IDENTIFICATION OF A DNA METHYLATION-DEPENDENT ACTIVATOR SEQUENCE IN THE PSEUDOXANTHOMA ELASTICUM GENE, ABCC6

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Running title: Transcriptional regulation of human ABCC6

ABCC6 encodes MRP6, a member of the ABC-protein family with unknown physiological role. The human ABCC6 and its two pseudogenes share 99% identical DNA sequence. Loss-of-function mutations of ABCC6 are associated with the development of pseudoxanthoma elasticum (PXE), a recessive hereditary disorder affecting the elastic tissues. Various disease-causing mutations were found in the coding region, however the mutation detection rate in the ABCC6 coding region of bona fide PXE patients is only approximately 80%. This suggests that polymorphisms or mutations in the regulatory regions may contribute to the development of the disease. Here, we report the first characterization of the ABCC6 gene promoter. Phylogenetic in silico analysis of the 5’ regulatory regions revealed the presence of two evolutionarily conserved sequence elements embedded in CpG islands. The study of DNA methylation of ABCC6 and the pseudogenes identified a correlation between the methylation of the CpG island in the proximal promoter and the ABCC6 expression level in cell lines. Both activator and repressor sequences were uncovered in the proximal promoter by reporter gene assays. The most potent activator sequence was one of the conserved elements protected by DNA methylation on the endogenous gene in non-expressing cells. Finally, in vitro methylation of this sequence inhibits the transcriptional activity of the luciferase promoter constructs. Altogether these results identify a DNA methylation-dependent activator sequence in the ABCC6 promoter.

Pseudoxanthoma elasticum (PXE, OMIM 264800 and OMIM 177850) is a rare heritable disorder, which affects the skin, the eye, the cardiovascular system and the gastrointestinal tract (1,2). Patients most commonly present dermal papules and/or angioid streaks of the retina detected during routine eye examination. Angioid streaks are marks of subretinal neovascularization, which is often accompanied by the loss of central vision, the most severe generally occurring symptom of PXE. Rarely, patients may suffer from other symptoms of vascular origin: gastrointestinal bleeding (3,4) or intermittent claudication (5). The observed dermal, vascular and ocular symptoms are accompanied by the accumulation of morphologically abnormal and mineralized elastic fibers in these tissues (6,7). It has been demonstrated that mutations in the ABCC6 gene (called hereafter ABCC6-PXE) are responsible for the development of PXE (8-11).

ABCC6-PXE is coding for MRP6, an ATP-Binding Cassette (ABC) transporter protein (12). The ABC-protein family consists of 48 members in the human genome (13). Most of the characterized
members of the family are involved in transmembrane transport; binding and hydrolysis of ATP are crucial for their transport-related function (13). We have previously demonstrated that MRP6 – similarly to other well characterized ABCC subfamily members – is capable of transporting glutathione conjugates (14). We have found that in the case of one set of missense mutations the loss of the ATP-dependent transport function may be responsible for the PXE phenotype (14). However, neither the physiological substrate of MRP6, nor the functional relationship between altered ABCC6-PXE gene products and the PXE phenotype is known.

Highest level of ABCC6-PXE transcript was found in the liver (12,15,16), which led to a hypothesis that PXE is a metabolic disorder (17). On the other hand, the widespread distribution of MRP6 in normal mouse tissues suggests that the protein may have multiple functions (16). Interestingly, the frequency of the most common PXE mutation (R1114X) was found to be 0.8% in the normal Caucasian population, while 3.2% in coronary artery disease patients (18). This significant difference makes the ABCC6-PXE gene a potentially important risk factor of atherosclerosis and indicates that it may play a crucial, but so far not understood, role in lipid metabolism. In this line, association of the R1268Q allele with variations of plasma triglyceride and HDL cholesterol level has been observed (19).

The ABCC6-PXE gene has been mapped to the 16p13.11 chromosomal domain and was found to be in a close proximity with tail-to-tail orientation to the ABCC1 gene (12). There are two pseudogenes (ABCC6psi-1 and ABCC6psi-2) also on the short arm of the Chr16 (20). To date 69 PXE-associated mutations have been identified in the human ABCC6-PXE gene (21-25). This high heterogeneity of PXE alleles in the population is comparable to that of other autosomal diseases. However, in patients with bona fide PXE the mutations are often undetectable in the coding region on one of the alleles, although the other allele harbors a recessive mutation (22,23). Moreover, in addition to the PXE developed due to mutations of the ABCC6-PXE gene, secondary PXE was also described in patients with chronic hemolytic disorder without mutations of the ABCC6-PXE gene (26).

These data suggest that polymorphisms and mutations of the transcriptional regulatory region of the gene may contribute to the development of PXE. Intriguingly, nothing is known about the transcriptional regulation of the ABCC6 gene. Here we report the first study on the promoter region of the ABCC6-PXE gene and the two ABCC6 pseudogenes. We identified two highly conserved potential regulatory regions in the 5' upstream 10 kb sequence of ABCC6 and found that the proximal one is under epigenetic control. Using DNA methylation analysis as a tool to identify important transcriptional regulatory sequences, we could locate positive and negative regulatory elements in the proximal region. Moreover, our functional data indicate which short regulatory elements can be the targets of epigenetic control of the ABCC6 gene.

Materials and Methods

Materials were purchased from Sigma-Aldrich (Budapest, Hungary) if not indicated in the text.

Cell culture – HepG2/TS and HepG2/F12 cells are HepG2 cells from two different laboratories. The TS subline was a generous gift from Dr Sára Tóth (Budapest, Hungary). These and the HL60 (acquired from ATCC) cells were maintained in RPMI medium. The F12 subline was a generous gift from Dr Ilona Koválszky (Budapest, Hungary). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) enriched with F12 (DMEM/F12). The HEK293 (acquired from ATCC) cells were maintained in DMEM. All the media were supplemented with 10% fetal calf serum, glutamine and antibiotics.

Bisulfite treatment – After genomic DNA extraction bisulfite treatment was performed according to the standard protocol (27,28). Briefly, DNA was denatured by 0.3 M NaOH treatment. Bisulfite solution was added to the samples before overnight incubation at 55°C. After desalting, the treatment was completed by desulphonation and neutralization. Samples were ethanol precipitated and used for PCR amplification.

PCR, cloning and sequencing of bisulfite-treated DNA – Fragments B1 (223 bp) and B2 (200 bp) were PCR amplified by using the following primers:

B1: -9186bs
TTTTTAGGTYGGTTGATAGTTTAGGTT and
-8963as
TTAAAACCTTCCCTATAAACACATACACCTT
B2: -391bs
TTTTATTAGATGAATTTTTGGAAATTGTT and
-171bas
TCRAAAAAACCCRAACAAATCCACACT

The primers were designed to amplify all three ABCC6 sequences with the same efficiency and to allow the identification of the genes by SNPs. Y stands for C or T and R stands for G or A (note that a methylable CpG cytosine is located at these positions in the original sequence). Degenerate primers were designed to amplify both the methylated and unmethylated sequences. (However, CpGs in the primers were not included in the analysis of DNA methylation.) The primer pairs were tested by the BiSearch web server for non-specific PCR amplification (29).

The AmpliTaqGold amplification system (Applied Biosystems) was used for the PCR of bisulfite treated samples. An initial 10 min long denaturation at 95°C was followed by 30 cycles with denaturation at 95°C for 30 sec, annealing for 30 sec at 56°C and 53°C (for B1 and B2, respectively) and elongation at 72°C for 90 sec. After A-tailing reaction (carried out according to the manufacturer’s instructions), the PCR products were subcloned into pGEM-Teasy vector (Promega) by T/A cloning and sequenced.

PCR amplification of ABCC6 transcripts – Total RNA was extracted by the TRIpur extraction kit from human cell lines. The following primers (30) were used to amplify and quantify the ABCC6-PXE mRNA by the LightCycler RNA Master SYBR Green I (One-step RT-PCR (Roche Diagnostics) kit: RT3062s GGCCCGGGCATCCAGGTT and RT3492as TTTCATCTACGGAGCATTTTCT
Cycling and reaction conditions were set up according to the supplier. Briefly, 150 ng total RNA per sample was amplified in a Light Cycler. Reverse transcription at 61°C during 20 minutes and denaturation at 95°C for 30 sec was followed by 43 cycles of 95°C 1 sec, 60°C 5 sec and 72°C and a single quantification step after each cycle at 80°C was included.

The expression of all three genes was analyzed in HepG2/F12 cells. Reverse transcription was performed with random hexamers on 1 µg of RNA using the MMLV-RT enzyme (Promega). The ABCC6 cDNAs were amplified at the annealing temperature of 60°C using AmpliTaqGold polymerase (Applied Biosystems). The following non-selective primers were used:
RTs144 TCCCATCTACCTCTCTTCATC
RTas340 AGCAGAGAGCCGTAACAC

Rapid Amplification of cDNA Ends – The determination of transcription initiation site was carried out according to (31). Briefly, total cDNA was synthesized as described in the previous section with the specific RTas340 AGCAGAGAGCCGTAACAC primer instead of random hexamers.

The cDNA was subjected to TdT (Promega) treatment to create a polyA-tail on the 3’ ends. Then the cDNA was amplified by Taq Polymerase (Promega) with nested primers: Adapter-oligorT
GAGACTGACATCGAT(17) and RTas361 GGAACACTCGCGCTCAGTTCAGTA in the first round and the same adapter-oligorT primer with RTas269 GCGACAGCCAGCAGGTACACA in the second round. Cycling conditions were as follows for both PCRs: 95°C for 30 sec and 72°C for 90 sec for 30 cycles. The PCR products were subcloned into pGEM-Teasy vector (Promega) by T/A cloning and sequenced.

Luciferase assays – Reporter plasmid vectors containing the ABCC6-PXE promoter sequences were constructed by PCR cloning. Human genomic DNA was used as template for amplification. The following sequence-specific primers were designed using Primer3 software (Biology WorkBench, workbench.sdc.edu):

downstream primer GAAAGAAGAGTGGG (position +72 relative to the translation start site) and upstream primers AGCGACACACGCAAGAG (−718), GATCTTGTTGACAGGG (−332) and CGATCCCAGCTGCAAT (−145). Restriction sites (KpnI site for upstream primers and HindIII site for downstream primer) were added to the 5’ end of primers and promoter sequences were amplified using high-fidelity thermostable DNA polymerase (Taq-Pfu mixture OptiTaq from EURx, Gdansk, Poland). PCR products were cloned into the pGL3-Basic vector (Promega, Madison, USA) to construct plasmids phABCC6(-718/+72)Luc, phABCC6(-332/+72)Luc and phABCC6(-145/+72)Luc, respectively. The
sequence of the cloned inserts was tested for the amplification of the ABCC6-PXE gene and the lack of errors by automated sequencing.

The plasmids were transfected by polyethyleneimine transfection reagent (Exgen500 from Fermentas, Vilnius, Lithuania). Cells were harvested and lysed 48 h after transfection. The activity of luciferase in cell lysate was determined in a Fluoroskan Ascent FL luminometric plate reader (Labsystems, Vantaa, Finland) using a commercial luciferase substrate (BD Biosciences, San Diego, USA). Results were normalised for transfection efficiency by cotransfection with a control reporter vector encoding secreted alkaline phosphatase under the control of a constitutive CMV-derived promoter (kind gift from Dr. S. Schlatter, Zurich). The level of alkaline phosphatase activity was determined spectrophotometrically.

For in vitro methylation of promoter sequences, the relevant sequence regions (corresponding to stretches -332/+72 and -145/+72 in the gene) have been excised from plasmids phABCC6(-332/+72)Luc and phABCC6(-145/+72)Luc using KpnI and HindIII restriction enzymes. The excised DNA molecules were gel-purified and subjected to in vitro methylation using SssI DNA methyltransferase (New England Biolabs, Beverly, USA) according to the manufacturer's protocol. Full methylation was confirmed by the resistance to HpaII and HinI digestion. The methylated promoter fragments were ligated back into the pGL3-Basic vector, the constructs were transfected into cells and luciferase activity was measured as described above.

Bioinformatics – For the in silico sequence analysis the following web servers were used: www.ensembl.org (32); www.ncbi.nlm.nih.gov/BLAST/ (33); Promoter 2.0 (34); Eponine (www.sanger.ac.uk/Users/td2/eponine (35); Consite (www.phylofoot.org) (36); Dialign bibiserv.techfac.uni-bielefeld.de/dialign (37); TESS (www.cbil.upenn.edu/tess (38)); Transfac (www.gene-regulation.com) (39).

RESULTS

In silico analysis
the translation start site of the human ABCC6 gene(s). R2 probably constitutes the proximal promoter: the most conserved segment is between -171 and -40 bp but the whole region is more extended with several dispersed stretches of highly conserved elements with a background of less conserved sequences and some inserted boxes specific to the human promoter. The R2 region ends at -400 bp, but two more stretches of highly conserved sequences were also found between -593 and -559 and between -730 and -683. The more upstream region of the human sequence considerably diverges from the rodent promoters. Both exon 1 and 2 were highly conserved, but only two stretches were conserved in the first intron. Interestingly, although both R1 and R2 regions were scanned for transcription factor binding sites by using various web server predictions (Consite, TESS, TRANSFAC (36,39)) combined with our phylogenetic data, but no clear-cut conserved response elements could be detected.

Identification of the transcription initiation site

The transcription initiation sites of the ABCC6 genes were also predicted by several web-based search engines (Promoter 2.0, Eponin (34,35)). Once again, no one (or more) clear-cut transcription initiation site(s) were proposed. Therefore, we determined the transcription initiation site experimentally. Total cellular RNA from HepG2/F12 cells was reverse-transcribed with a primer specific for the ABCC6 genes. Then a polyA tail was added at the 3' end of the cDNA, which served later as a sequence for primer annealing during subsequent semi-nested PCR experiments. These PCR products were subcloned and sequenced. The transcription initiation site was found to be located at -37 bp relative to the translation initiation site. Interestingly, none of the search engines predicted this position. Interestingly, this site aligns within 1 bp of the transcriptional start site of the ABCC1 gene, suggesting a similar mechanism of action of the basal transcriptional machinery (43).

Expression of the ABCC6 genes

Next, we investigated the ABCC6-PXE gene expression in HEK293, HepG2 and HL60 cell lines by RT-PCR. We used two different HepG2 sublines, called HepG2/TS and HepG2/F12 each grown in different culture conditions (see methods). The ABCC6 mRNA detection was carried out by semi-quantitative RT-PCR with the LightCycler technology using the SYBR Green detection method. In this experiment the primer pairs used recognized solely the ABCC6-PXE gene. The same amounts of total cellular RNA were used for each sample and water was included as negative control (see Figure 2). The crossing points were around 31.5 cycles for HEK293, HepG2/F12 and HL60 cells and the negative control, while approximately 26.5 cycles characterized the HepG2/TS cells. The ABCC6 mRNA was not detected in HL60 (myeloid leukemia) cells. The melting temperature analysis of these PCR products revealed the formation of primer dimers in each sample, but PCR product with the expected size appeared only in the HEK293 and HepG2 cells, as it is illustrated on Figure 2. Although these data do not allow precise quantification of the ABCC6 mRNA in the various cell lines, they strongly suggest a significantly higher expression level in the HepG2/TS cells than in the others. In other experiments we also detected the presence of ABCC6 mRNAs in DLD-1 (colon) and Caco-2 (enterocytes) cells and their absence in KATO (gastric) and HL60 cells (data not shown).

Expression of the pseudogenes

Next we asked, whether the pseudogenes may also be transcribed. In order to answer this question, we performed RT-PCR reactions on the HepG2/F12 cell line. In these experiments we used one common primer pair, since oligos could not be designed to amplify specifically the pseudogenes and ABCC6-PXE cDNAs due to the more than 99% identity of the three sequences. The PCR products were subcloned and 11 clones were sequenced. Ten of them contained fragments corresponding to cDNAs encoded by the ABCC6-PXE gene. Interestingly, two of these were splice variants and contained the entire intron 3. This transcript has a frameshift mutation. One clone contained an insert encoded by psi-2. None of the analyzed clones contained an ABCC6-psi-1 derived sequence.

DNA methylation of CGI/1 and CGI/2

We observed that several sequence deviations of the pseudogenes from the ancestral ABCC6-PXE gene correspond to CG/TG
transformation (Table 1). These data suggest that
the origin of these conversions is the deamination
of methyl-CpG dinucleotides, considered as
mutational hot spots. According to this hypothesis,
the \textit{ABCC6} genes are subject to hypermethylation
in some tissues. If this hypothesis is valid, the
\textit{ABCC6} gene transcription can be correlated to the
tissue-specific methylation of the gene and
furthermore, probably the most important
transcriptional regulatory regions are those which
undergo tissue-specific DNA methylation (44).

In order to test this hypothesis and to use
DNA methylation analysis as a tool to identify
 transcriptionally relevant regulatory sequences, we
investigated the extent of methylation of CGI/I and CGI/II (Figure 1B). These regions harbour the
highly conserved R1 and R2 regions, suggesting
their implication in the transcriptional regulation
of the gene(s). In the following experiments the
methylation of these regions were analyzed by the
genomic bisulfite sequencing method (27,28) in
HepG2/TS, HepG2/F12, HEK293 and HL60 cells.
This technique allows the methylation level
analysis of every single CpG dinucleotide in the
sequence of interest. In order to reveal potential
DNA methylation differences between \textit{psi-1}, \textit{psi-2}
and the \textit{ABCC6-PXE} genes, the respective PCR
fragments (B1 and B2) were designed to amplify
with at least one single nucleotide difference all
three sequences (Table 2).

The B1 fragment is 223 bp long and
contains 20 CpG dinucleotides in the original
sequence of the disease-causing gene and \textit{psi-1},
while 19 CpGs are present in the corresponding
\textit{psi-2} sequence. The region was almost completely
unmethylated in all the expressing cell lines in
both the pseudogenes and the \textit{ABCC6-PXE} gene
(Table 3). The same pattern was observed for the
non-expressing HL60 cell line between CpG-1 and
18. Interestingly, the last CpGs of the fragment,
CpGs-19 and 20, located already at the edge of a
less CpG dense sequence were methylated in
approximately 20% of the clones (Table 3).

The B2 region is 200 bp long and contains
20 CpGs in all the three sequences (Figure 3A).
Figure 3B shows the DNA methylation profile of
B2, which showed a strikingly different pattern
from B1. Both HEK293 and HepG2/TS cells were
completely unmethylated in all three genes,
similarly to the B1 region. The methylation
profiles in HepG2/F12 cells were different.

Although the studied region was generally
hypomethylated, CpG-1 was almost 50%
methylated and some residual methylation was
also observed at CpGs-2 and 3 in the \textit{ABCC6-PXE}
gene. \textit{Psi-1} and \textit{psi-2} seems to be less methylated
at CpG-1 than the \textit{ABCC6-PXE} gene but higher
background methylation was observed between
CpGs-2 and 10. Finally, the B2 region was almost
completely methylated in all three genes in the
\textit{ABCC6} non-expressing HL60 cell line. Altogether
these results support our initial hypothesis by
demonstrating the correlation between tissue-
specific gene expression and DNA methylation of
the \textit{ABCC6} genes. Our data suggest that the
proximal promoter of the \textit{ABCC6} gene(s) is
implicated in the tissue-specific transcriptional
regulation.

\textbf{Luciferase reporter gene assays}

These findings prompted us to further
study the proximal promoter region, by the
molecular dissection of the -718/+72 sequence
(relative to the first ATG). The detailed analysis
was carried out by luciferase reporter gene assays.
DNA fragments of the \textit{ABCC6-PXE} gene extending from -145 to +72, -332 to +72 and -718
to +72, were cloned into a pGL3 vector and
transiently expressed in HEK293, in HepG2/TS
and in HepG2/F12 cells.

The results of the reporter gene assays are
summarized on Figure 4, together with the map of
the schematic representation of the analyzed
promoter fragments (Figure 4D). All the cell lines
tested exhibited similar luciferase activity profiles
for the respective promoter constructs (Figure 4A-
C). The shortest promoter fragment resulted in a
moderate increase of the reporter gene expression
e.g. 3.5x in the HEK293 cells) over the cells
transfected with the pGL3 vector containing the
luciferase gene alone. The evolutionarily highly
conserved -332/+72 fragment contains the entire
region of the proximal promoter analyzed for
DNA methylation. After transient transfection, the
measured luciferase activity of this construct was
much higher than the activities measured for the
shortest one (e.g 6.1x vs 3.5x in the HEK293 cells)
(Figure 4 A-C). These data show that
transcriptional activator elements are present in the
-332/+72 region of the \textit{ABCC6} genes.

Finally, the -718/+72 construct was also
tested. In addition to the previous promoter
fragments, this construct contains two stretches of evolutionarily conserved elements embedded in a globally less conserved region. Interestingly, the measured luciferase activity was similar to that of the shortest construct in the case of all the tested cell types. The relative transcriptional activity of the longest fragment was significantly lower than observed for the -332/+72 construct (e.g. 4.1x vs 6.1x in HEK293 cells). These data suggest that transcriptional repressor sequences are located in the -718/-332 promoter region.

Luciferase reporter gene assays with methylated constructs

In order to analyze the functional role of DNA methylation in the regulation of ABCC6 gene transcription, we carried out luciferase reporter gene assays with methylated and unmethylated promoter constructs. We prepared in vitro SssI methylated -332/+72 and -145/+72 fragments. These fragments were ligated back into the pGL3 vector and directly transfected to HepG2/F12 and HepG2/TS cells without further passage into bacteria (which would lead to the loss of CpG methylation). We compared the measured relative luciferase activity of each methylated construct to the activity measured after the transfection of the unmethylated vector. Results are visualized on Figure 5. We observed the same relative luciferase activity for the shortest constructs in both HepG2 cell lines, independent of the methylation status. This finding suggests that the methylation of this region does not play a significant role in the transcriptional regulation of the gene. The analysis of the -332/+72 fragment revealed a considerably different picture. The in vitro methylated construct showed a significantly lower luciferase activity in both cell lines after the transfection as compared to the unmethylated plasmid. The decrease of transcriptional activity after DNA methylation was 25 % for HepG2/F12 and 40 % for HepG2/TS, clearly indicating that – in contrast to the shorter -145/+72 region – the methylation of this longer region may be crucial in the transcriptional regulation of the gene.

DISCUSSION

The aim of the present study was the identification of the main genetic and epigenetic transcriptional regulatory sequences of the human ABCC6-PXE gene. We adopted a strategy based on in silico sequence analysis and luciferase reporter gene assays. The regions to be investigated by the luciferase assays were selected after the in silico phylogenetic comparison and epigenetic studies. The DNA methylation analysis of the evolutionarily conserved promoter regions of the endogenous gene in human cell lines increased the probability of identifying physiologically relevant regulatory sequences probed after molecular dissection by luciferase assays.

A major challenge of this study was the specific analysis of the transcriptional regulation of ABCC6-PXE gene with the two pseudogene sequences in the background (20). The sequence homology of the three sequences is unusually high, it even caused diagnostic difficulties in the past (10,20). Indeed, the in silico analysis confirmed that the ABCC6 pseudogenes are both located in the vicinity of the disease-causing gene and share 99.5% sequence identity with the 5’ portion of ABCC6-PXE. These data suggest that the formation of psi-1 and psi-2 is a recent event at the evolutionary scale (see Figure 1A) (12,20,40). This is further supported by the absence of the pseudogenes in rodents. The study of the single point mutations revealed that several CG dinucleotides of a hypothetical common ancestral gene underwent sequence transformation and became TG (or CA, when the conversion took place initially on the antisense strand) (see Table 1) reflecting that these cytosines were subject to methylation. Indeed, methylated CG dinucleotides are known to be mutational hotspots (45). When the number of CG/TG transformations was compared between the two pseudogenes and to that of the total number of mutations we concluded that some regions display much less CG/TG conversions than others. This suggests that these particular regions are devoid of methylation. Moreover, some of them are characterized by a lower overall mutational rate implying that these sequences are highly conserved due to evolutionary pressure. This suggests that although these genes are considered as pseudogenes they may have physiological roles.

The results of two different in silico approaches pointed toward the potential regulatory role of two distinct regions. First, we carried out CGI prediction and identified two islands. The potential functional significance of the proximal
one was supported by the presence of a similarly located CGI in the highly homologous (over 40%) ABCC1 gene promoter (Ratajewski M, Pulaski L., unpublished results). As discussed above the ABCC6 genes are probably under epigenetic control as they might undergo DNA methylation in germ cells and somatic tissues prior to mutating. Since CGIs are frequently observed in promoters (41) and they are often methylated in somatic tissues when a gene is silenced (46-49) we considered the ABCC6 GC rich regions as potential target sequences of transcriptional regulation. A different approach of in silico sequence analysis also pointed toward the regulatory role of these regions. We hypothesized that the more important regulatory elements are conserved throughout the evolution. A phylogenetic comparison of the human, dog, rat and mouse ABCC6 genes indicated that two highly conserved regions are present in the investigated 10 kb upstream region. Both of them are embedded in the CGIs (see Figure 1B).

These regions were investigated in in vitro experiments after the selection of model cell lines. Three ABCC6-PXE expressing (HEK293 and two HepG2 lines) and one non-expressing (HL60) cell lines were chosen. In the HepG2/F12 subline we carried out a qualitative analysis of ABCC6 genes’ expression. We found the overrepresentation of ABCC6-PXE gene derived transcripts (10/11 clones). Interestingly, two of the sequences were splice variants and contained the entire third exon. This raises the possibility of a contamination by genomic DNA. However, this possibility was ruled out, since the forward primer recognized specifically the 2nd exon and the 2nd intron was missing from all the cloned inserts. We could report for the first time the expression of the ABCC6-psi-2 gene. In a previous study an unknown transcript, namely URG7 was detected in hepatitis B infected HepG2 cells (50). Our analysis of this mRNA molecule revealed that it was transcribed from the ABCC6-psi-2 gene. In a previous study an unknown transcript, namely URG7 was detected in hepatitis B infected HepG2 cells (50). Our analysis of this mRNA molecule revealed that it was transcribed from the ABCC6-psi-2 gene. However, in contrast to the transcript identified in our study, URG7 was incorrectly spliced.

Next, we carried out DNA methylation profile determination of the endogenous ABCC6 genes in the conserved promoter regions in order to detect potentially relevant regulatory regions. The bisulfite genomic sequencing technique was used in these experiments (27,28) to analyze the selected cell lines. This PCR based approach allows parallel analysis of the methylation pattern of the three ABCC6 genes. Moreover, since the bisulfite sequencing reflects the allelic methylation profiles, the method makes identification of various subpopulations in the cell lines possible. The same primer pairs were used to amplify all three ABCC6 genes, and the identification of SNPs during the bisulfite sequencing permitted the distinction between the pseudogenes and the disease-causing gene.

The distal CGI was almost completely unmethylated in each cell line in all three ABCC6 genes (see Table 3). Thus the behavior of this islands reflects that of the classical CpG islands (42). The methylation profile of this island was independent of the expression of ABCC6. Only the two 3' most CpGs (CpG-19 and 20) were significantly hypermethylated compared to the rest of the island in the HL60 cell line. However, it is questionable whether the almost 20% methylation of these CpGs has any potential physiological role.

The methylation profile in the proximal region contrasted the methylation profile of the distal CGI. The proximal island in the MRP6+ cells was hypomethylated, although the HepG2/F12 subline, which expressed the ABCC6-PXE gene at a significantly lower level than the other HepG2 cells had a subpopulation with CpG-1 hypermethylated (see Figures 2 and 3B). Moreover, various partially hypermethylated pseudogene allele populations were also detected, accounting for the unique methylation pattern differences between the three ABCC6 genes in the promoter region. This relative hypermethylation of the ABCC6 pseudogenes may be partially responsible for their lower expression level in HepG2/F12 cells. These data further stress the different characteristics of the two HepG2 sublines.

In contrast to the generally hypomethylated MRP6+ cells, the MRP6- HL60 cells were almost completely methylated (see Figure 3B), suggesting that the hypermethylation of the region inhibits the expression of the genes. These DNA methylation differences reveal a correlation between the expression of the ABCC6 genes and their methylation and suggest that the proximal conserved region may play a critical role in the regulation of ABCC6 transcription.
The molecular dissection of the proximal promoter region by luciferase reporter gene assay further supported this hypothesis. Our data revealed the presence of a transcriptional activator sequence between -332 and +72 bp (see Figure 4A-C). The fragment from -145 to +72 confers already partial transcriptional activity to the region. Interestingly, the region between -718 and -332 bp contains repressor elements as demonstrated by the luciferase experiments. When this fragment was added to the -332/+72 construct the luciferase activity dropped to a similar level measured after the transfection of the shortest construct. All these elements acted similarly in the three MRP6+ cell lines, suggesting that the main regulatory mechanisms in the proximal promoter region are similar in these cells.

Finally, we carried out luciferase assay on the methylated promoter to investigate the influence of DNA methylation on the transcriptional regulation of the gene (see Figure 5). The methylation of the shortest promoter fragment (-145/+72) had no effect on the luciferase activity in any of the HepG2 sublines. The luciferase activity in the cells transfected with the vector containing the medium length fragment characterized by the highest transcriptional potential was strongly diminished upon DNA methylation. Indeed, in HepG2/TS cells the transcriptional potential of the -332/+72 construct was reduced to approximately the same level as observed previously for the shortest and longest constructs. This suggests that the 129 bp sequence between the 5’ end of the shortest construct and the first CpG of the -332/+72 construct plays a crucial role in the transcriptional regulation of the gene. Our results on the CpG methylation of the endogenous gene strongly suggest that the detected activator role of the identified promoter fragment is physiologically relevant.

Loss of function mutations of the ABCC6 gene lead to the development of PXE (8-11). However, in almost 25% of the alleles from patients no mutation is detectable, when the entire coding region is investigated for diagnosis (22,23). In the light of the results presented in this paper we propose that polymorphisms or mutations of the proximal promoter and especially of the identified 129 bp long activator promoter region may contribute to the pathogenesis of PXE, and its analysis may give information of diagnostic and/or prognostic value.

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**FIGURE LEGENDS**

**Figure 1.** *In silico* analysis of the *ABCC6* genes. Chromosomal location and orientation of the genes are shown on scheme A. Ψ1 and Ψ2 stand for *ABCC6-psi1* and *psi2*, respectively. Scheme B represents the 10 kb 5' upstream region, first exon and a part of the first intron of the gene. Repeat elements and CpG islands (CGI - gray boxes) are indicated. R1 and 2 symbolize the evolutionarily conserved sequences. Numbering is relative to the translation start site. Not in scale.

**Figure 2.** Expression of the *ABCC6-PXE* gene in human cell lines. Detection by one-step RT-PCR of the *ABCC6-PXE* mRNA. Agarose gel migration of the 431 bp specific PCR product after the semi-quantitative analysis by the SYBR Green method (see text).

**Figure 3.** Methylation pattern analysis of the proximal CGI (B2 fragment) in the *ABCC6* genes by bisulfite genomic sequencing. (A) Relative positions of the 10 CpG dinucleotides (vertical bars) in the B2 fragment. The numbers indicate the distance of the first and last analyzed CpGs from the translation start site. (B) Each row symbolizes a single clone. The numbers after the rows indicate the number of clones sequenced with an identical methylation pattern. The black and white circles represent the methylated and unmethylated CpG dinucleotides, respectively. PXE, Ψ1 and Ψ2 stand for *ABCC6-PXE, psi1* and *psi2*, respectively.

**Figure 4.** Identification of transcriptional regulatory regions in the *ABCC6-PXE* promoter. Plasmids phABCC6(-145/+72)Luc, phABCC6(-332/+72)Luc and phABCC6(-718/+72)Luc containing fragments (in bold) of *ABCC6-PXE* promoter were transiently transfected into HEK293293 (A), HepG2/F12 (B) and HepG2/TS (C) cells. Transcriptional activity of the respective promoters was assayed by measuring luciferase activity in cell extracts. Normalised results are expressed as fold induction of transcriptional activity compared to the promoterless control plasmid (average±S.E.M., n=12 (A), 9 (B), 18 (C), * p<0.05). The different promoter fragments are visualized on panel (D).

**Figure 5.** Repression of *ABCC6* promoter transcriptional activity by CpG methylation. Plasmids phABCC6(-145/+72)Luc and phABCC6(-332/+72)Luc with unmethylated (empty bars) or SssI methylated (shaded bars) *ABCC6-PXE* promoter-derived sequences were transiently transfected into HepG2/F12 (A) and HepG2/TS (B) cells and transcriptional activity was assayed by measuring luciferase activity in cell extracts. Results are expressed as percentage of transcriptional activity with regard to the plasmids with unmethylated promoter regions (average±S.E.M., n=10, * p<0.05).
|  | ABCC6-PXE | ABCC6-psi1 | ABCC6-psi2 |
|---|---|---|---|
| -9275 | CG | CA | CG |
| -8970 | TG | TG | CG |
| -8879 | CG | TG | CG |
| -8567 | CG | CG | CA |
| -7844 | CG | CG | CA |
| -7715 | CG | TG | TG |
| -6847 | CG | TG | CG |
| -6686 | CA | CG | CG |
| -6636 | CA | CG | CG |
| -493 | CG | TG | CG |
| -138 | CG | CG | TG |
| 1540 (intron 1) | TG | CG | CA |
| 1758 (exon 2) | CG | CG | CA |
| 2360 (intron 2) | CG | CG | CA |
| 3424 (intron 2) | CG | CG | TG |
| 3500 (exon 3) | CG | CG | CA |
| 3780 (exon 4) | CG | CA | CG |
| 5704 (intron 4) | CA | CG | CA |

**Table 1.** CpG mutations of a hypothetical common ancestral *ABCC6* gene from -10 kb relative to the translation start site to the 4th intron. Only those hypothetical mutations are shown (in bold), which can be attributed to a CG/TG transformation on one of the two strands. Numbering is relative to the translation start site of the *ABCC6-PXE* gene.
| SNP position | PXE | psi1 | psi2 |
|--------------|-----|------|------|
| -9136        | TYg | A-g  | A-g  |
| -9110        | tT  | yG   | tT   |
| -9104        | G   | T    | T    |
| -345         | A   | G    | G    |
| -302         | A   | A    | T    |
| -218         | A   | T    | A    |

**Table 2.** SNP positions in the B1 and B2 amplicons. The polymorph nucleotides are shown in capitals as detected after the bisulfite treatment. Y stands for C or T and corresponds to a C in a CpG dinucleotide prior to bisulfite treatment. Nucleotides in small characters are shown to help the understanding of the sequence context, when necessary. Numbering is relative to the translation start site of the ABCC6-PXE gene.
| Cell line       | Total no of clones | Distribution of clones | Observed mCpG/CpG |
|-----------------|--------------------|------------------------|-------------------|
|                 |                    | PXE       | psi1       | psi2       |                      |
| HepG2/TS        | 34                 | 5         | 11         | 18         | 0/100 1/220 0/342   |
| HEK293          | 16                 | 6         | 5          | 5          | 0/120 0/100 0/95    |
| HepG2/F12       | 22                 | 4         | 8          | 10         | 1/80   0/160 0/190  |
| HL60            | 41                 | 14        | 13         | 14         | 8/280 3/260 2/266   |

**Table 3.** Methylation profile of the ABCC6 genes in the B1 region in human cell lines. The total number of clones, their distribution between the three genes and the corresponding total methyl-CpG versus total CpG dinucleotide number before bisulfite treatment are indicated for each cell line. PXE, psi1 and psi2 stand for ABCC6-PXE, psi1 and psi2, respectively.
Fig 1 A

Chr16p  \( \psi_2 \)  \( ABCC6 \)  \( \psi_1 \)

\[ \begin{array}{cccccc}
\text{tel} & 15 \text{ Mb} & 16 & 17 & 18 & 19 \\
\end{array} \]

Fig 1 B

\[ ABCC6 \text{ promoter} \]

| R1  | Alu    | Minisatellite | R2  |
|-----|--------|---------------|-----|
| -0.5 kb | -8.5  |               | -0.5 | 0.2 |
| CGI/I  |       |               | CGI/H |
Figure 2
Fig 3 A

-273 -172

Fig 3 B
Figure 4

(A) HEK
(B) HepG2/F12
(C) HepG2/TS

Transcriptional activity

Vector  -145/-72  -332/-72  -718/-72

(D) B2

-145/-72
-332/-72
-718/-72
ATG
Figure 5

A  HepG2/F12

B  HepG2/TS

Transcriptional activity [%]

Methylated  -  +  -  +

Transcriptional activity [%]

Methylated  -  +  -  +
Identification of a DNA methylation-dependent activator sequence in the pseudoxanthoma elasticum gene, ABCC6
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