

Identification of growth factor independent-1 (GFI1) as a repressor of 25-hydroxyvitamin D 1- α hydroxylase (CYP27B1) gene expression in human prostate cancer cells

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Abstract

The hormone 1,25-dihydroxyvitamin D (1,25D) may play a protective role in prostate cancer. 25-hydroxyvitamin D 1- α hydroxylase (CYP27B1) is the enzyme responsible for the regulation of cellular 1,25D levels. CYP27B1 is substantially repressed in prostate cancer cells. We have investigated the molecular basis for this inhibition. First, we identify a repressive region between –997 and –1200 in the human CYP27B1 promoter following transient transfection analysis in the prostate cancer cell lines DU145, PC3 and LNCaP. Next, we demonstrate a role for the transcription factor growth factor independent-1 (GFI1) in the repression of CYP27B1. Electrophoretic mobility assays with nuclear extracts from prostate cancer cell lines established binding of GFI1 to the sequence 5'-TGGTACAATCATAACTCACTGCAG-3' present at –997 to –1200 in the repressive region. Site directed mutagenesis of the core GFI1 binding sequence (5'-AATC-3') substantially increased while forced expression of GFI1 decreased the expression of the CYP27B1 reporter construct. Importantly, GFI1 repression is dependent on an intact GFI1 binding site in the –997 to –1200 region. GFI1 is an oncoprotein known to form a large protein complex with co-repressors that recruit histone deacetylases. We propose that the formation of such a repressive complex on the inhibitory domain of the CYP27B1 gene in prostate cancer cells could lead to silencing of either the nearby enhancer or proximal promoter domains and lead to cancer progression by reducing local production of 1,25D. These studies provide the basis for a more detailed understanding of CYP27B1 repression in prostate cancer cells and could provide a novel insight in future diagnosis and treatment.

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Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D (1,25D) plays a central role in calcium and bone metabolism, but more recent studies have revealed broadened properties for the hormone including anti-proliferation, prodifferentiation and immunosuppressive actions (Hewison & O'Riordan 1994, Christakos *et al.* 1996, Issa *et al.* 1998, Jones *et al.* 1998). Parental

vitamin D is synthesized from 7-dehydrocholesterol in the basal epidermal layer of the skin by a UV-radiation dependent process. Vitamin D subsequently acquires its biological activity via a series of sequential hydroxylations. The first of these occurs in the liver to generate 25-hydroxyvitamin D (25D), which is transported primarily to the renal proximal tubules, but also to extra-renal sites such as the bone, skin, immune system, small intestine, colon, breast and

prostate (Omdahl *et al.* 2002). At these tissue sites, 25D is converted to 1,25D by the mitochondrial cytochrome P450 enzyme, 25-hydroxyvitamin D 1 α -hydroxylase (CYP27B1) (Howard *et al.* 1981, Adams & Gacad 1985, Anderson *et al.* 2003). The molecular actions of 1,25D are mediated through the vitamin D receptor (VDR), which acts as a ligand-dependent transcription factor to regulate the expression of vitamin D responsive genes in a genomic and non-genomic manner (Carlberg & Polly 1998, Dwivedi *et al.* 2002, Norman *et al.* 2003).

While the precise mechanisms leading to the development of prostate cancer remain to be elucidated, there is evidence that 1,25D may have a protective role in the development and/or progression of the cancer. Such a role for 1,25D was first proposed by Schwartz & Hulka (1990), based on epidemiological studies that indicated an inverse relationship between UV-radiation exposure and mortality rate for prostate cancer. Furthermore, prostate epithelial cells have been demonstrated to express high-levels of functional VDR (Miller *et al.* 1992, Peehl *et al.* 1994) and treatment of various prostate cancer cell lines with 1,25D has an anti-proliferative effect, inducing both G₁ arrest and apoptosis (Zhuang & Burnstein 1998, Blutt *et al.* 2000), while also decreasing the invasiveness of prostate cancer cells *in vitro* (Peehl *et al.* 1994, Schwartz *et al.* 1997). However clinical trials have revealed the limited use of 1,25D as a therapeutic agent in the treatment of prostate cancer, due to an associated hypercalcemic effect (Osborn *et al.* 1995, Gross *et al.* 1998, Chen *et al.* 2000). Several less calcemic 1,25D analogues are being investigated as future therapeutic possibilities (Polek & Weigel 2002, Guyton *et al.* 2003), with the analogue EB1089 significantly reducing prostate tumor progression in nude mice without any apparent adverse side-effects (Gulliford *et al.* 1998).

Schwartz *et al.* (1998) first demonstrated the expression of CYP27B1 in human prostatic epithelial cells. Subsequently, it was shown that CYP27B1 enzyme levels are highest in normal prostate epithelial cells, somewhat lower in cells from benign prostatic hyperplasia (BPH) and highly suppressed in prostate cancer cells (Hsu *et al.* 2001). The reduced activity of CYP27B1 correlated with a reduced anti-proliferative activity of exogenous 25D (Hsu *et al.* 2001). This work supported the proposal that in normal prostate cells, 1,25D is locally produced by the action of CYP27B1 on 25D, with 1,25D acting in an autocrine/paracrine fashion to regulate epithelial cell growth. Therefore, the repressed level of CYP27B1 activity in prostate cancer cells could be important for prostate cancer

development and progression. The feasibility of this proposal has been demonstrated in transfected LNCaP cells (Whitlatch *et al.* 2002, Chen *et al.* 2003). When the cDNA sequence for CYP27B1 was introduced under the control of a viral promoter, and then 25D added to the culture media, the 25D was found to be as effective in inhibiting proliferation of LNCaP cells as the addition of 1,25D alone.

Reasoning that an understanding of the process whereby 1,25D synthesis is suppressed in prostate cancer cells could provide a novel insight to the diagnosis and treatment, we have investigated the molecular aspects of suppressed CYP27B1 activity in prostate cancer cells. In this study we have employed the upstream regulatory region of the human CYP27B1 gene and transient transfection analyses to localize a domain that is strongly inhibitory and have identified a transcription factor oncoprotein that plays a key role in the suppression mechanism.

Materials and methods

Materials

Restriction enzymes and T4 DNA ligase were purchased from New England Bio-labs (Beverly, MA, USA). Oligonucleotides were synthesized by Geneworks (Adelaide, South Australia). Cell culture media and associated tissue culture products were from Life Technologies (Grand Island, NY, USA). The dual-luciferase assay kit was supplied by Promega (Madison, WI, USA).

CYP27B1 5'-flanking-luciferase constructs and expression plasmids

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(-884)-Luc, pCYP27B1(-531)-Luc and pCYP27B1(-305)-Luc (Gao *et al.* 2002). Further 5'-deletions of the 5'-flanking sequence (together with 44 bp of CYP27B1-untranslated region) were generated by the polymerase chain reaction (PCR) during this study and named pCYP27B1(-1306)-Luc, pCYP27B1(-1200)-Luc and pCYP27B1(-1100)-Luc. For the PCR the following oligonucleotide primers were designed with a restriction enzyme site for XhoI (bold letters) so that the PCR products could be cloned into XhoI digested pGL3-basic vector: reverse primer (+24), 5'-CTTAGATCGCAGGATCT**CGAGGGTCTGG**-TTCAGGGTG-3'; forward primer-1 (-1318),

5'-TCCAGAACTCGAGAGCTAGCTGACTGGC-ACAGAG-3'; forward primer-2 (-1211), 5'-AAT-TTTTTCTCGAGACAGGCCCTCTCTCTGTTGC-C-3' and forward primer-3 (-1117), 5'-GTAAT-CCACCTCGAGCTCTCAAGGCACTACAGGC-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 CRE-I, 5'-CC-ATGCCTGGCTAATTTTTG-3' (-1075 to -1047); SD mCRE-I, 5'-CCATGCCTGTGTAATTTTTG-3' (-1075 to -1047); WT CRE-II, 5'-GCAGCCTT-GACCTCCGGGCTC-3' (-1041 to -1020); SD mCRE-II, 5'-GCAGCCTTGTGCTCCCGGGCTC-3' (-1041 to -1020); WTAP-1 like, 5'-CAATCATAACT-CACTGCAGCCTTG-3' (-1156 to -1132); SD mAP-1 like, 5'-CAATCATAACTTGTCTGCAGCCTTG-3' (-1156 to -1132); WT GFII, 5'-GTGCAGTGGTACAATCATAACTCAC-3' (-1167 to -1142); SD mGFII, 5'-GTGCAGTGGTACGGTCATAACTCAC-3' (-1167 to -1142); WT EBS-III, 5'-CCCAAAGTGTGGGATTACAGTCATG-3' (-973 to -948); SD mEBS-III, 5'-CCCAAAGTGTGTTATTACAGTCATG-3' (-973 to -948); WT EBS-II, 5'-CCTCATGCCATCCTCCTGCCTCAG-3' (-864 to -841); SD mEBS-II, 5'-CCTCATGCCAAACTCCTGCCTCAG-3' (-864 to -841); WT EBS-I, 5'-GAGCCACCA-CAGGATTCTGAGGC-3' (-812 to -790) and SD mEBS-I, 5'-GAGCCACCACATTATTCTGAGGC-3' (-812 to -790).

Mutations in the CAAT box, Sp1 binding site and EBS located in the -305 bp region of the CYP27B1 gene have been described earlier (Gao *et al.* 2002). The GFII over-expression clone, pcDNA3-Myc-GFII, has been described previously (Grimes *et al.* 1996a). The over-expression clone for prostate derived Ets factor (PDEF) was kindly donated by JP Brody, Whitaker Center for Biomedical Engineering, University of California, Irvine, USA. The expression clone for Ets-1 transcription factor (pEFBosEts-1) has been described previously (Dwivedi *et al.* 2000).

Maintenance and transfection of prostate cancer cells

DUI45, PC-3 and LNCaP (human prostate epithelial carcinoma cell lines) were provided by W Tilley (Hanson Institute, Adelaide, Australia). These cells

were maintained in RPMI-1640 supplemented with 5% fetal calf serum (FCS). All transient transfections were performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Human embryonic kidney cells (293T) were maintained with 10% FCS and transfections were carried out using DOTAP (Roche Diagnostics, USA). For transient transfection analysis, cells were grown in 175 cm² flasks to 60–70% confluency, washed once with phosphate buffered saline and removed by trypsinization. Cells (approximately 1 × 10⁴) were seeded into 24 well trays containing 400 µl RPMI-1640 supplemented with FCS. 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 thymidine kinase (TK) promoter directed *Renilla* luciferase plasmid (pRLTK-LUC) from (Promega) to normalize transfection efficiency. Cells were cultured overnight and harvested in 50 µl Passive Lysis Buffer (Promega). 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.

Electrophoretic mobility assays (EMSA)

Double-stranded oligonucleotides (shown below) were synthesized. The wild type CYP27B1GFII + AP-1 like oligonucleotide encompassed both the GFII core sequence (bold letters) and AP-1 like sequence (underlined). The core binding sequence of GFII and the AP-1 like binding sites were mutated (bold and underlined) in the respective oligonucleotides CYP27B1mGFII + AP-1 like and CYP27B1GFII + mAP-1 like oligonucleotides. An oligonucleotide that encompassed an authentic GFII binding site (located on the reverse strand) from the p21 promoter (p21GFII oligonucleotide) was employed as a control (Tong *et al.* 1998; the core is shown in bold letters: CYP27B1GFII + AP-1 like oligonucleotide, 5'-TGGTACAATCATAACT-CACTGCAG-3' and 3'-GACCATGTTAGTATT-GAGTGACGT-5'; CYP27B1mGFII + AP-1 like oligonucleotide, 5'-TGGTACGGTCATAACTCACTGCAG-3' and 3'-GACCATGCCAGTATTGAGTGCAGT-5'; CYP27B1GFII + mAP-1 like oligonucleotide, 5'-TGGTACAATCATAACTTGTCTGCAG-3' and 3'-GACCATGTTAGTATTGAACGACGT-5'; p21 GFII

oligonucleotide, 5'-CGAAGTACCGTGATTTTCAG-GCATGCACG-3' and 3'-GGCTTCATGGCACTAAA-GTCCGTACGTG-5'.

For EMSA experiments, nuclear extracts were prepared from 2×10^6 DU145 cells (Andrews & Fallor 1991). Each double-stranded oligonucleotide was labeled by end-filling with [α - 32 P] dCTP using Klenow enzyme and purified by ethanol precipitation. Binding reactions for the detection of bound GFI1 were carried out using 2 μ g nuclear protein and 1 μ g Poly (dI-dC) in a final volume of 12 μ l in Dignam Buffer C (20 mM HEPES buffer, (pH 7.6), 420 mM NaCl, 0.5 mM dithiothreitol, 5 mM MgCl₂, 0.5 mM EDTA and 20% glycerol) and were incubated on ice for 15 min. Radiolabeled probe (100 000 c.p.m.) was added and binding reactions were further incubated on ice for 30 min. EMSA competition binding assays were performed with unlabeled competitor oligonucleotide at fold molar excess concentrations (10- and 50-fold) by inclusion in the binding reactions. Binding reactions for the inhibition assays were carried out at 4 °C for 45 min using 8 μ l polyclonal goat antibody to GFI1 protein N20 (sc-8558X Lot 1702; Santa Cruz Biotechnology Inc.) and a polyclonal goat antibody for C/EBP α included as a control (sc-9315X; Santa Cruz Biotechnology Inc.). Retarded DNA nuclear protein complexes were resolved on a 4% non-denaturing polyacrylamide gel using pre-cooled low ionic strength gel running buffer (0.5 \times TBE) at 4 °C. The gel was dried and exposed to Kodak X-Omat AR film with an intensifying screen at -80 °C.

Western blot analysis

Total cell lysates were prepared from DU145 cells as described previously (Dwivedi *et al.* 2002). Proteins were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were probed with a polyclonal goat antibody to GFI1 protein N20 (sc-8558X Lot 1702; Santa Cruz Biotechnology Inc.) using standard procedures and immune complexes were detected by enhanced chemiluminescence as reported previously (Dwivedi *et al.* 2002).

Results

Deletion analysis of the CYP27B1 gene 5'-flanking region

An analysis of human CYP27B1 genomic clones showed that sequence upstream from -1501 corresponded to the 3'-untranslated region of an unrelated

gene, methyltransferase-like protein METTL1 (Bahar *et al.* 1999, Omdahl *et al.* 2001). On this basis, it seemed likely that most if not all CYP27B1 gene regulatory sites would lie within the first -1501 bp and this region has been studied in the present work. 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 designated as pCYP27B1(-1501)-Luc, pCYP27B1(-997)-Luc, pCYP27B1(-884)-Luc, pCYP27B1(-531)-Luc and pCYP27B1(-305)-Luc were introduced into human prostate carcinoma DU145 cells which have been shown to contain low levels of CYP27B1 (Hsu *et al.* 2001). Cells were cultured in RPMI-1640 media containing 5% FCS. Next day cells were harvested and luciferase activity was determined. The construct pCYP27B1(-305)-Luc directed significant expression that was not altered when the 5'-flanking length was increased to -531 but was almost doubled with the pCYP27B1(-884)-Luc plasmid (Fig. 1A). Further increasing the length to -997 led to the maximal expression level observed that represented an increase of nearly four-fold compared with pCYP27B1(-305)-Luc. When the entire 5'-flanking region of -1501 was investigated there was a marked overall inhibition of expression, with the level suppressed below that of pCYP27B1(-305)-Luc (Fig. 1A).

Expression of the 5'-flanking deletion constructs was also examined in PC3 and LNCaP human prostate cancer cell lines that also contain low levels of CYP27B1 (Hsu *et al.* 2001). In these two cell lines the profiles of expression were similar to that seen with DU145 cells (see Figs 1B and 1C). The level of repression in PC3 cells was about the same as that seen in DU145 cells although the repression was not as great in LNCaP cells. With the latter cells, the levels of luciferase activity expressed by the different constructs were low compared with the other two cell lines and this finding is consistent with the relative low enzyme activity of CYP27B1 exhibited by this cell line (Hsu *et al.* 2001). For comparison, the deletion constructs were also investigated in human embryonic kidney cells (293T). With these cells, most of the basal activity is achieved with the construct pCYP27B1(-305)-Luc (Fig. 1D) and there is almost no enhancer activity with pCYP27B1(-997)-Luc or repression with pCYP27B1(-1501)-Luc. This favours our hypothesis that the observed repression of the CYP27B1 gene may be specific to prostate cancer cells. In further studies with the prostate cancer cell lines we determined expression of the deletion constructs in serum-free medium. DU145, PC3 and LNCaP cells were initially

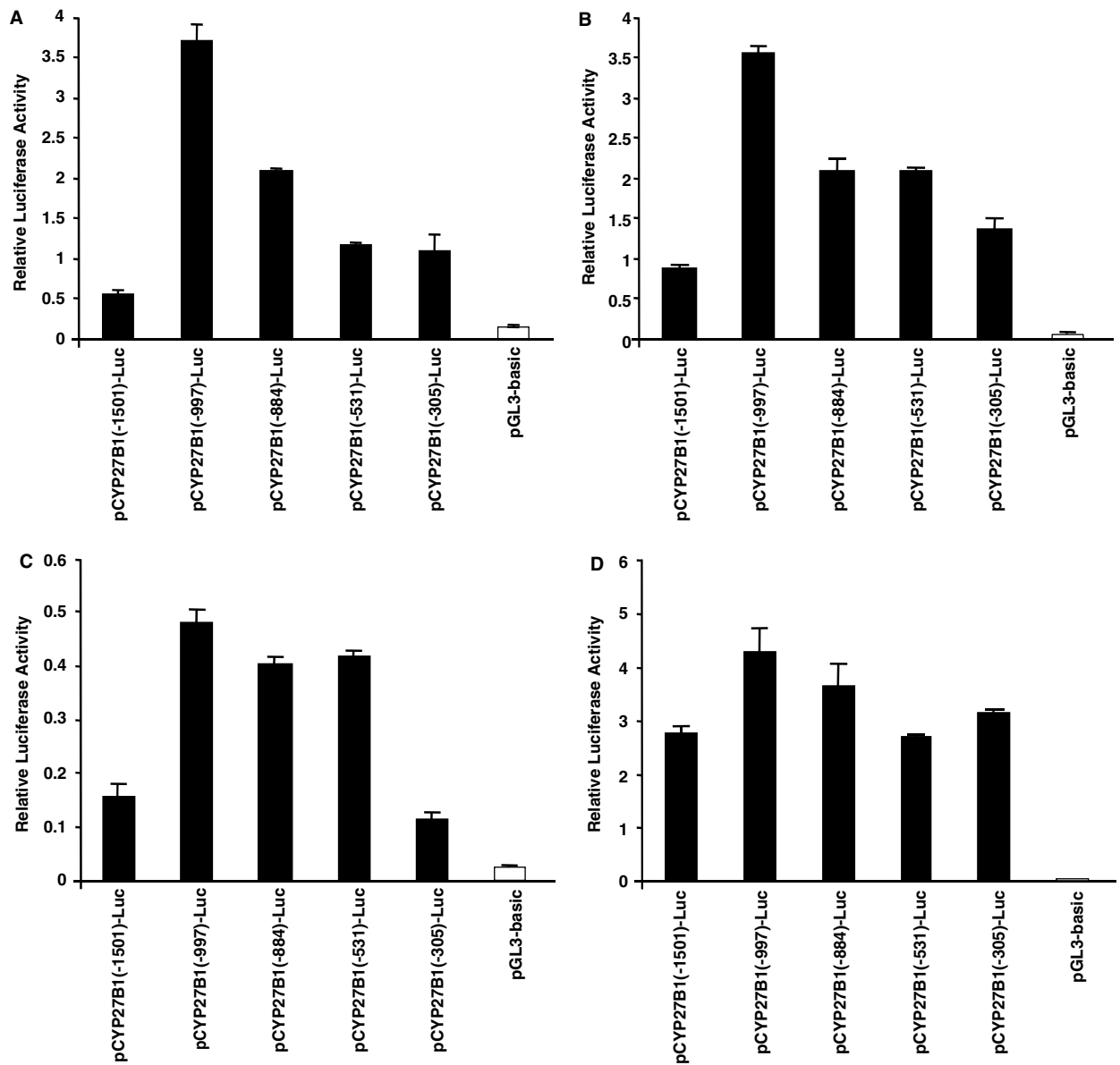


Figure 1 Localization of the proximal promoter, enhancer and repressive regions in the 5'-flanking region of the human CYP27B1 gene. Five deletion constructs containing -1501, -997, -884, -531 and -305 bp of 5'-flanking region were generated by PCR and restriction enzyme digestion as described previously (Gao *et al.* 2002). The constructs were transiently transfected into (A) DU145, (B) PC3, (C) LNCaP and (D) 293T cells. For comparison, the promoterless vector pGL3 Basic-Luc was also transfected. The relative luciferase activity shown represents the mean \pm s.d. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative experiment. The experiment was repeated five times.

cultured in media containing 5% fetal calf serum and then immediately transferred to serum-free RPMI-1640 medium when transfections were carried out. Interestingly, the absence of serum had no effect on the pattern or levels of expression of any of the CYP27B1 5'-flanking deletion constructs in these cell lines. Hence expression of the 5'-flanking region in

these cells is unaffected by growth factors and hormones present in serum.

The data in Figures 1A, B and C show that when the 5'-flanking sequence is extended from -997 to -1501 repression of CYP27B1 gene activity is observed. Further localization of the repressive region was carried out using DU145 cells and these cells were

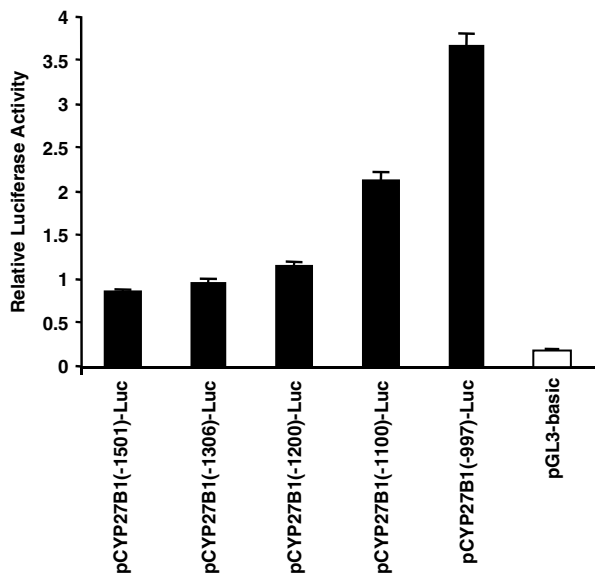


Figure 2 Further delineation of the repressive region. Three additional deletion constructs were generated using the PCR and CYP27B1(-1501)-Luc as a template and encompassed -1306, -1200, -1100 and -997 of the 5'-flanking sequence. The transient transfection analysis of these constructs was performed as described in Figure 1A–D. The relative luciferase activity shown represents the mean \pm s.d. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative experiment. The experiment was repeated three times.

employed for the remainder of the work reported here. Additional 5'-flanking deletion constructs were synthesized of length -1306, -1200, and -1100 and designated as pCYP27B1(-1306)-Luc, pCYP27B1(-1200)-Luc and pCYP27B1(-1100)-Luc, respectively. Transient transfection analysis performed in DU145 cells with these constructs demonstrated that most of the repression lies within the region from -997 to -1200 (Fig. 2). Overall the data from Figures 1A, B,

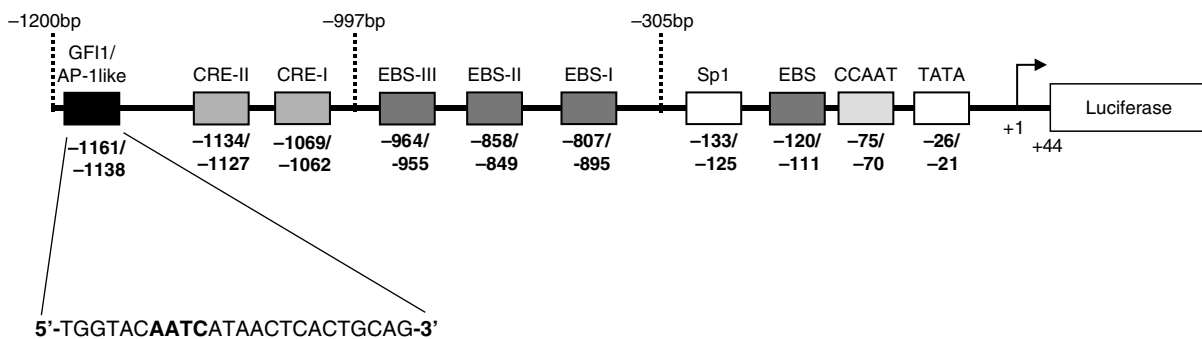


Figure 3 Schematic of the human CYP27B1 5'-flanking region (+44 to -1200). Transcription factor binding sites and their locations are indicated relative to the transcription initiation site of the gene. The sequence of the putative GF11/AP-1 like sequence is also shown with the GF11 core in bold and underlined.

C and Figure 2 show that the 5'-flanking region of the gene comprises an upstream inhibitory region between -997 and -1200, an enhancer region between -305 and -997 and a proximal promoter within the first -305 bp.

Characterization of the proximal promoter

Control elements present in the -305 bp proximal promoter have been previously identified by Gao *et al.* (2002) and are shown in Figure 3. A CCAAT box (5'-ATTGGCT-3') at -75 to -70 and a GC rich sequence that is an Sp1 binding site (5'-CCAGCCCCG-3') at -133 to -125, underlie basal expression in transfected kidney cells (Gao *et al.* 2002), with no significant contribution from a putative Ets protein binding site (5'-CTGTTCTGG-3'), designated as EBS at -120 to -111 on the antisense strand and located between the CCAAT box and GC rich sequence. An analysis of promoter mutant constructs in DU145 cells established that the CCAAT box binding site and the GC box are critically important for expression driven by the pCYP27B1(-305)-Luc construct in DU145 cells (Fig. 4A); inactivation of each site lowered expression by approximately 80%. However the Ets protein binding site did not contribute to expression (Fig. 4A). On the basis of earlier studies in which we showed that overexpressed Sp1 acted on the promoter through the GC box in an insect cell line (Gao *et al.* 2002), it seems reasonable to assume that Sp1 present in prostate cancer cells will bind the GC rich sequence. There is no information as to the identity of the functional protein that binds the CCAAT box.

Characterisation of the enhancer region

A computer analysis of the enhancer region from -305 to -997 using the transcription factor binding site database (TFSEARCH 2001–2002) revealed three

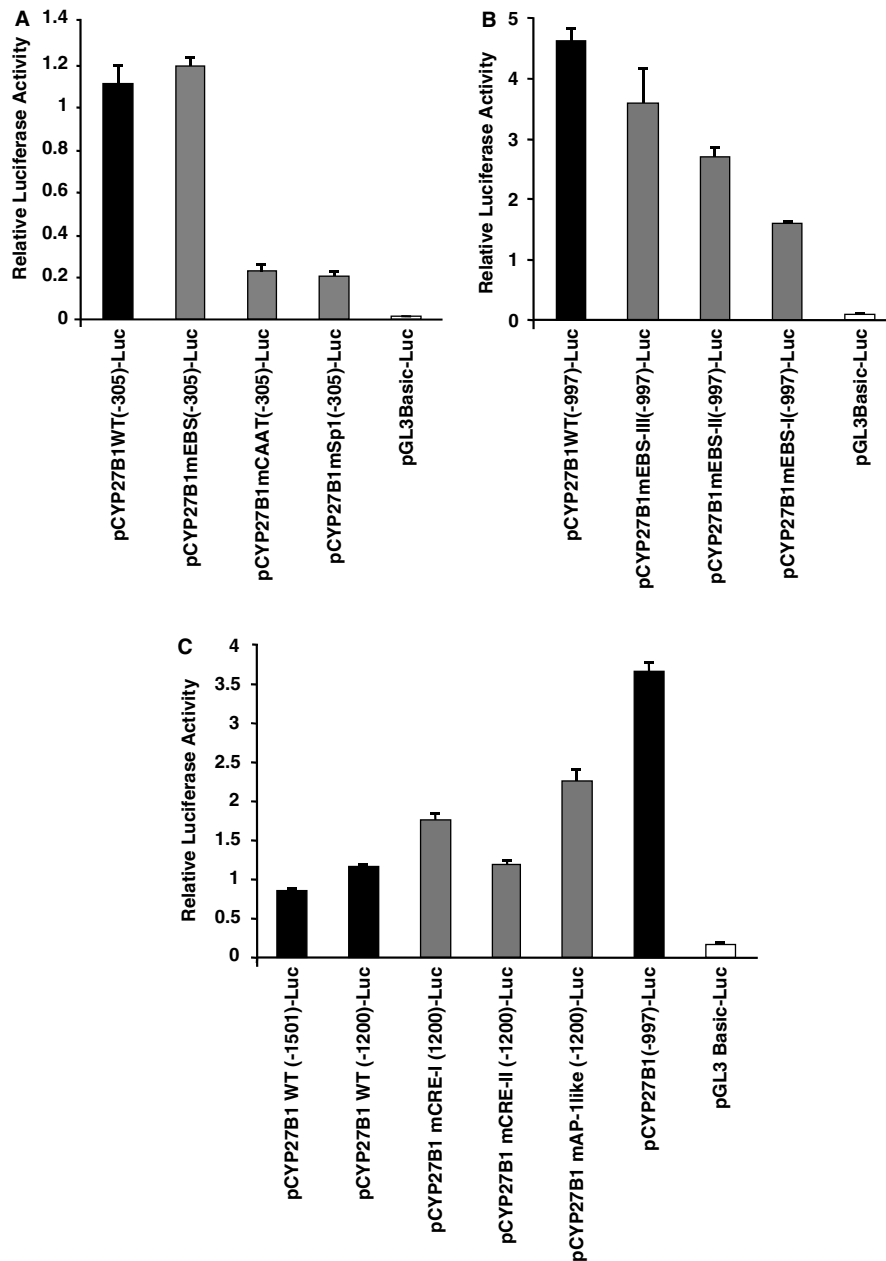


Figure 4 Mutational analysis of sites in the 5'-flanking region. (A) The CAAT box, Sp1 site and putative Ets binding site (EBS) in the proximal promoter activity were each mutated in the construct pCYP27B1 WT(-305)-Luc and transiently transfected into DU145 cells. (B) Each of the three putative Ets sites within the construct pCYP27B1(-997)-Luc were mutated by site directed mutagenesis and transiently transfected into DU145 cells. (C) The putative CRE-I, CRE-II and AP-1 like sites were each mutated in the construct pCYP27B1 WT(-1200) and transiently transfected into DU145 cells. In A, B and C the relative luciferase activity shown represents the mean \pm s.d. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative experiment. The experiment was repeated three times.

possible Ets protein binding sites (EBSs) that were similar in sequence and contained a 5'-GGAT-3' core. Two of these sites designated EBS-I (5'-CCACAG-GATTCTGA-3') and EBS-III (5'-TGTTGGGATTA-CAGT-3') were located on the sense strand, while the

third site, EBS-II (5'-ATGCCATCCTCCTG-3') was present on the non-coding strand (Fig. 3). Each of these sites was inactivated by site-directed mutagenesis within the pCYP27B1(-997)-Luc construct. It can be seen from Figure 4B that while each site contributed to

expression, EBS-I is the most important, with mutagenesis of this site lowering expression by about 70%. Similar results were seen in LNCaP cells with the EBS-I playing the major role (data not shown). A novel Ets transcription factor (PDEF) has been identified that is expressed predominantly in prostate epithelial cells, prefers a 5'-GGAT-3' core rather than a 5'-GGAA-3' core and has been shown to activate an upstream enhancer in the prostate specific antigen (PSA) gene (Oettgen *et al.* 2000). This raised the possibility that PDEF may be important for regulation of the CYP27B1 5'-flanking region. However, over expressed PDEF at plasmid concentrations of 200, 500 and 1000 ng had no effect on expression of the -997 construct in DU145 cells (data not shown). Similarly, over expression of another Ets family member, Ets-1, at these concentrations had no effect. Hence PDEF and Ets-1 are apparently not involved in enhancer activity in these cells.

Characterisation of the repressor region

Possible transcription factor binding sites were noted within the repressor region from -997 to -1200 (Fig. 3). Two putative cAMP responsive elements (CREs), CRE-I (5'-CTGGCTAA-3') and CRE-II (5'-TGACCTCC-3') were identified at -1069 to -1062 and -1134 to -1127, respectively, together with a putative AP-1 like sequence at -1150 to -1144 (5'-TAACTCA-3', underlined in Fig. 3). Each of these sites was inactivated within the context of the -1200 deletion construct. It can be seen that inactivation of the AP-1 like site (5'-TAACTCA-3' to 5'-TAACTTG-3') in construct pCYP27B1mAP-1 like (-1200)-Luc significantly reversed the inhibitory level of expression observed with the wild type -1200 construct while inactivation of CRE-I also led to increased expression (Fig. 4C). Alteration of the remaining putative site, CRE-II, did not affect expression. It is concluded that the CRE-I and the AP-1 like sites are important for mediating repression of the -1200 construct. We have focused on the AP-1 like site.

Initially, EMSA experiments were carried out using a double stranded oligonucleotide containing the AP-1 like sequence (5'-TAACTCACTGCAG-3') and binding was compared with a known AP-1 binding sequence (5'-CGCTTGAGTACTCAGCCGGAA-3') from the SV40 promoter (Santa Cruz Biotechnology Inc). A strong band was retarded on the authentic SV40 AP-1 probe but the AP-1 like probe from CYP27B1 did not significantly retard any nuclear protein complex in repeated experiments (data not shown). The lack of protein binding by the AP-1 like

probe was surprising since mutagenesis of the sequence (5'-TAACTCA-3' to 5'-TAACTTG-3') within the pCYP27B1(-1200)-Luc construct had been shown to reverse the inhibitory response (see Fig. 4C). This led us to examine nearby sequences that could be critical for binding of proteins to the AP-1 like sequence. We located upstream the sequence 5'-AATC-3' (see Fig. 3, bold and underlined) which can act as the core binding sequence for the zinc-finger transcription factor oncoprotein growth factor independent-1 (GFI1) (Zweidler-Mckay *et al.* 1996) and this sequence was further investigated.

Mutational analysis of the putative GFI1 core binding sequence

Mutagenesis of the putative GFI1 core sequence (5'-AATC-3' to 5'-GGTC-3') was carried out within the context of the -1200 deletion construct (pCYP27B1mGFI1(-1200)-Luc) and also in combination with the AP-1 like sequence (pCYP27B1mGFI1+mAP-1 like (-1200)-Luc). Transfection analysis of these constructs, together with pCYP27B1mAP-1 like (-200)-Luc, was carried out in DU145 cells (Fig. 5A). The data showed that inactivation of the putative GFI1 core sequence substantially reversed repression of the wild type construct pCYP27B1 WT (-1200) to a level even greater than that seen with the mutation in the AP-1 like sequence. However, mutations in both the GFI1 core and the AP-1 like sequence did not further increase expression of the construct compared with the mutation alone in the GFI1 core sequence. This favours the proposal that the AP-1 like sequence does not bind any protein on its own but serves as a flanking sequence for the nearby GFI1 core. To support the presence of endogenous GFI-1 in DU145 cells, a Western blot analysis was carried out using the GFI1 antibody (Fig. 5B). A major cross reacting band was observed with an approximate molecular size of 55-60 kDa that corresponds with that reported for GFI1 (Duan & Horwitz 2003), together with two lower molecular weight bands.

Binding of endogenous nuclear proteins to the GFI1 binding site

Nuclear protein binding to the possible GFI1 site in the CYP27B1 5'-flanking region was analysed by EMSA using nuclear extracts from DU145 cells and the probe designated CYP27B1 GFI1/AP-1 like oligonucleotide, which encompassed the AP-1 like sequence and core for GFI1 binding (5'-TGGTACAATCATAACTCACTGCAG-3'). For comparison,

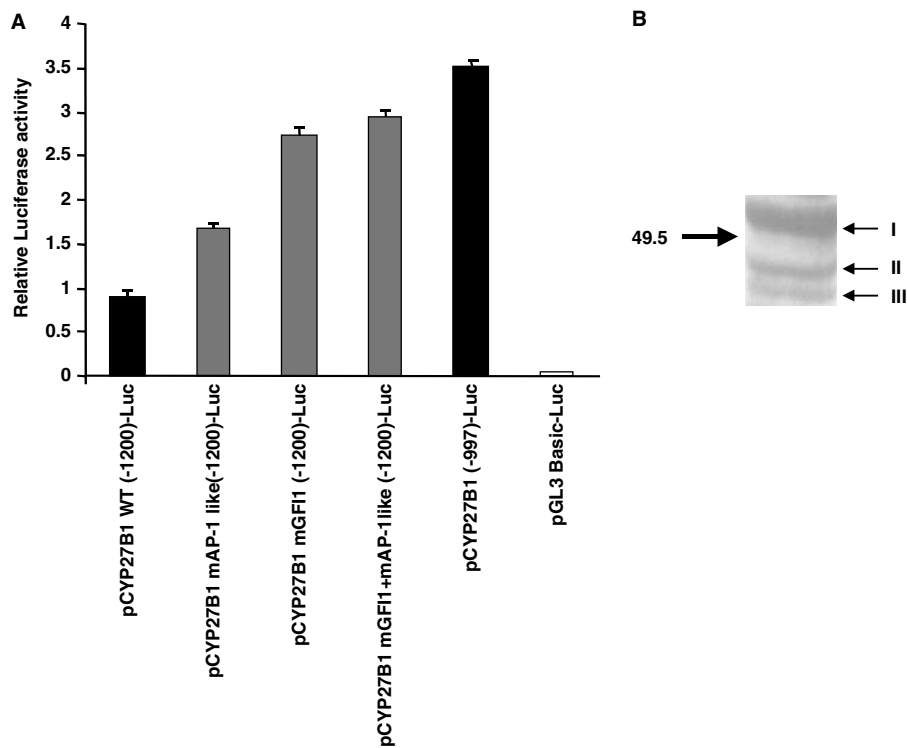


Figure 5 Mutagenesis of a putative GF11 binding site in the construct pCYP27B1(-1200) (A) The putative GF11 site and the AP-1 like site were either mutated singly or in combination in the construct pCYP27B1(-1200)-Luc. These constructs were analysed by transient transfection assays using DU145 cells. The relative luciferase activity shown represents the mean \pm s.d. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative experiment. The experiment was repeated three times. (B) A Western blot analysis was carried out using total DU145 cell extracts and GF11 polyclonal antibody. The arrow indicates the position of the molecular weight marker (49.5 kDa).

a p21 GF11 probe was also examined (5'-CGAAG-TACCGTGATTTTCAGGCATGCACG-3') that has been shown to strongly bind GF11 (Tong *et al.* 1998). The CYP27B1 GF11/AP-1 like probe retarded a major complex (C-I) and a minor complex (C-II) (Fig. 6A, lane 2) and the profile of binding was identical to that of the p21 GF11 probe (Fig. 6A, lane 1). When the SV40 probe (5'-CGCTTGATGACT-CAGCCGAA-3') was employed, a major band was observed but was of considerably lower mobility compared with complexes C-I and C-II (data not shown). Moreover, in other experiments C-I and C-II did not react with the following antibodies to AP-1 family members c-fos, c-jun, fra-1 and fra-2. Hence the protein complexes binding to the CYP27B1 GF11/AP-1 like sequence are unlikely to be members of the AP-1 family.

To further investigate this possibility, self-competition experiments were undertaken with the unlabeled CYP27B1 GF11/AP-1 like probe. At a 10-fold molar excess of probe, the binding of both complexes was markedly reduced with binding of C-II being

completely inhibited (Fig. 6A, lane 3) while at 50-fold molar excess, both C-I and C-II were totally abolished (Fig. 6A, lane 4). Hence C-I and C-II are specifically bound complexes. This result was further supported by competition experiments employing an unlabeled CYP27B1mGF11/AP-1 like oligonucleotide probe where the core sequence of GF11 (5'-AATC-3' to 5'-GGTC-3') was inactivated. This mutated oligonucleotide probe (CYP27B1mGF11/AP-1 like) at 10- or 50-fold molar excess did not affect binding of C-I or C-II (Fig. 6A, lanes 7 and 8), confirming the specificity of binding of these complexes to the GF11 binding sequence. Cross competition experiments were also carried out using 10- and 50-fold molar excess of the unlabeled p21 GF11 oligonucleotide probe and this competitor was found to completely abolish both the C-I and C-II complexes even at 10-fold molar excess (Fig. 6A, lanes 5 and 6). These results provide evidence that C-I and C-II retarded on the CYP27B1GF11/AP-1 like probe are GF11 proteins.

To confirm the presence of GF11 in C-I and C-II complexes, EMSA experiments were performed using a

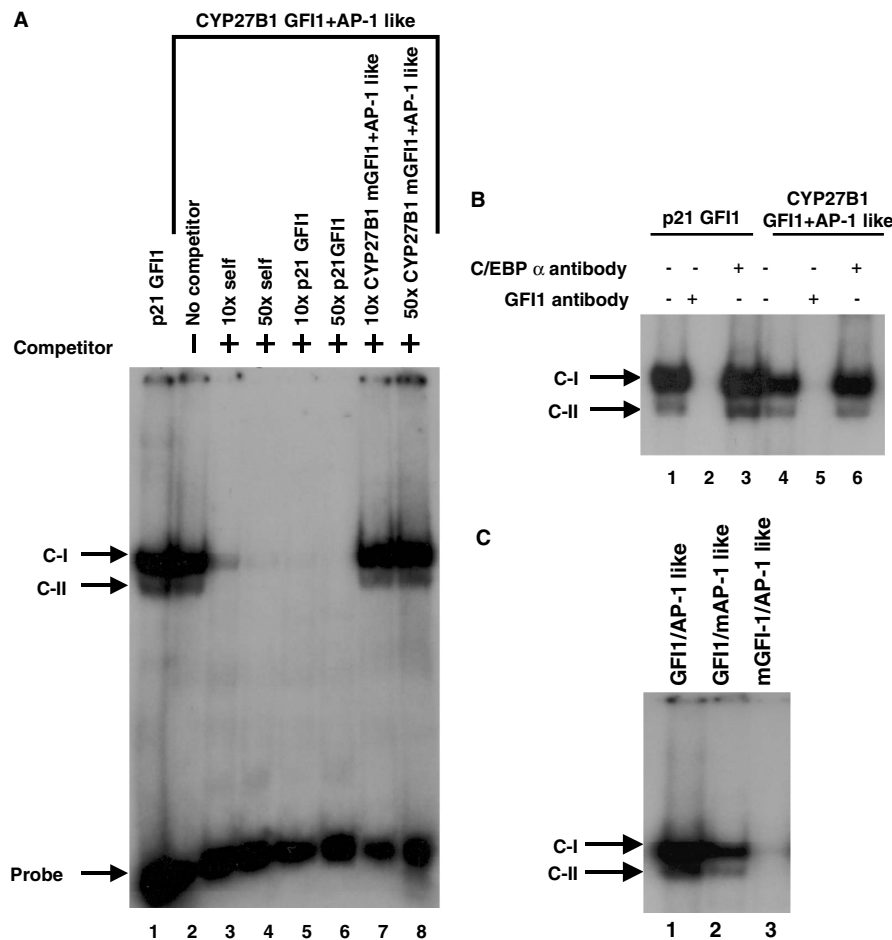


Figure 6 (A) Double stranded oligonucleotides for the GF1 binding site (CYP27B1 GF1/AP-1 like) and a control GF1 site (p21 GF1) were labeled by end filling with [α^{32} P] dCTP and incubated with nuclear extracts from DU145 cells. The retarded complexes are arrowed and numbered as C-I and C-II. Free probe is arrowed at the bottom of the gel. The formation of these complexes with radiolabeled CYP27B1 GF1/AP-1 like probe was competed with unlabeled self, p21 GF1 probe and mutated GF1 site in the CYP27B1 mGF1/AP-1 like probe. (B) A neutralization assay was carried out with GF1 antibody (lanes 2 and 5) and C/EBP α antibody (lanes 3 and 6) prior to addition of radiolabeled probes as indicated. (C). GF1 core and AP-1 like sequences were individually mutated and compared with the WT (-1200) CYP27B1 GF1/AP-1 like oligonucleotide for binding of endogenous GF1 in EMSA experiments. The retarded complexes C-I and C-II are indicated.

polyclonal antibody for GF1. This antibody totally reduced the formation of both complexes C-I and C-II on the p21 GF1 probe (Fig. 6B, lane 2) and the CYP27B1GF1/AP-1 like probe (Fig. 6B, lane 5). However, in a control experiment using the same amount of antibody for the unrelated transcription factor C/EBP alpha, the formation of these complexes was unaffected (Fig. 6B, lanes 3 and 6). These results confirm that C-I and C-II contain GF1 protein.

We next examined the contribution to GF1 binding of the AP-1 like sequence and the GF1 core. EMSA was carried out using the wild type (CYP27B1GF1/AP-1 like) probe and mutated GF1 and AP-1 like

probes (CYP27B1mGF1/AP-1 like and CYP27B1GF1/mAP-1 like; Fig. 6C). The mutation in the GF1 core sequence completely abolished binding (Fig. 6C, lane 3), however binding was only partially affected when the AP-1 like site was mutated (Fig. 6C, lane 2). These results support the notion that the AP-1 like sequence constitutes flanking sequence for optimal binding of GF1.

Repressive action of GF1

To examine directly whether GF1 can reduce expression of the CYP27B1 5'-flanking region, the effect of

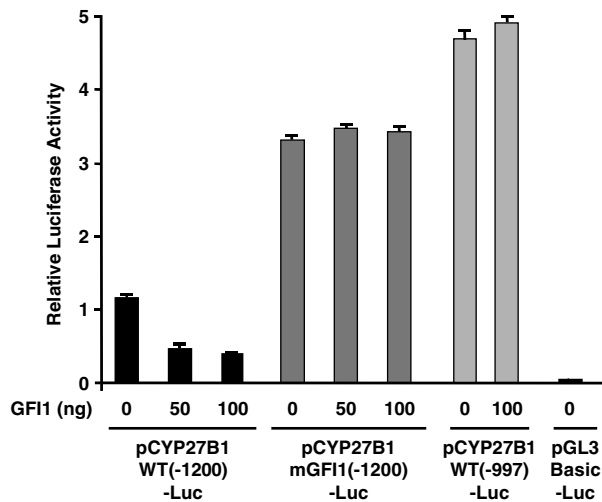


Figure 7 Effect of exogenously expressed GF11 in DU145 cells: effect of the transiently transfected GF11 expression clone pcDNA3-Myc-GF11 (50 and 100 ng) on expression of the pCYP27B1 WT(-1200)-Luc and pCYP27B1 mGF11(-1200)-Luc constructs was investigated in DU145 cells and compared with the pCYP27B1(-997)-Luc construct. The relative luciferase activity shown represents the mean \pm s.d. firefly luciferase: *Renilla* luciferase ratios of triplicate samples from a representative experiment. The experiment was repeated three times.

exogenous GF11 was examined (Fig. 7). As expected from our earlier results, the pCYP27B1(-1200)-Luc construct was repressed compared with pCYP27B1(-997)-Luc that does not contain the repressive region (Fig. 7). However, in the presence of over expressed GF11 at 50 ng and 100 ng, expression of the pCYP27B1(-1200)-Luc was further reduced by about 60% (Fig. 7). Importantly, when the GF11 core sequence was inactivated in the pCYP27B1mGF11(-1200)-Luc construct, exogenous GF11 had no effect on expression. This result demonstrates that GF11 can act as a repressor of the CYP27B1 gene in prostate cancer cells through the identified GF11 binding site. In a further control, over expression of GF11 had no significant effect on expression of pCYP27B1(-997)-Luc.

Discussion

The observation that 1,25D prevents proliferation of prostate cancer cells (Peehl & Feldman 2003) has attracted considerable interest because of the possibility of using vitamin D based therapies for prostate cancer (Chen *et al.* 2000, Chen & Holick 2003). A deficiency of CYP27B1 enzyme activity levels was

noted in prostate cancer cells compared with normal cells (Chen *et al.* 2003) and this correlated with the inability of exogenous 25D to inhibit cellular proliferation presumably because of insufficient local 1,25D production by CYP27B1 (Chen *et al.* 2003). In support of this proposal, when prostate cancer cells were transfected with a cDNA clone for human CYP27B1, exogenously added 25D regained the ability to inhibit cellular proliferation (Whitlatch *et al.* 2002, Chen *et al.* 2003). The data support the current contention that the reduced ability of prostate cancer cells to synthesize 1,25D contributes to their uncontrolled growth rate (Peehl *et al.* 1994, Schwartz *et al.* 1994).

The molecular basis for the lowered production of CYP27B1 enzyme in prostate cancer cell lines is currently of great interest. Chen *et al.* (2003) provided evidence that reduced CYP27B1 promoter activity was responsible but no repressive site was reported. In the current work, we have shown by transient expression analysis of deletion constructs in three different human prostate cancer cell lines cultured in serum free conditions that the 5'-flanking region of the human CYP27B1 gene comprises three regulatory domains. There is a proximal promoter region located within the first 305 bp, an enhancer region from -305 to -997 and a strong upstream inhibitory region from -997 to -1200. We report here for the first time a sequence within the inhibitory region at -1161 to -1138 that plays a substantial role in the repression of the 5'-flanking region of the CYP27B1 gene in prostate cancer cells. We provide evidence from EMSA experiments and over expression studies that the zinc finger transcription factor GF11 represses gene expression through binding to this sequence.

In initial studies, we investigated the role of an apparent binding site for AP-1 (Tong *et al.* 1998) that was located in the inhibitory domain. While mutagenesis of this AP-1 like sequence (5'-TAACTCAC-TGCAG-3' to 5'-TAACTTGCTGCAG-3') allowed significant reversal of gene inhibition in transient transfection studies, we were unable to detect binding of any protein to this sequence using EMSA experiments and DU145 cell nuclear extracts. The situation was clarified by the identification of a nearby core sequence for GF11 (5'-AATC-3') that was not included in the oligonucleotide used in the EMSA experiments. With an extended oligonucleotide (5'-TGGTACAAT-CATAACTCACTGCAG-3') we showed using a specific antibody that GF11 protein binds to this sequence in EMSA experiments employing DU145 nuclear extracts. A major and a minor protein complex were detected; the minor band may represent a degradation product. Competition studies using an unlabeled self

oligonucleotide confirmed that the binding of these proteins was specific, while an authentic GFI1 probe (p21 GFI1) competed with binding of the two complexes. We also showed that mutagenesis of the core within the context of the 5'-flanking region led to a marked reversal of CYP27B1 gene expression with this reversal being greater than that observed when the AP-1 like sequence was mutated. The AP-1 like sequence was therefore considered an important flanking sequence for functioning of the GFI1 core. In support of this concept, mutagenesis of the core sequence strongly prevented binding of GFI1 in EMSA experiments, while inactivation of the flanking AP-1 sequence inhibited binding, but to a lesser extent. In fact, DMS methylation interference experiments have previously indicated that GFI1 zinc fingers contact nucleotides at these positions flanking the core AATC sequence (Zweidler-McKay *et al.* 1996). Thus, both core and flanking regions are critical for GFI1 action on CYP27B1.

Both the major and minor protein complexes were also observed to bind to the p21 probe and to about the same extent as to the CYP27B1 probe (see Fig. 6A). A study of many potential GFI1 sites has reported that the GFI1 site in the p21 promoter has the highest affinity in EMSA studies (Duan & Horwitz 2003). There is almost no similarity in the flanking sequences surrounding the core in the p21 and CYP27B1 GFI1 sites and this is also interesting since we have identified flanking sequences as being particularly important for the binding and functional activity of the CYP27B1 GFI1 site. However, both sites contain nucleotides that were preferred in a random nucleotide selection with GFI1 zinc fingers (Zweidler-McKay *et al.* 1996).

There is evidence that GFI1 forms a large protein complex with its co-repressors ETO, msin3A and N-CoR and this acts as a scaffold for recruitment of histone deacetylases that can shut down gene expression through chromatin condensation (McGhee *et al.* 2003). Other corepressors may affect GFI1 repression, and such corepressors may lead to the formation of different sized GFI1 transcriptional multi-protein complexes. A key question then relates to the mechanism by which GFI1 represses the activity of the 5'-flanking region in prostate cancer cells. Perhaps a histone deacetylase containing multi-protein complex assembles on the far upstream GFI1 site leading to condensation of chromatin and silencing of the proximal promoter and/or the enhancer.

We have identified several important regulatory elements involved in positive and negative expression of the CYP27B1 5'-flanking region. The proximal

promoter region from +1 to -305 drives basal expression of the CYP27B1 gene through the action of a GC box (that likely binds Sp1) and a CCAAT box. Further upstream within the enhancer region there are three putative Ets protein binding sites with the core sequence 5'-GGAT-3'. One of these sites referred to here as EBS-I (at -807 to -895; Fig. 3), is particularly important since inactivation markedly lowered expression of the pCYP27B1(-997)-Luc construct. The identification of the protein that binds to this site is not known, but apparently is not the prostate specific protein, PDEF (Oettgen *et al.* 2000). We have also noted the presence of a CRE at -1069 to -1062 (see Fig. 3) that partially contributes to the repression of the CYP27B1 5'-flanking region, although maximum repression is only observed when the GFI1 site is included. Further studies are required to identify the CRE binding protein and its role in repression. There is recent evidence that NFkB can down regulate the CYP27B1 promoter in human embryonic 293 cells through a site in the CYP27B1 proximal promoter at -92 to -83 (Ebert *et al.* 2004). While this site clearly plays no role in the upstream repression phenomenon reported in the current work, it is possible that an NFkB site located in the far upstream region may be involved in repression, although such a site would be expected to contribute in only a minor way.

Our finding that GFI1 is important for repression of CYP27B1 gene expression in prostate cancer cell lines is novel. While GFI1 has been recognised as a transcriptional repressor from early studies (Grimes *et al.* 1996*a,b*), its functional role appeared to be confined to hemopoietic cells (Gilks *et al.* 1993) and its expression restricted to the bone marrow, thymus, spleen and testis and neurons (Gilks *et al.* 1993, Wallis *et al.* 2003). Recently, GFI1 expression was identified in primary human lung cancers, in association with neuroendocrine transcription factors and the neuroendocrine phenotype (Kazanjian *et al.* 2004). Our work extends the pattern of expression of GFI1 to include epithelial tumors of the prostate. Many putative GFI1 target genes have been suggested (Zweidler-McKay *et al.* 1996, Duan & Horwitz 2003) but the first and only report which thoroughly established a target for GFI1 (Doan *et al.* 2004), and which has been validated by others (Yucel *et al.* 2004), identified the GFI1 gene itself as a target of autoregulation in lymphocytes, but not myeloid lineage cells. Our work identifies CYP27B1 as a new target of the GFI1 oncoprotein. More work is needed to determine if GFI1 is oncogenic in prostate epithelial malignancies. If so, the repression of CYP27B1, and thus inactivation of the local tumor

suppressive effects of 1,25D, could be a mechanism for such oncogenic action.

In a recent report, Ma *et al.* (2004) confirmed that endogenous mRNA levels for CYP27B1 are reduced in DU145, PC3 and LNCaP cells correlating with reduced enzyme activity measured in these cells in earlier studies (Hsu *et al.* 2001). The finding of lowered CYP27B1 mRNA levels in these cell lines is in keeping with the present work where we have established reduced CYP27B1 promoter activity. However, it has been reported that in primary prostate cell cultures derived from normal and cancer tissues, there are equivalent amounts of CYP27B1 mRNA and protein. It is suggested that an unknown post-translational mechanism is responsible for the associated reduced CYP27B1 enzyme activity in the cultures from cancer tissue (Ma *et al.* 2004). Our studies reported here indicate that it would be of great interest to identify whether GF11 is expressed in human prostate cancer as a first step toward clarifying its postulated role in the disease.

Dedication

This paper is dedicated to the memory of John Omdahl.

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