Transcriptional Regulation and Characterization of the Promoter Region of the Human *ABCC6* Gene

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ABCC6, a member of the adenosine 5′-triphosphate-binding cassette family of genes, encodes multidrug resistance-associated protein 6, a putative transmembrane transporter expressed primarily in the liver and to a significantly lower extent in other tissues. Mutations in *ABCC6* result in pseudoxanthoma elasticum, a multi-system heritable connective tissue disorder with variable phenotypic expression. To examine the transcriptional regulation and tissue-specific expression of this gene, we cloned 2.6 kb of human *ABCC6* promoter and developed a series of 5′-deletion constructs linked to luciferase reporter gene. Transient transfections in a number of cultured cell lines of diverse origin identified a specific NF-κB-like sequence (−235/−226), which conferred high level of expression in HepG2 hepatoma cells, inferring liver specificity. The functionality of the promoter fragments was confirmed *in vivo* by tail vein injection followed by luciferase reporter assay. Testing of selected cytokines revealed that transforming growth factor (TGF)-β upregulated, while tumor necrosis factor (TNF)-α and interferon (IFN)-γ downregulated the promoter activity in HepG2 cells. The responsiveness to TGF-β was shown to reside primarily within an Sp1/Sp3 cognate-binding site at −58 to −49. The expression of the *ABCC6* promoter was also shown to be markedly enhanced by Sp1 protein, as demonstrated by cotransfection of *ABCC6* promoter–luciferase constructs and an Sp1 expression vector in *Drosophila* SL2 cells, which are devoid of endogenous Sp1. Furthermore, four additional transcription factors, with their cognate-binding sequences present in DNA, were shown to bind the 2.6-kb promoter fragment by protein/DNA array. Collectively, the results indicate that human *ABCC6* displays tissue-specific gene expression, which can be modulated by proinflammatory cytokines. These findings may have implications for phenotypic expression of heritable and acquired diseases involving abnormality in the *ABCC6* gene.

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INTRODUCTION

ABCC6 (GenBank nos. U91318 and AF076622) encodes MRP6, a member of the multidrug resistance-associated protein (MRP) family (Borst *et al.*, 1999). The interest in *ABCC6*/MRP6 has been recently heightened by demonstration of mutations in this gene/protein system in families with pseudoxanthoma elasticum (PXE), a multi-system disorder affecting the elastic structures in the skin, the eyes, and the cardiovascular system (Le Saux *et al.*, 2001; Ringpfeil *et al.*, 2001a; Uitto *et al.*, 2001). The clinical manifestations include loose and sagging skin, development of angioid streaks in the eyes, which can lead to loss of visual acuity, and development of early cardiovascular disease (Ringpfeil *et al.*, 2001a; Uitto *et al.*, 2001). As the primary site of *ABCC6* gene expression is the liver, it has been suggested that PXE is primarily a metabolic disorder due to altered function of MRP6 as a transmembrane transporter (Uitto *et al.*, 2001).

The *ABCC6* gene comprises 31 exons spanning ~73 kb of genomic DNA on the short arm of chromosome 16, locus 16p13.1. The transcribed messenger ribonucleic acid is ~6 kb, with a coding sequence of 4.5 kb, leading to translation of a polypeptide with 1503 amino acids. The function of MRP6 is currently unknown; however, it has been proposed to be a transmembrane transporter, as sequence analysis predicts three membrane-spanning domains 1–3 with five, six, and six transmembrane segments, respectively. There are two nucleotide-binding folds 1 and 2 (Uitto *et al.*, 2001), and these domains contain conserved Walker A and B

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Abbreviations: EMSA, electrophoretic mobility shift assay; HEK, human embryonic kidney; MRP, multidrug resistance-associated protein; PXE, pseudoxanthoma elasticum; 5′-RACE, 5′-rapid amplification of cDNA ends; SD, standard deviation; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; WT, wild type

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motifs critical for the adenosine 5′-triphosphate-binding function, while a conserved C motif, located between the A and B sequences, is critical for the function of the protein as a putative transmembrane transporter (Borst *et al.*, 1999).

A surprising finding in the context of the widespread clinical multi-system involvement in PXE was the observation that MRP6 is expressed predominantly in the liver (Belinsky and Kruh, 1999; Kool *et al.*, 1999). The biological function of MRP6 in the liver is currently unknown; however, MRP1, the prototype protein within the MRP family, functions as an efflux pump for amphipathic anionic conjugates (Borst *et al.*, 1999). Similarly, MRP6 has been recently shown to transport small-molecular-weight glutathione *S*-conjugates across the plasma membranes *in vitro*, but the physiological significance of the substrates being used, including leukotriene C_4 and ethylmaleimide S-glutathione, is currently unclear (Belinsky *et al.*, 2002; Iliás *et al.*, 2002).

All mutations in the *ABCC6* gene disclosed in families with PXE thus far are recessive, and most of them are premature termination codon-causing mutations, that is, either nonsense mutations, small insertions or deletions resulting in frameshift, or large deletions (Le Saux *et al.*, 2001; Ringpfeil *et al.*, 2001a, b; Uitto *et al.*, 2001; Miksch *et al.*, 2005). Also, a number of missense mutations affecting critical conserved amino acids within the nucleotide-binding folds have been disclosed as resulting in functional null alleles. Thus, most of the cases with classic PXE result from recessive null mutations in the *ABCC6* gene. However, in a number of patients with PXE, sequencing of the entire coding region and splice junctions failed to disclose pathogenetic mutations, raising the possibility that mutations in the regulatory regions of the *ABCC6* gene may underlie some cases. Furthermore, it has been suggested that some, but not all, heterozygous carriers of the mutations may show signs suggestive of PXE (Bacchelli *et al.*, 1999; Sherer *et al.*, 2001). The latter observations raise the issue of pathological consequences of subtle modulation of the *ABCC6* gene expression.

Little is known about the transcriptional regulation of the human *ABCC6* gene. A recent study (Arányi *et al.*, 2005) has identified a methylation-dependent activator sequence in the 5′ regulatory region of *ABCC6*, but nothing beyond that has been reported. In the present study, we have provided a comprehensive examination of the transcriptional regulation and tissue-specific expression of the human *ABCC6* gene.

RESULTS

High level of expression of the *ABCC6* gene in hepatoma cells

HepG2, a human hepatoma cell line, is expected to express *ABCC6*, since human liver shows a relatively high level of expression of this gene (Belinsky and Kruh, 1999; Kool *et al.*, 1999). We first performed reverse transcriptase-PCR with RNA isolated from HepG2 cells, as well as from a number of other cultured cells of mesenchymal or epithelial origin, including dermal fibroblasts, HaCaT (a transformed epidermal cell line), human embryonic kidney (HEK)293 (a kidney embryonic cell line), HT1080 (a fibrosarcoma cell line), and WISH (an amnionic epithelial cell line) cells. Utilization of

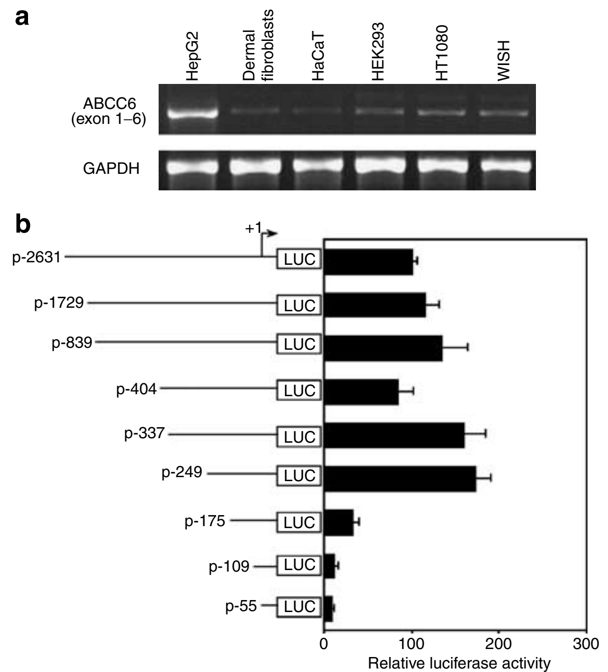


Figure 1. Expression of the human *ABCC6* gene in cells of various tissue origins and 5′ deletion analysis of the promoter in HepG2 cells. (**a**) Reverse transcriptase-PCR was performed using total RNA isolated from cultured HepG2, dermal fibroblast, HaCaT, HEK293, HT1080, and WISH cells using primer pairs specific for the human *ABCC6* and *GAPDH* sequences, as described under Materials and Methods. (**b**) The *ABCC6* promoter fragments correspond to regions with the 5′ ends as indicated in the figure and 3′ ends fixed at +30 were fused to the luciferase (*Luc*) reporter gene in pGL3 vector. Each construct (0.8 μg) was cotransfected with 0.2 μg of pRSV-β-galactosidase plasmid into HepG2 cells using FuGENE6 reagent (Roche), and 48 hours later cells were lysed and assayed for luciferase and β-galactosidase activities. Luciferase activity was divided by β-galactosidase activity to correct for transfection efficiency, and the results were expressed as relative luciferase activity. The data are presented as the mean$\pm$SD of three independent experiments each performed in triplicate. The activity of the p-2631 promoter construct was set arbitrarily as 100.

primers corresponding to exons 1–6 resulted in a strong band of the expected size (~650 base pairs (bp)) in HepG2 cells, and a much weaker band of the same size was noted in the other cell lines tested (Figure 1a).

Identification of a putative liver-specific *cis*-element in the *ABCC6* promoter

To search for regulatory *cis*-elements that might be critical for expression of the *ABCC6* gene in HepG2 cells, we developed a series of 5′ deletion constructs consisting of *ABCC6* promoter linked to luciferase reporter gene. The largest construct had its 5′ end at –2631 (p-2631 construct) upstream from the transcription initiation site (+1; Figure 1b). The transcription initiation site was determined by 5′-rapid amplification of cDNA ends (5′-RACE), which revealed that the transcription site (+1) resides 30 nucleotides upstream from the A in the ATG translation initiation codon of the gene. Transfection of the 5′ deletion constructs into HepG2 cells in culture revealed robust expression with the construct p-2631 as well as with constructs containing deletions down

to −249 (Figure 1b). However, further deletion of the 5′ sequences to −175 resulted in about 80% reduction of the luciferase activity, and further reduction was noted with p-109 and p-55 constructs. These observations suggest that the segment between −249 and −176 contains elements that confer high level of expression of the *ABCC6* gene in HepG2 cells.

To examine whether the sequence −249/−176 is a specific requirement for high level of expression in HepG2 cells, we compared the activity of three constructs (p-2631, p-249, and p-175) in HepG2 cells with that in HEK293 and fibrosarcoma cells (HT1080), which expressed *ABCC6* at a very low level (see Figure 1a). Transfection experiments confirmed that deletion of the −249/−176 sequence from the promoter significantly (>80%) reduced the activity in HepG2 cells, while no statistically significant difference was noted in the two other cell lines tested with the constructs p-249 and p-175 (Figure 2). It should be noted that the basal promoter activity with the p-2631 construct was about 50 times higher in HepG2 cells than in HEK293 and HT1080 cells, the relative luciferase activity being $43.6 \pm 1.5 \times 10^6$ *vs* $0.9 \pm 0.06 \times 10^6$ and $1.0 \pm 0.05 \times 10^6$ in HepG2, HEK293, and HT1080 cells, respectively (mean ± standard deviation (SD)), after correction of the transfection efficiency. Thus, the nucleotide segment −249/−176 of the *ABCC6* confers high level of expression in the hepatoma HepG2 cells.

Examination of the nucleotide sequence within the −249/−176 segment revealed the presence of an NF-κB-like sequence, which differs from the consensus NF-κB-binding site by substitution of T by C in the sixth position of the 10-bp consensus sequence (GGGAMTNYCC; M = A or C; N = any nucleotide; Y = C or T) (see Figure 3). No homology with previously published liver-specific *cis*-elements was noted within this segment (Hayashi *et al.*, 1999).

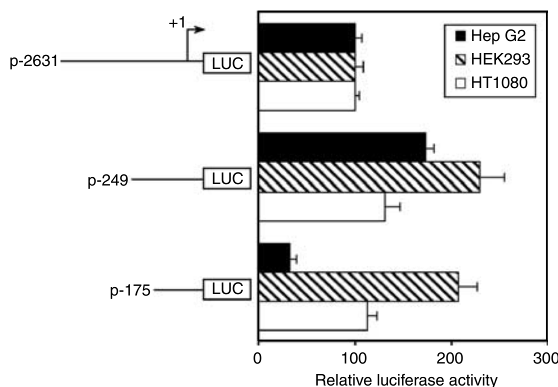


Figure 2. Liver-specific expression of the *ABCC6* promoter construct depends on −249/−176 segment. Three truncated promoter constructs (p-2631, p-249, and p-175) were cotransfected with pRSV-β-galactosidase plasmid into HepG2, HEK293, and HT1080 cells, as described in the legend to Figure 1. At 48 hours after transfection, cells were lysed and assayed for luciferase and β-galactosidase activities, and the results were expressed as relative luciferase activity. The data are presented as the mean ± SD of three independent experiments, each performed in triplicate. The activity of p-2631 promoter construct was set arbitrarily as 100 for each cell type.

To examine for the presence of transacting factors that might bind to the NF-κB-like sequence, an 18-bp probe, −239/−222, was synthesized and used in electrophoretic mobility shift assay (EMSA) with nuclear proteins isolated from HepG2 cells. In addition to nonspecific bands, a radiolabeled DNA/protein complex was noted in electrophoresis (Figure 3). Addition of unlabeled probe −239/−222 as a competitor abolished this binding in 100-fold excess, while the nonspecific complexes were not affected. However, addition of a 22-bp unlabeled oligonucleotide containing the consensus NF-κB-binding site, in 100-fold excess, failed to significantly reduce the specific DNA/protein complex and did not affect the nonspecific binding (Figure 3). Nevertheless, slight reduction in the intensity of the specific complex was noted with 350-fold excess of the NF-κB oligomer (not shown). To examine the sequence requirements

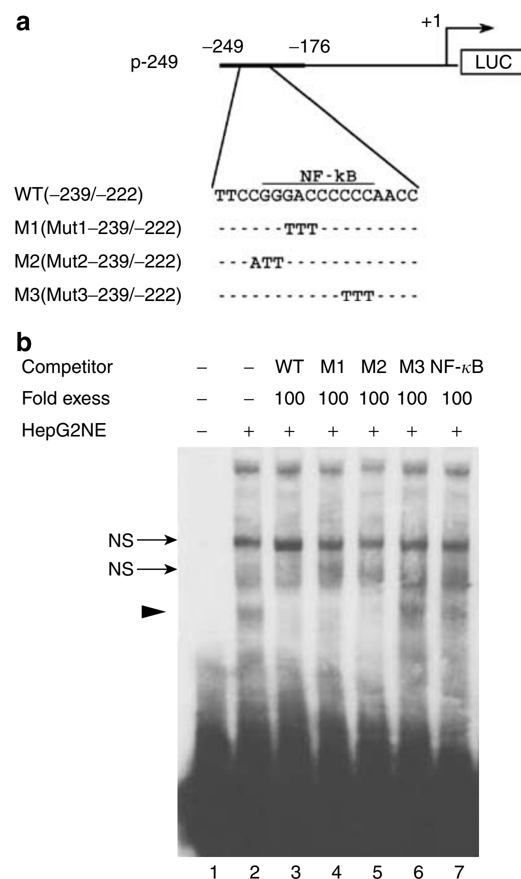


Figure 3. Binding of HepG2 nuclear proteins to the −239/−222 sequence of the human *ABCC6* promoter. (**a**) Sequences for the WT sense strand of −239/−222 oligonucleotide (WT), and the mutant −239/−222 oligonucleotides (M1, M2, and M3), with nucleotide substitutions within the NF-κB-like sequence as delineated by a horizontal bar, are shown. (**b**) WT−239/−222 oligonucleotide was labeled with [γ-^{32}P]dATP and used as a probe in EMSA with nuclear extracts (NE) prepared from HepG2 cells. Competition experiments were performed with 100-fold excess of the unlabeled WT and mutated (M1-M3), as well as consensus NF-κB-binding, oligonucleotides. Specific complexes are indicated by an arrowhead and nonspecific (NS) complexes by arrows. The widened bar at the 5′-end of the p-249 promoter construct indicates the presence of the −249/−176 segment.

for nuclear protein binding within the NF-κB-like sequence in further detail, three mutant oligomers (M1–M3) were synthesized, which harbored 3-bp substitutions affecting different parts of the wild-type (WT) sequence (Figure 3). Using these oligomers as competitors in 100-fold excess revealed that M1 and M2 probes were able to compete for the protein binding to the same extent as the WT probe (Figure 3b). However, mutations in probe M3 abolished its ability to compete for protein binding. Collectively, these findings suggest that the NF-κB-like sequence provides high level of expression in liver cells to the *ABCC6* promoter. It should be noted that two putative CCAAT/enhancer-binding protein cognate sites (Hayashi *et al.*, 1999) were identified at positions −375 to −366 and −336 to −330. However, elimination of these sequences (compare constructs p-404, p-337, and p-249) did not alter the *ABCC6* promoter activity (Figure 1b).

The function of promoter *in vivo*

To further study the liver-specific characteristics of the human *ABCC6* promoter, we examined the activities of the full-length (p-2631) and the shorter (p-249) promoter–luciferase constructs *in vivo*, utilizing a rapid tail vein injection technique (Zhang *et al.*, 1999) (Figure 4). An efficient gene transfer can be achieved in mouse liver by a rapid tail vein injection of a large volume of plasmid DNA solution (hydrodynamics-based transfection). The gene transfer efficiency was confirmed by β-galactosidase staining of liver after transfer of pCMV-LacZ construct into liver with this technique (Figure 4b). Injections of 100 μg *ABCC6* promoter–luciferase construct DNA in 2 ml Ringer's solution were performed on sets of three mice per construct. The results shown in Figure 4a indicate that both promoters are functional *in vivo*, and demonstrate significantly elevated activity compared to the controls injected with 2 ml of Ringer's solution alone. However, the full-length promoter construct (p-2631) yielded significantly higher luciferase expression and was about 13.6-fold higher than the expression of the p-249 construct. *In vitro*, the ratio of expression of these two constructs was ∼0.7. This may reflect the fact that gene regulation *in vivo* is a more complex process than in isolated cells in culture, and more regulatory elements/factors are probably involved in the expression of *ABCC6 in vivo*.

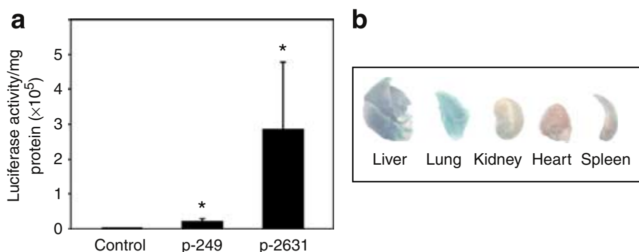


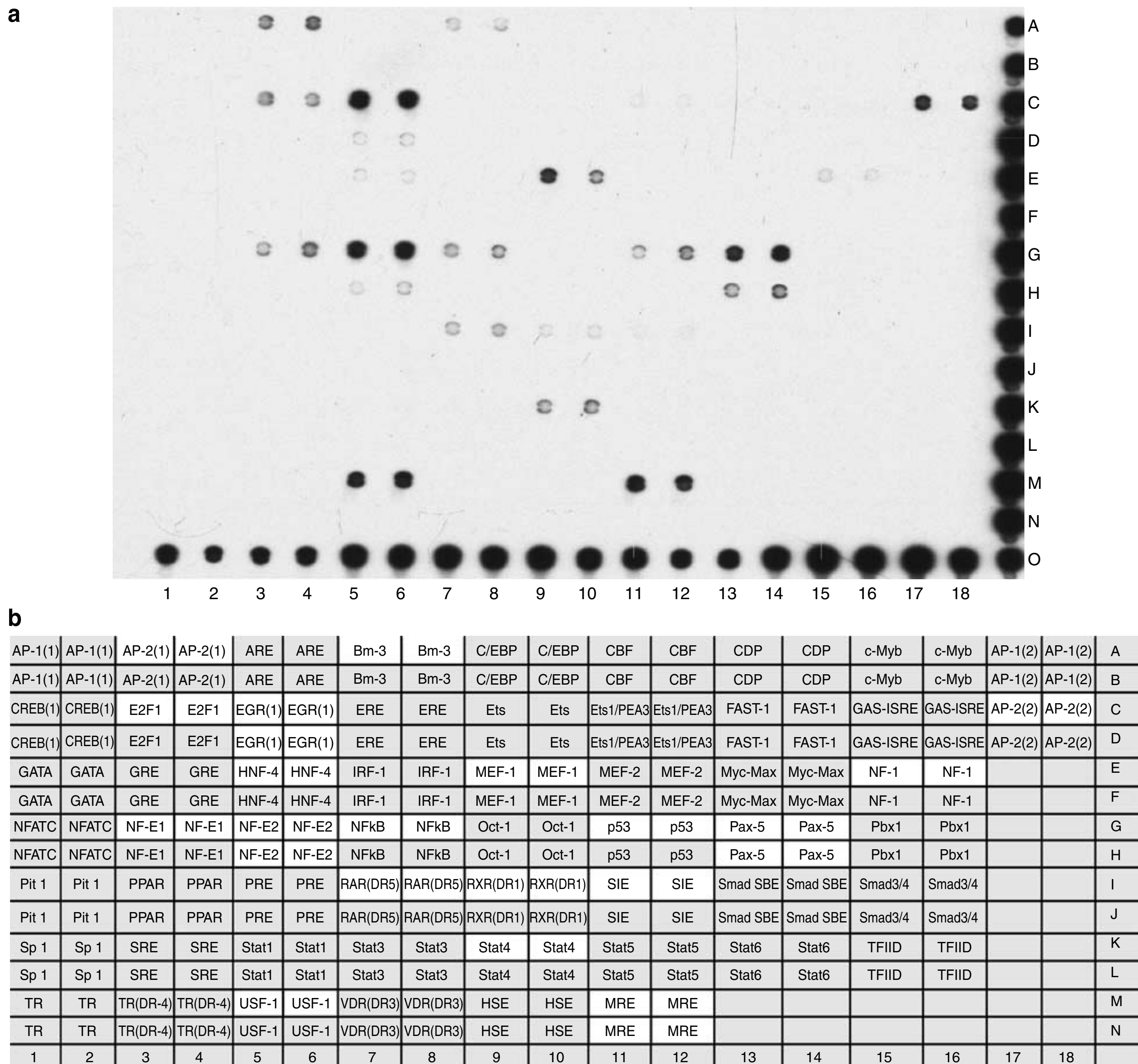
Figure 4. Demonstration that the human *ABCC6* promoter constructs are functional *in vivo*. The promoter–reporter gene constructs were infused into the tail vein of 3.5-month-old mice using a hydrodynamics-based transfection method (Zhang *et al.*, 1999). After 24 hours, the mice were euthanized and examined for the expression of the reporter gene. (**a**) In all, 100 μg of the promoter construct, either p-249 or p-2631, linked to a luciferase reporter gene was injected into the tail vein in 2 ml of Ringer's solution. The relative luciferase activity was determined and normalized to mg of liver protein. The values are mean ± standard error of the mean ($n=3$; each assay performed in triplicate). *$P<0.01$, Student's *t*-test, as compared to controls injected with 2 ml of Ringer's solution only. (**b**) pCMV-lacZ promoter–reporter gene construct (100 μg) was injected to the tail vein of mice as described in (**a**). The efficiency of gene delivery to the liver was confirmed by β-galactosidase staining at 24 hours after injection. As shown, most of the injected DNA was expressed in the liver, while a low level of expression was noted in other organs examined.

Search for binding proteins on the promoter

For a systematic search of the transcription factors that bind to the *ABCC6* transcriptional regulatory region, we used the p-2631 construct consisting of *ABCC6* promoter linked to luciferase reporter gene to isolate a 2661-bp DNA fragment spanning nucleotide −2631 to +30. This fragment was used to isolate nuclear proteins which bind to this region of promoter, and the identities of the transcription factors were determined by a protein/DNA array-based procedure, as described in Materials and Methods. The results, shown in Figure 5, identified a total of 18 transcription factors in the pulled-down samples. However, among them, only four factors were found to have the corresponding consensus-binding sequences within this region of DNA, based on computer searches, *viz.*, activator protein-2, USF-1, NF-κB, and epidermal growth receptor (Figure 9).

Cytokine modulation of *ABCC6* gene expression and the role of Sp1/Sp3 in transforming growth factor-β responsiveness

Although there is no evidence that PXE involves tissue inflammation, PXE-like cutaneous findings have been encountered in a number of metabolic, both acquired and heritable, disorders, some of which involve immunologic and inflammatory aberrations (Ringpfeil and Uitto, 2005). Since inflammatory cytokines play a role in a number of pathological conditions by regulating the expression of genes at the transcriptional level, we examined the prototypic pro-inflammatory cytokines, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, and interferon (IFN-γ), for their effects on the *ABCC6* promoter activity in HepG2 cells transiently transfected with the p-2631 promoter–reporter gene construct. The results indicated that addition of TGF-β (0.1–10 ng/ml) increased the promoter activity in a dose-dependent manner up to about 2.5-fold (Figure 6a). At the same time, addition of TNF-α (0.1–10 ng/ml) or IFN-γ (10–1,000 U/ml) suppressed the promoter activity (Figure 6a). Further analysis utilizing the 5′-deletion constructs revealed that the constructs with their 5′-end either at −337 or at −175 similarly responded to TGF-β (10 ng/ml), depicting over two-fold increase. Careful search for sequence homology for known TGF-β response elements, including the SMAD-binding site (Shi *et al.*, 1998; Zawel *et al.*, 1998), revealed the presence of a CAGA box-like sequence, CAGACAGA, superimposed on the transcription initiation site (−3 to +7) (Figure 7). Furthermore, at the position −58 to −49 upstream from the transcription initiation site, there was a consensus Sp1-binding site (Figure 7). To examine the



AP-1(1)	AP-1(1)	AP-2(1)	AP-2(1)	ARE	ARE	Bm-3	Bm-3	C/EBP	C/EBP	CBF	CBF	CDP	CDP	c-Myb	c-Myb	AP-1(2)	AP-1(2)	A
AP-1(1)	AP-1(1)	AP-2(1)	AP-2(1)	ARE	ARE	Bm-3	Bm-3	C/EBP	C/EBP	CBF	CBF	CDP	CDP	c-Myb	c-Myb	AP-1(2)	AP-1(2)	B
CREB(1)	CREB(1)	E2F1	E2F1	EGR(1)	EGR(1)	ERE	ERE	Ets	Ets	Ets1/PEA3	Ets1/PEA3	FAST-1	FAST-1	GAS-ISRE	GAS-ISRE	AP-2(2)	AP-2(2)	C
CREB(1)	CREB(1)	E2F1	E2F1	EGR(1)	EGR(1)	ERE	ERE	Ets	Ets	Ets1/PEA3	Ets1/PEA3	FAST-1	FAST-1	GAS-ISRE	GAS-ISRE	AP-2(2)	AP-2(2)	D
GATA	GATA	GRE	GRE	HNF-4	HNF-4	IRF-1	IRF-1	MEF-1	MEF-1	MEF-2	MEF-2	Myc-Max	Myc-Max	NF-1	NF-1			E
GATA	GATA	GRE	GRE	HNF-4	HNF-4	IRF-1	IRF-1	MEF-1	MEF-1	MEF-2	MEF-2	Myc-Max	Myc-Max	NF-1	NF-1			F
NFATC	NFATC	NF-E1	NF-E1	NF-E2	NF-E2	NFkB	NFkB	Oct-1	Oct-1	p53	p53	Pax-5	Pax-5	Pbx1	Pbx1			G
NFATC	NFATC	NF-E1	NF-E1	NF-E2	NF-E2	NFkB	NFkB	Oct-1	Oct-1	p53	p53	Pax-5	Pax-5	Pbx1	Pbx1			H
Pit 1	Pit 1	PPAR	PPAR	PRE	PRE	RAR(DR5)	RAR(DR5)	RXR(DR1)	RXR(DR1)	SIE	SIE	Smad SBE	Smad SBE	Smad3/4	Smad3/4			I
Pit 1	Pit 1	PPAR	PPAR	PRE	PRE	RAR(DR5)	RAR(DR5)	RXR(DR1)	RXR(DR1)	SIE	SIE	Smad SBE	Smad SBE	Smad3/4	Smad3/4			J
Sp 1	Sp 1	SRE	SRE	Stat1	Stat1	Stat3	Stat3	Stat4	Stat4	Stat5	Stat5	Stat6	Stat6	TFIID	TFIID			K
Sp 1	Sp 1	SRE	SRE	Stat1	Stat1	Stat3	Stat3	Stat4	Stat4	Stat5	Stat5	Stat6	Stat6	TFIID	TFIID			L
TR	TR	TR(DR-4)	TR(DR-4)	USF-1	USF-1	VDR(DR3)	VDR(DR3)	HSE	HSE	MRE	MRE							M
TR	TR	TR(DR-4)	TR(DR-4)	USF-1	USF-1	VDR(DR3)	VDR(DR3)	HSE	HSE	MRE	MRE							N
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	

Figure 5. Identification of transcription factors binding to human *ABCC6* promoter region by a protein/DNA array. (**a**) Hybridization signals of the transcription factors bound to the 2661-bp promoter fragment identified in nuclear extracts of HepG2 cells. The TransSignal protein/DNA filter contains 54 transcription factors, each tested in duplicate (adjacent horizontal dots) and in two different concentrations (adjacent vertical dots, 1:10 dilution). (**b**) The transcription factors identified by the protein/DNA array as binding to *ABCC6* promoter region are indicated on white background. No signal was noted for those factors shown on the shadowed background.

role of these two *cis*-elements in TGF-β responsiveness, discrete 2- or 4-bp mutations were introduced into these sequences within the promoter–reporter gene construct p-337, and the WT and mutated promoters were transfected to HepG2 cells, followed by incubation with or without TGF-β (10 ng/ml) (Figure 7). In accordance with the findings reported above, the WT p-337 promoter construct responded to TGF-β with about 2.2-fold enhancement of luciferase activity. Introduction of 2-bp mutations in the CAGA box-like sequence somewhat reduced, but did not entirely abolish, the TGF-β responsiveness (Figure 7). However, introduction of mutations to the putative Sp1-binding site entirely abolished the upregulation of the promoter activity by TGF-β. These observations suggest that the consensus Sp1-binding site plays the primary role in TGF-β response within the *ABCC6* promoter.

To examine the role of the Sp1 cognate sequence in further detail, EMSA was performed with a radiolabeled probe extending from −62 to −45 (Figure 8a). Incubation of HepG2 nuclear extracts with the radiolabeled probe revealed a double band as well as a faster moving band, which were shown to be specific DNA/protein complexes (Figure 8b, left arrows). Supershift with antibodies recognizing Sp1 protein epitopes resulted in disappearance of one of these bands,

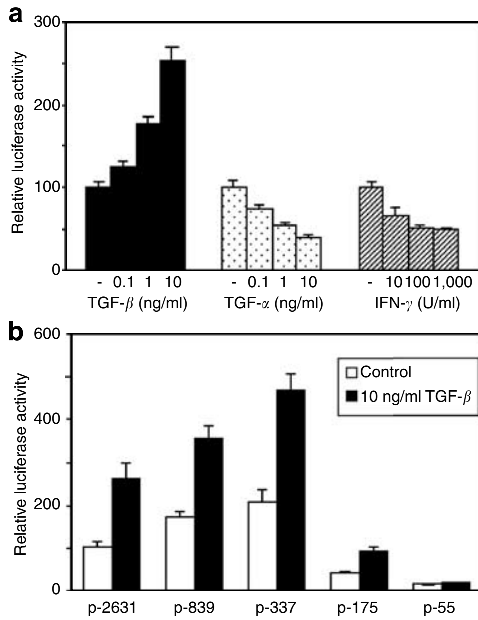


Figure 6. Effects of selected cytokines on the expression of human *ABCC6* promoter in HepG2 cells. (**a**) p-2631 construct (0.8 μg) was cotransfected with pRSV-β-galactosidase plasmids (0.2 μg) into HepG2 cells using FuGENE6 reagent (Roche). At 18 hours after transfection, HepG2 cells were placed in serum-free minimal essential medium for 6 hours, after which TGF-β (■), TNF-α (□), or IFN-γ (▨) was added in the concentrations indicated. After incubation for 24 hours, HepG2 cells were lysed and assayed for luciferase and β-galactosidase activities, and the results, expressed as relative luciferase activity, are presented as mean±SD of three independent experiments, each performed in triplicate. The activity of p-2631 promoter without cytokines was set arbitrarily as 100. (**b**) Promoter constructs p-2631, p-839, p-337, p-175, and p-55 were transfected in HepG2 cells and the cells were then incubated with (■) or without (□) of 10 ng/ml of TGF-β as described. The results, expressed as relative luciferase activity, are presented as the mean±SD of three independent experiments, each performed in triplicate. The activity of p-2631 promoter without TGF-β (control) was set arbitrarily as 100.

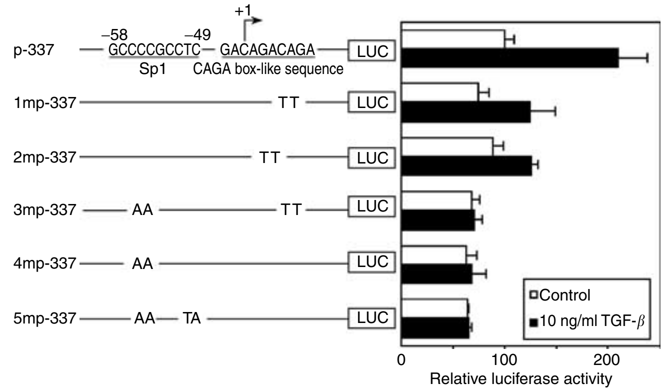


Figure 7. The functional role of Sp1-binding site in TGF-β stimulation of *ABCC6* gene expression. Transfection experiments were carried out using the WT plasmid construct p-337, as well as mutated plasmids (1m to 5m p-337) developed by site-directed mutagenesis with mutant primers, as described in Materials and Methods. The mutated sequences within the Sp1-binding site and the CAGA box-like sequence are indicated in the figure. The cells were incubated with TGF-β (10 ng/ml) as described in the legend to Figure 6. The results are expressed as relative luciferase activity, mean±SD of three independent experiments, each performed in triplicate. The activity of p-337 promoter without TGF-β was set arbitrarily as 100.

Table 1. Cotransfection of the *ABCC6* promoter-luciferase constructs and a human Sp1 expression vector in *Drosophila* SL2 cells

Promoter construct	Luciferase activity (U × 10^4) −Sp1	Luciferase activity (U × 10^4) +Sp1	-Fold stimulation[1]
p-337	1.35±0.022	100.77±20.47	74.6
p-249	2.43±0.044	141.22±15.94	58.1

Construct p-337 or p-249 (0.4 μg) was cotransfected into SL2 cells simultaneously either with 0.4 μg of pPAC vector alone (−Sp1) or the pPACSp1 vector (+Sp1). The *ABCC6* promoter activity was measured and expressed as luciferase units (U), mean±SD (*n*=3).

[1]Fold stimulation is calculated as the ratio of +Sp1/−Sp1 for each promoter.

while two different bands disappeared with the Sp3 antibodies with concomitant formation of supershift complexes (Figure 8b). The same three bands were dissolved by the addition of 50-fold excess of unlabeled −62/−45 oligomer (WT) or addition of an oligomer containing the Sp1 consensus-binding site (Figure 8b). Interestingly, addition of the Mut1−62/−45 probe (M1) containing a 2-bp mutation in Sp1-binding sequence partially competed for the WT −62/−45 probe binding to the nuclear proteins. However, introduction of a 4-bp substitution in the Sp1 sequence (Mut2−62/−45 (M2) probe) abolished the ability to compete for the WT probe to bind to Sp1/Sp3 proteins. Collectively, these observations attest to the role of Sp1/Sp3 transacting factors in regulation of *ABCC6* gene expression and its responsiveness to TGF-β.

Activation of the *ABCC6* promoter in *Drosophila* SL2 cells by the human Sp1 transcription factor

As Sp1 is expressed in virtually all mammalian cells, we utilized *Drosophila* SL2 cells, an established *in vitro* model lacking endogenous SP-1 activity, to determine whether the transcription factor specifically activates the *ABCC6* promoter. This was done by transfecting *ABCC6* promoter constructs p-337 and p-249 to SL2 cells. Very low level of expression was noted when these reporter gene constructs were expressed together with a control pPAC (-Sp1) vector (Table 1). However, cotransfection of the *ABCC6* promoters with a construct expressing Sp1 full-length cDNA under the *Drosophila* actin promoter resulted in enhancement of luciferase activity up to ∼75-fold (Table 1).

DISCUSSION

The *ABCC6* gene encoding MRP6 is expressed primarily in the liver and to a lesser extent in the kidneys (Belinsky and Kruh, 1999; Kool *et al.*, 1999), while very low level of expression has been observed in a number of other tissues (Beck *et al.*, 2003). Immunohistochemical staining with

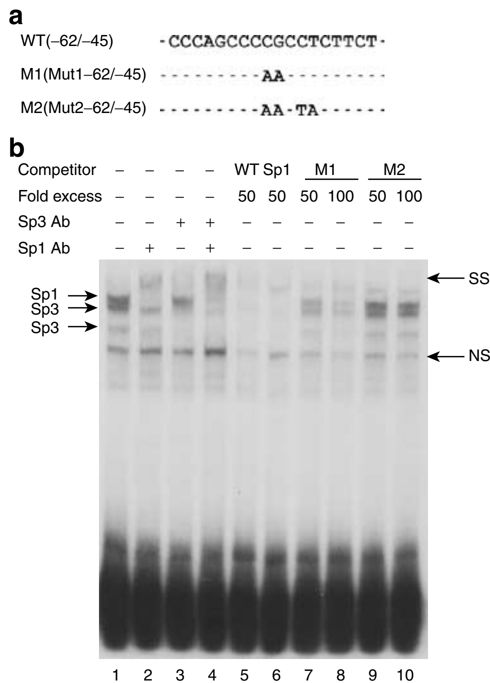


Figure 8. Sp1 and Sp3 proteins bind to the proximal promoter region of the human *ABCC6* gene. (**a**) Sequences of WT−62/−45 and the corresponding mutant (M1, M2) oligonucleotides are shown. (**b**) WT−62/−45 oligonucleotide was labeled with [γ-^{32}P]dATP and used as a probe in EMSA, with nuclear extracts prepared from HepG2 cells (all lanes). Supershift experiments (lanes 2–4) were performed by 1 hour preincubation of the reaction mixture with 2 μg of each antibody prior to the addition of the radiolabeled probe. Competition experiments (lanes 5–10) were performed with 50- or 100-fold excess of the unlabeled WT−62/−45, mutated (M1, M2), or consensus Sp1-binding oligonucleotide. Arrows on the left indicate the positions of specific Sp1 or Sp3 DNA/protein complexes. Arrows on the right refer to supershift (SS) or nonspecific (NS) DNA/protein complexes.

antibodies recognizing MRP6 has located it to the basolateral plasma membrane of hepatocytes (Scheffer *et al.*, 2002), a feature consistent with the presence of three membrane-spanning domains in the protein. Highly sensitive multi-round reverse transcriptase-PCR and RNase protection assay approaches have suggested expression also in other tissues, including skin and vessel wall, albeit at a much lower level (Bergen *et al.*, 2000). Consequently, the gene potentially has transcriptional *cis*-elements that confer high liver-specific expression to this gene. In this study, we first explored liver specificity of the *ABCC6* expression by utilizing HepG2 cells, a hepatoma cell line that has an expression profile characteristic of hepatocytes (Khalil *et al.*, 2001). We developed a number of 5′ deletion promoter–reporter gene constructs and transfected them into HepG2 cells, as well as to a number of other established cell lines of mesenchymal or epithelial origin. Transient transfections revealed significantly, >50-fold, higher expression of the promoter constructs in HepG2 cells as compared with HEK293 cells or HT1080, a fibrosarcoma cell line. Thus, the *ABCC6* promoter apparently contains features that confer high level of expression in the liver.

Initial scanning of the nucleotide sequence information within 2631 bp upstream from the transcription initiation site of the *ABCC6* gene revealed the presence of two sequence motifs with homology to the consensus CCAAT-binding site. The corresponding enhancer-binding protein has been shown to be a liver-enriched transcription factor presumably participating in hepatic differentiation and involved both in determination and maintenance of the hepatic phenotype (Hayashi *et al.*, 1999). Use of 5′-deletion constructs in transient transfection assays revealed, however, that elimination of these CCAAT elements (at −375 to −366 and −336 and −330) from the promoter had no appreciable effect on the promoter activity in HepG2 cells. In contrast, elimination of the promoter segment between −249 and −176 resulted in significant (>80%) reduction in its activity. This finding was similar to that reported by Arányi *et al* (2005), who noted ~50% reduction when the sequence from −332 to −145 was eliminated from their promoter construct. The sequence between −249 and −176 was found to contain an NF-κB-like segment (−235 to −226), which differed from the NF-κB consensus sequence by one nucleotide substitution. This sequence was shown to bind, in a specific manner, nuclear proteins isolated from HepG2 cells, and competition assays suggested the importance of distinct cytosine residues within the 3′-end of this 10-bp sequence. This NF-κB-like sequence represents a novel liver-specific element. It should be noted that careful sequence analysis failed to identify other, previously established sequences for liver-enriched transcription factors, including hepatocyte nuclear factors 1, 3, and 4 (Hayashi *et al.*, 1999). It is conceivable, therefore, that the presence of this novel *cis*-element is responsible for the high level of expression of *ABCC6* in the liver.

Liver-specific expression of p-2631 construct was also alluded to by high level of expression *in vivo* following its injection to the tail vein of normal mice. The rapid injection of the large volume of fluid enables DNA delivery to the liver by causing a transient right-sided congestive heart failure and backpressure to the liver vessels (Zhang *et al.*, 1999). Unexpectedly, the expression of the p-249 construct, which showed somewhat higher level of expression in HepG2 cells in culture, was significantly lower than that of p-2631. These findings may reflect the fact that expression of genes in *in vivo* may be more complex and involves a number of additional factors (Figure 9). Nevertheless, our results indicate that the expression of the p-2631 construct containing human *ABCC6* promoter segment is high both *in vitro* and *in vivo*. In this context, it should be noted that human *ABCC6* has at least two pseudogenes, *ABCC6*-Ψ1 and *ABCC6*-Ψ2, that extend from exon 1 to exon 9 and to exon 4, respectively, and contain 5′-flanking sequences. Although *ABCC6*-Ψ1 is 99.995% homologous with *ABCC6*, there are a number of differences in the 5′-region which allow distinction between the pseudogene and the functional WT gene (Pulkkinen *et al.*, 2001).

We further examined the capacity of the −2631 to +30 promoter region to bind transcription factors using a protein/DNA array. This recently developed technology is a significant improvement over gel mobility-shift assays, and

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          ▼P-2631
      --------GG CGTACAGCCA CTGTGATACT AGGAGTAATA TAGTAGTATC

-2593 TCCCCAGGAT ATAACAAATA ATATCACAAG GTGTACATGC ACTGTGATAT

-2543 TAAAGGAAAT AATATCTCTT CAGGATATAA TGAATAATAT CACAAAGTAT
          HNF-3b                 Oct-1
-2493 ACACCCACTG TGATATTAGG GGTAATACTA TATCTCCAAG ATATAGTGAA
                                                     Pit-1a
-2443 TAATATCACA GGGGGTACAC CCACAGTATA TTAGGGGTAA TAGTATATCC

-2393 CCAGAATATA ACCAATAATA TCACAGCCTG TACACCCACC CTCACAATAT

-2343 GGATAATATC TTCCAGGATG TTACGAATAG TATCAAAGAG TATACACCCA

-2293 CTGTGATATT AGGGGCAATA TCTCCACAGA ATATTACAAA TAATATCAAA
                     GATA
-2243 AGATGTACAC GCACTGTGAC ATTAGGGGTA ATATCACCCA AAAATATTAC
                     CRE-BP
-2193 AAATAATATC ACAGCATGTA CACAATGGTG TACGTTCATT GTGATATTAT

-2143 GATATCCATA GGGTATTACA AATAATAGCA CAGGGTGTAC CCCCACTGTG

-2093 ATATTAGGAG TCATATCTTT CTGGGAGGTC ACAGCGTGTA CACGCATGGT

-2043 GTAAATTCAC TGGGATATTA GGAGTAATAT CGCCCTAGAA TATTTCGAAT

-1993 CATATCACAG GGTGTACACC CACTGTGATA TTAAAAGGAA TATCTTTCTA

-1943 GAACATTACA AATAGTATCA CAAGGTGTAC ACCCACTATG AGATTAGGAG

-1893 TAACATCTCC CTAGAATATT ATGAATAATA TCACAGTGTG TACAGGCACT

-1843 GTGATTTTAG GAGTACTACC TTCTTAAGAT ATTATGAATA ATATCATAGG

-1793 GTATACACCC ACTGTGAAAT TAAAAGCAAT AGCTCCCTAC GATATTACGA
                ▼P-1729
-1743 ATAATATCAC GCAGTGTACA CTCAAGGTGA TATTAGGAGT AGTATCTCCC

-1693 TAGGAAATTA CGAATATTAT CACAGAGTGT ACACCCACTG TGATATTGAA

-1643 AGTATTATCT TTCTAGGATA TTATGAATAA TATTACAGGG TGTACTCCCT

-1593 CTGTGATATT AGGAGTTATA TCTCCCTAGG ATATTACAAA TCATATCACA

-1543 GGGTGTACAC CCACTGTGGT ATTAGGTGTA GTATCTCCCT AGGATATAAC
                AML-1a
-1493 AAATAATATC ACAGGGTATA CACCTACGGT TATATCAGGA GTTATATCTT

-1443 CATAGAATAT TATGAATAAT ATCACAGGGT GTACACCCTC TGTGATATTA

 1393 AAAGTAATAT CTTTCTAGGA TAGTATGAAT AATATCACAG GGTGTACTCT

-1343 CACTGTGATA TTAGCGGTAA TAACTCCCTA GGATATCACG AATTCTATGA

 1293 CAGAGTGTAC ACCCACCATG AGATTTGGAG TAATATATCT CTAGCATATT

-1243 ATGAATAATA TCACAGGTTG TACACCTACT GTGATATTAG GAGTAAAATC

-1193 TCCCTCGGAT ATTACAAATA ATATCACAGT GTGTACTCCC ACTGTGATAT
                           Oct-1
-1143 TAGGAGTAAA ATCTCCTTCA GATATTACGA ATAATATCAC AGGATGTACA
                                                c-Ets
-1093 CCCACTGTCA TATTACCAGT AATATCTCCC TTGGATATTT CTAATAATAC

 1043 CACAGCGTTT ACACACATGG TGTTCACCCA CTGGGATGTT AGGATAATAT

 -993 CTCCCTTGGA TATTACCAAT CGTATCAGAG GCTGTACACA CGTGGTGTTC
                                           USF, Myc-Max
 -943 ACCCAGCGTG ATATTAGGAA TAATATCTCC CTCGGATATT AGGAACAGTC

 -893 TCTCCCTGGG ACTTAGCAGA ACGTAGCCCC CCAGTGTTAA ATAAGCGACC
          ▼P-839
 -843 ACAGCAAGAG GTAGAGGCGC AGGGACTGAA ATGAAGACAA ATATCTGACA
     AML-1a
  793 AGTTTAAAAC ACGTATTTAA AATAGAATTT ATAAAATGCT TAATCTGCCG

 -743 ACTCAGGAGC CCGCGGTGCA GGGTGGGGTG GGAGTTGGCA GGTGACCGCT
                 CACCC-binding factor
 -693 ACACCAGCAG AGTAAGACTG CAGGGCTGCG GCCCTCCCTC CTATGCCCTT

 -643 CTGTTCAGAC ACCCGAGGGC CCCATGTGCA CTCCTGGCAT CATACGACCA

 -593 GAAAACAGGA CCCTAGAGGT TTCTTGAGTT TCTGCTTACC AGGGCGGCTG

 -543 GGTATAGCCC TGCCAGCCCA TTGCATAATC TTCTAAGTTC TCCCCACCAC
                          C/EBP
 -493 CCTCCATTCC AGCAGCGAGG TGCCTGTGAC GCGGTCTCCA TCCTCTCGGC
                                                 ▼P-404
 -443 CTCGACCCGG TGGTCCCCGC GATTGGACGC TTAGGCGGTC ACCAGGCCTC

 -393 CTTGTTACTA ACGTGTGCAC ACCTTTCAGT TCTCTCATCA CGATGAACTC
         ▼P-337
 -343 CTGGAAATTG CTGGGTCCAA AGTGTTTAGG AAGTCTGGAG TGATTCTTGT
        C/EBP                                       ▼P-249
 -293 TGCAGGGGGA AGAGGGAACT ATGGAGGTGT CACTGAACTT TCAGGGGTTC
                                                        ER
 -243 CGGGACCCCC CAACCCGGTG CCCGTCCCAG CTCCCCGAGC GCCTCTCTTC
     STATx,NF-kB           ▼P-175
 -193 CCCCATCCCC CACCTCGCCT GTTTTCACCT CCCGTGGCCT CACTCCCGCC
        GATA                                          ▼P-109
 -143 GCGCAGCTGG ACCTTGCCCG GGGCCTCCCG ATCCCGGAGC TCGAATCCCA
                   c-Myc/Max,AP-2
                                                ▼P-55
  -93 GCCGGACCAG CCCAGCCCGA CCAGCCCAGC CCCGCCTCTT CTCCGGCAGG
                                        Sp1           ⮕+1
  -43 ATCGCGGCCG AGCAGTCTGC CCAGAGACTT AGCGACAGAC AGACGCTGGG

   +8 ACCCACGACG ACAGAAGGCG CCGATGGCCG
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Figure 9. Nucleotide sequence of human *ABCC6* promoter region and positions of selected cognate transcription factor-binding sites. The sequence extending from −2631 to +30 was scanned for transcription factor-binding sites by using ConSite (Softberry, Inc., version 2.2004) combined with TFSEARCH (version 1.3, Kyoto University, Japan). The putative recognition sites for selected transcription factors, including those identified by a protein/DNA array (see Figure 5 and text), are underlined. The translation initiation codon (ATG) is in bold, and the transcription initiation site is referred to by +1. The 5′ ends of deletion constructs developed in this study are indicated by arrowheads.

allows functional analysis of dozens of eukaryotic transcription factors at a time. This approach identified 18 putative transcription factors in the sample bound to DNA, but their counterpart cognate-binding sequences were identified only in case of four of them, activator protein-2, USF-1, NF-κB, and epidermal growth receptor. The functionality of NF-κB was suggested by 5′ deletion analyses as well as by mutation analysis altering the NF-κB binding (see Results), while the putative functions of the other three factors are currently untested. It should be noted that computer searches for 14 transcription factors identified by the protein/DNA array approach did not find the corresponding cognate-binding sequences in DNA. This could be explained in some cases by the possibility that these factors do not directly bind to DNA but form complexes with other factors. The second explanation may reside in the fact that the stringency of the search did not allow recognition of binding sites with $<80\%$ homology with the consensus sequence. Finally, there may be some crosshybridization between the families of transcription factors. For example, while binding signals for PAX-5 and hepatocyte nuclear factor-4 were noted (see Figure 5), no consensus-binding sites for these factors were identified. However, consensus sites for PAX-4 and hepatocyte nuclear factor-3β were identified with partial sequence homologies.

The complexity of the transcriptional regulation of *ABCC6* has also been attested by a recent study identifying a DNA methylation-dependent activator sequence in *ABCC6* (Arányi *et al*., 2005). Specifically, these authors identified both activator and repressor sequences in the proximal promoter region. The most potent activator sequence consisted of conserved elements protected by DNA methylation in nonexpressing cells (Arányi *et al*., 2005). These findings, together with our results, raise the possibility that mutations in the regulatory regions of the *ABCC6* gene may underlie some cases of PXE. However, besides large genomic deletions affecting the promoter region, no regulatory mutations have been disclosed in the *ABCC6* gene (Miksch *et al*., 2005; our unpublished results).

Another novel observation derived from the present study is that cytokines, including TGF-β, TNF-α, and IFN-γ, are able to modulate the *ABCC6* promoter activity. In particular, TGF-β, a profibrotic cytokine in the liver (Bissell *et al*., 2001), significantly upregulated the *ABCC6* promoter activity. Scanning of the promoter sequence for putative TGF-β response elements identified a CAGA box-like sequence (GACAGACAGA) overlapping the transcription initiation site (−3 to +7). This segment has similarity to the consensus motifs for Smad binding (GTCTAGAC, so-called Smad-binding element, and AG(C/A)CAGACAC, so-called CAGA box). Both sequences contain the core motive AGAC, which represents the optimal binding sequence for Smad3 and Smad4 (Shi *et al*., 1998; Zawel *et al*., 1998), a sequence also present in the CAGA box-like sequence in the *ABCC6* promoter. However, mutation of the CAGA box-like sequence by 2-bp substitutions (see Figure 7) failed to abolish the TGF-β responsiveness of the promoter. The inability of the CAGA box-like sequence to serve as a functional TGF-β

response element through Smad binding may relate to its position within the gene.

In contrast to the CAGA box-like sequence, an upstream Sp1-binding site at –58 to –49 was shown to be critical for TGF-β response within the *ABCC6* promoter. Specifically, 2- or 4-bp substitutions within the Sp1 consensus sequence entirely abolished the responsiveness to TGF-β. EMSA with supershift using specific antibodies identified Sp1 and Sp3 as nuclear proteins specifically binding to the Sp1 cognate sequence in the *ABCC6* gene promoter. Previous studies have suggested that Sp1 binding is necessary for TGF-β1-induced expression by a number of genes, as exemplified by proα2(I) collagen and β5 integrin (Lai *et al.*, 2000; Poncelet and Schnaper, 2001), and it has been suggested that gene activation in these cases may involve cooperation between Smad3 and Sp1. However, Sp1-binding site has been shown to function as a distinct TGF-β responsive element for promoter expression and Sp1 by itself can mediate this response (Li *et al.*, 1998). In case of *ABCC6* promoter, site-directed mutagenesis of the Sp1 site alone was able to abrogate the TGF-β responsiveness, suggesting a critical role for this *cis*-element in regulation of the corresponding gene expression. The expression of the *ABCC6* promoter was also shown to be dependent on Sp1 protein by the use of *Drosophila* SL2 cells. This is an established *in vitro* model to study the role of Sp1, since these cells are devoid of endogenous Sp1, while all mammalian cells contain this protein (Courey and Tjian, 1988). Specifically, transfection of two promoter–luciferase constructs (p-337 and p-249), both of which contain the Sp1 sequence at −58 to −49, to SL2 cells resulted in low level of expression. However, cotransfection with a construct expressing Sp1 under the *Drosophila* actin gene promoter resulted in up to 75-fold enhancement of the *ABCC6* promoter activity. These observations clearly attest to the importance of Sp1 in regulation of the *ABCC6* promoter activity and its responsiveness to TGF-β.

MATERIALS AND METHODS

Cell culture

All mammalian cell lines and human dermal fibroblasts, obtained from neonatal foreskin, were cultured in medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Cellgro, Mediatech, Inc., Herndon, VA). In case of human HepG2 hepatoma cells, the medium was minimal essential medium (Cellgro), while human dermal fibroblasts, HaCaT-transformed epidermal cells, HEK293 cells, HT1080 fibrosarcoma cells, and WISH amniotic cells were cultured in Dulbecco's modified Eagle's medium (Cellgro). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Drosophila SL2 cells (kindly provided by Dr James Jaynes, Thomas Jefferson University, Philadelphia, PA) were grown at room temperature in *Drosophila* Schneider cell medium (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum.

The experiments were approved by the Institutional Review Board, at Thomas Jefferson University, and they adhere to the Declaration of Helsinki Principles.

Reverse transcription-PCR

Total RNA was isolated from cultured cells using TRIzol reagent, as recommended by the manufacturer (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed using 1 μg total RNA, oligo (dT) primer (Promega, Madison, WI), and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The following primers were used: 5′-ATGGCCGCGCCTGCT GAGCCCTGC-3′ (sense) and 5′-CCAGTCTCTGGACAGGGGTTA GACTGC-3′ (antisense) for *ABCC6*; 5′-GGTGAAGGTCGGAGT CAACGGA-3′ (sense) and 5′-AGGTCCACCACCCTGTTGCTGT-3′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase as an internal control. A 50 μl PCR reaction mixture consisted of 1 × PCR buffer, 1 × Q-buffer, 2.5 U Taq polymerase (Qiagen, Valencia, CA), 200 μM nucleotide mix, 15 pmol each primer, and 1 μl of reaction mixture containing first-strand cDNA. The amplification conditions were 94°C for 5 minutes, followed by 38 cycles of 94°C for 45 seconds, 68°C for 45 seconds, and 72°C for 1 minute, and one cycle of 72°C for 10 minutes. PCR products were separated by gel electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

5′-rapid amplification of cDNA ends analysis

5′-rapid amplification of cDNA ends (RACE) analysis was performed with SMART RACE cDNA amplification kit (Clontech Laboratories, Inc., Palo Alto, CA) using 1 μg total RNA isolated from the human HepG2 hepatoma cells. First-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen), SMART II oligonucleotide, and 5′-RACE cDNA synthesis primer, according to the manufacturer's instructions (Clontech Laboratories, Inc.). 5′-RACE PCR was performed using a universal primer contained in the kit, *ABCC6* gene-specific primer (MRP6-1: 5′-CCAGTCTCTGGACAGG GGTTAGACTGC-3′) located in exon 5, and Advantage 2 polymerase. The amplified products were diluted 50-fold, and a volume of 1 μl was used in nested 5′-RACE with nested universal primer and the second *ABCC6* gene-specific primer (MRP6-2: 5′-GGAACACTGCG AAGCTCATCGTGG-3′) located at the exon 3–4 border, according to the manufacturer's instructions (Clontech Laboratories, Inc.). The products were cloned into the pCR4-TOPO vector as recommended by the manufacturer (Invitrogen). Recombinant plasmids were purified with Miniprep Kit (Qiagen) and subjected to nucleotide sequence analysis using ABI 377 DNA sequencer.

Promoter plasmid constructs

A 2661-bp fragment spanning from −2631 to +30 of the promoter region of the human *ABCC6* gene was prepared by PCR amplification of the human genomic DNA using a sense primer (−2631F: 5′-GTGGTACCAAGGCGTACAGCCACTGTGA-3′) containing a *Kpn*I restriction site and an antisense primer (+30R: 5′-TACTCGAGTTC TGTCGTCGTGGGTCCCAGCGT-3′) containing an *Xho*I restriction site. The PCR products were separated by agarose gel electrophoresis and extracted from a gel slice (Qiagen). The purified fragment was digested with *Kpn*I and *Xho*I, and cloned into pGL3 basic luciferase vector (Promega) between *Kpn*I and *Xho*I sites to generate the p-2631 construct. Additional reporter gene constructs containing sequentially truncated fragments from the 5′-end of p-2631, spanning from −1729, −839, −404, −337, −249, −175, −109, and −55 to +30 of the *ABCC6* promoter region were similarly prepared using sense primers containing a *Kpn*I restriction site and the antisense

primer +30R. Mutagenesis of the Sp1-binding site and CAGA box-like sequence in p-337 reporter construct, spanning from −337 to +30, was performed by site-directed mutagenesis, as described by Ho *et al*. (1989) using a sense primer (−337F: 5′-GCGGTACCTGGA AATTGCTGGGTCCA-3′), antisense primers (+30R-1mCAGA: 5′-TA CTCGAGTTCTGTCGTCGTGGGTCCCAGCGTC**AA**TCTG-3′, +30R-2m CAGA: 5′-TACTCGAGTTCTGTCGTCGTGGGTCCCAGCGTCTG**AA** TG-3′) containing an *Xho*I restriction site, and the mutant oligonucleotides (mutated nucleotides are underlined in bold). The final constructs were sequenced in both directions to ensure correct nucleotide sequence. The sequence of the insert in p-2631 construct confirmed its fidelity with human *ABCC6* database sequence (Figure 9). Computer analysis of the promoter region of *ABCC6* was conducted to detect putative *cis*-acting elements using transcription factor databases (TFSEARCH, Kyoto University, version 1.3) and ConSite (nsiteM, Softberry, Inc., version 2.2004; www.phylofoot.org).

Transient transfections and luciferase assay

Plasmid constructs used for transient transfections were prepared using a purification kit (Qiagen). HepG2, HEK293, and HT1080 cells were plated on 35-mm dishes 24 hours prior to transfection and grown to approximately 80% confluency. The cells were transfected with 0.8 μg of experimental plasmid and 0.2 μg of pRSV-β-galactosidase plasmid as an internal control of transfection efficiency, using FuGENE 6 transfection reagent according to the manufacturer's instructions (Roche Diagnostic Co., Indianapolis, IN). For Sp1 cotransfections in Schneider *Drosophila* SL2 cells, the same transfection methods were used, except that 0.4 μg of the *ABCC6* promoter–luciferase constructs were cotransfected with 0.4 μg of pPACSp1 (Sp1 expression vector) or pPAC (empty vector as control), which were a generous gift from Dr Robert Tjian, University of California, Berkeley. In addition, in each experiment, 0.2 μg of pHSPLacZ was used as an internal control of transfection efficiency (Kadonaga *et al*., 1987).

For experiments with cytokines, HepG2 cells were washed twice with sterile phosphate-buffered saline 18 hours after transfection and then incubated in serum-free minimal essential medium for 6 hours prior to addition of the cytokines for 24 hours. Incubations were performed with human TGF-β (R&D systems, Minneapolis, MN), human TNF-α (R&D systems), and human interferon-γ (IFN-γ, Roche Diagnostic Co.).

The transfected cells were harvested in reporter lysis buffer (Promega) and used to measure the luciferase activity with the Luciferase Assay Reagent (Promega) using Lumat LB 9507 luminometer (Berthold, Wildbad, Germany). The β-galactosidase activity was determined according to standard protocols (Sambrook *et al*., 1989), and luciferase activity (arbitrary units) was divided by β-galactosidase activity in the same sample (densitometric units at 420 nm) to correct for transfection efficiency and expressed as relative luciferase activity. Luciferase assays were carried out in triplicate, and each experiment of transfection was repeated at least three times. Data shown in the figures represent the mean ± SD of three independent experiments.

To investigate the activity of *ABCC6* promoter–luciferase reporter gene construct *in vivo*, ~3.5-month-old male FVB/N mice were used for tail vein injections with the constructs. A CMV–lacZ construct was injected as a positive control to check gene transfer efficiency to different tissues, and Ringer's solution was injected as a negative control. The injections were peformed using a 27-gauge needle and 100 μg of each DNA construct in 2 ml of Ringer's solution within 7 seconds. Several tissues, including the entire liver, were collected from each injected mouse at day 2 postinjection for luciferase assay or for β-galactosidase staining according to standard protocols (Manthorpe *et al*., 1993; Zhang *et al*., 1999). The luciferase activity (arbitrary units) was normalized to per mg of liver extract protein and expressed as relative luciferase activity. Each assay was performed in triplicate.

EMSA

Nuclear extracts were prepared from HepG2 cells according to an established protocol (Andrews and Faller, 1991) and stored at −80°C until use. The protein concentration in the extracts was determined by a commercial assay kit (Bio-Rad Laboratories, Hercules, CA). Double-stranded oligonucleotides (−239/−222 and −62/−45) were end-labeled with [γ-^{32}P]dATP by T4 polynucleotide kinase (Promega). In all, 6 μg of the nuclear extract was incubated in the binding buffer (10 mM Hepes (pH 7.6), 4% glycerol, 1% Ficoll, 25 mM KCl, 1 mM dithiothreitol, 0.5 mM EDTA, and 25 mM NaCl) containing 1 μg poly(dI-dC) (Roche Diagnostic Co.) and 20 μg bovine serum albumin on ice for 10 minutes. End-labeled oligonucleotides, 60,000 c.p.m., were added to the mixture and incubated at room temperature for 20 minutes. For competition experiments, 50- or 100-fold molar excess of unlabeled oligonucleotides were added to the binding reaction mixture 10 minutes prior to the addition of the labeled probe. Oligonucleotides containing Sp1 or NF-κB consensus-binding site were purchased from Promega. For supershift experiments, 2 μg of antibodies against Sp1 or Sp3 (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with the binding reaction mixture on ice for 1 hour before the labeled probe was added. The DNA–protein complexes were separated by electrophoresis on 4% polyacrylamide gel in 0.5 × TBE at 200 V for 2 hours at 4°C, fixed for 30 minutes in 30% methanol and 10% acetic acid, vacuum-dried, and autoradiographed.

Protein/DNA array

A 2661-bp fragment of *ABCC6* promoter region, extending from −2631 to +30, was excised from p-2631 construct with restriction enzymes *Kpn*I and *Xho*I, and labeled with biotin using Bio-16-dUTP (Roche, Mannheim, Germany) and the Klenow fragment of DNA polymerase I (Invitrogen). Unincorporated Bio-16-dUTP was removed by a spin column and the biotin-labeled DNA fragment was coupled to the M-280 streptavidin magnetic beads (Dynal Biotech, Oslo, Norway) under the conditions suggested by the manufacturer. The DNA-coupled magnetic beads were incubated with 500 μg protein in HepG2 nuclear extracts for 2 hours at 4°C in the binding buffer (4% Ficoll, 20 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl, 0.05% Triton X-100, 10% glycerol) with an excess amount of dC-dI for competition of nonspecific binding. After several washes, the bound proteins were dissociated from the DNA-coupled beads by incubation in a buffer containing 2 M NaCl, for 60 minutes on ice.

The bound proteins extracted from the 2661-bp promoter region fragment were incubated with the TranSignal (Panomics, Redwood, CA) probe mix, a set of 54 biotin-labeled DNA-binding oligonucleotides corresponding to the consensus sequences for the correspond-

ing transcription factors, respectively, to allow the formation of DNA/protein complexes. The transcription factor-bound probes were isolated and then dissociated from the DNA/protein complexes, and used to hybridize to the TranSignal Array that had been spotted with complementary consensus-binding sequences of the transcription factor probes, at 42°C for 8 hours. Hybridization signals were visible after exposure to X-ray film following chemiluminescent detection. Array hybridization was repeated using the nuclear extracts prepared separately and the same results were obtained.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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