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Liver-specific enhancer in *ABCC6* promoter—Functional evidence from natural polymorphisms

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ABSTRACT

Pseudoxanthoma elasticum (PXE) is a heritable connective tissue disease caused by mutations in the *ABCC6* gene that encodes a transmembrane transporter of unknown function, expressed mainly in the liver. It has been suggested that some PXE patients for whom no mutations can be found in the coding region of *ABCC6* probably suffer from insufficient level of active protein due to lowered gene expression. Here we report the functional analysis of previously reported natural polymorphisms found in the *ABCC6* gene promoter. The only polymorphism known to be significantly more common in PXE patients was located within one of the PLAG transcription factor binding sites located by us previously. This mutation negatively influenced PLAG-mediated induction of *ABCC6* promoter in a reporter gene system. Moreover, site-directed mutagenesis of an analogous sequence within another PLAG-binding site in the promoter both depressed PLAG binding and specifically repressed *ABCC6* promoter activity in cells of liver origin. Thus, we have identified novel sequence determinants of liver-specific transcription of the *ABCC6* gene with direct relevance for at least some PXE patients.

Introduction

The human *ABCC6* gene encodes a transmembrane transporter of unknown function which localizes in basolateral membrane of polarized cells, mainly hepatocytes. Mutations in the coding region of *ABCC6* have been identified as a major cause of connective tissue illness known as *pseudoxanthoma elasticum* (PXE). PXE is characterized by mineralized and fragmented elastic fibers in the skin, Bruch's membrane in the retina, and vessel walls [1]. PXE was recently conclusively identified as a metabolic disease with disease symptoms localized away from its site of expression and activity in the liver [2], which generated increased interest in genetic determinants of its tissue-specific expression pattern. Differences in severity of disease between patients probably depend on other genetic modulatory factors that might exert a positive or negative modulatory effect on the course of PXE [3,4].

ABCC6 expression in human body is restricted to liver and in lesser extend to kidneys what is probably linked with the very specific function of this protein [5]. Studies on mechanisms of transcriptional regulation of *ABCC6* gene might lead researchers to find potential substrates for this protein, since this strategy was successful in the cases of other ABC transporters [6], and can be even more important for PXE patients suffering from insufficient level of expression of active ABCC6. Previously, we have reported that *ABCC6* repression in tissues that do not express this transporter is caused by hypermethylation of a CpG island located in the proximal fragment of its promoter region. We have also found that 9-*cis*-retinoic acid influences positively the activity of promoter as well as ABCC6 level *in vivo* [7]. Unfortunately it is difficult to expect that these findings can be useful for planning potential therapeutical interventions due to relatively broad spectrum of activities of compounds affecting identified signaling pathways. From this point of view, much more promising is our recent discovery of involvement of PLAG proteins in the regulation of *ABCC6* expression [8].

It has been estimated that the ratio of PXE patients without specific mutations in the coding region of *ABCC6* gene might be as high as 25% [9]. Some polymorphisms have recently been found in the proximal fragment of *ABCC6* gene promoter: −127C > T, −132C > T, and −219A > C [10]. Authors of the cited work reported that only differences in frequencies of transversion at position −219 between PXE patients and healthy controls were statistically significant. Interestingly, −219A > C is located in the liver specific activatory sequence stretch of *ABCC6* promoter identified previously by us [11] and what is even more important, it resides within the distal binding site for PLAG transcription factors, albeit it does not overlap the binding consensus itself [8]. Based on these initial concepts, we decided to test the effect of all 3 reported polymorphisms on the activity of *ABCC6* promoter using a reporter gene system and EMSA.

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Materials and methods

Cell culture. HepG2 (hepatocellular carcinoma), Hep3B (hepatocellular carcinoma), HEK293 (artificially transformed human embryonic kidney epithelial cells) and A549 (alveolar epithelial non-small cell lung cancer) cell lines were obtained from ATCC (Manassas, USA) and maintained in standard conditions (Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 37 °C, 5% CO_2).

Promoter constructs and transfections. All used reporter constructs were described previously [11,7] and the luciferase measurements were performed as described [8] and standardized as described [11]. Cotransfections of reporter constructs and plasmids carrying cDNA for selected transcriptional factors were performed analogously to previous work [8]. Expression plasmids were bought from Origene Technologies Inc. (Rockville, USA).

Site-directed mutagenesis. Mutagenesis was performed directly on plasmids (pUC18) containing 217 bp (region −145/+72—all nucleotide locations are reported relative to the A in the ATG translation initiation codon) and 404 bp (region −332/+72) long fragments of *ABCC6* promoter, using the PCR based method, following by removal of the template by DpnI digestion. For mutagenesis, the following primer pairs were used: 5′-CTCCCGATCCCGGAGCTTGAATTCCAGCCGGAC-3′ (mG1f), 5′-GTCCGGCTGGAATTCAAGCTCCGGGATCGGGAG-3′ (mG1r); 5′-GAGCGCCTCTCTTCCCCCCTCCCCCACCTCGCCT-3′ (mG2f), 5′-AGGCGAGGTGGGGGAGGGGGGAAGAGAGGCGCTC-3′ (mG2r), 5′-GCAGCTGGACCTTAAAAGGGGCCTCCCGATCCC-3′ (m3f), 5′-GGGATCGGGAGGCCCCTTTTAAGGTCCAGCTGC-3′ (m3r). The mutated sequences were verified by automated sequencing and then recloned into pGL3-basic vector using KpnI and HindIII restriction endonucleases to construct: phABCC6mG1(−145/+72)Luc, phABCC6mG2(−332/+72)Luc and phABCC6m3(−332/+72)Luc.

Nuclear extracts and EMSA. Nuclear extracts were prepared using a modification of the technique described by Dignam et al. [12] and subsequently used in EMSA. EMSA was performed with infrared dye-labeled oligonucleotide probes IRDye700-*ABCC6* representing the sequences: 5′-CTCCCGATCCCGGAGCTCGAATCCCAGCCGGAC-3′ (wt) 5′-CTCCCGATCCCGGAGCTTGAATTCCAGCCGGAC-3′ (mG1), 5′-GAGCGCCTCTCTTCCCCCATCCCCCACCTCGCCT-3′ (wt) 5′-GAGCGCCTCTCTTCCCCCCTCCCCCACCTCGCCT-3′ (mG2), 5′-GCAGCTGGACCTTGCCCGGGGCCTCCCGATCCC-3′ (wt), 5′-GCAGCTGGACCTTAAAAGGGGCCTCCCGATCCC-3′ (m3). 2 μg of HepG2 cell nuclear extract were incubated with 10 fmoles of IRDye-labeled probes in buffer containing: 5 mM Tris (pH 8.0), 50 mM NaCl, 5 mM DTT, 5 mM $MgCl_2$, 0.5% Igepal, 25% glycerol, 100 μM $ZnCl_2$, 2.5 μg salmon testis DNA. For the competition assay, 40- and 200-fold molar excess of unlabeled oligonucleotides was added to the reaction mixture. Binding reactions were resolved by electrophoresis in 7.5% polyacrylamide gels in 0.5% Tris–acetate EDTA buffer.

Computational analysis and statistics. For computational analysis of the *ABCC6* promoter region, Matinspector software [13] was used. Testing for statistical significance was done by one-way ANOVA followed by Tukey's test as appropriate. A P value of 0.05 or lower was considered statistically significant.

Results and discussion

Using a reporter gene-based medium-throughput screening assay we have previously identified PLAG factors as important regulators of *ABCC6* gene expression [8]. Two sites for PLAG protein binding have been identified: one more conserved at the position −163/−145 bp (the proximal site) and a second one, less conserved at −223/−206 bp (the distal site). Both of them are located in the transcriptional activator sequence of *ABCC6* gene promoter and are probably involved (perhaps indirectly) in the liver specific pattern of expression of investigated gene. In our previous work, we provided evidence that PLAG-mediated transactivation of the *ABCC6* promoter depends predominantly on the proximal site as mutations in it completely abolished the promoter response to PLAG overexpression, although we did not exclude the importance of the second site [8]. The −219A > C polymorphism identified by Schulz et al. [10] is located inside the distal PLAG site, between two parts of the PLAG1 consensus, within the central nucleotide stretch thought to be irrelevant to PLAG binding (Fig. 1). The remaining reported promoter polymorphisms (−127C > T and −132C > T) were identified in a fragment of the core promoter, close to a putative Sp1/Sp3 binding site reported by another group [14].

We have first verified the impact of promoter polymorphisms on the transcriptional activity of the *ABCC6* promoter in a reporter gene system. The two proximal polymorphisms reported as insignificantly linked to PXE (−127C > T and −132C > T) were introduced into a fragment containing the basal (core) promoter (−145/+72) [11]. Reporter gene assays have clearly shown that both transitions did not influence the basal activity of *ABCC6* promoter in four tested cell lines of various tissue origin (Fig. 2). Moreover, the positive response of the core promoter to Sp1 overexpression in HepG2 cells was unchanged for the mutated sequence (with 1.33 ± 0.05 fold induction of phABCC6(−145/+72)Luc plasmid and 1.35 ± 0.07 fold induction of phABCC6mG1(−145/+72)Luc in the presence of overexpressed Sp1). This suggests strongly that these polymorphisms do not interfere with transcription factor binding, as confirmed also by electromobility shift assay (EMSA), where we did not find any differences in protein binding between wild type sequence and mutated one containing both polymorphisms (Fig. 3A). We observed only one band (as the effect of shift of the probe on polacrylamide gel by proteins from nuclear extract) and its specificity was proven in a competition assay. We have not seen any band supershift after Sp1 antibody addition, suggesting that Sp1 is not involved in protein–DNA complex formation at this particular sequence stretch. We conclude that −127C > T and −132C > T promoter polymorphisms are unlikely to impact *ABCC6* expression levels, in line with the conclusions drawn by Schulz et al. [10].

The remaining polymorphism, −219A > C transversion, was expected to be more practically relevant based on its overrepresentation in the PXE cohort and on its location within an important regulatory sequence (PLAG binding consensus). Even though we have previously proven the secondary role of this distal PLAG site in relation to the proximal one in *ABCC6* regulation [8], we were now able to show direct binding of HepG2 nuclear proteins to this sequence by EMSA, including the appearance of supershift after application of anti-PLAGL1 antibodies (Fig. 3B). The specificity of observed DNA binding was proven using competition assay in which an excess of unlabeled probe resulted in elimination of protein binding to the labeled probe. It is important to point out that we have not seen a decrease of intensity of the shift band in the supershift assay, which can be explained by the premise that PLAGL1 (target of the antibodies) is not a major interactor of this sequence. It is probable that out of PLAG factors, PLAG1 binds more strongly to this DNA fragment, since the PLAG1 consensus within the −223/−206 sequence is more conserved than that for PLAGL1 [8] (this would also explain the stronger negative effect of the −219A > C mutation on PLAG1 inducibility of *ABCC6* promoter). Unfortunately, the lack of a reliable anti-PLAG1 antibody performing efficiently in supershift precluded a similar proof being made for this TF.

The basal promoter activity in the reporter gene system was, surprisingly, unchanged in the presence of the −219A > C mutation in all tested cell lines (Fig. 2), including those of hepatic origin.

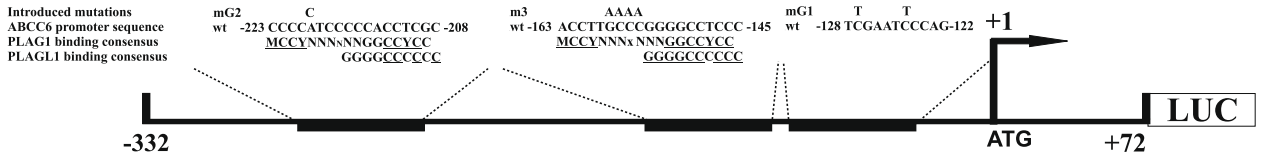

Fig. 1. Model of proximal fragment of the human *ABCC6* gene promoter and location of PLAG binding sites and introduced mutations. PLAG1 and PLAGL1 consensus binding sequences are overlaid on the wild type sequence with underlined conserved nucleotides.

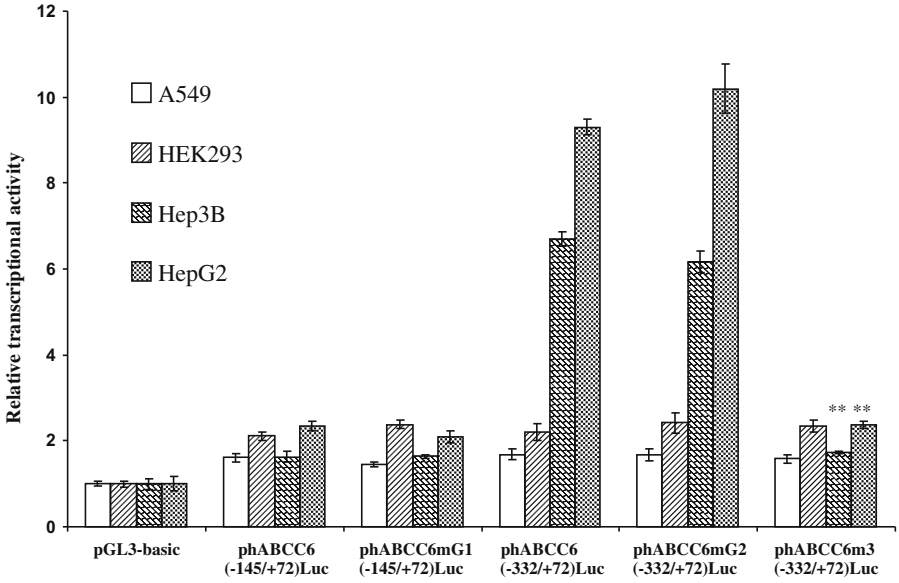

Fig. 2. Transcriptional activity of *ABCC6* promoter constructs in cells of different tissue origin. Results of luciferase assay are standardized against control reporter activity (pCMV-SEAP) and expressed as fold induction over control value (activity of promoterless pGL3-basic vector), $^{**}p < 0.001$, mean ± SEM ($n = 5$).

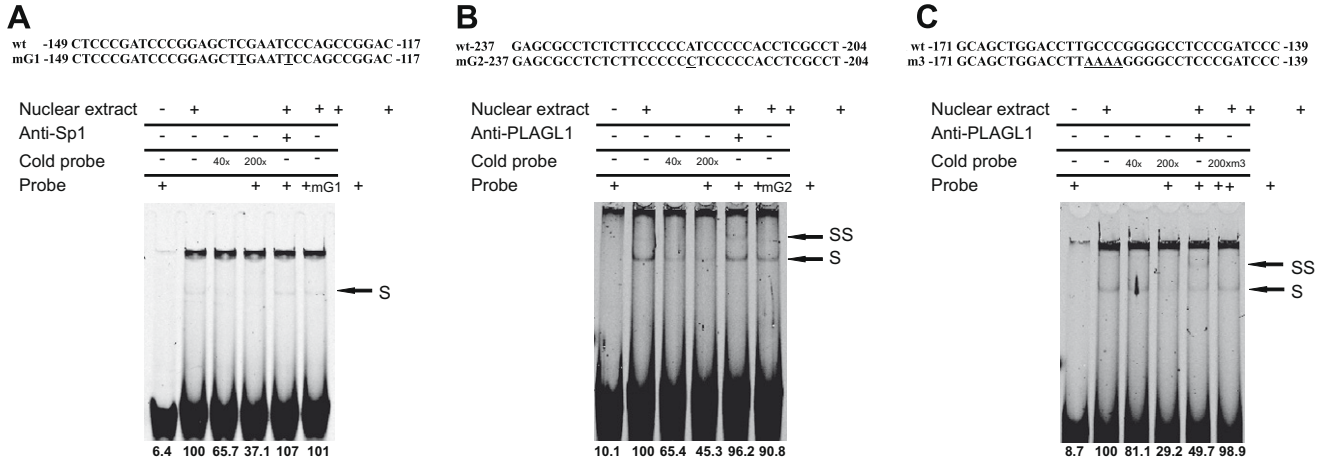

Fig. 3. Results of EMSA performed on nuclear extracts from HepG2 cells with fluorescently labeled probes. Numbers below scans represent densitometric quantification of the shifted (S) band in each lane (normalized to the value for sample containing only probe and nuclear extract assumed as 100). Competitor is fluorescently unlabelled probe and numbers in its description represent molar fold excess of competitor with regard to labeled probe. Each panel shows sequences of wild type and mutated probes.

However, even though nucleotide exchange at this location is not computationally predicted to influence PLAG binding to the surrounding consensus, we have seen a significant negative effect of this transversion on inducibility of the gene promoter by PLAG overexpression (20% decrease of induction when PLAGL1 was overexpressed and 30% decrease of induction in the case of PLAG1—Fig. 4). On the other hand, the −219A > C mutated sequence showed only a slight difference (ca. 10%) from the wild type one in binding of proteins from HepG2 cells nuclear extract (Fig. 3B). This might be the effect of limitations of resolution of the EMSA technique which is not able to show the differences in binding of protein at the level of 20% *in vitro* (when the amount of protein is relatively high), or the possibility that this site interacts somehow *in vivo* (by stabilization or DNA bending) with the PLAG transcription factors occupying the proximal site and/or other undefined factor(s) responsible for liver specificity of *ABCC6* expression. This kind of interaction of course could not be reconstructed using EMSA [15]. However, since the mutation is located

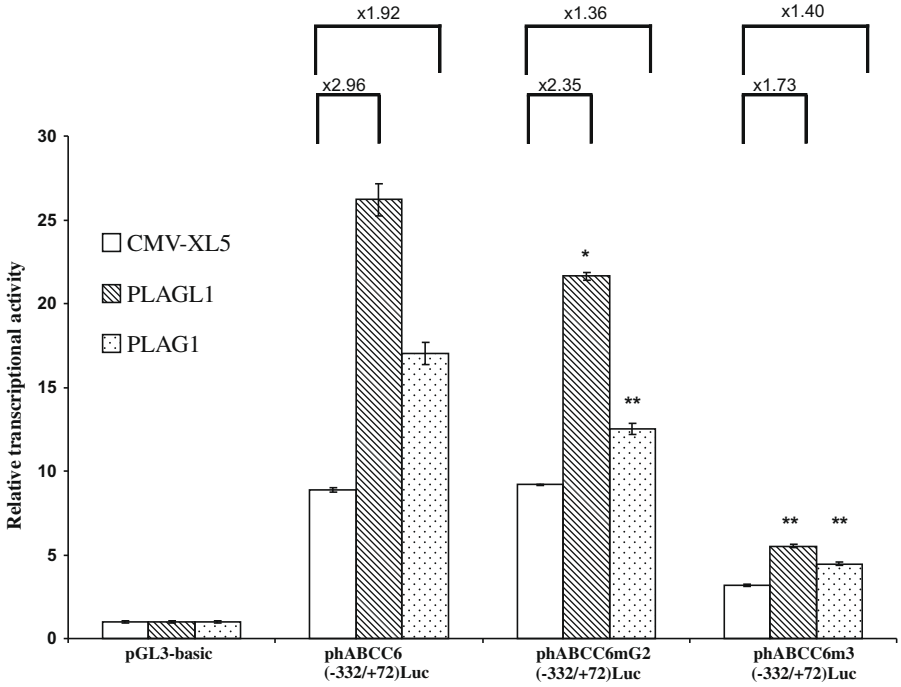

Fig. 4. Induction of transcriptional activity of the *ABCC6* gene promoter reporter constructs by overexpression of PLAGL1 and PLAG1 transcriptional factors in the HepG2 cell line. $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 5$.

within the internal sequence not known to be important for PLAG binding, it is possible to speculate that PLAG binding is, in fact, nearly unaffected by the polymorphism and that the observed effect on transactivation is due to decreased site-specific binding of another, liver-specific factor (perhaps a coactivator).

In order to test our hypothesis of a liver-specific regulatory sequence within the PLAG site, we decided to mutagenise the corresponding location in the proximal PLAG binding site that is more relevant to PLAG-mediated inducibility of *ABCC6*. We exchanged all four nucleotides (−158/−155) that aligned with variable positions in the consensus and could functionally correspond to −219A. Strikingly, this mutation led to complete abolishment of liver specific activity of phABCC6(−332/+72)Luc in both tested liver-derived cell lines (HepG2, Hep3B) (Fig. 2), where the transcriptional activity of this construct decreased to levels seen for other cells (A549, HEK293). For the non-hepatic cell lines, the mutated construct phABCC6m3(−332/+72)Luc possessed the same transcriptional activity as the one carrying wild type sequence, further proving the liver specificity of this effect. Moreover, phABCC6m3(−332/+72)Luc has a decreased (but not abolished) inducibility by overexpression of PLAG factors (compared to the wild type plasmid). Mutation of the −158/−155 nucleotide stretch decreased the response of *ABCC6* promoter to PLAGL1 by c.a. 40% and to PLAG1 by c.a. 25% (Fig. 4). This strongly supports the hypothesis of liver-specific cooperative activation of *ABCC6* promoter by PLAG factors and other proteins binding in the middle of the PLAG consensus. Further evidence is gathered from EMSA results where despite very specific PLAG binding to the −171/−139 probe, mutating the 4 central nucleotides was enough to abolish the ability of the cold probe to compete with labeled probe even at two hundred molar excess (Fig. 3C).

It is notable that the liver-specific regulatory sequences thus identified can be explained as major determinants of tissue specificity, previously proposed to be located in this region of the promoter but elusive to exact pinpointing. In the present study, we confirmed that plasmid phABCC6(−332/+72)Luc is much more active transcriptionally than the shorter core promoter-containing phABCC6(−145/+72)Luc exclusively in cells of hepatic origin (HepG2, Hep3B), while in other cells where *ABCC6* is naturally expressed at a low level (HEK293, A549) the activities of both plasmids are indistinguishable (Fig. 2). This cell type specificity determinant of the *ABCC6* gene is localized to a region overlapping a previously identified CpG island [11]. However, computational analysis and medium-throughput reporter gene screening assay failed to identify any known transcription factor from LET (liver enriched transcriptional factor) family [16] that would be able to interact with the *ABCC6* promoter. Another study [14] suggested that NFκB may be involved in liver specific expression of *ABCC6*. We have failed to confirm this hypothesis: overexpression of active NFκB or RelA did not affect *ABCC6* promoter activity [8], although it strongly induced other NFκB-regulated genes in the same experimental setup (data not shown). Treatments activating NFκB-mediated signaling pathways (lipopolysacharide, H_2O_2) gave the same negative results at the level of promoter as well on *ABCC6* mRNA measured by real-time RT-PCR (data not shown). The distal liver-specific regulatory sequence identified by us is, however, located very close to the putative NFκB binding site of Jiang et al. [14], perhaps explaining the observed discrepancy.

In summary, our results hint strongly at cooperative binding of an undefined liver-specific transactivator with PLAG proteins to the investigated DNA fragments (proximal and distal PLAG sites). The possibility of this kind of interaction has been already shown for PLAGL1 and p53 [17]. In the case of *ABCC6* promoter we suppose that this binding cooperation is the major molecular determinant of liver specific expression of *ABCC6* and that this complex mechanism is the reason why previous attempts to find liver-specific transcription factors regulating *ABCC6* failed. Our hypothesis is also supported by the fact that in non-hepatic cells with low *ABCC6* level, overexpression of PLAGs had always a smaller stimulatory effect (by about 30–40%) on *ABCC6* promoter activity in comparison to HepG2 or Hep3B cells ([8] and data not shown). This is easily explained by the lack or low levels of the liver-specific coactivator in these cells.

The most important aspect of our study is the fact that the location of the liver-specific regulatory sequences were located by us

based on a naturally occurring polymorphism (−219A > C), previously linked epidemiologically to PXE pathogenesis [10]. It corroborates for the first time the notion that *ABCC6* promoter polymorphisms may be functionally linked to PXE etiology. This finding may have also repercussions for future treatments of PXE patients suffering from insufficient level of expression of normal allele of *ABCC6* since it might affect the response of *ABCC6* gene to potential treatments or internal stimuli that induce PLAGs in order to maintain the high expression of this gene in liver *in vivo*. It may also give new impulse to searching for new mutations in the *ABCC6* promoter in PXE patients. Further study will be necessary to define the factors that bind to sequence −158/−155 and the molecular mechanism by which they coactivate the transcription of *ABCC6* gene. Such investigations are now in progress in our laboratory.

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