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Hormonal regulation and characterisation of the mouse *Cyp4b1* gene 5′-flanking region☆

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Abstract

The *Cyp4b1* structural gene comprises 12 exons that span ~23 kb. The transcriptional initiation site, mapped by 5′-RACE and primer extension analyses from kidney mRNA, was found to be 34-bp upstream of the translation initiation site. *Cyp4b1* mRNA expression was found restricted to kidney and androgenic up-regulation in several mouse strains was observed. Spatial location investigated by in situ hybridisation revealed abundant and specific expression of *Cyp4b1* mRNA in the proximal tubules of the renal cortex. A 1.8-kb murine *Cyp4b1* 5′-flanking region, which encompasses a TATA box-like sequence and several putative transcription factor-binding sites, was isolated. Transient transfection studies with different *Cyp4b1* promoter constructs indicated that this flanking region exhibits promoter activity when expressed in different cell lines and that a 98-truncated fragment represents the minimal sequence required for basal transcription. Androgen responsiveness was examined by cotransfection with an androgen receptor expression vector, in the presence of androgens.

Keywords: Androgen; Cytochrome P450; Mouse kidney; Promoter analysis; Sexual dimorphism

The mammalian CYP4 family is composed of *CYP4A*, *CYP4B*, and *CYP4F* subfamilies [1,2]. CYP4B1 gene, the single representative of subfamily *4B* to date, was associated with xenobiotic-metabolising and mutagenic activation of some procarcinogens [3–5], rather than ω-hydroxylation of fatty acids and eicosanoids, as is characteristic of *CYP4A* and *CYP4F* members [6,7]. Recent reports have suggested an alternative role for CYP4B1 proteins, which are capable of hydroxylating endogenous metabolites such as lauric acid [8] or arachidonic acid [9]. These latest studies defined new emerging physiological functions for the CYP4B1 enzyme, positioning it at the interface of xenobiotic and endogenous substrate metabolism [10].

Rat and rabbit were the first species from which CYP4B1 cDNAs were cloned and sequenced [11]. More recently, the corresponding orthologues from mouse and human were also cloned from various sources [5,12,13]. Although the overall amino acid sequence identity shared by the CYP4B1 proteins from these four species is relatively high (84–90%), they exhibited significant interspecies differences in substrate specificity, particularly the human isoform, as well as in their induction and expression levels which follow a sex-, tissue-, and species-specific pattern [11]. Mouse *Cyp4b1* was purified and cloned from kidneys of male mice and found to be present in pulmonary microsomes of both males and females [5]. Like the rest of animal CYP4B1 isoforms, it is mainly involved in the bioactivation of several structurally diverse xenobiotics and procarcinogens, but may also participate in endogenous metabolism, as noted [14].

In this report we describe that mouse *Cyp4b1* is restricted to the kidney and, to a lesser extent, to the lung. Moreover, we show a dramatic androgenic control of *Cyp4b1* expression, at the mRNA level, in kidney. The mechanisms regulating the sexually differentiated gene expression in this tissue, where a number of proteins including other CYPs display an androgen-mediated

☆ *Abbreviations:* CYP, cytochrome P450; DHT, 5α-dehydrotestosterone; SDS, sodium dodecyl sulphate; SSC, saline-sodium citrate; SSPE, sodium phosphate–sodium chloride–EDTA; PIPES, 1,4-piperazine-bis-(2-ethanosulphonic acid); DTT, dithiothreitol.

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regulation [15–18], are poorly understood. As an initial step towards elucidating *Cyp4b1* androgenic transcriptional regulation, we document here the cloning and nucleotide sequence of the murine *Cyp4b1* gene proximal 5′-flanking region. The transcription start site and several upstream putative *cis*-regulatory elements were identified and a minimal promoter region responsible for efficient transcriptional activity in renal cell lines of proximal tubule origin was delineated.

Materials and methods

Materials. [α-^{32}P]dCTP and [γ-^{32}P]dATP were purchased from Amersham–Pharmacia. Restriction enzymes were purchased from Roche. General materials for cell culture and media components were obtained from Life Technologies and Biological Industries. Oligonucleotides used in this study were synthesised at TIB MOLBIOL-Shyntheselabor (Germany). All other chemicals and reagents were of the maximum purity available.

Animals. BALB/c, 129/Sv, and C57BL/6 (B6) mice were obtained at 6–7 weeks of age from IFFA CREDO and housed in the animal facilities as described elsewhere [19]. When required, male mice were castrated as previously reported [18]. All experiments were conducted in accordance with institutional guidelines for laboratory animal treatment and housing.

Library screening and DNA analysis. A mouse 129/Sv genomic library in λFIXII (Stratagene) was screened with a 1.8 kb probe corresponding to mouse Cyp4b1 full-length cDNA previously obtained in our laboratory. Several positive phage clones were purified to homogeneity and characterised using standard protocols [20]. Genomic clone sequencing was carried out using ABI PRISM Big dye terminator chemistry following the manufacturer's instructions (PE Applied Biosystems).

Primer extension analysis. Primer extension assays were performed following standard techniques [21]. Two *Cyp4b1*-specific primers: 'mCYP 103L' and 'mCYP4B1 218' (see Table 1), complementary to first and second exons, respectively, were end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega) and annealed with mouse kidney total RNA. A sequencing reaction for comparison with the primer extension product was performed and run alongside the samples. Finally, the gel was dried and exposed to Kodak X-Omat film at −70 °C.

5′ Rapid amplification of cDNA ends. The 5′ rapid amplification of cDNA ends (5′-RACE) analysis was also used to confirm the position of the transcription start site. Essentially, 1 μg of male kidney total RNA was reverse-transcribed using SuperscriptII (Life Technologies) with antisense primer 'mCyp4b1 478r' (relative positions in cDNA: nt 458–478). Gene-specific antisense primers 'mCYP4B1 218' (nt 197–218) and 'Cyp103L' (nt 83–103) were used for first PCR and second nested PCR, respectively. The PCR amplified products were sequenced and the transcription initiation site was assigned as nucleotide position +1.

Construction of reporter plasmids. All plasmid constructs were generated using standard methods [20]. Different fragments of the murine *Cyp4b1* 5′-flanking region containing the *Nco*I restriction site were synthesised by PCR. *Nco*I-digested PCR products were ligated into pGL3 basic vector (Promega) generating a series of chimeric cypb1-luciferase (*luc*) gene reporter constructs. Correct orientation and sequence of each construct were verified by sequencing. The human androgen receptor expression plasmid pSVAR$_0$ was described previously [22]. Construct 1xAREcyp98*luc* was generated by insertion of the double-stranded oligonucleotide corresponding to the consensus ARE element: GATCTG**AGAACAtgaTGTTCT**G in the *Bgl*II site of the cyp-98/+17 *luc* construct, and used as an androgen-responsive positive control.

Cell culture. Mouse kidney proximal tubule cells, PKSV-PCT and PKSV-PR, kindly provided by Dr. Vandewalle (INSERM, Paris), were cultured as previously described [23]. Generation and establishment of PCT3 and PR10 clonal-derivatives have also been described [24]. MDCK, HEK293, HepG2, and CV-1 cell lines, grown in DMEM supplemented with 10% FCS, 2 mM glutamine, and antibiotics were kindly provided by Drs. Garcia de Herreros (IMIM, Barcelona), Bruno Stieger (Zürich University, Zürich), and Emili Itarte (UAB, Barcelona), or purchased from the American Type Culture Collection (ATCC), respectively.

Transient transfection and reporter gene assays. For each transfection experiment using the *luc* reporter plasmid, cells were seeded (5×10^4/well for PCT3 and PR10 and 150×10^4/well for all the remaining cell lines) in 24-well plates. Twenty-four hours later, co-transfections were performed with 0.3 μg of each appropriate promoter reporter construct and 0.012 ng of the internal control vector (SEAP2, Secreted alkaline phosphatase) for normalisation of transfection

Table 1
Oligonucleotides used for primer extension, RT-PCR, and chimeric plasmid construction

Primer	Sequence (5′–3′)[a]	Orientation
Cyp −83/N	gtccatggTCAGTGCCCCGTATCCAGA	Forward
Cyp −1022/N	ctccatggCAGTTCCCTTTGCCAATGTT	Forward
Cyp −153/N	gcccatggCTTTGAGGCAGCTATCTCCG	Forward
Cyp −115/N	caccatggTTTAAGCTGCTGCTAGCTG	Forward
Cyp −675/N	tgccatggTGGGCCTGAGTGGTTCTATC	Forward
Cyp −1.2/N	gtccatggGGGTTGGCAACTCACCATTTT	Forward
Cyp −272/N	gtccatggCTGCTCCCTGTTCTCTGCTC	Forward
ARE	gatctgAGAACATGATGTTCTga acTCTTGTACTACAAGActctag	ds[b]
mCYP 103L	GGATCACTACAGAAGCCCACA	Reverse
mCYP4B1 218	AGGCCTCCTGTCTTCTGGATCT	Reverse
mCyp4b1 459f	TGATGTGCTGAAGCCCTATG	Forward
mCyp4b1 478r	CATAGGGCTTCAGCACATCA	Reverse
mCYP4B1 1695r	AAAGCCCAGGCATTGAATC	Reverse
mCypA up	ATGGTCAACCCCACCGTG	Forward
mCypA low	CAGATGGGGTAGGGACG	Reverse

[a] In lowercase, added restriction sites.
[b] Double-stranded oligonucleotide.

efficiency, using the LIPOFECTAMINE plus reagent (Promega), following the supplier's recommendations. For experiments testing the androgen-responsive capacities of the *Cyp4b1* promoter constructs, transfected cells were cultured in steroid-free serum-containing medium. Where required, medium supplemented with 10^{-6} M DHT was added to the cultures for 48 h before harvesting.

Luciferase assays. At 48 h of transfection cells were harvested and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Blank luciferase activity was measured using the same volume of mock-transfected cell lysates and subtracted from the readings to obtain relative light units. All results are based on at least three independent experiments.

Secreted alkaline phosphatase (SEAP) activity was used to normalise luciferase values and measured in the corresponding culture medium following the manufacturer's instructions (Great EscAPe SEAP, Clontech).

RT-PCR and Northern blot. Five hundred nanograms of total RNA isolated from various tissues of male 129/Sv mice was used in each RT-PCR using the SuperScript One-Step RT-PCR System (Life Technologies), using specific primers for Cyp4b1 459f (sense) and 1685r (antisense) (459–1685 of the cDNA). Primers for mouse Cyclophilin A (CypA) cDNA amplification, used as an internal control for RNA integrity and quantity, had been previously described [24]. Semi-quantitative RT-PCRs were performed under conditions that permitted the analysis at the linear range of the PCR for each primer set. The identity of the ~1.2-kb Cyp4b1 amplified product was confirmed by sequencing. Northern blot assays were performed using 15 μg/lane of total kidney RNA and hybridized with a ^{32}P-labelled 0.48-kb Cyp4b1 cDNA fragment (corresponding to nt 1–478). The hybridisation conditions and washing stringency were performed as described in [18].

In situ hybridisation. Kidneys were removed and snap frozen in liquid nitrogen. Cryostat sections were hybridised with riboprobes and synthesised according to the directions of the digoxigenin RNA labelling kit (Roche) which expanded from nt 23 to 478 of mouse Cyp4b1 cDNA. For signal development, the slides were immersed in developing buffer containing the substrates NBT and BCIP and allowed to stand at room temperature in the dark for 1–2 h. Colorimetric reaction was observed under microscope and terminated by immersion in PBS. The sections were examined with an Olympus G50 microscope.

Results and discussion

Tissue distribution and androgen-stimulated expression of Cyp4b1 mRNA in kidneys of different strains of mice

Cyp4b1 gene expression was investigated by RT-PCR in a wide panel of adult male mice tissues (Fig. 1A). *Cyp4b1* mRNA was exclusively expressed in the kidney and, to a lesser extent, in the lung and its expression was completely negative in the remaining tissues. Amplification of the internal control *CypA* gene demonstrates that all tissue samples are qualitatively comparable in terms of template RNA integrity and therefore that the differences observed in *Cyp4b1* mRNA levels are truly related to tissue specificity. Although Imaoka et al. [5] had previously shown that *Cyp4b1* was expressed in renal and pulmonary microsomes, we have further extended the concept that *Cyp4b1* mRNA is exclusively expressed in kidney and lung. Although detected in rabbit liver by others [11], we were not able to detect it in murine liver, thereby demonstrating an important species difference in tissue-specific expression patterns.

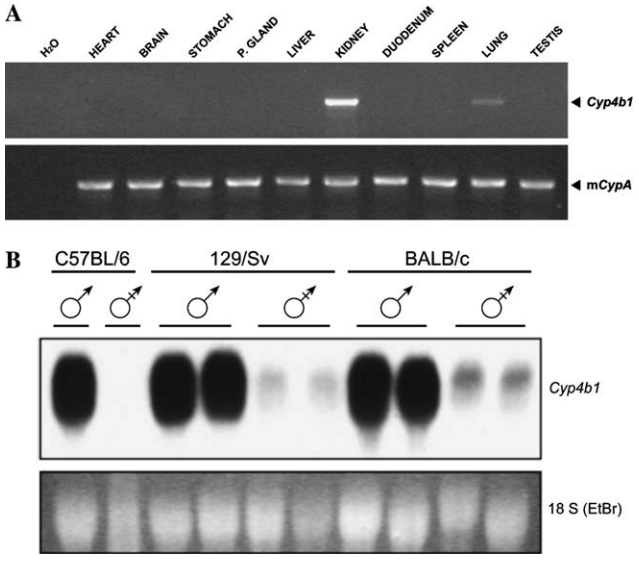

Fig. 1. Tissue distribution of *Cyp4b1* mRNA expression and androgen up-regulation in kidneys of several mouse strains. (A) Expression profile of *Cyp4b1* in mouse tissues analysed by RT-PCR. Total RNA (500 ng) from various murine tissues (indicated at the top) was reverse-transcribed and PCR-amplified using specific *Cyp4b1* primers (459f and 1685r). As an internal control, mouse cyclophilin A (*mCypA*) primers were used (bottom panel). (B) Northern blot analysis of *Cyp4b1* mRNA in kidneys of different inbred mouse strains. Total RNA (15 μg) from intact and castrated male kidneys of C57BL/6, 129/Sv, and BALB/c (two animals each) was electrophoresed and transferred to nylon membranes. The hybridisation was performed with mouse *Cyp4b1* cDNA under the conditions described in Materials and methods. A single 2.0-kb transcript was significantly detected in intact males and weakly expressed in castrated animals in all three mouse strains tested; the 18S ribosomal RNA band (from ethidium bromide-stained gel before transfer) is also shown as a control of sample loading.

Since it has been noted that significant strain-, sex-, and age-related differences may exist among individual members of the CYP family, we examined by Northern blotting whether sex steroids mediate sex-differentiated *Cyp4b1* gene expression in the kidney of different inbred mice strains such C57BL/6, BALB/c, and 129/Sv. As shown in Fig. 1B, we observed that in all strains tested, *Cyp4b1* mRNA hybridisation signals did significantly decrease after castration, confirming the renal sex difference described earlier at the level of protein and activity by Imaoka et al. [5] and demonstrating that these differences occur at mRNA level.

Location of Cyp4b1 mRNA by in situ hybridisation

In situ hybridisation of frozen mouse kidney sections revealed that *Cyp4b1* transcripts were detected in renal cortex. Comparison of the in situ sections (Fig. 2) with

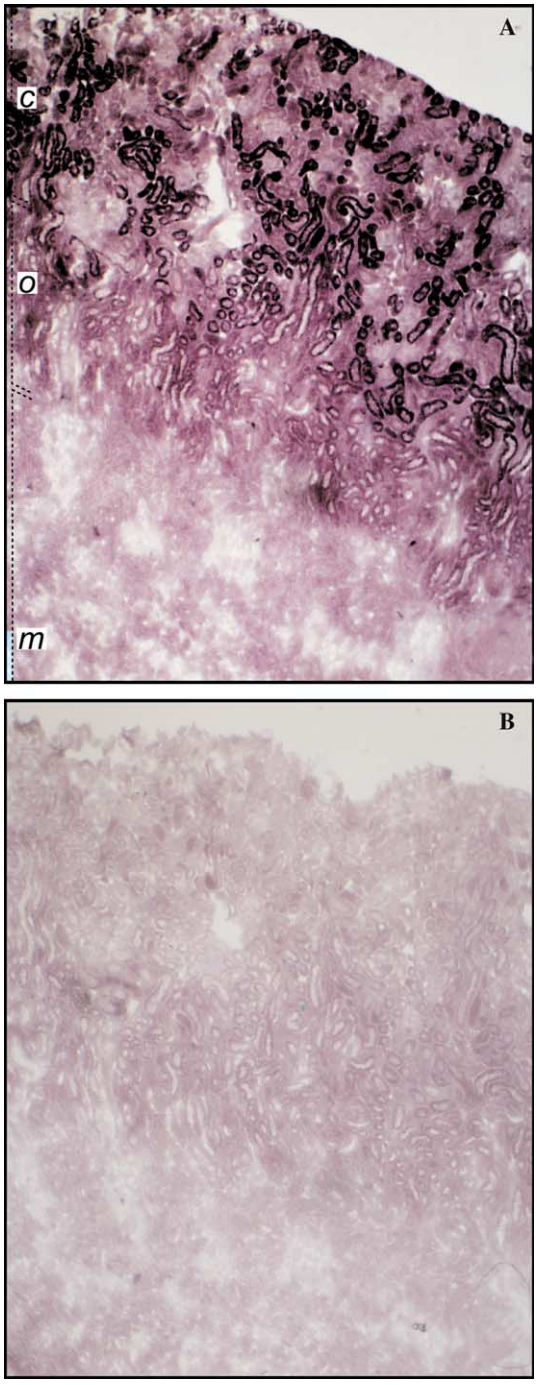

Fig. 2. Distribution of *Cyp4b1* transcripts in mouse kidney by in situ hybridisation. Mouse kidney sections stained with digoxigenin-labelled antisense *Cyp4b1* riboprobe showing intense labelling in the renal cortex and the outer stripe of the outer medulla (A). No signal was obtained with the corresponding sense riboprobe under identical conditions (B). The signal is concentrated in proximal tubules of the S1 and S2 segments (c, cortex), a weak labelling is present in adjacent S3 segments of the outer medulla (o, outer stripe of outer medulla), glomeruli or distal tubules (m, medulla). Magnification 4× (A and B).

PAS (periodic acid–Schiff reagent)-stained serial kidney sections (not shown) demonstrated that the gene is expressed mainly in proximal tubular cells.

The finding that androgens control the expression of the *Cyp4b1* gene in these cells is particularly interesting since Imaoka et al. [5] reported that CYP4B1 plays a major role in the mutagenic activation of the potent procarcinogen, 3-methoxy-4-aminoazobenzene. Metabolism of this and other substrates such as 2-aminofluorene and 2-aminoanthracene, among others [3,4], may affect homeostasis of the kidney and its susceptibility to disease.

It has been reported that lung carcinomas exhibit higher levels of *CYP4B1* mRNA than normal lung tissues [25] and that androgenic regulation of *CYP4B1* is responsible for mutagenic activation of bladder carcinogens in rat bladder [26]. Taking this information into account, we find provocative the possible association of the androgenic up-regulation of this member of the cytochrome P450 enzyme family in kidney with the well-documented higher incidence of renal cell carcinomas in men than in women. Interestingly, the great majority of these tumours develop from epithelial cells of proximal convoluted tubules which are, in turn, the cellular targets for androgen action.

Identification of the transcription start site and 5′-flanking sequence analysis

Relatively little is known regarding the molecular mechanisms of tissue-specific gene expression in the kidney compared with other organs such as the liver. In liver, a sexually dimorphic gene expression pattern for several P-450s has been related to the growth hormone (GH) secretion pattern [27]. In relation to *Cyp4b1* and to gain insight into the molecular mechanisms controlling its androgen and cell-specific expression in the kidney, we aimed to clone and characterise its proximal promoter region. Initially, the transcription initiation site of the *Cyp4b1* gene was mapped by primer extension and 5′-RACE. Primer extension using 'mCyp103L' rendered two products of 119 nt (major band) and 116 nt (minor band) which indicated the existence of two transcription start sites, with the larger one being more prominent than the smaller one (Fig. 3). The major transcription start site, confirmed when using the 'mCYP4B1 218' primer (not shown), was designated +1, at 34-bp upstream from the translation initiation ATG codon. To further determine the transcription start site, 5′-RACE assays were performed. Upon sequencing the single product raised, the estimated cap site was located at 16 nucleotides upstream from the 5′-end of the known *Cyp4b1* cDNA sequence (GenBank Accession No. NM_002873), which corresponds to 34-bp upstream from the ATG codon, as noted.

Next, we attempted to begin study of the *Cyp4b1* gene 5′-flanking region. Screening of a mouse genomic library rendered a clone that contained 1.8-kb of the 5′-flanking upstream region, which was isolated and sequenced by

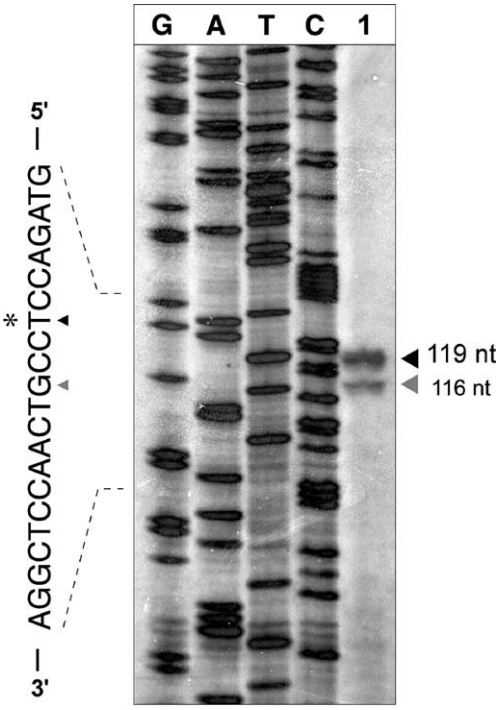

Fig. 3. Identification of the transcriptional start sites of the mouse *Cyp4b1* gene. The nucleotide sequence flanking the transcription initiation sites is illustrated on the left of the figure, with transcription initiation sites obtained by primer extension (arrowheads) and by 5′-RACE (asterisk). The arrows on the right represent the major (bold) and minor (grey) transcription start sites as mapped by primer extension, obtained using primer cyp103L and mouse kidney total RNA as a template (lane 1). The sizes of the extended bands are indicated. For further details see Materials and methods.

primer walking. This fragment includes 1797 nt of the 5′-flanking region and extends to the *Nco*I site in the first exon (nt +35) (Fig. 4). The arrow in Fig. 4 depicts the transcriptional start site mapped by 5′-RACE and the asterisks indicate the sites mapped by primer extension. This region was examined for the presence of typical eukaryotic promoter elements. A TATA-like motif (TATAA) was identified at position 25 bp upstream of the transcription start site, positioned at a good distance for having promoter function. Closer inspection of its sequence vicinity failed to reveal any evident consensus CAAT box. Further analysis of the 5′-flanking sequence was conducted using the MatInspector V2.2 program (http://transfac.gbf.de) to detect putative gene regulatory elements. Several potential consensus transcription factor binding sites were identified. A myeloid zinc finger (MZF-1) was found at −54 and two GC-boxes suitable for Sp1 binding were found centred at −100 and −115 positions. A cluster including several nuclear factor activating T cells (NFAT), MZF-1, and GATA factor potential binding sites was also located from positions −344 to −588. Finally, from −1025 position to the end of the promoter region analysed, a few more scattered NFAT and GATA potential binding sites were found.

Transcriptional activity of the mouse Cyp4b1 proximal promoter

A series of 5′-truncated promoter fragments, containing from −1.8 kb to −34 nt upstream from the transcriptional initiation site and extending to nt +34, were assayed in the mouse early proximal tubule (*pars convoluta*)-derived PCT3 cells [24]. As shown in Fig. 5, the full-length promoter fragment (−1.8 kb) is able to drive transcription, conferring ∼6-fold transcriptional activity compared to the control plasmid pGL3-basic. An additional control construct was prepared by subcloning the same sequence inserted in the reverse orientation into the pGL3-basic vector (REV1.8*luc*). As expected, this construct failed to drive reporter transcriptional activity, indicating that the −1.8 kb region displays basal *Cyp4b1* promoter activity. Deletion of 549-bp from the most distant 5′-region rendered a −1.2*luc* construct which was able to increase transcriptional activity ∼23-fold. These results indicated that, at the very least, some repressor elements present within the −1.8-kb fragment were missing. Subsequent deletions were essentially maintaining this elevated level of activation, except for construct −641*luc* that produced a 53% decrement in relation to the −950 nt fragment. Interestingly, deletions of the −641 construct further increased the transcriptional capacity of the promoter, being maximal with the −137*luc* construct, which gave a ∼32-fold induction over basal levels obtained with promoterless pGL3 basic vector.

Truncation of the −137 promoter fragment to nucleotide −98 resulted in a 12-fold reduction in luciferase activity. Finally, a further truncation to nucleotide −48 completely abolished activity, indicating that a minimal promoter defined from −48 to −98 bp contains essential sequences for constitutive expression of mouse *Cyp4b1* gene. Sequence analysis of that fragment revealed a putative MZF-1 site located at −54. The fact that no other sites were found in this promoter construct suggests that this factor might be of functional significance. MZF-1 has been considered as a haematopoietic transcription factor expressed in differentiating myeloid cells (for a review see [28]). In vitro, MZF-1 has been shown to repress reporter gene expression in non-haematopoietic cell lines and activate expression in haematopoietic cell lines, which suggests that the presence of tissue-specific regulators/adaptors or differential MZF-1 modifications might determine MZF-1 transcriptional regulatory function [29]. Cyp4F5 gene, another member of the family, expressed in liver, kidney, and brain bears a putative MZF-1 binding site in its 5′-flanking region for which no functional data have yet been published [30]. Maximal luciferase expression achieved with the −137*Cyp4b1* construct, which includes two putative Sp1 sites, indicates that this transcription factor might also contribute to *Cyp4b1* expression.

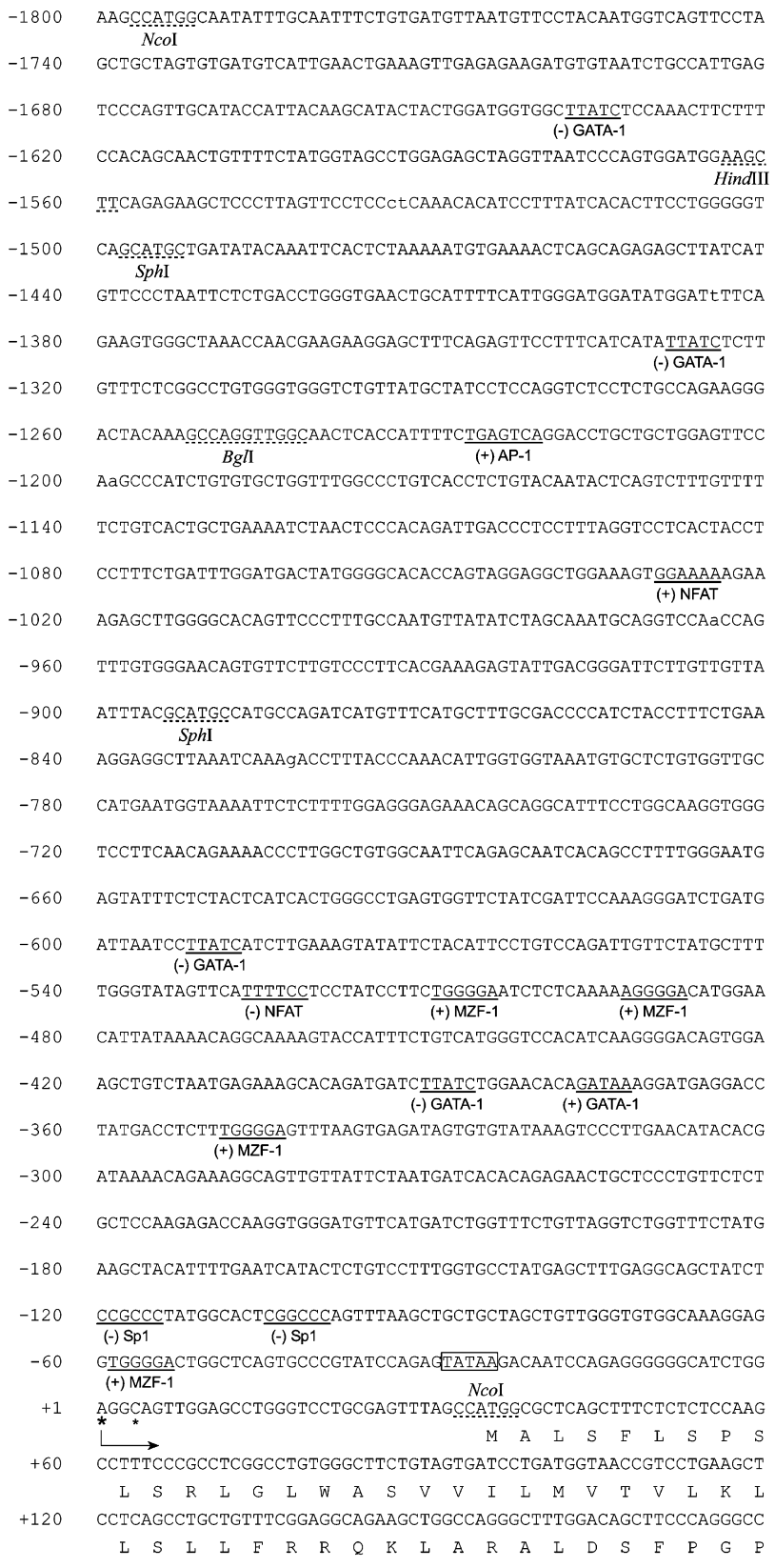

Fig. 4. Nucleotide sequence of the 5′-flanking region of the murine *Cyp4b1* gene. Numbering of nucleotides is relative to the transcription initiation site (nt +1). Bent arrow and asterisks represent the transcriptional start sites mapped by 5′-RACE and by primer extension, respectively. Restriction enzyme sites *Bgl*I, *Nco*I, *Hin*dIII, and *Sph*I are marked. Putative binding motifs for various transcription factors are indicated (with recognising core sequences underlined). An apparent TATA-box motif is boxed. The analysis of transcription factor binding sites was performed with the Matinspector program [33].

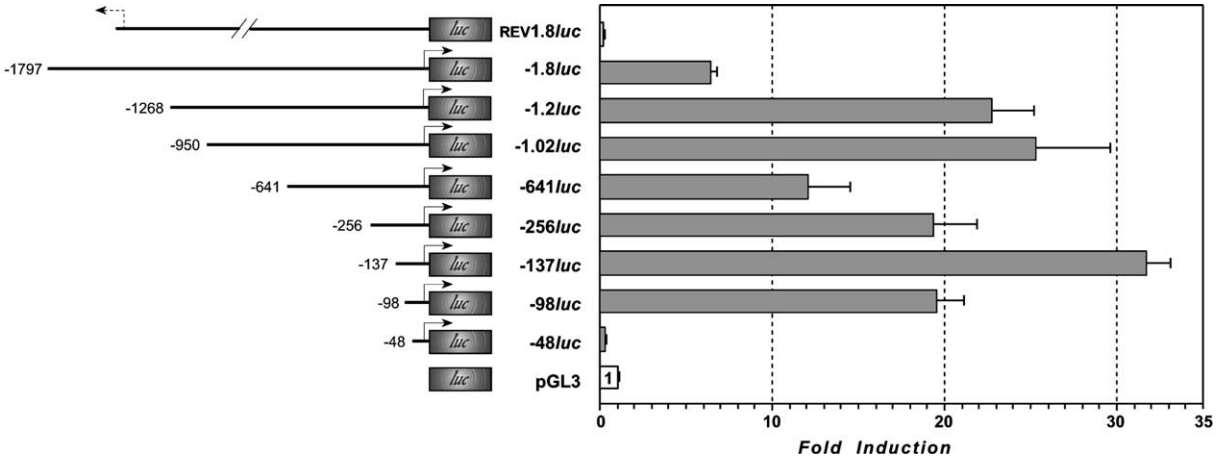

Fig. 5. Functional analysis of the mouse *Cyp4b1* proximal promoter in transfected proximal tubule PCT3 cells. Various fragments of mouse *Cyp4b1* 5′-flanking region from ≈1.8 kb to −43 nt upstream and extending to nt +32 downstream were subcloned into the luciferase reporter vector pGL3 to estimate their corresponding transcriptional activity. Schematic representation of the constructs used for transfection experiments is indicated. The plasmid constructs were cotransfected with pSEAP2 into mouse renal PCT3 cells and activities were normalised against SEAP activity. Data are means ± SD (standard deviation) from quadruplicate determinations and the results are representative of three independent experiments.

Androgen responsiveness evaluation of the mouse Cyp4b1 proximal promoter

In view of the observed in vivo up-regulation of *Cyp4b1* renal expression by androgens, we aimed to determine whether the isolated *Cyp4b1* proximal promoter was able to sustain androgen responsiveness in cultured cells. Although detailed characterisation of the molecular elements mediating androgen-responsive gene expression in the kidney has always been hampered by the lack of appropriate cultured cell systems suitable for DNA transfection studies, a recent work from our laboratory demonstrated that the immortalised differentiated PCT3 and PR10 proximal-tubule cells, originally derived from L-PK/Tag1 transgenic mice, represent valuable ex vivo cell systems for analysing hormone-specific regulation of androgen-regulated kidney-specific genes [24]. A series of 5′-deletion fragments of *Cyp4b1* promoter fused to luciferase gene were transiently transfected into the renal proximal tubule cell line, PCT3. Androgen receptor-encoding plasmid, $pSVAR_0$, was also cotransfected to provide for maximal ligand-dependent transactivation of the reporter constructs. The androgen responsiveness of these constructs was tested by incubating the transfected cells in the absence or presence of 1 μM DHT for 48 h. This treatment results in minimal changes in luciferase induction, with only a slight ∼2-fold increase in activity for constructs −137*luc*, −641*luc*, and −1.8*luc*, but with less absolute activity in the latter two (Fig. 6). The positive control, '1xARE-AREcyp98*luc*,' resulted in a substantially elevated reporter induction of ∼10-fold, while the promoterless pGL3-basic plasmid showed no significant change in luciferase activity.

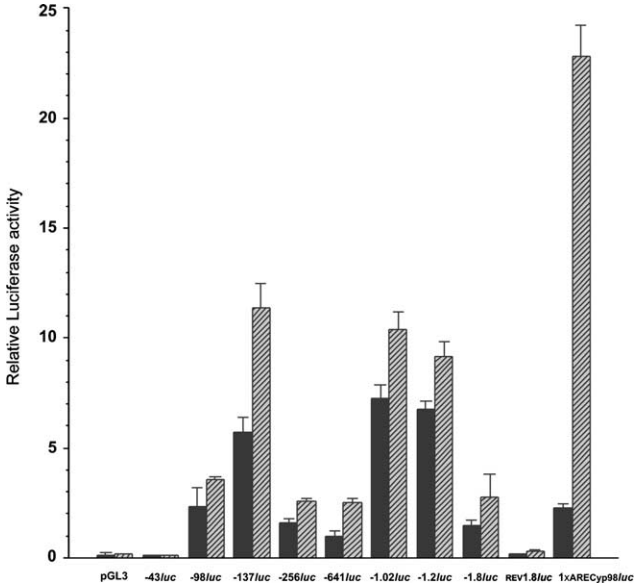

Fig. 6. Transcriptional regulation of the mouse *Cyp4b1* proximal promoter by AR and DHT. The same Cyp4b1-luc construct set, indicated in Fig. 5, was cotransfected with $pSVAR_0$ in PCT3 cells in the absence (solid bars) and presence (dashed bars) of 10^{-6} M DHT. Values were standardised relatively to secreted alkaline phosphatase activities. Each bar is the mean ± SD from four determinations.

Despite the absence of canonical androgen response element (ARE) motifs in the promoter fragments tested, indirect mechanisms could be involved in androgen-dependent activation of the promoter. Up-regulating and/or increasing the DNA binding activity of other as yet unidentified transcription factor/s may constitute, among others, a mechanism described for members of the steroid hormone receptor superfamily [31]. Following the rationale that MZF-1 could represent a putative repressor of the *Cyp4b1* promoter, we might speculate that androgens would induce expression of an adaptor

which, on binding to MZF-1, will prevent DNA-binding of MZF-1 to the *Cyp4b1* promoter, thereby allowing transcription. If androgens inhibit the repressive activity of MZF-1 and the factor would be missing in the cells, no strong effect on transfected constructs will be expected on androgen activation of PCT3 cells. Still, there is a possibility that the promoter fragments tested do not participate in the androgen up-regulation of the *Cyp4b1* gene in vivo and that other functionally active sequences further up in the promoter or located in the intervening sequences will be identified.

The sex-related steady-state accumulation of mRNA may be due, at least in part, to post-transcriptional regulation of the gene, as reported for other CYPs in kidney [32]. At this point, we cannot rule out this possibility since we do not yet have information on transcriptional rates of the *Cyp4b1* gene between male and female mice.

Analysis of the Cyp4b1 promoter in different cell lines

To evaluate whether the activity of the *Cyp4b1* promoter was cell-specific, the construct −137*luc*, which showed full promoter activity in PCT3 cells (see Fig. 5), and the 1.8-kb promoter-containing fragment −1.8*luc* were tested by transfection in several renal and non-renal cell lines (Fig. 7). Negligible luciferase activity was observed for control vector transfections in all cell lines. Mouse *Cyp4b1* promoter sequences −137 to +32 were able to direct notable luciferase gene expression, not only in PCT3 but also in mouse late proximal tubule (*pars recta*)-derived PR10 cells and human embryonic kidney-derived HEK293 cell line, with near inactivity in MDCK (canine kidney-derived), CV-1 (simian kidney-derived), and HepG2 (human hepatoblastoma-derived). In general, the longer promoter-fragment construct showed less activity in all cell lines than the shorter segment.

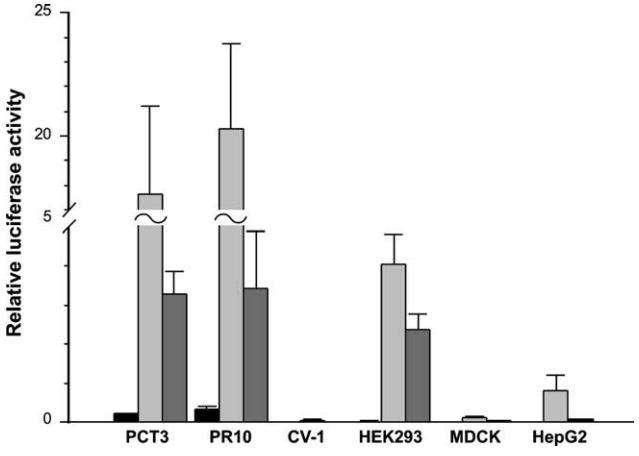

Fig. 7. Functional analysis of the *Cyp4b1* proximal promoter in different cell lines. The promoterless pGL3 basic (solid bars), the −1.8-kb (dark grey bars), and −137-bp (light grey bars) Cyp4b1-luciferase constructs were transfected into mouse proximal tubule (PCT3 and PR10), human embryonic, and monkey kidney (HEK293 and CV-1, respectively), canine kidney distal tubule-like MDCK, and human hepatoblastoma HepG2 cell lines. Luciferase activities for each construct on each cell line were measured and values were normalised for differences in transfection efficiency using secreted alkaline phosphatase activity as an internal control. For each instance, data from three independent experiments were used. Each bar is the mean ± SD from quadruplicate determinations.

Several studies have shown that PKSV-PCT and PKSV-PR, from where PCT3 and PR10 clones derived [24], conserved the main features of the parental cells from which they derived in transgenic mice [23]. The high constitutive promoter activity exhibited by the −137*luc Cyp4b1* construct in PCT3 and PR10 renders these cells valuable models to further identify the factors controlling *Cyp4b1* mRNA in an androgen-dependent or -independent fashion.

In summary, the results of this study present the in vivo regulation and preliminary characterisation of the 5′-regulatory region of mouse *Cyp4b1* gene. Understanding of its transcriptional regulation will shed light on delineating of the molecular mechanisms underlying androgenic regulation of specific expression in the kidney and, hopefully, the mechanisms of androgen-dependent carcinogenesis in this tissue.

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