

Tissue-Specific Expression of the ABCC6 Gene

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The ABCC6 gene encodes MRP6, a member of the multidrug resistance-associated protein (MRP) family. Interest in ABCC6/MRP6 derives, in part, from the fact that mutations in this gene/protein system have been identified in families with pseudoxanthoma elasticum (PXE). Early studies indicated that ABCC6 is expressed primarily in the liver and to a lesser extent in the kidney, but more recently a widespread distribution has been suggested. To explore the tissue-specific expression of ABCC6, we first examined various mouse tissues by RT-PCR. The results indicated high levels of mRNA in the liver, whereas low level of expression was noted in the kidney and small intestine. To explore other tissues in which initial RT-PCR was essentially negative, a second-round nested PCR was performed, which revealed expression also in the brain, tongue, stomach, and eye. Unexpectedly, however, distinct PCR products of smaller molecular weight were noted in these tissues. Subcloning and sequencing of these PCR products indicated that they reflected aberrant splicing in the 3' end of the ABCC6 mRNA, resulting in each case in a premature termination codon. Similar results were noted with RT-PCR analysis using RNA isolated from cultured human epidermal keratinocytes and dermal fibroblasts. Collectively, our results confirm high level of expression of ABCC6 in the liver and the kidney, whereas very low level of expression in a variety of other tissues was noted. The results have implications for mutation detection strategies in PXE by RT-PCR, and they further support the notion that PXE is a primary metabolic disorder.

Key words: ATP-binding cassette genes/multiple drug resistance-associated proteins/pseudoxanthoma elasticum/RT-PCR analysis

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ABCC6 belongs to the family of genes encoding proteins with an ATP-binding cassette, and this gene specifically encodes MRP6, a member of the family of multidrug resistance-associated proteins (MRP) (Borst *et al*, 1999). Although the precise physiologic function of MRP6 is currently unknown, it is thought to be a transmembrane transporter due to its high degree (~41%) homology with the prototype protein, MRP1, an efflux transporter expressed in the liver (Belinsky and Kruh, 1999; Kool *et al*, 1999). The sequence information derived from full-length ABCC6 cDNA and from genomic sequences predicts that MRP6 consists of 1503 amino acids, characterized by the presence of three membrane spanning domains (MSD1-3), comprising 5, 6, and 6 transmembrane segments, respectively (for review, see Uitto *et al*, 2001). MRP6, in analogy to other ABC transporter proteins, has two intracellular nucleotide binding folds (NBF1, NBF2), each domain containing conserved Walker A and B motifs critical for ATP binding and the function of the protein as a transmembrane transporter. The ABCC6 gene consists of 31 exons spanning ~73 kb of

DNA on the short-arm of human chromosome 16, at locus 16p13.1. The corresponding mRNA is ~6 kb in size, and it has an open reading frame of 4.5 kb.

The interest in the ABCC6/MRP6 gene/protein system has been heightened by the discovery that mutations in the ABCC6 gene underlie pseudoxanthoma elasticum (PXE), a heritable systemic disorder affecting elastic structures in the skin, eyes, and the cardiovascular system (see Ringpfeil *et al*, 2000, 2001a; Le Saux *et al*, 2001). The extent of ABCC6 expression in various tissues has been controversial. During early characterization of ABCC6 by northern analysis, it was suggested that this gene is expressed predominantly in the liver and to a lesser extent in the kidney, whereas a number of other tissues tested were essentially devoid of the corresponding mRNA transcript (Belinsky and Kruh, 1999). This and related observations led us to conclude that PXE may be primarily a metabolic disorder resulting from the lack of activity of MRP6 as a putative transmembrane transporter in the liver (Uitto *et al*, 2001). At the same time, highly sensitive RNase protection assays (Kool *et al*, 1999) and RT-PCR (Bergen *et al*, 2000) suggested that ABCC6 mRNA may be present in other tissues as well, albeit in much lower abundance, including the skin, the eye, and the vessel wall, i.e., sites of pathology in PXE. Furthermore, a combination of RNase protection assays, *in vitro* hybridization, and immunohistochemical analyses demonstrated the presence of MRP6 not only in epithelial and endothelial cells in a variety of tissues but also in neurons

Abbreviations: MRP, multidrug resistance-associated protein; MSD, membrane spanning domain; NBF, nucleotide binding fold; PTC, premature termination codon; PXE, pseudoxanthoma elasticum

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and leukocytes (Beck *et al*, 2003). These observations suggested a more complex multifunctional role for ABCC6 and further implied that perturbations in the physiology of local cellular milieu in the absence of MRP6 activity may mechanistically contribute to the pathogenesis of PXE.

To address the controversy regarding the tissue-specific expression of the ABCC6 gene, this study was designed to define the expression profile of this gene. Specifically, we have examined various mouse tissues and different cell types by a highly sensitive two-step PCR strategy consisting of first-step RT-PCR, followed by a second-step nested PCR. Our results confirm that the ABCC6 gene is expressed predominantly in the liver and the kidney, but low level of expression can also be observed in a variety of other tissues. The low level of expression, however, in several tissues is accompanied by extensive aberrant splicing, particularly in the 3' end of the mRNA, and many of these transcripts predict the synthesis of non-functional MRP6 polypeptides.

Results

ABCC6 mRNA abundance in the mouse liver and the kidney The ABCC6 gene has been shown, on the basis of Northern analysis (Belinsky and Kruh, 1999), to be expressed predominantly in the liver and the kidney, but more widespread tissue distribution has also been reported by more sensitive techniques (Kool *et al*, 1999; Beck *et al*, 2003). In this study, we examined ABCC6 gene expression profile in a variety of mouse tissues by a highly sensitive two-step PCR strategy, consisting of the first-step RT-PCR and followed by a second-step nested PCR. We first isolated total RNA from several mouse tissues and subjected to RT-PCR with primers corresponding to exon 22 (forward primer) and the 3'-untranslated sequence within exon 31 (reverse primer) (see Table S1). After 30 cycles of amplification, a clear band of the expected size, ~ 1.7 kb, could be noted with RNA isolated from the liver and to a lesser degree in the kidney (Fig 1A). A faint band was also noted with RNA isolated from the small intestine, whereas the remaining tissues, including the skin and the eye, were essentially negative. Amplification of glyceraldehyde-3-phosphatase hydrogenase (GAPDH) sequences using the same RNA preparations as templates revealed the presence of a band of approximately equal intensity in all samples, attesting to the presence and integrity of mRNA in these specimens (Fig 1A).

Evidence for very low level of expression of ABCC6 in a number of mouse tissues To pursue further the expression of ABCC6 in tissues that showed low or undetectable levels of mRNA abundance by the first-round RT-PCR, an aliquot of the first-round product was subjected to a second round of amplification using nested primers within exons 23 and 31 (see Fig 2, *top panel*). After an additional 30 cycles, bands of the expected size, ~ 1.5 kb, were noted in the kidney, brain, small intestine, stomach and eye, however, no signal was obtained from the skin RNA (Fig 1B). In addition to the band of the expected size, additional bands of smaller molecular mass were noted in the brain, stomach and eye (Fig 1B). Furthermore, tongue mRNA resulted in a single PCR product, which was smaller than expected (~ 1.2 kb).

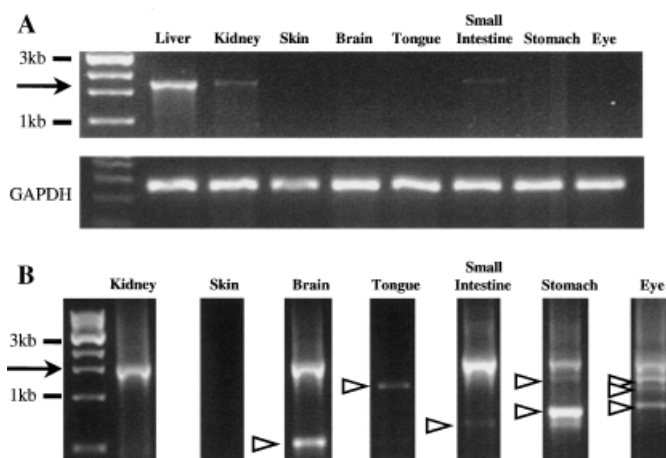


Figure 1
Expression of the ABCC6 gene in various mouse tissues. (A) RT-PCR was performed using total RNA (1 μ g) isolated from various mouse tissues (liver, kidney, skin, brain, tongue, small intestine, stomach, and eye) as template. Amplification with the ABCC6 specific primer pair (see Table S1) was shown to result in a fragment of the expected size, ~ 1.7 kb (arrow), encoding exons 22–31, in the liver, kidney and small intestine (*upper panel*). Glyceraldehyde-3-phosphatase hydrogenase mRNA was amplified as a control to ensure the presence and integrity of mRNA (*lower panel*). (B) Nested PCR was carried out using 1 μ L of each PCR product from the first-round RT-PCR. Amplification with the nested primer pair resulted in a product of the expected size, ~ 1.5 kb (arrow), encoding exons 23–31. In addition, bands smaller than expected were noted in several tissues (arrowheads).

Aberrant splicing in the 3'-end of the mouse mRNA The products that were smaller than expected from the second round nested PCR (1.5 kb) were subcloned and subjected to automated sequencing. Sequence comparison of the products with full-length ABCC6 mRNA sequence, as obtained using liver mRNA as template and validated by the genomic sequences, revealed that these bands represented aberrantly spliced mRNA transcripts (Fig 2). The smaller band obtained from brain mRNA was clearly devoid of exons 24–29, whereas tongue mRNA yielded a PCR product missing exons 27–29. Similarly, the small intestine had a weak band representing a PCR product devoid of exons 24–29, and in case of stomach mRNA, the predominant band was devoid of exons 25–29 but contained an insertion of 75 nucleotides representing intron 29 sequences (Fig 2). Finally, eye mRNA, in addition to the PCR products of the expected size, had three different products devoid of exons 28 and 29, 25–27, and 25–29, respectively. All these deletions are expected to affect the function of MRP6, as they predict deletion of sequences within the NBF2, a critical functional component of the protein, which is encoded by exons 28–30 (Fig 2, *top panel*). Furthermore, all the observed deletions are out-of-frame and are predicted to result in premature termination codon (PTC) of translation, in most cases within the 5' end of exon 31 encoding the C-terminal end (CT) of the protein (Fig 2).

Low level of expression and aberrant splicing of the ABCC6 mRNA in human skin cells To examine whether similar aberrant splicing could be observed in human cells as well, RT-PCR was performed on total RNA isolated from cultured epidermal keratinocytes and dermal fibroblasts,

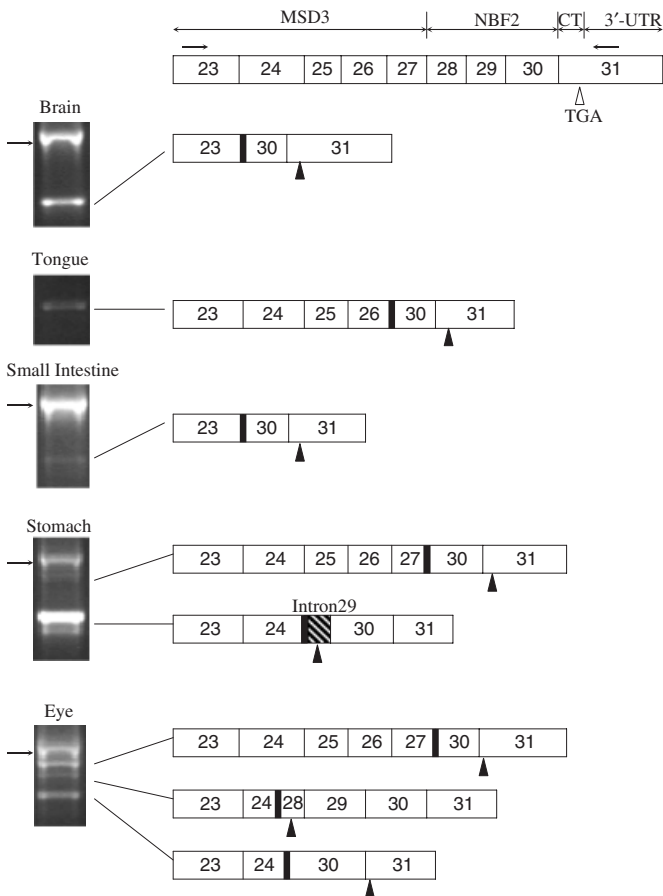


Figure 2
Aberrant splicing affects the 3'-end of the mouse ABCC6 gene. Bands smaller than expected in the second-round nested PCR (see Fig 1) were subcloned and subjected to automated sequencing, which revealed aberrant splicing in tissues as indicated. Schematic representation shown on the top depicts exons 23–31 (open boxes) resulting from normal splicing. This segment of the mRNA encodes the third membrane spanning domain (MSD3, exons 23–27), the second nucleotide binding fold (NBF2, exons 28–30), and the C-terminal end of the polypeptide (CT, partial exon 31) and contains the 3'-untranslated region (3'-UTR). The positions of the primer pair mE23F and mE31R2 used in the nested PCR (short arrows) and the natural termination codon (TGA, open arrowhead) are shown. The regions of aberrant splicing are shown as solid vertical bars between exons, and positions of premature termination codons (PTC) are marked by solid arrowheads. Smaller, and predominant, PCR products in stomach mRNA included partial sequences of intron 29 (striped box) containing a PTC.

the two principal cell types present in the skin. Initial amplification utilizing primers within exons 23 and 31 revealed no distinct bands with 30 cycles of amplification (not shown). Utilization of nested primers in second-round PCR covering segments spanning exons 23–26, 25–29, and 29–31, however, resulted in distinct bands, some of them being smaller than expected from the full size PCR product (Fig 3). Specifically, subcloning and sequencing of the keratinocyte-derived PCR products revealed the absence of exons 24 and 30, whereas in case of fibroblasts deletion of exons 24, 26, and 28 was noted. In case of both keratinocyte and fibroblast PCR products, a PTC for translation was predicted within exon 27 (Fig 3). Thus, although ABCC6 mRNA sequences can be detected both in epidermal keratinocytes and in dermal fibroblasts by nested sec-

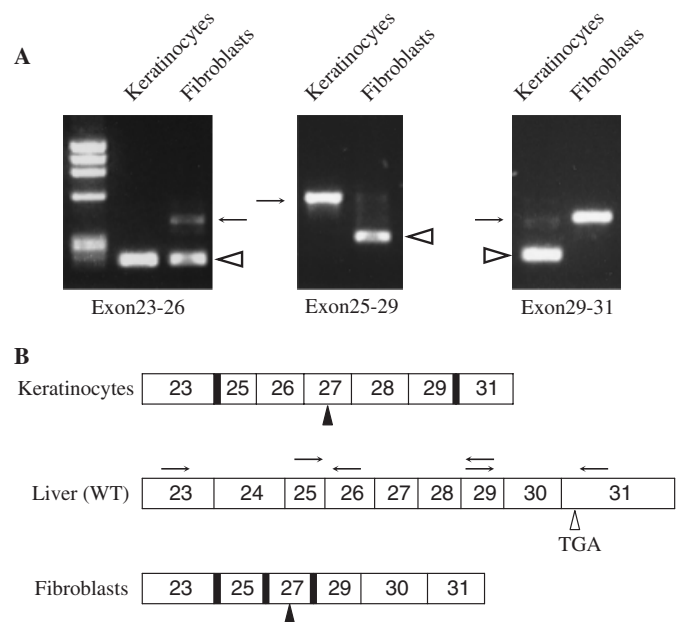


Figure 3
Expression and aberrant splicing of the human ABCC6 gene in cultured keratinocytes and fibroblasts. (A) Nested PCR was performed using 1 μ l of the first-round RT-PCR products obtained with total RNA from the cultured human epidermal keratinocytes and dermal fibroblasts as templates. Amplification with the nested primer pairs (E23U and E26L, E25F and E29L, and E29F and E31L, see Table S1) resulted in bands of the expected size (arrows) as well as in bands that were smaller than expected (arrowheads). (B) The smaller bands were subcloned and subjected to automated sequencing, which showed aberrant splicing in both types of cultured cells. Schematic representation indicates the wild-type sequence of exons 23–31 (WT, open boxes) reflecting normal splicing. The positions of the nested primer pairs (arrows), the natural termination codon (TGA, open arrowhead), and premature termination codons in exon 27 due to aberrant splicing (solid arrowheads) are indicated. The regions of aberrant splicing are shown as solid bars between the exons.

ond round PCR, indicating a very low level of expression, sequence analysis of the PCR products suggests the presence of predominantly truncated mRNA species, which are predicted to result in the synthesis of non-functional MRP6 protein.

Similar RT-PCR analyses for detection of possible alternative splicing in the 5' region (exons 1–22) of the ABCC6 mRNA in human epidermal keratinocytes and dermal fibroblasts were also performed. In fibroblasts, a minor PCR product representing alternatively spliced exon 7 was noted, whereas no evidence for aberrant splicing affecting the remaining exons (1–6 and 8–22) was obtained (not shown). Furthermore, no evidence for alternative splicing affecting exons 1–23 was obtained with keratinocyte RNA. Thus, the alternative splicing in case of ABCC6 mRNA is limited essentially to the 3' end of the transcript.

Discussion

PXE is a heritable disorder with characteristic phenotypic manifestations in the skin, the eyes, and the cardiovascular system (Neldner, 1988; Uitto and Pulkkinen, 2002). The unifying pathologic feature in these three principal tissues

affected in PXE is accumulation of abnormal elastic structures with aberrant mineralization. Based on these observations, PXE was originally thought to be a heritable connective tissue disease with primary abnormalities in elastin or associated microfibrillar components of the elastic structures (McKusick, 1972). Analyses of the candidate genes, including elastin on chromosome 7, and several components of the elastin associated microfibrils on chromosomes 5 and 15, however, were unyielding (see Ringpfeil *et al*, 2001b). Subsequently, positional cloning approaches localized the gene mutated in PXE to chromosomal region 16p13.1 (Le Saux *et al*, 1999; Cai *et al*, 2000), and sequencing of the candidate genes within the critical interval revealed mutations in the ABCC6 gene, a member of the ATP-binding cassette family of genes (Bergen *et al*, 2000; Le Saux *et al*, 2000; Ringpfeil *et al*, 2000; Struk *et al*, 2000). The protein known as MRP6 was initially included in the MRP family due to its structural homology with MRP1, a well-characterized transmembrane efflux pump primarily transporting amphipathic anionic conjugates, such as glutathione-S conjugates as well as glucuronidated and sulfated compounds (Borst *et al*, 1999). On the basis of structural homology, it was suggested that the function of MRP6 could also relate to cellular detoxification.

MRP6 consists of 1,503 amino acids, the primary sequence predicting the presence of three membrane spanning domains (MSD1-3) and two NBF (NBF1, NBF2). The membrane spanning domains are essential for proper topographic insertion of the protein into the plasma membrane, and NBF's play a critical role for MRP6 to serve as a putative metabolic transporter. Most of the mutations encountered in families with PXE consist of PTC, due to nonsense mutations or out-of-frame insertions or deletions, or of missense mutations affecting critical amino acid residues either within the NBF2 or in the intracellular peptide segment connecting two transmembrane segments within MSD3 (see Le Saux *et al*, 2001; Ringpfeil *et al*, 2001a; Uitto *et al*, 2001). These mutations are predicted to result in inactivation of the putative function of MRP6. Interestingly, all mutations characterized thus far are recessive, and inactivation of both alleles of the ABCC6 gene is required for clinical phenotype.

Considering the multi-system nature of PXE, a surprising finding was that the ABCC6 gene was expressed in high abundance in the liver and to a lesser extent in the kidney. In fact, northern hybridizations with RNA from a number of other human tissues, besides liver and kidney, failed to find expression (Belinsky and Kruh, 1999). This and related observations led us to suggest that PXE may be primarily a metabolic disorder at the environment-genome interface, and that absence of MRP6 activity in the liver may result in metabolic perturbation that facilitates calcification of elastic structures in the target tissues (Uitto *et al*, 2001). In support of this interpretation were the clinical observations that PXE, even though clearly a heritable disorder with high degree of penetrance, is not present at birth or during the early childhood, but the diagnosis is established usually in mid to late teens (Neldner, 1988). Furthermore, the disease is slowly progressing, consistent with accumulation of a noxious metabolite(s). At the same time, more sensitive analyses using RT-PCR and RNA protection assays have suggested

more wide-spread tissue expression of the ABCC6 gene, including the presence of the corresponding mRNA transcripts in the skin and vessel wall, in skin fibroblasts and in neural tissues and leukocytes (Bergen *et al*, 2000; Le Saux *et al*, 2000; Beck *et al*, 2003). These observations have led to the suggestion that the ABCC6/MRP6 gene/protein system may play a role in protecting the extracellular milieu against oxidative stress by serving as a transporter of free radical scavengers in the local microenvironment of the skin, the eye, and the vessel wall. In the absence of MRP6 transport activity, oxidative stress damages the connective tissue and promotes calcification. This suggestion is based on the observation that one of the substrates transported by MRP6 in *in vitro* systems is glutathione conjugated to leukotriene C4 and *N*-ethylmaleimide, an established free radical quencher (Belinsky *et al*, 2002; Iliás *et al*, 2002).

Considering the uncertainty regarding the molecular mechanisms involved in development of PXE phenotype at tissue level as a result of ABCC6 mutations, we sought to clarify the tissue expression profile of ABCC6. Our results, obtained by an exquisitely sensitive two-step PCR strategy, clearly demonstrate that liver is the predominant site of expression of this gene, with lower levels of the ABCC6 mRNA in the kidney and small intestine. These data agree with previous observations obtained by using northern analysis and mRNA protection assays (Belinsky and Kruh, 1999; Kool *et al*, 1999). Using the sensitive second-round PCR we were able to show expression of ABCC6 in a number of additional tissues, yet the expression was extremely low. A surprising finding in tissues expressing ABCC6 at very low levels was the presence of mRNA transcripts that were shown to reflect aberrant splicing, particularly in the 3' end of the mouse mRNA affecting the region corresponding to exons 23–31. In some instances, the aberrantly spliced variant was the exclusive (tongue) or predominant (stomach) species of mRNA. Detailed analysis of the splice variants by subcloning and nucleotide sequencing revealed that the deleted exons encode segments of MSD3 or NBF2, domains critical for the function of MRP6 as a putative transmembrane transporter. Furthermore in each case, the deletions were out-of-frame, causing generation of missense sequences and a PTC. Thus, the protein translation products generated from the aberrantly spliced mRNAs are predicted to result in synthesis of a non-functional protein.

Tissue-specific expression of genes is the basic mechanism of development and differentiation in multicellular eukaryotes. Therefore, genes can be classified into either tissue-specific or housekeeping genes. The tissue-specific genes encode proteins involved in functional and phenotypic characteristics of a cell type, and consequently, transcripts of tissue-specific genes are considered to exist only in the corresponding tissues. We show in this study that a highly tissue-specific gene, ABCC6, which is primarily expressed in the liver, is also expressed as spliced transcripts in nonspecific tissues. This finding is consistent with the suggestion that any gene may be transcribed at a very low level in any cell type (Chelly *et al*, 1989). In addition, since aberrant RNA splicing is observed in a high proportion of genes, it is not surprising that alterations in RNA splicing

can cause or be associated by a disease (Faustino *et al*, 2003). Finally, it should be emphasized that the expression of the ABCC6 gene, in tissues other than liver, kidney, and the intestine, as determined at the mRNA level, is extremely low and may well represent illegitimate transcription (Chelly *et al*, 1989). Thus, the extremely low level of expression, combined with demonstration of transcripts predicting synthesis of non-functional protein, all emphasize the point that ABCC6 is expressed predominantly, if not exclusively, in the liver and the kidney.

Examination of cultured epidermal keratinocytes and dermal fibroblasts established from human skin revealed the presence of similar aberrant splicing within the 3' end of the ABCC6 mRNA corresponding to exons 23–31. It should be noted that cultured keratinocytes did not express exon 24, whereas a previous study (Bergen *et al*, 2000) was able to amplify ABCC6 mRNA segments in the skin with an exon 24-specific primer. One explanation for this difference is that cultured keratinocytes might not fully reflect the gene expression spectrum of skin. Alternatively, skin contains a number of other cell types besides keratinocytes, which could contain transcripts retaining exon 24. Again, critical exons encoding MSD3 and NBF2 segments were deleted, and the deletions resulted in a PTC within exon 27 predicting synthesis of a non-functional protein. It is of interest that similar analysis of the remaining 5'-segment of the mRNA, exons 1–23, did not reveal evidence of significant level of aberrant splicing in keratinocytes or fibroblasts. The reasons for the concentration of aberrant splicing to the 3' end of the mRNA are not clear. There is a possibility, however, that aberrant splicing within the exons 1–23 of the transcript may generate PTCs which, when present in the 5' segment of the mRNA, result in rapid loss of the corresponding mRNA transcripts through nonsense mediated mRNA decay (Vasudevan *et al*, 2002). The aberrantly spliced transcripts may not be present to serve as template for RT-PCR, provided that the nonsense mediated decay is exceptionally rapid.

The results presented in this study may complicate molecular diagnostics of PXE. First, mutation detection strategies have been developed to identify pathogenetic lesions in the ABCC6 gene, and a large number of such mutations have been identified using genomic DNA as a template for PCR amplification (see Le Saux *et al*, 2001; Ringpfeil *et al*, 2001b). It should be noted that such analyses have been complicated by the presence of at least two pseudogenes which harbor sequences corresponding to exons 1–9 of the authentic ABCC6 (Pulkkinen *et al*, 2001). It has been suggested that this complication could be avoided by utilizing RT-PCR for mutation detection with RNA as template. Demonstration of extensive aberrant splicing may, however, complicate mutation detection using mRNA as a template. Secondly, our findings that ABCC6 is expressed in high abundance in the liver, as previously demonstrated by others (Belinsky and Kruh, 1999; Kool *et al*, 1999), to a lesser extent in the kidney, and at very low level in the remaining tissues tested, further lend support to the notion of PXE as a metabolic disorder. Even though low level of expression can be found in a number of other tissues, and shown in our study also in cultured keratinocytes and fibroblasts, the two principal cell types in human skin, the predominant species of

ABCC6 mRNA in these cells are aberrantly spliced predicting the synthesis of non-functional protein. It is conceivable, therefore, that the absence of MRP6 in the liver in patients with PXE results in metabolic perturbation which phenotypically affects the target tissues rich in elastic structures.

Materials and Methods

Cell culture Human epidermal keratinocytes were isolated from neonatal foreskin and grown in serum-free keratinocyte growth medium (KGM, Clontec, San Diego, California) as described previously (Tamai *et al*, 1995). Human dermal fibroblasts were isolated from same tissue specimens and maintained in Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU per mL penicillin, and 100 µg per mL streptomycin (Cellgro, Mediatech, Herndon, Virginia).

The use of human tissues was approved by Thomas Jefferson University Institutional Review Board, and all experiments adhere to the principles of Helsinki Declaration Guidelines.

RT-PCR Eight-wk-old male C57BL/6 mice were sacrificed by CO₂ asphyxiation and tissues were immediately excised and homogenized in 1 mL TRIzol reagent (Invitrogen, Carlsbad, California) per 100 mg tissue. Total RNA was isolated from these tissues, as well as from cultured epidermal keratinocytes and dermal fibroblasts, using the TRIzol reagent, as recommended by the manufacturer. First-strand cDNA synthesis was performed by the use of 1 µg total RNA, oligo(dT) primer (Promega, Madison, Wisconsin), and Superscript II reverse transcriptase (Invitrogen), according to the manufacturers' instructions.

Cloning of the 3'-end of the ABCC6 cDNA Sequences of the primer pairs used to detect the mouse exons 22–31 transcripts (mE22F and mE31R, first-round RT-PCR), the mouse exons 23–31 sequences (mE23F and mE31R2, second round nested PCR), the human exons 23–31 (E23F and E31R, first-round RT-PCR), and the human exons 23–26 (E23U and E26L), exons 25–29 (E25F and E29L), and exons 29–31 (E29F and E31L) sequences (all for second-round nested PCR), as well as the primers used for amplification of GAPDH as a control, are shown in Table S1. The annealing temperatures of PCR primers and the expected sizes of the products are also listed in Table S1. A 25 µl PCR reaction mixture consisted of 1 × PCR buffer, 1 × Q-buffer, 1.25 U Taq polymerase (Qiagen, Valencia, California), 200 µM nucleotide mix, 15 pmol each primer, and 1 µl each first-strand cDNA. The amplification conditions were 94°C for 5 min, followed by 30 cycles of (94°C for 45 s, the annealing temperature (52–60°C; see Table S1) for 45 s, and 72°C for 2 min), and one cycle of 72°C for 10 min. Second-round nested PCR was performed using 1 µl each PCR product from the first-round RT-PCR as template. The PCR products were separated by gel electrophoresis on 1.5% agarose gels and stained with ethidium bromide. The products were cloned into the pCR4-TOPO vector as recommended by the manufacturer (Invitrogen). Recombinant plasmids were purified with the Miniprep Kit (Qiagen) and subjected to nucleotide sequence analysis using ABI 377 DNA sequencer.

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Supplementary Material

The following material is available online for this article.

Table S1 Primers used in first-round RT-PCR and in second-round nested PCR of ABCC6 mRNA

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