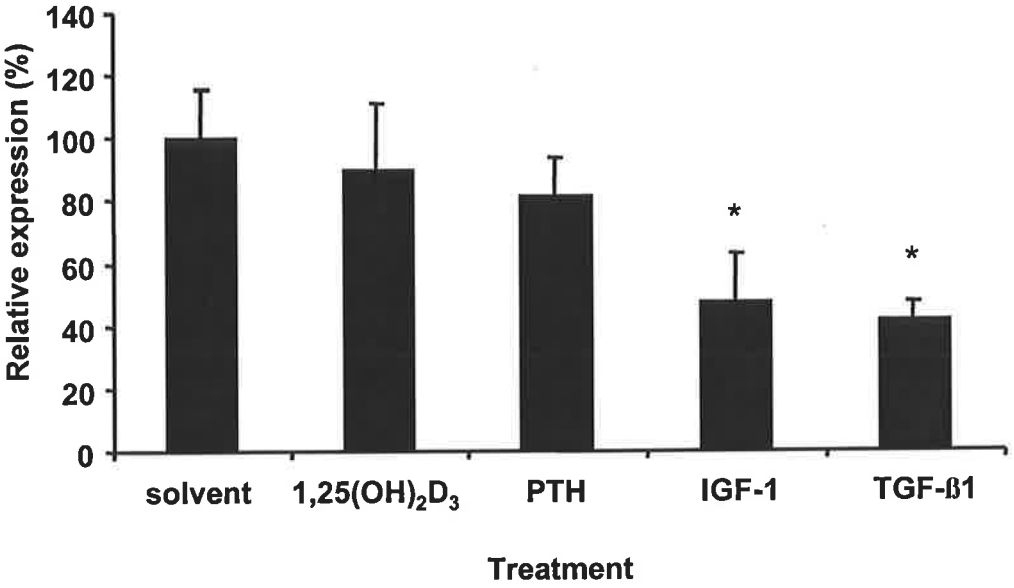

Figure 5.1: Effect of exogenous factors on expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. ROS 17/2.8 cells were transiently transfected with the pCYP27B1(-1501)-Luc construct and then treated with either PTH (10^{-7}M), $1,25(OH)_2D_3$ (10^{-7}M), TGF-ß1 (1ng/ml), IGF-1 (10^{-9}M) or vehicle for 24 hours in serum free media before measuring luciferase activity. The ratio of firefly luciferase: *Renilla* luciferase was calculated and then converted to a percentage relative to the appropriate solvent treated culture. Data are expressed as the mean ± S.D. of triplicate samples and are a representative of a minimum of 3 experiments. One way analyses of variance were performed to identify significant differences between groups, * = $p<0.01$ vs. solvent.

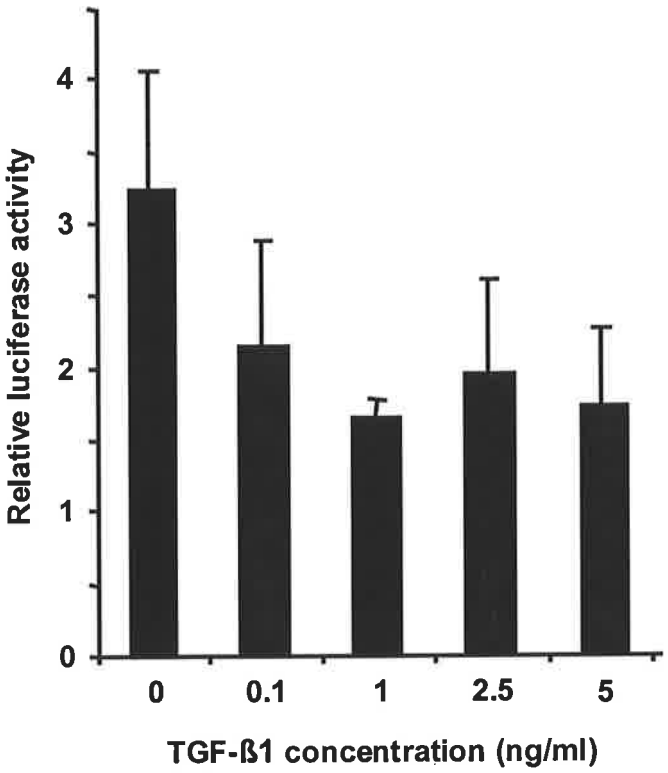

Figure 5.2: Dose dependency of the response of the 5'-flanking region of the *CYP27B1* gene to TGF-β1. ROS 17/2.8 cells were transiently transfected with the pCYP27B1(-997)-Luc construct and then treated with varying concentrations of TGF-β1 in serum free media for 24 hours. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

2.5ng/ml and 5ng/ml did not equate to increased repression. Consequently, a TGF-β1 concentration of 1ng/ml was used for all experiments described hereafter.

5.4.3 Localisation of a TGF-β inhibitory region

The next aim was to localise more precisely the elements within the 5'-flanking region of the *CYP27B1* gene that were responsible for the inhibitory effect of TGF-β1. The deletion constructs pCYP27B1(-531)-Luc and pCYP27B1(-305)-Luc were first investigated. The results show that expression of the pCYP27B1(-1501)-Luc and pCYP27B1(-531)-Luc constructs were both lowered by approximately 50% by TGF-β1 at a concentration of 1ng/ml (Figure 5.3). However expression of the pCYP27B1(-305)-Luc construct was lowered to a lesser extent. This experiment was performed in triplicate six times and the median level of repression of the pCYP27B1(-305)-Luc construct was 20%.

5.4.4 Putative regulatory elements within the TGF-β inhibitory region

Hence a significant component of the TGF-β inhibitory region is localised between –531 and –305. The regulatory elements that could be mediating the repression phenomenon were next investigated (Figure 5.4). As has been described previously, TGF-β signalling is often mediated by Smad transcription factors that recognise specific response elements (SBEs) within the promoters of target genes (Chapter 1.8.4). Therefore, the only SBE (-307/-304) within this region was considered a candidate, together with an adjacent Fra-1 binding sequence (-303/-297) that was identified using the web-based software 'TESS: Transcription Element Search

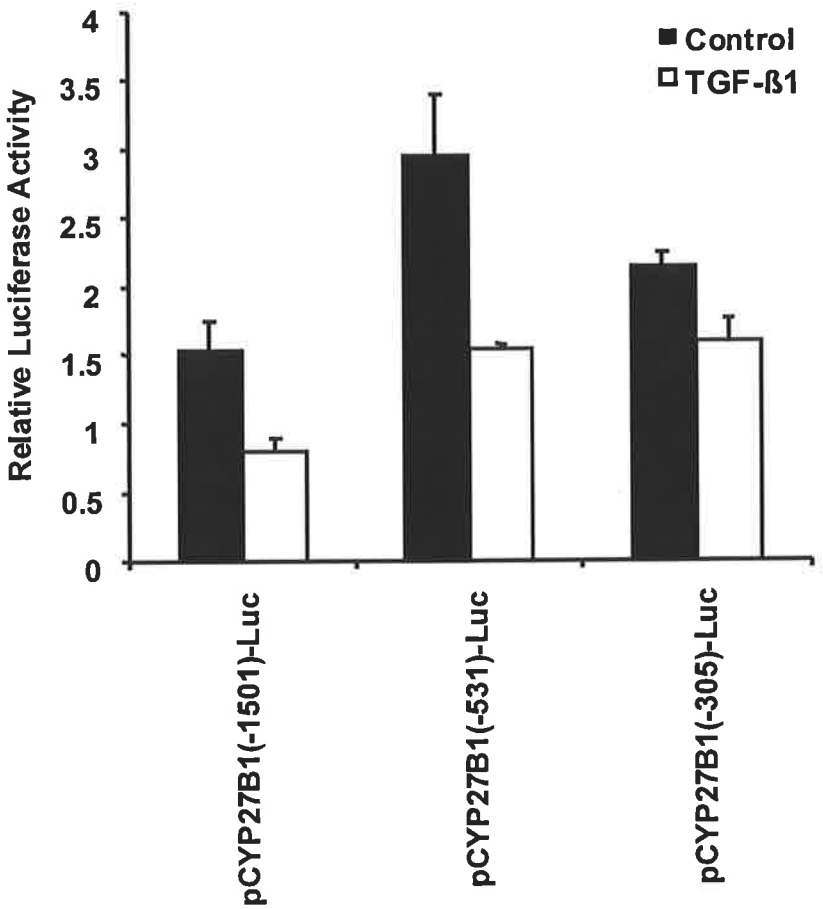

Figure 5.3: Isolation of the TGF-β1 inhibitory region within the 5'-flanking region of the *CYP27B1* gene. ROS 17/2.8 cells were transiently transfected with the pCYP27B1(-1501)-Luc, pCYP27B1(-531)-Luc and pCYP27B1(-305)-Luc constructs and then treated with TGF-β1 (1ng/ml) or solvent for 24 hours in serum free media. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

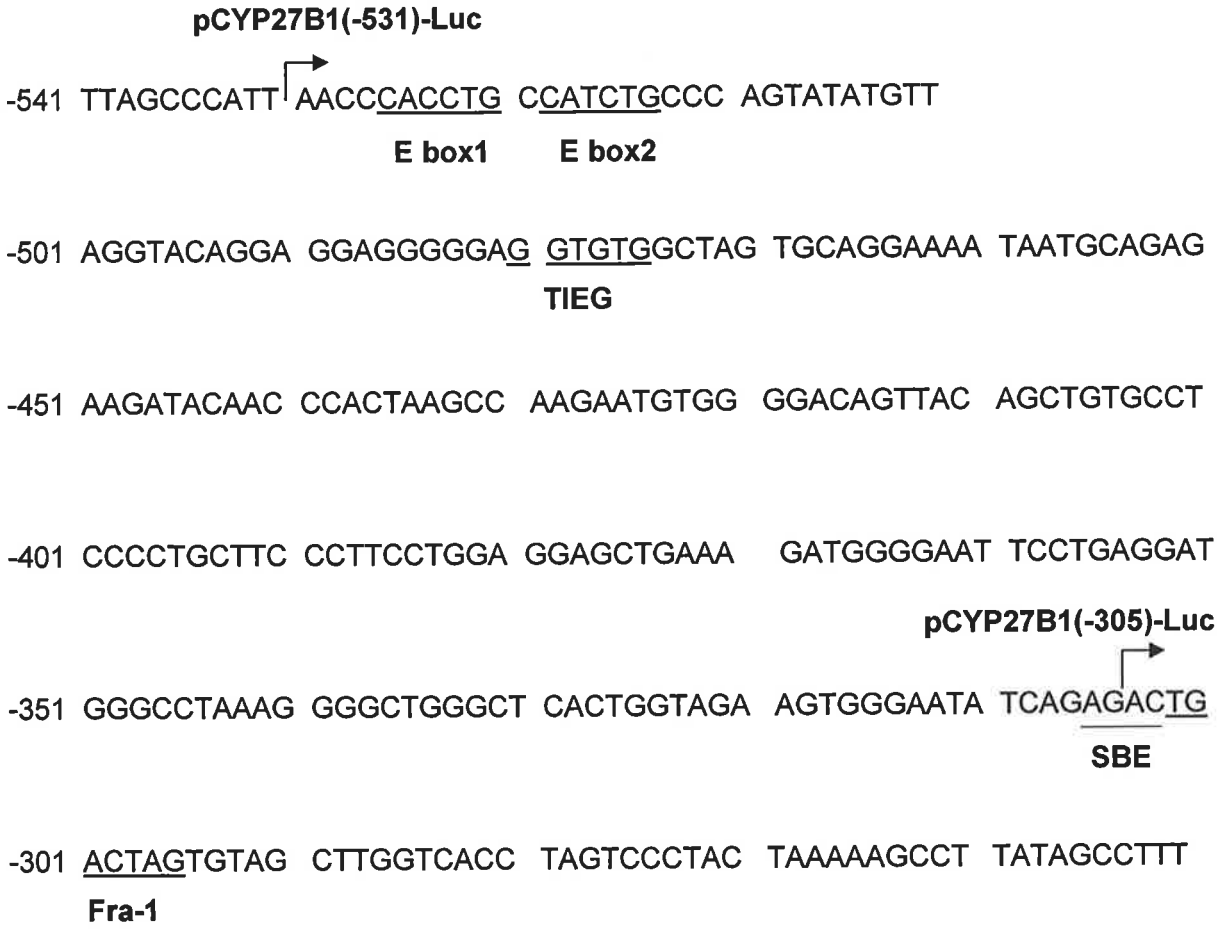

Figure 5.4. Sequence of the TGF-β inhibitory region (-531 to -305) within the 5'-flanking region of the *CYP27B1* gene. Putative transcription factor binding sites are underlined.

System'. These elements were inactivated by site-directed mutagenesis within the pCYP27B1(-531)-Luc construct (see 5.3.1), and both the wild type and mutant reporter constructs were transiently transfected into ROS 17/2.8 cells. As shown in Figure 5.5, the wild type pCYP27B1(-531)-Luc construct is repressed approximately 50% by treatment with TGF-β1. Inactivation of the putative Fra-1 element substantially increased the basal level of expression but did not inhibit the repressive action of TGF-β1 (Figure 5.5). Similarly, inactivation of the putative SBE raised basal expression but did not reverse the effect of TGF-β1 (Figure 5.5).

TGF-β can also effect repression by inducing expression of the *TGF-β-inducible early gene* (*TIEG*) which encodes the transcriptional repressor TIEG (Cook and Urrutia, 2000). Inspection of the 5'-flanking sequence within the TGF-β inhibitory region revealed a consensus binding site for TIEG at -482/-477. The putative TIEG element was inactivated within the pCYP27B1(-531)-Luc construct (see 5.3.1) and both the wild type and mutant reporter constructs were transiently transfected into ROS 17/2.8 cells. The results showed that inactivation of the putative TIEG element did not reverse repression by TGF-β1, but caused a modest increase in the level of basal expression (Figure 5.6).

A review of the literature also highlighted the presence of a pair of adjacent E-boxes at -527/-522 and -520/-515 (Murayama *et al.*, 2004). These elements have been shown to mediate the repressive action of $1,25(OH)_2D_3$ on the 5'-flanking region of the *CYP27B1* gene in kidney cells. However TGF-β has also been shown to interfere with the expression of promoters that are activated through E-box elements (Liu 2001). Therefore E-box 1 and 2 were inactivated together within the pCYP27B1(-

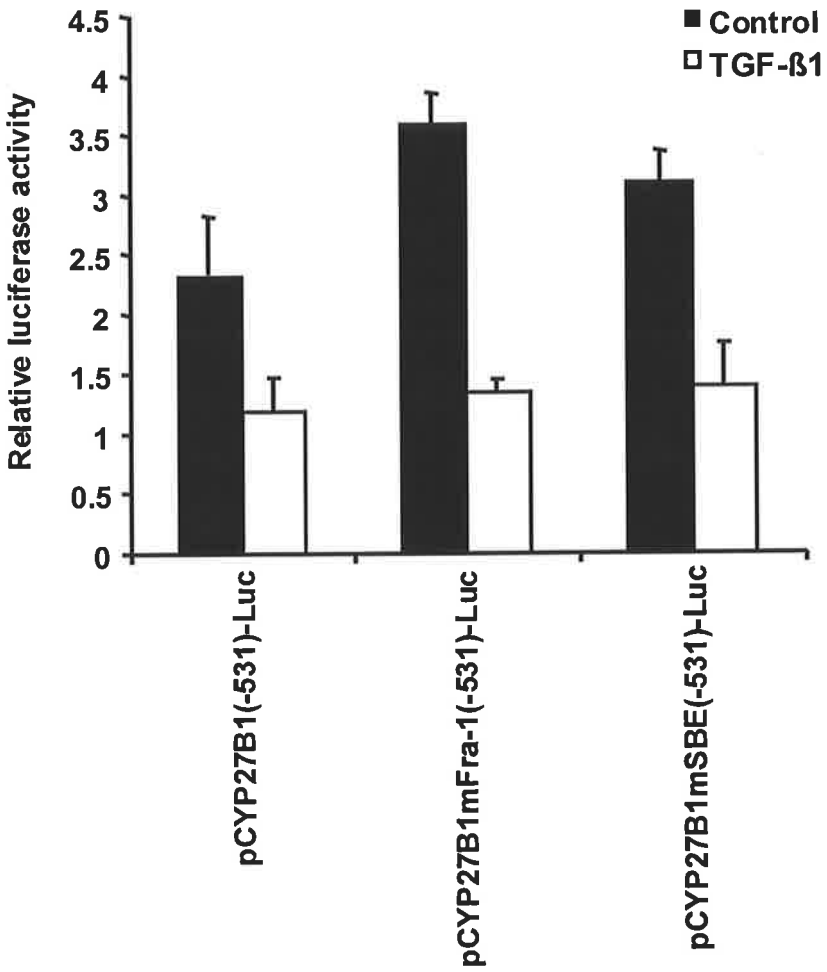

Figure 5.5: Mutational analysis of a putative Fra-1 site and a putative SBE within the TGF-β1 inhibitory region. The putative Fra-1 site and putative SBE were individually inactivated within the pCYP27B1(-531)-Luc construct. ROS 17/2.8 cells were transiently transfected with the wild type or mutant constructs and then treated with TGF-β1 (1ng/ml) or solvent for 24 hours in serum free media. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

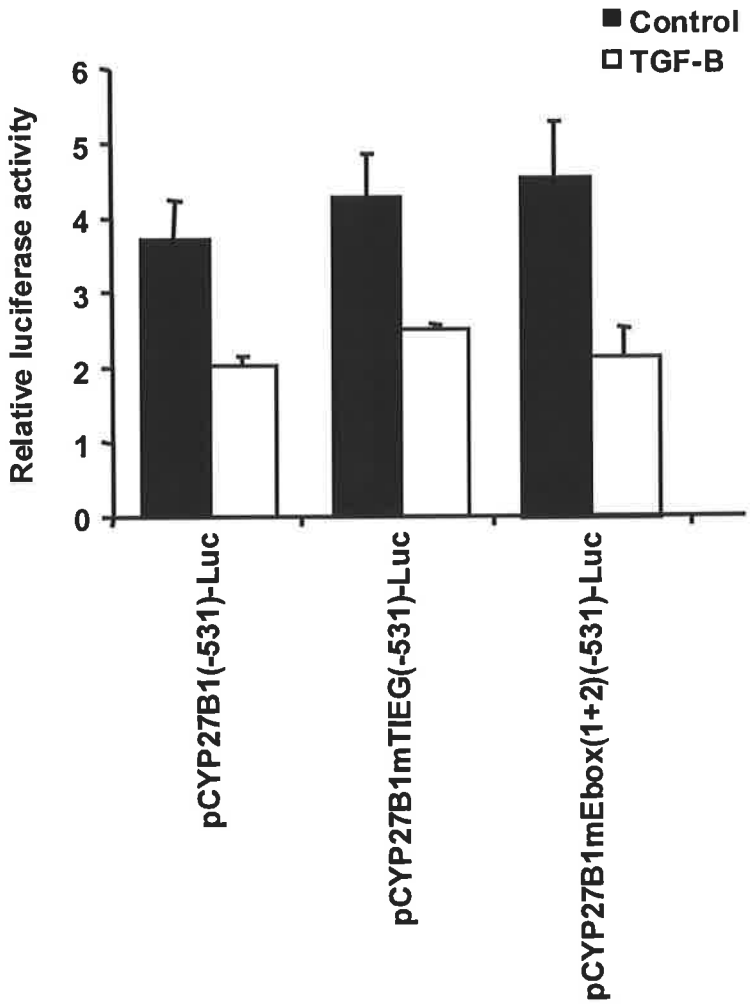

Figure 5.6: Mutational analysis of a putative transcription factor binding sites within the TGF-β1 inhibitory region. The putative TIEG site and two E-boxes were inactivated within the pCYP27B1(-531)-Luc construct. ROS 17/2.8 cells were transiently transfected with the wild type or mutant constructs and then treated with TGF-β1 (1ng/ml) or solvent for 24 hours in serum free media. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

531)-Luc construct (see 5.3.1) and transfected into ROS 17/2.8 cells. Mutagenesis of the E-boxes resulted in a small increase in basal expression illustrating that these elements did not mediate activation within osteoblast cells (Figure 5.6). It was not surprising then that treatment of the cells with TGF-β1 inhibited both the wild type and mutant pCYP27B1(-531)-Luc construct to a similar extent.

5.4.5 Non-genomic pathways and repression by TGF-β

The contribution of non-genomic pathways to repression by TGF-β1 was investigated using dominant negative clones for ERK1 (ERK1K71R), Ras (Ras 17N), MKK4 (MKK4K116R) and MEK5 (MEK5A) (see Methods 5.3.3). Clones were cotransfected with the pCYP27B1(-997)-Luc construct which as described earlier, was used because it was the most highly expressing reporter construct. As shown in Figure 5.7, cells over expressing 500ng of empty vector as control, were repressed nearly 50% by TGF-β1 relative to vehicle treated cultures. When the dominant negative plasmids were over expressed (200ng and 500ng), the level of repression by TGF-β1 remained the same. Similarly, treatment of ROS 17/2.8 cells with the p38 kinase inhibitor SB203580 (5uM and 10uM) did not affect the level of repression by TGF-β1 compared to control treated cells (Figure 5.8).

5.5 DISCUSSION

Over recent years evidence has accumulated of extra-renal expression of the *CYP27B1* gene in a variety of tissues, including bone (see Chapter 1.3). The emerging view is that extra-renal synthesis of $1,25(OH)_2D_3$ may serve an as yet

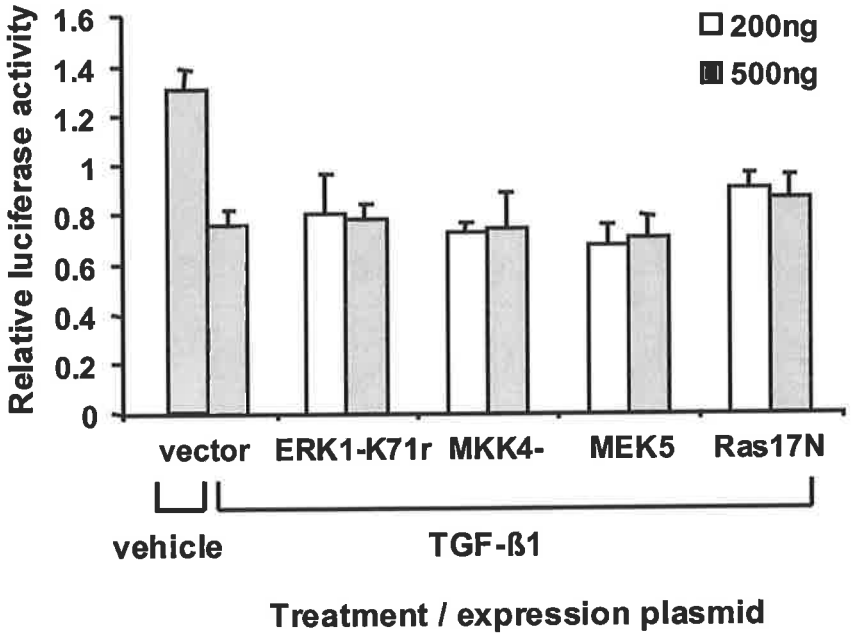

Figure 5.7: The possible contribution of non-genomic signalling to repression by TGF-β1. Dominant negative expression plasmids (200ng and 500ng) were transiently transfected into ROS 17/2.8 cells along with the pCYP27B1(-997)-Luc construct. Cultures were then treated with TGF-β1 (1ng/ml) or solvent for 24 hours in serum free media. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

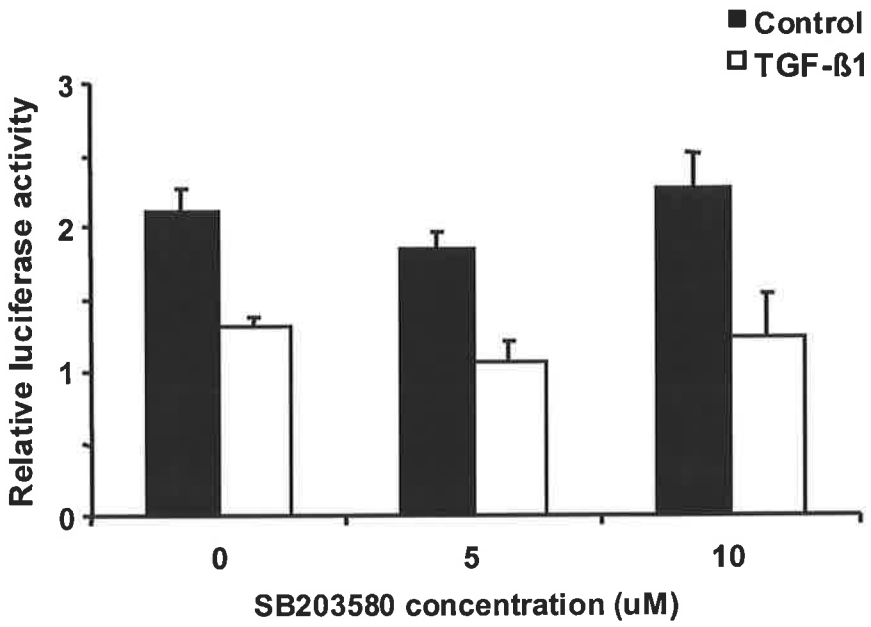

Figure 5.8: The effect of the p38 kinase inhibitor SB203580 on repression by TGF-β1. ROS 17/2.8 cells were transiently transfected with the pCYP27B1(-997)-Luc construct. Cultures were then treated with TGF-β1 (1ng/ml) or solvent for 24 hours in serum free media, as well as SB203580 (0uM, 5uM and 10uM). The relative luciferase activity shown represents the mean ± S.D. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative of a minimum of 3 experiments.

unidentified autocrine or paracrine role (Dusso *et al.*, 2005). This concept is supported by the observation that the factors regulating CYP27B1 activity in non-renal cells such as macrophages, appear to differ from those in kidney cells (see Chapter 1.4.2). However the factors that regulate *CYP27B1* gene expression in bone cells are unknown. This is the first study to investigate hormonal and growth factor regulation of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells.

This study examined a range of biological factors that were predicted to regulate expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. In kidney cells, *CYP27B1* gene expression is regulated predominantly by the calciotropic factors $1,25(OH)_2D_3$ and PTH. Accordingly, these factors were predicted to modulate expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. The observation in macrophages that immune cell regulators control CYP27B1 activity raises the possibility that local factors may regulate expression of the 5'-flanking region of the *CYP27B1* gene in osteoblasts. Therefore, TGF-β and IGF-1 were investigated as these growth factors are secreted locally in bone, and modulate osteoblast gene expression *in vitro*.

Although not statistically significant, the observation that PTH marginally decreased expression of the pCYP27B1(-1501)-Luc in ROS 17/2.8 osteoblast cells is in contrast to the reported action of PTH in kidney cells (Gao et al., 2002; Murayama et al., 1998). However a negative association between serum PTH levels and bone *CYP27B1* mRNA has been previously reported *in vivo* in rats (Anderson *et al.*, 2005). This negative effect of PTH on *CYP27B1* gene expression would suggest that the synthesis of $1,25(OH)_2D_3$ by osteoblasts serves a function unrelated to calcium

homeostasis. Treatment of the cultures with $1,25(OH)_2D_3$ did not significantly alter expression of the pCYP27B1(-1501)-Luc construct. This result is also in contrast to that reported in MCT kidney cells (Murayama *et al.*, 1998). Taken together, these data indicate that the calciotropic factors $1,25(OH)_2D_3$ and PTH have different actions on expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast and kidney cells and are consistent with findings *in vivo* (Anderson *et al.*, 2005).

The addition of TGF-β1 or IGF-1 to the culture media resulted in transcriptional repression of the pCYP27B1(-1501)-Luc construct. Both growth factors decreased expression by approximately 50% with TGF-β achieving a slightly greater effect. Interestingly, $1,25(OH)_2D_3$ increases the synthesis of both TGF-β (Wu *et al.*, 1997) and IGF-1 (Chenu *et al.*, 1990) by cultured bone cells. As such, repression of the 5'-flanking region of the *CYP27B1* gene by TGF-β and IGF-1 may represent a feedback response. Alternatively, the physiological role of repression of the *CYP27B1* gene may be to limit the local production of $1,25(OH)_2D_3$ by proliferating osteoblasts. As described in Chapter 1.6.1, *in vitro* studies have demonstrated that treatment of immature osteoblast cells with $1,25(OH)_2D_3$ can inhibit their development and consequently, the osteoblasts do not synthesize a mineralised extracellular matrix (Owen *et al.*, 1991).

Repression by TGF-β1 was then investigated further. A dose response experiment was performed in order to establish the TGF-β1 concentration that produced maximum repression. The results indicated that TGF-β1 at 1ng/ml caused the greatest level of repression and is consistent with the dose used in other studies

(Leong et al., 2001; Nagarajan et al., 1999). Therefore this concentration was used in future experiments.

In order to elucidate the location of the TGF-β inhibitory region, deletion constructs of the 5'-flanking region of the *CYP27B1* gene were tested. The observation that expression of the pCYP27B1(-531)-Luc construct but not the pCYP27B1(-305)-Luc construct was lowered by approximately 50% by TGF-β1 indicated that the elements responsible for mediating the repressive phenomenon lie between -531 and -305. As described in Chapter 1.8.6, transcriptional repression by TGF-β is not well understood, though a number of mechanisms have been reported. For instance, TGF-β represses the rat osteocalcin promoter in ROS 17/2.8 cells through an AP-1 like binding sequence that interacts with Fra-2 (Banerjee *et al.*, 1996). Inspection of the 5'-flanking region of the *CYP27B1* gene revealed a putative AP-1-like Fra-1 element at -303/-297 and an adjacent canonical SBE sequence at -307/-304. These adjacent elements span the junction at which the distal end of the -305 base pair promoter meets the sequence of the pGL-3 plasmid within the pCYP27B1(-305)-Luc construct. Such a site was consistent with the observation that in repeated experiments, the pCYP27B1(-305)-Luc construct was partially repressed by TGF-β1. As such, the putative SBE/Fra-1 elements were of particular interest. However individual inactivation of each these sites within the pCYP27B1(-531)-Luc construct demonstrated that neither site contributed to repression by TGF-β1. Interestingly, mutagenesis of the putative SBE and Fra-1 elements increased basal expression. These data suggest that a repressive complex may associate with these elements under basal conditions, however this observation was not investigated further.

An alternate mechanism by which TGF-β can repress transcription is by inducing expression of the TGF-β-inducible early-response genes *TIEG1* and *TIEG2*, whose products act as transcriptional repressors (Cook and Urrutia, 2000). A consensus DNA binding element for the TIEG proteins was identified within the TGF-β inhibitory region but changing the core sequence 5'-GGTGTG-3' to 5'-GGCTTG-3' did not reverse repression by TGF-β1. Therefore this element was also ruled out of mediating the response to TGF-β1.

Two adjacent E-boxes exist within the TGF-β inhibitory region and are proposed to mediate negative regulation of the 5'-flanking region of the *CYP27B1* gene by $1,25(OH)_2D_3$ in MCT kidney cells (Murayama *et al.*, 2004). Murayama *et al.* (2004) propose that upon stimulation by PTH, the basic helix-loop-helix (bHLH) proteins bound to the E-boxes recruit a co-activator complex to promote transcription. However upon $1,25(OH)_2D_3$ treatment, liganded VDR associates with the bHLH factors and the co-activator complex is replaced with a histone deacetylase (HDAC) co-repressor complex. It was hypothesised here that a similar interaction may occur upon treatment with TGF-β1, whereby activated Smads may bind the bHLH proteins that associate with the E-boxes to recruit either a co-repressor, or disrupt a co-activator complex. The later mechanism is predicted to mediate the inhibition of myogenic differentiation by TGF-β. More specifically, Smad3 binds the activator MyoD which prevents MyoD from forming a dimer with E12 and subsequently activating target promoters through E-boxes (Liu *et al.*, 2001). However transfection data did not support this model in ROS 17/2.8 cells since inactivation of the E-boxes did not reverse repression by TGF-β1.

Finally, TGF-β is known to signal through three subgroups of the mitogen activated protein (MAP) kinase superfamily; the extracellular signal regulated kinases (ERK), the c-Jun N-terminal kinases (JNK) and the p38/MAPKs (Javelaud and Mauviel, 2005) (Figure 5.9). To date, the ERK5 subgroup has not been shown to be involved in TGF-β signalling. Activation of MAP kinase pathways directs the phosphorylation of nuclear transcription factors to modulate gene expression. Dominant negative expression plasmids and the p35 kinase inhibitor SB203580 were employed to disrupt non-genomic pathways and assess their involvement in repression by TGF-β1. However, over expression of the plasmid encoding a dominant negative Ras protein did not reverse the effect of TGF-β1 indicating that the downstream MAP kinases ERK1 and ERK2 were not involved in the repression. Not surprisingly then, a dominant negative ERK1 expression plasmid had no effect on the repression phenomenon either. Similarly over expression of the plasmid encoding a dominant negative MKK4 protein (which is an activator of JNK – Figure 5.9) did not affect the level of repression. Although ERK5 has not previously been demonstrated to mediate TGF-β signalling, a dominant negative MEK5 kinase (an upstream activator of ERK5 – Figure 5.9) was also investigated. Again however, the level of repression was not affected. Finally, the possibility that TGF-β1 may induce p38 signalling was tested using the p38 inhibitor SB203580. As with the dominant negative MAP kinases, this inhibitor was unable to interfere with repression by TGF-β1 indicating that the p38 pathway is not involved either.

Therefore, there is no evidence that non-genomic signalling mediates the repressive effect of TGF-β1. It is acknowledged however, that the kinases investigated were not exhaustive. For instance, other JNK activators such as MKK7 were not tested.

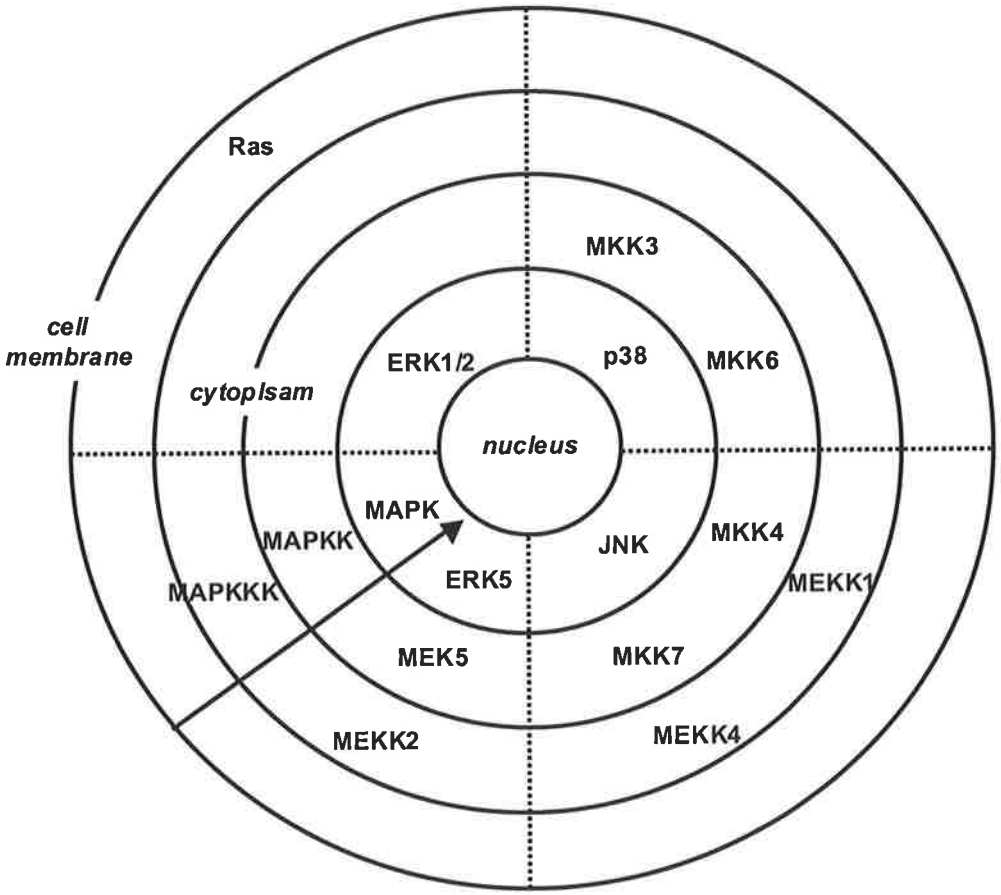

Figure 5.9: Overview of non-genomic signalling pathways. TGF-β1 is known to activate ERK, JNK and p38 signalling. The black arrow indicates the direction of the TGF- β1 signal from the cell membrane to the nucleus. Adapted from Javelaud and Mauviel (2005).

Moreover, signalling through MAPK pathways is complex, and involves a cascade of phosphorylation events and these signalling cascades have not yet been fully described. Therefore, a contribution of non-genomic pathways to the repressive signal initiated by TGF-β1 remains a possibility.

In summary, the present work has provided evidence that the factors that regulate expression of the *CYP27B1* gene in osteoblasts are different to those that regulate the renal *CYP27B1* gene. Although PTH and $1,25(OH)_2D_3$ are important regulators of the renal *CYP27B1* gene, their effects on the 5'-flanking region of the *CYP27B1* gene were not repeated in osteoblast cells. Moreover, the greatest effect on expression of the 5'-flanking region of the *CYP27B1* gene in osteoblasts was produced by the exogenous growth factors TGF-β1 and IGF-1 and these factors are known to be locally secreted by these cells. Overall, these observations are consistent with the hypothesis that the synthesis of $1,25(OH)_2D_3$ by osteoblasts serves an autocrine/ paracrine role as opposed to the endocrine actions of $1,25(OH)_2D_3$ produced in the kidney. Accordingly expression of the *CYP27B1* gene in osteoblast cells is controlled by local factors that are most likely associated with the function of locally produced $1,25(OH)_2D_3$.

Interestingly, no exogenous factor has yet been identified that up regulates expression of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. The fact that *CYP27B1* gene expression is not subject to substantial up- and down-regulation underscores the importance of the transcription factors that determine its basal expression (as described in Chapters 3 and 4). The molecular mechanism whereby TGF-β1 represses expression of the 5'-flanking region of the *CYP27B1* gene in

osteoblast cells was not identified. However the elements responsible for a significant proportion of the repression are predicted to lie within an inhibitory region between -531 and -305. These experiments cannot rule out the possibility that a number of sites contribute to the repressive action of TGF-β.

5.6 CONCLUSIONS

5.6.1 The 5'-flanking region of the *CYP27B1* gene in osteoblast cells appears to be regulated by different factors to those in the kidney. This concept is consistent with the hypotheses that regulation of *CYP27B1* gene expression is tissue specific, and that the factors that regulate the synthesis of $1,25(OH)_2D_3$ in osteoblasts are related to a possible function of $1,25(OH)_2D_3$ in cell growth and differentiation rather than contributing to the regulation of calcium homeostasis.

5.6.2 A TGF-β inhibitory region was identified between -531 and -305. The elements that mediate repression are yet to be identified, with the present work eliminating many candidate transcription factor binding sites.

Chapter 6

Summary and Conclusions

The classical action of vitamin D hormone ($1,25(OH)_2D_3$) is to maintain calcium and phosphate homeostasis by targeting such tissues as the intestine and bone. However more recent studies have revealed new roles for this hormone including the regulation of cellular proliferation and differentiation of numerous cell types and this is reviewed by Dusso *et al.* (2005). Moreover, vitamin D deficiency is associated with an increasing number of diseases, in particular cancers (Grant, 2002). In recent years it has also become clear that the *CYP27B1* gene is expressed in a number of $1,25(OH)_2D_3$ target tissues (Hendrix *et al.*, 2004; Zehnder *et al.*, 2001). As such, many of the non-classical actions of $1,25(OH)_2D_3$ are hypothesised to be autocrine or paracrine.

Therefore the regulation of *CYP27B1* gene expression in non-renal tissues is an important question, although at present, this regulation is poorly understood in non-renal tissues. The broad aim of the present study was to investigate the regulation of the 5'-flanking region of the *CYP27B1* gene in osteoblast-like cells. More specifically, to identify regulatory elements in the gene that determined the basal level of transcription in osteoblast-like cells, and to investigate whether various exogenous hormones and growth factors could alter the control of *CYP27B1* gene expression in these cells. The aims of the thesis are listed in Chapter 1.10.

Deletion analysis of the 5'-flanking sequence of the *CYP27B1* gene identified three regulatory regions. These regions are depicted in Figure 6.1 along with the putative regulatory elements that have been identified in this study. The first -305 base pairs was sufficient to drive a modest level of expression and was designated the proximal promoter. An enhancer region was identified between -997 and -531 and an upstream repressive region was identified from -1501 to –997. The majority of repression was localised to a region from –1100 to -997. This pattern of expression differed in one important aspect from that previously described in the kidney cell line AOK-B50 (Gao *et al.*, 2002) (Aim 1). In kidney cells, a weak upstream repressive region was identified between -1501 and -997. However the present study detected a strong inhibitory region between -1100 and -997. A strong inhibitory region was also reported in DU145 prostate cancer cells but this region was located between –1200 and –1100 (Dwivedi *et al.*, 2005), further upstream from the repressive region in ROS 17/2.8 cells (Aim 1).

Expression of the enhancer region was dependent upon intact CCAAT box and GC box binding sites located within the proximal promoter. These data mirrored the situation in kidney cells where the CCAAT box and GC box are important for expression of the proximal promoter. Maximal expression of the enhancer region also required two intact Ets protein binding sites (EBSI and EBSII). These sites have previously been shown to contribute to the enhancer region in DU145 prostate cancer cells (Dwivedi *et al.*, 2005). However a third Ets protein binding site (EBSIII) that functions in DU145 did not contribute to expression of the enhancer in ROS 17/2.8 cells.

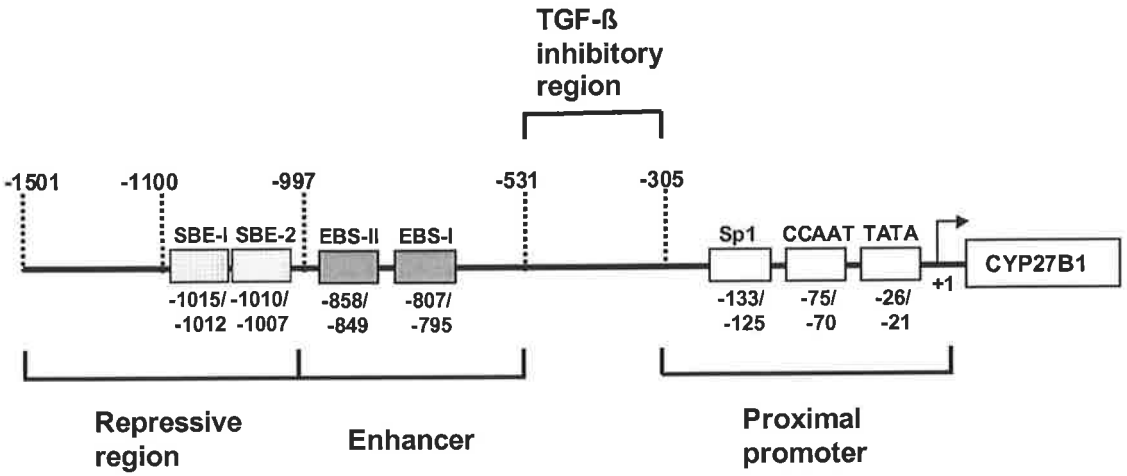

Figure 6.1: Putative regulatory elements within the 5'-flanking region of the human *CYP27B1* gene. Basal expression of the *CYP27B1* gene 5'-flanking region comprises three regulatory regions within osteoblasts; a proximal promoter, an enhancer and a repressive region. A TGF-ß inhibitory region was also identified between -531 and -305. The location of putative transcription factor binding sites are indicated relative to the transcription initiation site of the gene.

Repression of the 5'-flanking sequence of the *CYP27B1* gene was shown to be mediated by the palindromic sequence 5'-GTCTCAGAC-3' at -1015/-1007 (Aim 2). This sequence contains two canonical Smad binding elements (SBEs), 5'-GTCT-3' and 5'-AGAC-3', which were designated SBE1 and SBE2 respectively. The repressive sequence closely resembles a palindrome that has been previously shown to mediate basal repression of the Smad7 gene promoter (Denissova and Liu, 2004). Mutagenesis of either SBE within the 5'-flanking region of the CYP27B1 gene reversed repression as did changing the cytosine that separates SBE1 and SBE2 to an adenine. Electrophoretic mobility shift assays (EMSAs) showed that SBE1 could bind GST-Smad4 (Aim 3), but other components of the co-repressor complex were not identified.

The successful identification of DNA binding proteins by EMSA analysis requires some prior knowledge of the proteins involved. Therefore in order to elucidate the proteins that associate with SBE1 and SBE2, an alternative approach such as mass spectrometry could be employed. Drewett *et al.* (2001) used a DNA-pull down strategy to capture proteins isolated from HEK-293 cells that bound oligonucleotides corresponding to the c-fos serum response element. These proteins were then separated by SDS-PAGE and identified by mass spectrometry. A similar approach could be used to identify the proteins that interact with regulatory elements within the 5'-flanking region of the *CYP27B1* gene such as SBE1 and SBE2 within the repressive region, as well as the Ets binding sites within the enhancer and CCAAT and GC boxes within the proximal promoter.

Regarding repression through SBE1 and SBE2, these sites represent another difference between regulation of the 5'-flanking sequence of the *CYP27B1* gene in osteoblast and prostate cells. In DU145 prostate cells, the majority of repression has been attributed to a Growth factor independent-1 (GFI-1) protein binding site at -1161 to -1138 (Dwivedi *et al.*, 2005). Hence the basal level of expression of the *CYP27B1* gene in osteoblast cells reflects a complex balance between positive factors that bind to the promoter and enhancer, and negative factors that bind to the repressive region.

This study also sought to determine what exogenous hormones and growth factors regulate the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. Importantly, the results demonstrated that the actions of parathyroid hormone (PTH) and $1,25(OH)_2D_3$ on the 5'-flanking sequence of the *CYP27B1* gene in osteoblast cells differed from their reported actions in kidney cells. Specifically, treatment with PTH caused a modest down-regulation of expression of the pCYP27B1(-1501)-Luc construct in ROS 17/2.8 cells, whereas PTH induces *CYP27B1* gene expression in kidney cells (Armbrecht *et al.*, 2003a; Gao *et al.*, 2002). A negative association between serum PTH levels and bone *CYP27B1* mRNA has been previously reported in vivo in rats (Anderson *et al.*, 2005). Similarly, $1,25(OH)_2D_3$ had no effect on expression of the pCYP27B1(-1501)-Luc construct in ROS 17/2.8 cells despite being shown to repress expression of the *CYP27B1* 5'-flanking sequence in MCT kidney cells (Murayama *et al.*, 2004). Together these data indicate that the calciotropic factors PTH and $1,25(OH)_2D_3$ are unlikely to be important modulators of *CYP27B1* gene expression in osteoblast cells (Aim 4). These observations are consistent with the hypothesis that the synthesis of $1,25(OH)_2D_3$ by osteoblasts serves a function unrelated to calcium homeostasis.

In order to predict what other factors were likely to regulate *CYP27B1* gene expression in osteoblast cells, data obtained from macrophage cells were considered. In macrophage cells CYP27B1 activity is controlled by immune cell regulators such as lipopolysaccharide (LPS) (Reichel *et al.*, 1987b), and cytokines such as interferon-γ (IFNγ) (Adams *et al.*, 1989; Monkawa *et al.*, 2000). Therefore it was hypothesised that factors that regulate osteoblast function may also regulate *CYP27B1* gene expression in ROS 17/2.8 cells. Accordingly, the growth factors TGF-β1 and IGF-1 (which are secreted locally) were investigated and both were observed to repress expression of the pCYP27B1(-1501)-Luc construct in the order of 50% (Aim 4). Repression by TGF-β1 was investigated further and a TGF-β inhibitory region was isolated between -531 and -305, however the mechanism of repression remained unresolved (Aim 5). Overall, experiments investigating regulation of the *CYP27B1* gene by exogenous factors indicated that expression is largely unaffected by calciotropic factors in osteoblasts. Rather, the greatest effect on expression of the 5'-flanking region of the *CYP27B1* gene in osteoblasts was produced by the exogenous growth factors TGF-β1 and IGF-1, growth factors that are locally secreted by these cells. These observations are consistent with the hypothesis that the synthesis of $1,25(OH)_2D_3$ by osteoblasts serves an autocrine or paracrine role as opposed to the endocrine actions of $1,25(OH)_2D_3$ produced in the kidney.

However the physiological role of extra-renal $1,25(OH)_2D_3$ synthesis is unclear. Within bone, locally produced $1,25(OH)_2D_3$ may control osteoblast proliferation, differentiation and mineralisation. As described in Chapter 1.6.1, the production of a mineralised extracellular matrix by osteoblasts is dependent on the ordered

development of proliferating osteoblasts into a mature phenotype. Treatment of mature osteoblasts in culture with $1,25(OH)_2D_3$ can stimulate in vitro mineralisation (Matsumoto *et al.*, 1991). Therefore *CYP27B1* gene expression by osteoblast cells may produce a local supply of $1,25(OH)_2D_3$ that drives mineralisation.

Importantly however, when immature osteoblast cells are treated with $1,25(OH)_2D_3$ their development is interrupted and an extracellular matrix does not form (Owen *et al.*, 1991). Hence it may be necessary for osteoblasts to limit their exposure to $1,25(OH)_2D_3$ during early stages of development. Given that bone remodelling is an ongoing process, osteoblasts of all stages of development should co-exist in bone. *CYP27B1* gene expression in the kidney supplies $1,25(OH)_2D_3$ to the circulation but cannot control which osteoblasts are exposed to its endocrine actions. However such specificity may be achieved by autocrine/paracrine $1,25(OH)_2D_3$ production if only mineralising osteoblast cells express the *CYP27B1* gene.

The need to limit $1,25(OH)_2D_3$ in immature osteoblasts illustrates the potential significance in this study of the identification of repressive regions within the 5'-flanking region of the *CYP27B1* gene. It is possible that repression of the *CYP27B1* gene is limited to the early stages of osteoblast development and is alleviated during mineralisation. Such a hypothesis could be tested by comparing *CYP27B1* mRNA levels of normal osteoblast cells in culture as they progressively differentiate to form an extracellular matrix (Gronthos *et al.*, 1999). It is anticipated that *CYP27B1* mRNA levels would be higher in mineralising cultures than in proliferating cultures.

An alternative interpretation of the repression phenomena could be that they are characteristic of the cancer phenotype. It is important to note that ROS 17/2.8 is a cancer cell line, as is DU145, the cell line in which basal repression has been investigated in prostate cells. It is possible that the decreased synthesis of $1,25(OH)_2D_3$ that results from repression of the *CYP27B1* gene disturbs the processes of cell growth and proliferation in normal cells. In support of this concept $1,25(OH)_2D_3$ inhibits the proliferation of various cancer cell cultures including leukemia (Abe *et al.*, 1981), breast (James *et al.*, 1996) and prostate (Zhao *et al.*, 2000b). Moreover, CYP27B1 activity has been reported to be lower in prostate cancer cells compared to normal prostate cells (Hsu *et al.*, 2001). Therefore it would be of considerable interest to know if repression of *CYP27B1* gene is observed in normal cells. This question could be investigated by using a viral vector to transfect normal osteoblast cells with the CYP27B1 reporter constructs described in this study (Forsayeth and Garcia, 1994). Regardless of whether repression of *CYP27B1* gene expression is a function of normal osteoblast development, or a characteristic of the cancer phenotype, the molecular mechanisms involved are clearly important questions.

This is the first study to investigate regulation of the 5'-flanking region of the *CYP27B1* gene in osteoblast cells. The results demonstrate that *CYP27B1* gene expression in osteoblast cells involves a complex interaction of enhancer and repressor elements. Importantly, the data indicate that different mechanisms regulate *CYP27B1* gene expression in osteoblast, kidney and prostate cells. This is consistent with the concept that the control of *CYP27B1* gene expression is tissue specific. The results of this study also demonstrate that expression of the *CYP27B1* gene in

osteoblast cells is determined by the concentration of local factors. This local regulation of gene expression supports the hypothesis that the production of $1,25(OH)_2D_3$ by osteoblast cells serves an autocrine or paracrine role.

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