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Regulation of the 5′-flanking region of the human *CYP27B1* gene in osteoblast cells

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ABSTRACT

Synthesis of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) is catalysed by the enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (CYP27B1). Regulation of *CYP27B1* gene expression is poorly understood, particularly in non-renal tissues including bone where 1,25$(OH)_2D_3$ is hypothesised to serve autocrine/paracrine roles. Transient transfection of ROS 17/2.8 osteoblast-like cells with reporter gene constructs containing deletions of the 5′-flanking region of the human *CYP27B1* gene revealed a proximal promoter, enhancer region and strong upstream repressive region. Putative CCAAT and GC boxes, as well as Ets protein binding sites were shown to contribute to promoter and enhancer activities respectively in common with kidney and prostate cells. Inhibition of basal expression was largely attributed to a palindrome 5′-$\underline{\text{GTCT}}$C$\underline{\text{AGAC}}$-3′ (−1015/−1007 bp) that contains two putative canonical Smad binding elements. We conclude that repression of *CYP27B1* gene expression may be a common event but the novel inhibitory elements we have identified may be unique to osteoblasts.

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1. Introduction

The classical action of the biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) is to maintain calcium homeostasis (Dardenne et al., 2001; Dusso et al., 2005). More recently, vitamin D has also been shown to be important in other processes such as the regulation of cellular proliferation and differentiation (Atkins et al., 2003, 2007; Matsumoto et al., 1990), immunity (DeLuca and Cantorna, 2001) and reproduction (Panda et al., 2001). The rate limiting step in the bioactivation of 1,25$(OH)_2D_3$ is catalysed by the mitochondrial enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (CYP27B1). This enzyme is expressed in the kidney which is responsible for the endocrine action of this hormone by determining the circulating levels of 1,25$(OH)_2D_3$ (Omdahl et al., 2002). Production of 1,25$(OH)_2D_3$ by the kidney is highly regulated by parathyroid hormone (PTH) which up regulates expression of CYP27B1 (Shinki et al., 1997). There is evidence that in kidney cells, PTH acts 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 *CYP27B1* gene in the kidney using vitamin D receptor (VDR) and a novel mechanism (Murayama et al., 2004).

The *CYP27B1* gene is also expressed in a number of non-renal tissues including bone (Anderson et al., 2005; van Driel et al., 2006). In such tissues, many of the non-classical actions of locally synthesised 1,25$(OH)_2D_3$ are hypothesised to be autocrine/paracrine (Anderson et al., 2003; van Driel et al., 2006). Importantly, recent studies have demonstrated that cultured osteoblasts incubated with 25$(OH)D_3$ require expression of *CYP27B1* with the subsequent production of 1,25$(OH)_2D_3$ to induce osteoblast gene expression, stimulate mineralization and modulate osteoblast proliferation (Atkins et al., 2007; van Driel et al., 2006). These results strongly suggest that expression of the *CYP27B1* gene in osteoblasts has physiological significance. We and others have previously provided evidence that the *CYP27B1* gene is regulated differently in osteoblast cells compared with kidney cells (Turner et al., 2007; van Driel et al., 2006). However at present regulation of the *CYP27B1* gene in bone remains poorly understood. The aim of this study was to investigate the regulation of the 5′-flanking region of the human *CYP27B1* gene in the osteoblast cell line ROS 17/2.8 and to compare this with the regulation of expression in other cell lines.

2. Materials and methods

2.1. Materials

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Oligonucleotides were synthesised by Geneworks (Adelaide,

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Australia). Cell culture media and associated tissue culture products were from Life Technologies (Grand Island, NY). The dual-luciferase assay kit was supplied by Promega (Madison, WI). Recombinant human type 1 transforming growth factor was purchased from R and D Systems (Minneapolis, MN).

2.2. CYP27B1 5′-flanking–luciferase reporter constructs

5′-Deletion constructs of the *CYP27B1* 1501 bp 5′-flanking region fused to the firefly luciferase reporter gene in the pGL3 vector have been previously described and are designated as pCYP27B1(−1501)-Luc, pCYP27B1(−997)-Luc, pCYP27B1(−531)-Luc and pCYP27B1(−305)-Luc (Gao et al., 2002). A further 5′ deletion of the 5′-flanking sequence (together with 44 bp of *CYP27B1* 5′-untranslated region) was generated by PCR during this study and named pCYP27B1(−1100)-Luc. For the PCR, the following oligonucleotide primers were designed with a restriction enzyme site for XhoI (in bold) so that the PCR products could be cloned into XhoI digested PGL3-basic vector.

Reverse primer
(+24) 5′-CTTAGATCGCAGGAT**CTCGAG**GGTCTGGTTCAGGGTG-3′
Forward primer
(−1117) 5′-GTAATCCAC**CTCGAG**CTCTCAAGGCACTACAGGC-3′

2.3. Site-directed mutagenesis

Mutations in putative transcription factor binding sites were introduced using the Quik-Change Site Directed Mutagenesis protocol (Stratagene, La Jolla, USA). Oligonucleotides for site-directed (SD) mutagenesis were designed as follows. The core sequence is underlined in each wild type sequence and altered nucleotides are in bold letters.

WT CCAAT	5′-CAGGAGGAGGGATTGGCTGAGGAGCTTG-3′ (−85/−58)
SD mCCAAT	5′-CAGGAGGAGGGA**GCTC**CTGAGGAGCTTG-3′
WT GC box	5′-GAAGTCAGCCCCAGCCCCGCCTACTGTTC-3′ (−143/−115)
SD mGC box	5′-GAAGTCAGCC**AA**AGC**TTT**GCCTACTGTTC-3′
WT EBS-I	5′-GAGCCACCACAGGATTCTGAGGC-3′ (−812/−790)
SD mEBS-I	5′-GAGCCACCACA**TT**ATTCTGAGGC-3′ (−812/−790)
WT EBS-II	5′-CCTCATGCCATCCTCCTGCCTCAG-3′ (−864/−841)
SD mEBS-II	5′-CCTCATGCC**AAA**CTCCTGCCTCAG-3′ (−864/−841)
WT SBE1	5′-GCCCAGGCTGGTCTCAGACTCCTGAGC-3′ (−1025/−999)
SD mSBE2	5′-GCCCAGGCTGGT**AA**CAGACTCCTGAGCT-3′
WT SBE2	5′-AGGCTGGTCTCAGACTCCTG-3′ (−1021/−1002)
SD mSBE2	5′-AGGCTGGTCTC**TT**ACTCCTG-3′
WT SBE1 + 2	5′-GCCCAGGCTGGTCTCAGACTCCTGAGC-3′ (−1025/−999)
SD mSBE1 + 2	5′-GCCCAGGCTGGT**AA**C**TT**ACTCCTGAGC-3′
WT SBE(c)	5′-CAGGCTGGTCTCAGACTCCTGAGC-3′ (−1022/−999)
SD mSBE(c)	5′-CAGGCTGGTCT**A**AGACTCCTGAGC-3′

2.4. Maintenance and transfection of osteoblast-like cells

ROS 17/2.8 (rat osteosarcoma) cell line was provided by the Garvan Institute, Sydney. These cells were maintained in DMEM/Ham's F12 media supplemented with 10% fetal calf serum (FCS). All other cell lines were maintained in DMEM plus 10% FCS. All transient transfections were performed using DOTAP or Fugene 6 (Roche Diagnostics, USA) according to the manufacturer's instructions. For transient transfection analysis, cells were grown in 75 cm^2 flasks to 60–70% confluency, washed once with phosphate buffered saline and removed by trypsinization. Cells (approximately 5×10^4) were seeded into 24-well trays containing 400 µl. Following attachment of cells, the media were changed to RPMI-1640. Within an experiment, transfections were carried out in triplicate and the data averaged; each transfection was performed using 200 ng of each of the CYP27B1 deletion constructs, together with 50 ng of the TK promoter directed *Renilla* luciferase plasmid (pRLTK-LUC) (Promega) to normalise transfection efficiency and harvested in 50 µl of Passive Lysis Buffer (Promega). For experiments involving TGF-β, cells were treated with recombinant human TGF-β1 at 1 ng/ml or solvent (1 mg/ml BSA in 4 mM cysteine hydrochloride) for 24 h prior to harvest. Luciferase activity in cell lysates was determined as described previously (Gao et al., 2002) using the Dual Luciferase Reporter assay kit (Promega) and measured using a luminometer model TD 20/20 (Turner Design Instruments, Sunnyvale, CA, USA). All experiments reported here were repeated on at least three separate occasions and the data shown are from one representative experiment.

3. Results

3.1. Deletion analysis of the human CYP27B1 gene 5′-flanking region

An earlier analysis of human *CYP27B1* genomic clones showed that the sequence upstream of −1501 corresponded to the 3′-untranslated region of an unrelated gene, methyltransferase-like protein METTL1 (Bahr et al., 1999; Omdahl et al., 2002). On this basis it seemed likely that most if not all of the *CYP27B1* gene regulatory sites would lie within the first −1501 bp. In support of this concept we have demonstrated this length of flanking region can direct appropriate tissue-specific expression in transgenic mice (Hendrix et al., 2004). In the current study we have investigated the transcriptional activity of this −1501 bp region in ROS 17/2.8 cells by deletion analysis. Firstly we identified that this cell line expresses endogenous *CYP27B1* mRNA at a relatively high level using quantitative RT-PCR (13,500 copies/µg total RNA) using methods as previously described (Anderson et al., 2008).

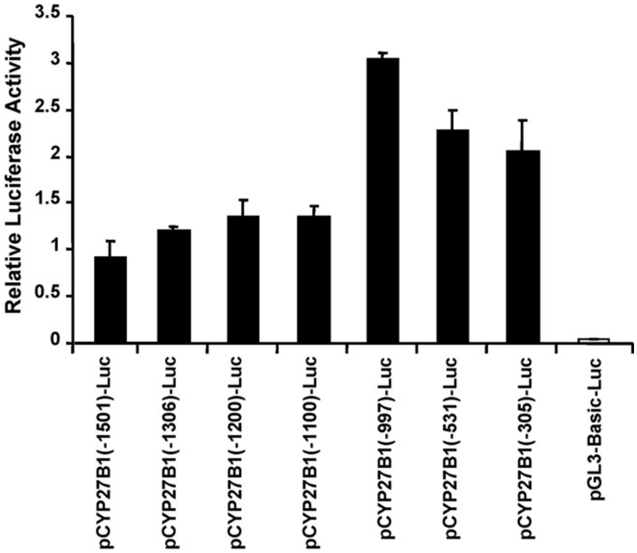

Fig. 1. Expression of the 5′-flanking region of the human *CYP27B1* gene in ROS 17/2.8 cells. Deletion constructs containing −1501, −1100, −997, −531 and −305 bp of 5′-flanking region were transiently transfected into ROS 17/2.8 cells. For comparison the promoter-less pGL-3 Basic-Luc was also transfected. The relative luciferase activity shown represents the mean ± S.D. firefly luciferase:*Renilla* luciferase ratios of triplicate samples from a representative experiment.

Thus different lengths of the 5′-flanking region, together with 44 bp of the *CYP27B1* gene 5′-untranslated region, were fused to the fire fly luciferase gene as reporter. These deletion 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. The constructs were introduced into ROS 17/2.8 cells using DOTAP as the transfectant reagent. ROS 17/2.8 cells were initially seeded in DMEM/Ham's F12 plus serum. Following attachment of the cells, the medium was changed to serum free RPMI-1640 prior to transfection. After an overnight incubation (approximately 16 h), cells were harvested and luciferase activity determined using the dual luciferase protocol. As can be seen in Fig. 1, the construct pCYP27B1(−305)-Luc directed significant basal expression that was unaltered when the 5′-flanking length was increased to −531. Increasing the length to −997, led to maximal expression that was about 50% greater than the pCYP27B1(−305)-Luc construct. When the length of the 5′-flanking region was extended to −1101, there was a marked overall inhibition of expression with the level suppressed below that of pCYP27B1(−305)-Luc. Expression remained repressed in 5′-flanking sequence lengths of −1200 and −1306 and was lowest when the entire 5′-flanking region of −1501 was investigated (Fig. 1). Overall these data indicate that the 5′-flanking region of the *CYP27B1* gene is driven by a proximal promoter within the first −305 and an enhancer between −531 and −997 but this expression is strongly down regulated by an upstream inhibitory region located primarily between −997 and −1100 (Fig. 2).

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