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Compound Heterozygosity for a Recurrent 16.5-kb *Alu*-Mediated Deletion Mutation and Single-Base-Pair Substitutions in the *ABCC6* Gene Results in Pseudoxanthoma Elasticum

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Pseudoxanthoma elasticum (PXE) is a systemic heritable disorder affecting the elastic structures in the skin, eyes, and cardiovascular system, with considerable morbidity and mortality. Recently, mutations in the *ABCC6* gene (also referred to as "*MRP6*" or "*eMOAT*") encoding multidrug-resistance protein 6 (MRP6), a putative transmembrane ABC transporter protein of unknown function, have been disclosed. Most of the genetic lesions delineated thus far consist of single-base-pair substitutions resulting in nonsense, missense, or splice-site mutations. In this study, we examined four multiplex families with PXE inherited in an autosomal recessive pattern. In each family, the proband was a compound heterozygote for a single-base-pair–substitution mutation and a novel, ~16.5-kb deletion mutation spanning the site of the single-base-pair substitution in *trans*. The deletion mutation was shown to extend from intron 22 to intron 29, resulting in out-of-frame deletion of 1,213 nucleotides from the corresponding mRNA and causing elimination of 505 amino acids from the MRP6 polypeptide. The deletion breakpoints were precisely the same in all four families, which were of different ethnic backgrounds, and haplotype analysis by 13 microsatellite markers suggested that the deletion had occurred independently. Deletion breakpoints within introns 22 and 29 were embedded within *Alu*Sx repeat sequences, specifically in a 16-bp segment of DNA, suggesting *Alu*-mediated homologous recombination as a mechanism.

Introduction

Pseudoxanthoma elasticum (PXE [MIM 177850 and MIM 264800]), a systemic heritable connective-tissue disorder, affects the elastic structures primarily in the skin, eyes, and cardiovascular system (McKusick 1972; Neldner 1988). The characteristic skin findings consist of yellowish papules that tend to coalesce into larger plaques of inelastic skin, with primary predilection in the flexural areas, such as the neck, antecubital fossae, and the groin. Histopathology of biopsies from the clinically affected skin shows accumulation of morphologically altered, often fragmented-appearing, "elastotic" material primarily in the mid dermis (Uitto et al. 1983). Examination of these biopsies by special stains (e.g., von Kossa) reveals that the elastic structures are calcified. In addition, a number of proteins, including osteonectin, fibronectin, and vitronectin, as well as various proteoglycans, have been shown to accumulate in the lesional areas of skin, in association with the elastic fibers (Martinez-Hernandez and Huffer 1974; Baccarani-Contri et al. 1996). Thus, PXE has been traditionally considered as a prototype of heritable connective-tissue diseases with primary involvement of elastic fibers (McKusick 1972).

PXE was originally described as a distinct entity, apart from xanthomas, >100 years ago (Darier 1896), but its molecular basis had remained unresolved. Initially, several candidate genes, including elastin and elastin-associated proteins on chromosomes 5, 7, and 15, were excluded by genetic linkage analysis (Christiano et al. 1992). More recently, genetic linkage to chromosomal region 16p13.1, utilizing positional cloning approaches, was reported (Struk et al. 1997; van Soest et al. 1997), and, subsequently, the interval containing the PXE locus was narrowed to ~500 kb (Le Saux et al. 1999; Cai et al. 2000). Examination of the sequence covering the critical interval revealed the presence of four candidate genes, and, subsequently, we and others disclosed pathogenetic mutations in the *ABCC6* gene (also referred to as "*MRP6*" [multidrug-resistance protein 6] or "*eMOAT*") in a limited number of families with PXE (Bergen et al. 2000; Germain et al. 2000; Le Saux et al. 2000; Ringpfeil et al. 2000; Struk et al. 2000). The majority of the genetic lesions disclosed thus far consist of single-base-pair substitutions, resulting in missense,

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nonsense, or splicing mutations in the *ABCC6* gene. In this study, we have delineated a 16.5-kb, apparently *Alu*-mediated deletion mutation, which is detected in four multiplex families with PXE that are of different ethnic backgrounds.

Material and Methods

PCR Amplification of ABCC6 *Sequences*

Total genomic DNA, obtained from members of four multiplex families with PXE (table 1), was used as a template for PCR amplification of individual exons of *ABCC6,* with *Taq* DNA polymerase with Q-buffer (Qiagen) being used according to the manufacturer's recommendations. The primers were placed on flanking intronic sequences, ⩾32 nucleotides away from the intron-exon borders. The PCR conditions were as follows: 95°C for 3 min; followed by 38 cycles of 95°C for 15 s, annealing temperature of the primers (55°C–60°C) for 30 s, and 72°C for 45 s; and 72°C for 5 min. The sequence information (introns plus exons) of the *ABCC6* gene was obtained from bacterial artificial chromosome (BAC) clone A-962B4 (GenBank accession number U91318), and the intron-exon borders were deduced by comparison with the published cDNA sequence (Kool et al. 1999; GenBank accession number AF076622). The PCR products were examined on 2% agarose gels.

Mutation Detection by Conformation-Sensitive Gel Electrophoresis (CSGE) and Nucleotide Sequencing

The PCR products corresponding to the *ABCC6* exons were first subjected to heteroduplex analysis by CSGE on 6%–12% bis-acrylamide gels (Ganguly et al. 1993; Körkkö et al. 1998). In order to detect homozygous mutations, the PCR products from the affected family members were mixed with an equal amount of the corresponding PCR product from an unrelated, clinically normal control individual (Christiano et al. 1994). If CSGE revealed heteroduplexes, the corresponding PCR products were subjected to direct automated sequencing (ABI 377 sequencing system; PE Biosystems).

Verification of Mutations

Nucleotide changes that were potentially pathogenetic mutations were documented as segregating either with the disease or with obligate heterozygote carriers, by restriction-enzyme digestions, if the sequence variant altered a restriction site, and their absence was verified in 50 unaffected, unrelated healthy control individuals. The digestion products of the restriction-enzyme analysis were examined on either 2%–4% agarose gels or 6% polyacrylamide gels (6% TBE Gels; Novex). Alternatively, the corresponding amplicons were examined by CSGE analysis and/or by direct nucleotide sequencing.

Haplotype Analysis

Microsatellite markers spanning ~9 cM on chromosome 16p13.1, indicated in figure 1 and in table 2 (Genome Database; also see Cai et al. 2000), were used for haplotype analysis by PCR amplification. The PCR products were end-labeled with γ-[^{32}P]dATP and were examined on a 6% polyacrylamide sequencing gel (SequaGel; National Diagnostics). In particular, microsatellite marker D16B9622, residing ~0.7 kb downstream from the translation termination codon within the *ABCC6* gene, was used (Cai et al. 2000).

Characterization of the Deletion Mutation

In order to find the putative deletion, a series of Southern blot analyses were performed. For this purpose, 7 μg of genomic DNA from each of the affected individuals

Table 1

Features of Families with PXE That Have the 16.5-kb Deletion Mutation in *ABCC6*

Proband (Ethnicity)	Age (years)		No. of Affected Family Members	Complementary Mutation	Phenotype[a]
	When Studied	At Disease Onset			
1 (German)	61	9	2	R1164X	Skin—cobblestoning on neck, antecubital fossae, and wrists, sagging skin in axillae and bilateral groin; eyes—angioid streaks, central vision loss; CVS—claudication, ischemic attack; other—ovarian cancer
2 (British)	60	Unknown	3	R1164X	Skin—moderate to severe involvement; eyes—loss of vision
3 (British)	41	Unknown	3	R1141X	Skin—cobblestoning on neck, axillae, and bilateral groin; eyes—angioid streaks; CVS—coronary artery disease, GI bleeding
4 (Greek)	60	51	2	3736−1G→A	Skin—cobblestoning in axillae, bilateral groin, and antecubital fossae; eyes—angioid streaks, central vision loss, macular degeneration; CVS—angina, abdominal pain, loss of peripheral pulses; other—depression, chronic fatigue syndrome

[a] CVS = cardiovascular system; GI = gastrointestinal.

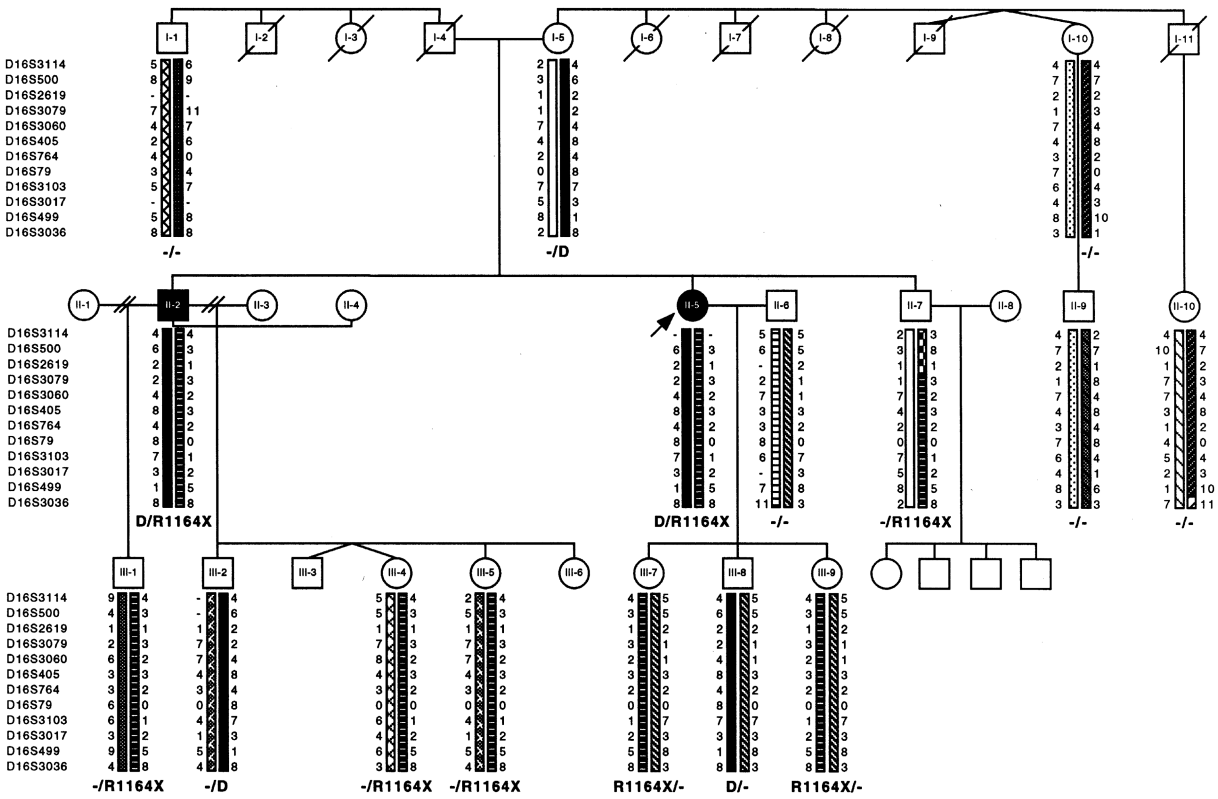

Figure 1 Pedigree of family 1 with PXE. The proband (II-5 [*arrow*]) and her older brother (II-2) were clinically affected. Haplotype analysis with 12 microsatellite markers (*left side*) spanning ~9 cM of 16p13.1 allowed assignment of phase of a deletion mutation (D) and a nonsense mutation (R1164X), as indicated at the bottom of the haplotypes.

and each normal control was digested with different restriction enzymes, including *Spe*I and *Hin*dIII. The digestion products were separated on 0.8% agarose gels and were transferred to nylon membranes (Hybond N+; Amersham). The membranes were hybridized with cDNA, and genomic probes were labeled with α[^{32}-P]dCTP, by use of a Prime-It RmT Random Primer labeling kit (Stratagene). The restriction-enzyme patterns were compared with those obtained from BAC clone A-962B4 containing the *ABCC6* gene. If an altered band was observed, the corresponding region was carefully studied by hybridizations with additional cDNA or genomic probes, to refine the location of the breakpoints.

PCR Detection of the Deletion

After the exact positions of the breakpoints had been confirmed by PCR with a series of primer pairs within introns 22 and 29 (see the Results section), the following primers were designed for diagnostic detection of the deletion mutation in PXE patients: primer 1, 5′-GGA ATA CTC AAG GCG AAT GG-3′; primer 2, 5′-TCT TGA AGC AGC AGT GAG TC-3′; and primer 3, 5′-TTG AGC AGG CTG ACT GTA GG-3′. These three primers were used in a single PCR reaction, with standard conditions being used for amplification. In the case of normal sequence, a 552-bp PCR product is generated by primers 2 and 3, whereas in the case of the deletion mutation primers 1 and 3 result in a 652-bp product. The annealing temperature for these primers was 57°C.

RT-PCR Amplification of ABCC6 *mRNA*

Total RNA was extracted, by use of standard procedures, from dermal fibroblast cultures established either from a carrier of the deletion mutation (see the Results section) or from unrelated controls. cDNA was synthesized (Access RT-PCR system; Promega), and PCR amplification of the *ABCC6* cDNA was performed under the conditions described above for genomic DNA amplification. The primers revealing the deletion were as follows: forward, 5′-GTG AAG GCC ACA GTG CAC C-3′; and reverse, 5′-TCA GAC CAG GCC TGA CTC C-3′. The annealing temperature for these primers was 60°C.

Clinical Assessment of Families with PXE

Members of families 1, 3, and 4 were personally examined at least by one of the authors; information on

Table 2

Haplotypes of Affected Individuals in Four Unrelated Families with PXE

	Haplotype for[b]			
	del/R1164X		del/R1141X;	del/3736−1G→A;
Marker[a]	Family 1	Family 2	Family 3	Family 4
D16S3114	4 4	10 5	9 6	9 4
D16S500	6 3	4 10	4 8	6 10
D16S2619	2 1	3 2	2 3	2 2
D16S3079	2 ***3***	2 ***3***	1 9	8 7
D16S3060	4 ***2***	8 ***2***	7 4	5 8
D16S405	8 ***3***	4 ***3***	4 8	3 4
D16B9622	2 ***2***	2 ***2***	1 2	3 1
D16S764	4 ***2***	3 ***2***	2 2	3 2
D16S79	8 0	3 3	3 3	3 2
D16S3103	7 1	3 1	9 3	7 4
D16S3017	3 2	4 1	5 4	2 4
D16S499	1 5	5 1	8 7	5 8
D16S3036	8 8	7 7	8 11	4 6

[a] The distances between the listed markers are as follows: telomere, D16S3114 (1.9 cM) D16S500 (0.5 cM) D16S2619 (0.7 cM) D16S3079 (0.5 cM) D16S3060 (22 kb) D16S405 (430 kb) D16B9622 (0.7 kb) *ABCC6* (317 kb) D16S764 (8 kb) D16S79 (1.5 cM) D16S3103 (0.4 cM) D16S3017 (0.9 cM) D16S499 (1.5 cM) D16S3036, centromere. The markers flanking the *ABCC6* locus are underlined.

[b] In each column, the numerals to the left denote the alleles harboring the deletion mutation, whereas those to the right denote the complementary alleles that contain a single-base-pair substitution. The shared haplotype in alleles containing the R1164X mutation in families 1 and 2 is in boldface italics.

family 2 was provided by Dr. F. Michael Pope (University Hospital of Wales) (table 1). The diagnosis of PXE was made on the basis of cutaneous manifestations, together with ophthalmologic and cardiovascular findings. In the affected individuals, the diagnosis was confirmed by histopathology of the skin, by hematoxylin-eosin, Verhoeff-van Gieson, and von Kossa stains. Blood samples were obtained from the family members after informed consent had been given, and DNA was isolated by standard procedures.

Results

Strategy for Mutation Analysis and Identification of Single-Base-Pair Substitutions

Families consisting of individuals with definitive diagnosis of PXE were subjected to mutation detection in the *ABCC6* gene. Each of the four families reported here had two or more affected individuals with biopsy-proved PXE, the family pedigrees being consistent with autosomal recessive inheritance. DNA from the affected individuals in each family, as well as from their clinically unaffected family members, was subjected to PCR amplification of the *ABCC6* gene. The PCR products were then subjected to heteroduplex scanning analysis by CSGE, and, if evidence for a heteroduplex was noted, thereby suggesting the presence of a sequence variant, the corresponding PCR product was subjected to automated nucleotide sequencing.

Family 1.—The proband, a 61-year-old female (II-5) with characteristic clinical features of PXE, was first subjected to analysis, together with her mother (I-5) and her three clinically unaffected children (III-7, III-8, and III-9) (fig. 1). Initial heteroduplex scanning of *ABCC6* by CSGE suggested the presence of a sequence variant within exon 24. Comparative sequencing of the proband's PCR product revealed an apparently homozygous single-base-pair substitution, 3490C→T, which resulted in replacement of a codon for arginine by a stop codon, a mutation designated "R1164X" (fig. 2*A*). This mutation, which abolishes an *Aci*I restriction-enzyme site (fig. 2*B*), was not present in 50 unrelated control individuals.

CSGE and sequence analysis of other members of this family indicated that the older, clinically affected brother (II-2) of the proband similarly appeared homozygous for the mutation R1164X. However, the proband's mother (I-5) was apparently homozygous for the normal sequence (fig. 2*A*); DNA was not available from the proband's father, who was deceased. The proband's maternal aunt (I-10) and paternal uncle (I-1) showed the normal allele only, whereas the proband's younger brother (II-7) was clearly heterozygous for the mutation R1164X. Sequence analysis of the children (generation III) of the two affected individuals revealed that five of them were heterozygous for the mutation R1164X, whereas two of them (III-2 and III-8) were apparently homozygous for the normal sequence.

Haplotype analysis in this family was first performed with 12 microsatellite markers flanking the *ABCC6* locus on chromosome 16p13.1 (fig. 1). This analysis clearly indicated that the two affected individuals had inherited one allele from their mother (I-5); the other allele was presumably of paternal origin (i.e., from individual I-4). The inheritance of the latter allele conferred heterozygous carrier status of the mutation R1164X to individuals II-7, III-1, III-4, III-5, III-7, and III-9 (fig. 1). At the same time, inheritance of the maternally derived allele from both affected individuals (II-2 and II-5) to their respective offspring (III-2 and III-8) resulted in apparent homozygosity for the normal allele. Collectively, these findings suggest that the proband (II-5) and her clinically affected older brother (II-2) had inherited from their father a nonsense mutation R1164X in exon 24 of the *ABCC6* gene, and they had inherited from their mother a deletion mutation spanning exon 24 of the gene, thus reducing the paternal nonsense mutation to hemizygosity.

Family 2.—The proband was a 60-year-old male with characteristic clinical features of PXE, including loss of vision and extensive cutaneous involvement. His older

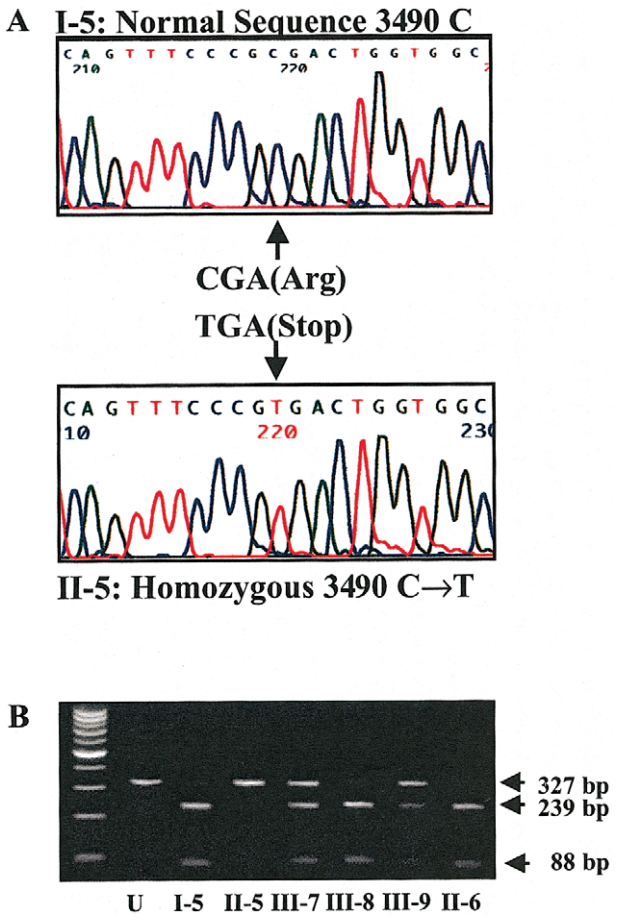

Figure 2 Identification of a nonsense mutation in *ABCC6* in family 1 with PXE. *A,* Results of automated sequencing of the proband's PCR product, which revealed an apparently homozygous single-base-pair substitution, 3490C→T (*bottom*), in comparison with her mother's sequence (*top*), which showed an apparently homozygous normal sequence, 3490C. Sequencing of PCR products of III-7 and III-9 (see fig. 1) showed heterozygous 3490C/T (not shown). The C→T transition changed the codon for Arg (CGA) to a stop codon (TGA). *B,* The C→T substitution abolished an *Aci*I restriction site. Digestion of the 327-bp PCR product (uncut [*lane U*] resulted, in the case of the normal allele, in 239- and 88-bp fragments. Digestions with *Aci*I confirmed that the proband, II-5, is homozygous for the mutant allele, that her mother, I-5, is homozygous for the wild-type allele, and that two of the proband's children, III-7 and III-9, are heterozygous carriers, whereas another son, III-8, is apparently homozygous for the normal sequence.

brother was similarly affected. The proband had three children, and the oldest one, a 39-year-old female, was not affected. The proband's second child, a son, was reported to have clinical findings consistent with PXE, but he was not available for examination. The youngest child, also a son, was subjected to clinical examination at the age of 14 years and was reported to have signs of PXE, but the diagnosis was not confirmed by skin biopsy. Initial mutation detection by PCR amplification of *ABCC6,* followed by CSGE and nucleotide sequencing, demonstrated the presence of an apparently homozygous R1164X mutation in both the proband and his older brother. This mutation is identical to that in family 1. Restriction-enzyme digestion with *Aci*I revealed that, of the proband's three children, the daughter and the younger son showed evidence for the wild-type allele only, while the middle child was clearly heterozygous for the R1164X mutation. The two children of the proband's daughter, who were examined at the ages of 12 and 10 years, respectively, were clinically normal and did not show evidence for the R1164X mutation. If the proband were truly homozygous for the R1164X mutation, his children would be expected to be heterozygous carriers of this mutation. Since this mutation was detected in only one of his three children, it was postulated that the proband may harbor a deletion mutation in his other *ABCC6* allele

Families 3 and 4.—We have previously described two families with PXE for which haplotype analysis suggested a deletion mutation in one of the *ABCC6* alleles, while point mutations were segregating in the other allele (Ringpfeil et al. 2000). In these families, designated here as "family 3" (family 3 in Ringpfeil et al. 2000) and "family 4" (family 4 in Ringpfeil et al. 2000), a nonsense mutation (R1141X) and a splicing mutation (3736−1G→A) are located in exon 24 and in the 3′ acceptor splice site of intron 26, respectively. Haplotype analysis suggests that the putative deletion mutation in both families affected the regions spanning exons 24 and 27, respectively, reducing the single-base-pair–substitution mutations to hemizygosity.

Characterization of the Deletion Mutation

In order to delineate the extent of the putative deletion mutation in family 1, microsatellite markers flanking *ABCC6* and intragenic polymorphisms were utilized (see table 2). First, D16B9622, which resides ~0.7 kb downstream from the translation termination codon, indicated that individuals who carry the putative deletion mutation are distinctly heterozygous for the maternal and paternal alleles (table 2). This finding suggests that the deletion, which clearly affects exon 24, does not extend beyond the 3′ end of the gene. Second, examination of an intragenic polymorphism (2490C→T) in exon 19 revealed heterozygosity in the proband's DNA, suggesting that the deletion breakpoints reside between exon 19 and the marker D16B9622 at the 3′ end of the gene.

To pinpoint the location of the breakpoints resulting in the deletion mutation, a series of Southern blot analyses were performed. First, hybridization of *Hin*dIII restriction-enzyme digests with a cDNA probe spanning exons 27–31 revealed two bands, 11.9 and 3.7 kb in size, in normal control individuals, whereas parallel digestions and hybridizations with DNA from the pro-

bands in families 1–4 revealed an additional band, 8.8 kb in size (fig. 3*B*). Hybridizations of the *Spe*I digests with the cDNA probe corresponding to exons 27–31 further suggested that the 3′ breakpoint resides within intron 29. Similar hybridizations of *Spe*I restriction-enzyme digests, with a cDNA spanning exons 17–26, revealed the presence of several constant bands both in the controls and in the affected individuals (not shown). However, an additional band, of ~11 kb, was noted in the four probands, suggesting that the deletion is identical in all four families. The 11-kb band was also seen when the *Spe*I digests were probed with a cDNA corresponding to exons 22 and 23, localizing the 5′ breakpoint to this general region. Finally, hybridization of *Hin*dIII digests with the latter cDNA probe refined the location of the 5′ breakpoint as residing between a *Hin*dIII site within intron 22 and the beginning of exon 23. Collectively, these results suggest that the probands in all four families harbor a deletion mutation in one of their alleles, most likely involving breakpoints within introns 22 and 29.

In order to identify the precise breakpoint sequences, a series of primer pairs corresponding to sequences of intron 22 and of intron 29 were synthesized and were used for PCR amplification of genomic DNA both from controls and the probands in families 1–4. Sequencing of the PCR products resulted in identification of the breakpoints within intron 22 (nucleotide 47322) and within intron 29 (nucleotide 30869). This deletion was calculated to be 16.5 kb and was predicted to delete exons 23–29 from the gene (fig. 3*A*). Identification of these breakpoints allowed us to develop an amplification system consisting of three primers, as shown in figure 3*A*, with two of the primers (i.e., primers 1 and 3) being placed just outside the breakpoints, whereas the third primer (i.e., primer 2) was localized inside the deletion. In the case of normal sequence a 552-bp PCR product is generated by primers 2 and 3, whereas in the case of

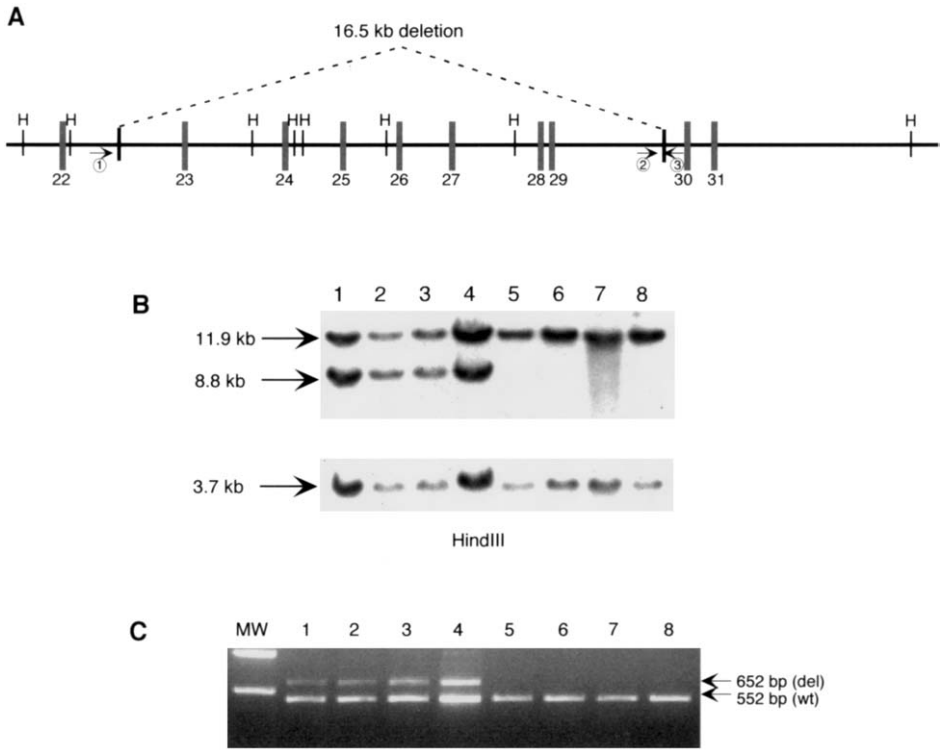

Figure 3 Characterization of the 16.5-kb deletion mutation in *ABCC6* in PXE. *A*, Schematic representation of the 3′ end of *ABCC6*, including exons 22–31. Positions of *Hin*dIII restriction enzyme sites (H) are indicated. Note that the deletion mutation extends from intron 22 to intron 29. The positions of the three primers used for PCR detection of the deletion mutation are indicated (*circled numbers*) *B*, Southern analysis of *Hin*dIII digests of DNA isolated from the probands in the four families with PXE (families 1–4 [*lanes 1–4, respectively*]) and from four healthy unrelated controls (*lanes 5–8*). The hybridization was performed with a cDNA probe extending from exon 27 to exon 31. Note the presence of two constant bands, of 11.9 and 3.7 kb, whereas the individuals with PXE demonstrate an additional, 8.8-kb band. *C*, PCR analysis of the deletion mutation in patients with PXE. Three PCR primers at positions shown in panel *A* and employed in the same PCR reaction were used for amplification of genomic DNA either from patients with PXE (*lanes 1–4*) or from unrelated healthy controls (*lanes 5–8*). In control individuals, amplification with primers 2 and 3 results in a 552-bp band (wt), whereas, in case of the heterozygous deletion mutation, primers 1 and 3 result in an additional band, of 652 bp (del).

the deletion a 652-bp PCR product is observed with primers 1 and 3 (fig. 3*C*). This PCR assay provides a rapid means to identify the presence of this 16.5-kb deletion in families with PXE. Consequently, the deletion breakpoints, as determined by sequencing of the PCR products generated by primers 1 and 3, were found to be identical in all four families reported here. Examination of a cohort of 26 additional families with PXE did not reveal the presence of this deletion. Thus, the frequency of this deletion mutation in the patient population studied is 1/15. The incidence of PXE has been reported to be 1:160,000 (Sherer et al. 1999). On the basis of this estimate, the frequency of carriers of PXE is calculated to be 1/200, and the frequency of the deletion mutation delineated in this study is 1/3,000. Finally, extensive Southern analyses did not reveal evidence for other large deletions in the DNA of other patients with PXE (data not shown).

Consequences of the Deletion at the mRNA Level

To verify the consequences that the genomic deletion has at the mRNA level, reverse transcriptase–PCR using skin fibroblast RNA as template was performed with primers placed on exons 22 and 31 of the corresponding mRNA. Sequencing of the PCR product clearly indicated the absence of 1,213 nucleotides corresponding to exons 23–29 (nucleotides 2997–4208; GenBank accession number AF168791) (fig. 4*A*). This mutation predicts deletion of 405 amino acids, encoded by exons 23–29, from the MRP6 protein, followed by a 60-amino-acid missense sequence and a premature termination codon (PTC) for translation within exon 30. This results in elimination of most of the third transmembrane spanning domain (TMSD3) sequence, as well as of the entire second nucleotide-binding fold (NBF2) sequence (fig. 4*A*). Elimination of TMSD3 and NBF2 sequences predicts synthesis of a nonfunctional polypeptide.

Haplotype Analysis of Families 1–4

The recurrent 16.5-kb genomic deletion in one allele of patients with PXE could reflect the presence of a founder effect, or alternatively, it could be a recurring event. To examine these two possibilities, a panel of 13 microsatellite markers flanking the *ABCC6* locus was utilized for haplotype analysis. These microsatellites span ~9 cM of DNA, and their distances are specified in the legend to table 2. Note that the distance between the two markers closest to the *ABCC6* locus is 390 kb, indicating that the likelihood of recombination is significantly less than 1%. Examination of the haplotype of the alleles, specifically those of the two closest markers (D16B9622 and D16S764) containing the deletion mutation indicated that each proband had a distinct haplotype (table 2). Thus, there is no evidence of common ancestry, and the deletion mutation appears to have arisen independently in these four families.

The deletion mutation in each of these four families is associated with single-base-pair substitutions, two of them being the same one—R1164X in families 1 and 2. Haplotype analysis of the allele containing this nonsense mutation revealed a similar haplotype for five markers directly flanking the PXE-gene locus, between D16S3079 and D16S764, spanning ~1.5 cM (table 2). Thus, a founder effect for the R1164X mutation cannot be excluded with certainty, despite the apparently diverse ethnic backgrounds of these two families (table 1).

Discussion

The *ABCC6* gene encoding an ABC transporter protein, MRP6, has recently been shown to harbor mutations underlying PXE, most of the genetic lesions being single-base-pair substitutions resulting in nonsense, missense, or splice-site mutations (Bergen et al. 2000; Germain et al. 2000; Le Saux et al. 2000; Ringpfeil et al. 2000; Struk et al. 2000). The gene consists of 31 distinct exons spanning ~73 kb of DNA, and the corresponding mRNA, ~6 kb in size with an open reading frame of 4.5 kb, encodes a polypeptide of 1,503 amino acids with a calculated molecular mass of 165 kD (Belinsky and Kruh 1999; Kool et al. 1999). MRP6 has a high degree of identity (~62%) with MRP1, the prototype within the MRP family of proteins (Belinsky and Kruh 1999). The function of MRP6 is currently unknown, but, in analogy to other members of the MRP family of transmembrane transporter proteins, it has been proposed to serve as an efflux pump eliminating metabolic compounds from the intracellular milieu.

The utility of strategies to identify PXE mutations in *ABCC6* has been demonstrated in this study in four multiplex families with PXE. In each family, the proband was initially shown to be apparently homozygous for a nucleotide substitution, resulting in either a nonsense mutation (R1164X, R1164X, and R1141X in families 1, 2, and 3, respectively) or a splice-site mutation (3736−1G→A in family 4). These nucleotide substitutions were not present in 50 unrelated control individuals, excluding these substitutions from being polymorphisms. Interestingly, however, some children of the affected individuals in families 1, 2, and 4 showed the presence of the normal sequence only. It was concluded, therefore, that the affected individuals were compound heterozygotes for a single-base-pair–substitution mutation and a deletion mutation in *ABCC6,* and the presence of the deletion mutation reduces the single-base-pair–substitution mutation to hemizygosity.

The recurrent deletion mutation, delineated in this

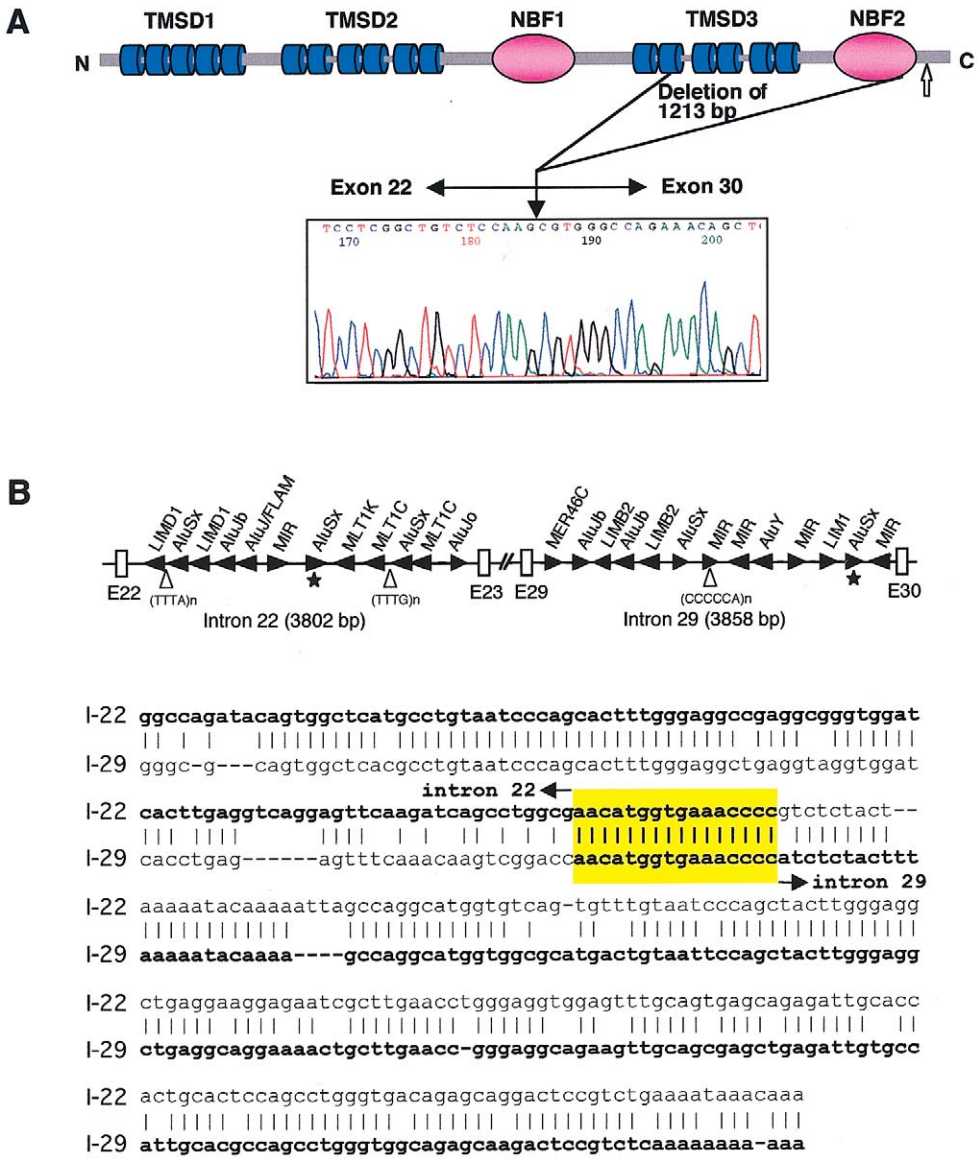

Figure 4 Schematic structure of the MRP6 protein, consisting of three TMSDs (TMSD1–TMSD3) and two NBFs (NBF1 and NBF2) (*A*) and the repetitive sequences surrounding the breakpoints in *ABCC6* (*B*). *A,* 16.5-kb genomic deletion, resulting in out-of-frame elimination of 1,213 bp from the *ABCC6* mRNA corresponding to exons 23–29. At the protein level, deletion of these exons is predicted to result in elimination of most of the sequences within TMSD3 as well as of the entire NBF2. A downstream PTC for translation, resulting in truncation of the polypeptide at amino acid position 1463, is indicated by a white arrow. *B,* Abundance of repetitive sequences within introns 22 and 29. *Alu* and other interspersed repetitive elements are denoted by black arrows along the intronic sequences, and the types of these elements are identified the arrows. The white arrowheads denote the positions of simple repeats, and the asterisks denote the positions of the *Alu*Sx repeats containing the deletion breakpoints (*top*). Note the overall sequence homology in *Alu*Sx repeats, including a 16-bp sequence containing the deletion breakpoints (*yellow*), within introns 22 and 29 (*bottom*). The boldface type represents continuation of the DNA strands after the deletion event.

study of four unrelated families, has been shown to span ~16.5 kb of the *ABCC6* gene, from intron 22 to intron 29. This deletion is predicted to result in an MRP6 polypeptide, which is shortened by 505 amino acids, the last 60 amino acids being missense sequences. This polypeptide, which is devoid of intact TMSD3 and NBF2, is nonfunctional. In all four families examined, the breakpoints within intron 22 and intron 29 have been shown by direct nucleotide sequencing to be precisely the same.

Analysis of the sequences spanning the deletion breakpoints to detect possible repetitive elements was

performed by the RepeatMasker Documentation program and suggested that the deletion mechanism is *Alu* mediated. Specifically, a recombination event between two highly homologous *Alu*Sx repeats within introns 22 and 29 is likely responsible for the occurrence of the deletion, and the site of the crossover event lies within a 16-bp sequence (aacatggtgaaacccc) (fig. 4*B*). Similar analysis of the entire *ABCC6* gene revealed a high content of repetitive elements, which account for ~60% of all intronic sequences. Specifically, the introns 22 and 29 harbor 14 repetitive sequence elements each, accounting for 67% and 75% of the intronic sequences, respectively. These repetitive elements are depicted in figure 4*B,* and include, in addition to *Alu* and other interspersed repetitive elements, three simple repeats, $(TTTA)_n$, $(TTTG)_n$, and $(CCCCCA)_n$. *Alu*-mediated rearrangements have been shown to be a cause of a number of inherited diseases, such as Fanconi anemia (Morgan et al. 1999), familial hypercholesterolemia (Lehrman et al. 1987), and hereditary angioedema (Stoppa-Lyonnet et al. 1990), as well as a cause of predisposition to colon cancer (Nyström-Lahti et al. 1995) and breast cancer (Puget et al. 1999). In addition, acute myeloid leukemia can be caused by *Alu*-mediated recombinational events in somatic tissue (Strout et al. 1998). It is conceivable that, because of the abundance of repetitive sequence elements, the *ABCC6* gene is prone to *Alu*-mediated rearrangements, such as deletions, insertions, duplications, or inversions. These possibilities should be taken into account when one is planning strategies for the search of the gene defects in PXE.

The MRP6 protein consists of three TMSDs (TMSD1–TMSD3), and it has two intracellular NBFs, both critical for the function of the protein as a transmembrane transporter (Belinsky and Kruh 1999) (fig. 4*A*). Exon 24 encodes a segment of MRP6 residing within TMSD3, and the mutations R1141X and R1164X are predicted to result in the synthesis of a truncated polypeptide entirely devoid of NBF2 (fig. 4*A*). In addition, these PTC mutations could result in reduction of the MRP6-transcript levels, through nonsense mutation–mediated mRNA decay (Hentze and Kulozik 1999). Similarly, the splicing mutation 3736−1G→A (Ringpfeil et al. 2000) predicts in-frame splicing of exon 27, resulting in deletion of 49 amino acids within the NBF2 domain. As a consequence, no functional MRP6 is predicted to be synthesized, which results in the PXE phenotype.

The specific biological function of MRP6, which is primarily expressed in the liver and the kidneys, is currently unknown (Belinsky and Kruh 1999; Kool et al. 1999). However, MRP1, the best-characterized protein within the MRP family, functions as an efflux pump for amphipathic anion conjugates. One could speculate that the mechanisms involved in the development of PXE could be analogous to those in Dubin-Johnson syndrome, an autosomal recessive disorder characterized by conjugated hyperbilirubinemia with otherwise normal liver function (MIM 237500). In this condition, MRP2, another ABC transporter within the MRP family of proteins, is inactivated by mutations affecting the NBFs (Wada et al. 1998; Toh et al. 1999). As a result of deficiency in MRP2, up-regulation of MRP3 has been observed (König et al. 1999). Since MRP2 is located apically whereas MRP3 resides basolaterally (Paulusma et al. 1996; König et al. 1999), the metabolites are not removed from hepatocytes to the bile; instead, the compounds are conjugated in the liver and transported back to the systemic circulation. Thus, MRP6 function may relate to cellular detoxification, and, when genetically altered to be nonfunctional, it may cause accumulation of yet-unidentified compounds that potentially lead to progressive calcification of elastic structures in the target tissues. If this hypothesis is proved to be correct, then PXE could be considered to be a metabolic disease, rather than a primary heritable connective-tissue disorder (Uitto et al. 2001).

PXE demonstrates considerable clinical and genetic heterogeneity (Pope 1975; Neldner 1988). Although many families with PXE present with autosomal recessive inheritance, an autosomal dominant pattern also has been reported, and many of the cases appear to be sporadic. It has been suggested that, in families in which PXE is autosomal recessive, the heterozygous carriers may show partial manifestations of the disease (Altman et al. 1974; Neldner 1988; Bacchelli et al. 1999). However, clinical identification of the affected individuals and potential carriers with partial manifestations is complicated by delayed onset of the clinical findings, as well as by the fact that cutaneous signs mimicking PXE are frequently encountered in sun-damaged areas of skin in older individuals. Furthermore, other diagnostic manifestations of PXE, such as cardiovascular involvement, are prevalent in the general population, although usually at a later age (Mendelsohn et al. 1978; Neldner 1988). This diagnostic dilemma in PXE can now be overcome by delineation of specific mutations in the *ABCC6* gene, which allows both unequivocal identification of the affected individuals and DNA-based carrier detection in families with a history of PXE.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank Overview, http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html (for BAC clone A-962B4 [accession number U91318], published cDNA sequence [accession number AF076622], and nucleotides 2997–4208 of exons 23–29 [accession number AF168791])

Genome Database, The, http://www.gdb.org (for microsatellite markers)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for PXE [MIM 177850 and MIM 264800] and for Dubin-Johnson syndrome [MIM 237500])

RepeatMasker Documentation, http://ftp.genome.washington.edu/RM/RepeatMasker.html

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