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Spectrum of Mutations in the Gene Encoding the Adrenoleukodystrophy Protein

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Summary

X-linked adrenoleukodystrophy (ALD) has been associated with mutations in a gene encoding an ATP-binding transporter, which is located in the peroxisomal membrane. Deficiency of the gene leads to impaired peroxisomal β-oxidation. Systematic analysis of the open reading frame of the ALD gene, using reverse transcriptase–PCR, followed by direct sequencing, revealed mutations in all 28 unrelated kindreds analyzed. No entire gene deletions or drastic promoter mutations were detected. In only one kindred did the mutation involve multiple exons. The other mutations were small alterations leading to missense (13 of 28) or nonsense mutations, a single amino acid deletion, frameshifts, or splice acceptor–site defects. Mutations affecting a single amino acid were concentrated in the region between the third and fourth putative transmembrane domains and in the ATP-binding domain. Mutations were detected in all investigated ALD kindreds, suggesting that this gene is the only gene responsible for X-linked ALD. This overview of mutations is useful in the determination of structurally and functionally important regions and provides an efficient screening strategy for identification of mutations in the ALD gene.

Introduction

X-linked adrenoleukodystrophy (ALD) is a peroxisomal disorder characterized by impaired peroxisomal β-oxidation of very-long-chain fatty acids (VLCFAs), which probably is a consequence of reduced activation of the VLCFAs. This results in demyelination of the nervous system and adrenocortical insufficiency. The phenotype of ALD is highly variable, the most frequent phenotypes being childhood cerebral ALD, adrenomyeloneuropathy (AMN), and adrenocortical insufficiency (Addison disease). All male ALD patients and most carriers have an elevated level of VLCFA in plasma and fibroblasts, making the diagnosis of ALD relatively straightforward (for overview see Moser and Moser 1989).

Recently, a gene responsible for the disease in ALD patients was identified, using positional cloning, on the basis of its localization in Xq28 close to the color-vision genes. The ALD gene belongs to the superfamily of ATP-binding cassette (ABC)–transporter genes and encodes a protein, ALDP, containing six putative membrane-spanning regions and one ATP-binding domain (Mosser et al. 1993). ALDP shows 30% identity with PMP70, a peroxisomal membrane protein also belonging to the ABC-transporter family (Kamijo et al. 1990). Monoclonal antibodies raised against part of ALDP showed the protein to be located in the peroxisomal membrane. This suggests that this protein plays a role in the active transport of enzymes, cofactors, and/or substrates involved in the β-oxidation of VLCFA over the peroxisomal membrane (Contreras et al. 1994; Mosser et al. 1994). Intragenic deletions were initially found in ~6% of the unrelated ALD patients analyzed (Mosser et al. 1993). Also, two point mutations, one leading to a missense and the other to a nonsense mutation, have been described more recently (Cartier et al. 1993; Uchiyama et al. 1994). Moreover, ALDP could not be detected in lymphoblast and fibroblast lines of several ALD patients (Contreras et al. 1994; Mosser et al. 1994).

We report the first systematic analysis of ALD mutations, over the whole protein-coding region, in a large group of patients. We identified ALD mutations in all 28 independent kindreds studied. This overview of the type and position of the mutations gives more insight into the structurally and functionally important regions of the protein and facilitates the design of a strategy for the identification of mutations in other ALD kindreds.

Material and Methods

Oligonucleotide Primers

For nomenclature of the primers, the positions of the 5′ end of the oligonucleotides are indicated, followed by "F," for forward, or "R," for reverse primers. Positions refer to those given by Mosser et al. (1993) (EMBL database Z21876). Primers followed by an asterisk are located

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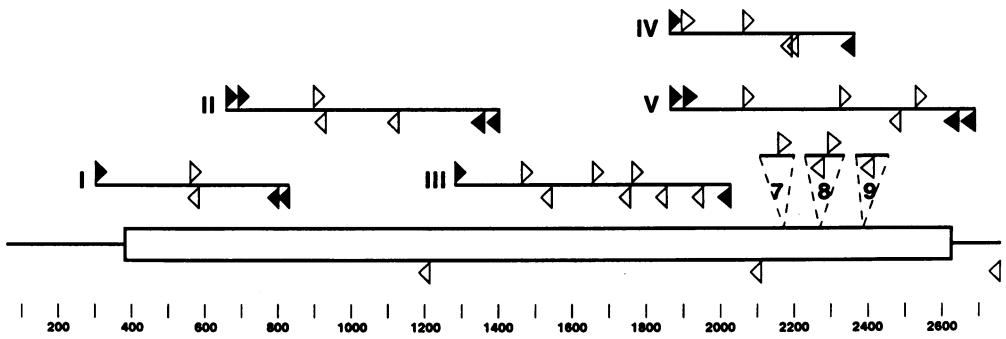

Figure 1 Schematic representation of primer positions. The box represents the open reading frame of the ALD mRNA. The lines before and behind the box represent the 5′ UTR and 3′ UTR, respectively. Lines 7–9 represent introns in which primers were chosen to confirm the mutations on genomic DNA (not to scale); lines I–V represent the overlapping PCR products that cover the entire open reading frame of the cDNA. Blackened triangles represent primers used in the amplification of the cDNA and sequencing (see also table 1); and unblackened triangles indicate primers used for sequencing of these PCR products. Primers depicted in the intron sequences and below the cDNA are used only in the confirmation of the mutations on genomic DNA. Triangles pointing to the right indicate forward primers, and those pointing to the left indicate reverse primers. The approximate position of the primers is indicated by the scale bar, denoting the nucleotide number of the cDNA (see Mosser et al. 1993). Primer sequences are as follows: 303F, AGCAACAATCCTTCCAGCC; 576F, AAAGCTGGCATGAACCGGG; 685F, CCTTGGTGAGCCGCACCTTC; 702F, TTCCTGTCGGTGTATGTGGC; 914F, CCAGCAGACCTACTACCGGG; 1300F, TGCTACAGCGCTCCTACCAG; 1479F, GTGAAGAAGGCAGCCTTGGA; 1681F, GTCACTTCAAGAGGCCCAGG; 1781F, CCAGGTGGTGGATGTGGAA; 1870F, TCAGGGTGGAGGAAGGCAT; 1880F, GGAAGGCATGCATCTGCTC; 1890F, CATCTGCTCATCACAGGCCC; 2061F, TACCCGGACTCAGTGGAGGA; 2312F, GATCTTCCAGGCGGCCAAGG; 2527F, AGGAGCTCTGCCAGATCCTG; 593R, CCGGTTCATGCCAGCTTTGGC; 821R, GGTAGCAGGGAGGGCGATGA; 840R, GGATGGCACTGTTGACGAAGG; 931R, CGGTAGTAGGTCTGCTGGGA; 1145R, GAGGAACACCACGAGGCCGG; 1231R, TGTAGCGCAGCTCCCCCTTC; 1384R, AACTGCTCCAGCATAACATACCAC; 1409R, GGCGCTCCACACATACTTCA; 1542R, TGAAGGCTTCTGTGCGCTC; 1752R, GGACACCAGACCGGCCTAT; 1861R, CTGGCCACCACCACCTCT; 1975R, ACACCACCGTACGTGGGC; 2016R, GCGGGATGTAGAACATGC; 2114R, CAGGTCCTGCTCCGAGTAGC; 2194R, TCCTTCCAGTCACACATAGCCTC; 2204R, CGACAGGACGTCCTTCCAGT; 2366R, CCGGTGGGTGATGGAGAG; 2478R, GCTGCTTCTCCTCCGTCAG; 2669R, TGTGATCCGAGCTTGGGG; 2686R, TGCTGCTGTCTCCTTCATGTG; 2742R, GGGTTTTCTAGGAGGAGGGG; 544F*, CAGGGGCCCTGTCGTCACAG (intron 7); 849F*, TGGAGGGTGCACAGACTCTC (intron 8); 876R*, GCCGAGAGGAGAGTCTGTGC (intron 8); and 1078R*, AGGCCACCTCCTCCCCTCAG (intron 9).

in introns, and the numbers refer to their position in a partial genomic DNA sequence encompassing part of intron 7 through exon 10 (Sarde et al. 1994). For a schematic presentation of the primer positions and sequences, see figure 1.

Cell Lines, RNA Isolation, Reverse-Transcriptase–PCR, and Sequencing

Fibroblast lines from 25 male patients and 3 female carriers, who were diagnosed on the basis of clinical findings and elevated levels of VLCFAs in plasma or fibroblasts from 28 unrelated ALD kindreds, were cultured in Dulbecco's modified Eagle medium, supplemented with 10% FCS and 50 μg gentamycin/ml or 50 U penicillin/ml and 50 U streptomycin/ml. On trypsinization, the cells were washed twice in ice-cold PBS. RNA was isolated using guanidinium isothiocyanate, as described elsewhere (Kemp et al. 1994*b*) or RNAzol (Biotecx), according to the protocol supplied by the manufacturer. Reverse transcription was performed with random primers, using Maloney murine leukemia virus–reverse transcriptase (Gibco-BRL). The cDNA was amplified as five overlapping fragments (see fig. 1 and table 1). When two subsequent PCR reactions were used, 5 μl of the first reaction was transferred to the second reaction, making an end volume of 100 μl. PCR reactions of fragment 1 were performed in 10 mM Tris-HCl pH 8.4, 2.25 mM $MgCl_2$, 50 mM NaCl, 0.01% gelatin, 0.1% Triton X-100, 10% dimethylsulfoxide (DMSO), 0.2 μg BSA/μl (Boehringer-Mannheim), 0.65 mM dNTP, 1 ng each forward and reverse primer/μl, and 0.05 U AmpliTaq DNA polymerase/μl (Perkin-Elmer). For fragments 2 and 5, 67 mM Tris-HCl pH 8.8, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10mM β-mercaptoethanol, 10% DMSO, 0.2 μg BSA/μl, 0.65 mM dNTP, 1 ng each forward and reverse primer/μl, and 0.05 U AmpliTaq DNA polymerase/μl were used. PCR was performed for 30 cycles (1 min at 93°C, 1.5 min at 55°C, and 2 min at 72°C for fragment 1; and 1 min at 94°C, 1.5 min at 59°C, and 2 min at 72°C for fragments 2 and 5). PCRs of fragments 3 and 4 were performed as described by Kemp et al. (1994*b*). To remove free primers and nucleotides, products of fragments 1, 2, and 5 were separated on agarose gels, extracted from the gel, and precipitated. For fragments 3 and 4, the QIAquick-spin PCR purification kit (Qiagen) was used.

Sequencing of fragments 1, 2, and 5 was performed with the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems and Perkin-Elmer), using the primers indicated in figure 1 and table 1, according to the protocol supplied by the manufacturer. The sequence reactions were run and analyzed, using an automatic sequencer (ABI 373A). Sequencing and analysis of fragments 3 and 4 were performed as described by Kemp et al. (1994*b*).

Table 1

Primers Used for PCR and Sequencing of ALD cDNA

cDNA Fragment[a]	First-Step PCR	Second-Step PCR	Forward Sequencing	Reverse Sequencing
I	303F–840R	303F–821R	303F, 576F	593R; 840R
II	685F–1409R	702F–1384R	702F, 914F	931R; 1145R; 1384R
III	1300F–2016R	Not done	1300F; 1479F; 1681F; 1781F; 1880F	1542R; 1752R; 1861R; 1975R
IV	1870F–2366R	Not done	1880F; 2061F	2194R; 2204R
V	1870F–2686R	1890F–2669R	2061F; 2312F; 2527F	2478R; 2669R

NOTE.—Numbers followed by "R" or "F" indicate primers used in the PCR.

[a] Five overlapping fragments in which the open reading frame of the cDNA was amplified.

Confirmation of Mutations on Genomic DNA

Chromosomal DNA was isolated from EDTA-anticoagulated blood or fibroblasts as described by Kemp et al. (1994*b*) or using the salt-extraction method (Miller et al. 1988). Amplification of 100 ng of genomic DNA was performed in 100 μl, using oligonucleotide primers on both sides of the mutation (see table 2). Amplification with primers 303F and 821R was performed, as described above for fragment 1 of the cDNA, for primers 1479F and 1861R, and 1781F and 1861R conditions were used as described by Kemp et al. (1994*b*). The remaining PCRs were performed as described for fragment 2 and 4 of the cDNA. Mutations previously detected on cDNA were confirmed by *Hpa*II digestion (T1045C and C929A), by analysis of the length of a radioactively labeled PCR product on a polyacrylamide gel (del 1801–1802) (Kemp et al. 1994*b*) or by direct sequencing of the PCR product as described above for the cDNA.

To increase the specificity for genomic ALD sequences in Xq28, the amplification was performed in two subsequent steps for some of the PCRs (table 2, note g). For the first step, 0.1 ng DNA/μl was amplified in 10 cycles. Subsequently, 5 μl product was transferred to the second reaction, with a final volume of 100 μl. For all sequence reactions spanning exons 8–10, the annealing temperature in the cycle sequencing reaction was increased to 60°C, which is identical to the elongation temperature of the reaction. These conditions were used to exploit sequence differences between the ALD gene and homologous sequences that are not located on Xq28.

Results

Detection of Mutations Affecting the ALD mRNA

Mutations in the ALD gene were analyzed in one affected person from each of 28 kindreds, by sequencing the ALD cDNA derived from male patients or female carriers. Overlapping PCR fragments representing the entire open reading frame were sequenced directly on both strands (for primers see fig. 1 and table 1). Only when a mutation resulting in a truncated protein product was detected and confirmed on genomic DNA (see below) was sequencing of the entire open reading frame not completed.

Fragments representing ALD mRNA were obtained in all fibroblast lines. Since the PCRs were not necessarily performed in the linear range of amplification, and since this method of detection is able to reflect even very low amounts of ALD mRNA, the level of transcription of the ALD gene cannot be judged. Therefore, this method enabled us merely to detect qualitative alterations or complete absence of the mRNA.

In all kindreds, a mutation of the ALD gene was detected. The nucleotide alteration was different for 24 of 28 patients analyzed. No indication of more than one mutation was obtained in each case. An overview of all different mutations is given in table 2 and figure 2. Mutations are ordered 5′ to 3′, in five different classes: missense mutations (13), amino acid deletion (1), nonsense mutations (4), and frameshift mutations caused either by deletion of one or more nucleotides (8) or by splice site mutations (2).

Apart from these mutations, a G/A polymorphism at position 1934 (no amino acid change) and a C/G polymorphism at position 2632 (downstream of the stop codon) were observed (Kemp et al. 1994*b*). In all cDNAs sequenced, the C at position 754 was replaced by a T, altering an alanine to a valine residue.

Analysis of Mutations on Genomic DNA

All mutations were confirmed in PCR products derived from genomic DNA of the patient or an affected family member, using the information about the exon-intron organization of the ALD gene (Sarde et al. 1994). Mutations C929A and T1045C were verified by digestion of a genomic PCR product by *Hpa*II. The mutation C929A destroys a *Hpa*II recognition site. Complete digestion was monitored by using a PCR product containing an additional *Hpa*II site (primers 702F and 1145R). The mutation T1045C created a novel *Hpa*II site, which was confirmed

Table 2

Mutations in the Putative ALD Gene in Patients Studied

Type of Mutation and cDNA Alteration[a]	Amino Acid Alteration[b]	Exon[c]	Genomic-PCR Primers	Genomic-Mutation Detection[d]	Phenotype[e]	Kindred Reference Number
Missense:						
C696T[f]	R104C (R)	1	303F + 821R	303F, 821R	AMN	17
G832A	S149N (N)	1	702F + 1145R	702F, 931R	AMN	8
G841C	R152P (K)	1	702F + 1145R	702F, 931R	ChALD	27
G874A[f,g]	R163H (R)	1	702F + 931R	702F, 931R	SympCar	14
G966C	D194H (D)	1	685F + 1145R	914F, 1145R	ChALD	12
T1045C	L220P (L)	1	914F + 1145R	*Hpa*II	AMN	7
G1182A[f]	G266R (G)	1	702F + 1231R	914F, 1231R	AMN	24
G1552A[f]	R389H (R)	3	1479F + 1861R	1479F, 1752R	AMN	20
(2×):						
G2211A[f]	E609K (E)	8	544F* + 1078R*[h]	544F*, 876R*	AMN	13, 18
A2212G	E609G (E)	8	544F* + 1078R*[h]	544F*, 876R*	ChALD	5
C2235T[f]	R617C (R)	8	544F* + 2742R	544F*, 876R*	ChALD	23
C2364T[f]	R660W (R)	9	544F* + 2742R	2312F, 1078R*	AMN	21
Amino acid deletion:						
del 2355–2357	del I657 (V)	9	849F* + 2478R[h]	2312F, 1078R*	ChALD	6
Nonsense:						
C783T[f]	Q133[h]	1	702F + 931R	702F, 931R	ChALD	26
G797A	W137[h]	1	685F + 1145R	702F, 931R	ChALD	10
C855T	Q157[h]	1	702F + 1145R	702F, 931R	AMN	9
C929A	Y181[h]	1	702F + 1145R	*Hpa*II	ChALD	15
Frameshift:						
del C442	A19>	1	303F + 821R	303F, 593R	ChALD	2
del C663	G92>	1	303F + 840R	576F, 821R	ChALD	22
del 1171–1178	F261>	1	702F + 1231R	914F, 1231R	ChALD	28
(4×):						
del 1801–1802	E471>	5	1781F + 1861R	Polyacrylamide gel	ChALD, AMN	3, 4, 16, 25
alt 1989–2377	P534>	6–9	1890F + 2669R	1890F, 1078R*	AMN	11
Splice defect:						
del 2021–2054	R545>	SA 7	1880F + 2132R	1880F, 2114R	ChALD	1
ins 8 bp 2251[f]	R622>	SA 9	849F* + 1078R*[h]	849F*, 1078R*	AMN	19

[a] Nucleotide numbers refer to Mosser et al. (1993), EMBL database Z21876.

[b] Amino acid numbers refer to Mosser et al. (1993) (start codon is +1); in case of a frameshift or splice-defect mutation, the last amino acid residue that is not altered in the expected protein product is indicated. The corresponding residue in the human PMP70 protein is indicated in parenthesis.

[c] Exon number affected by the mutation. SA = alteration of the splice acceptor site (for details see Kemp et al. 1994*a*).

[d] Numbers indicate primers used for sequencing. *Hpa*II = mutation confirmed by *Hpa*II digestion; and polyacrylamide gel = deletion monitored as a decrease in size of the PCR product.

[e] Phenotype of the patient tested in this family—ChALD = childhood ALD; and SympCar = a symptomatic carrier.

[f] Mutation that might be the result of deamination of methylated CpG.

[g] Mutation detected in a female carrier and for which no other family material was available.

[h] PCR input material was derived from PCR with primer 544F and primer 2669R (10 cycles). (See Material and Methods.)

using a comparable PCR product (primers 914F and 1145R). The dinucleotide deletion at positions 1801 and 1802 was monitored by analysis of radioactively labeled PCR products overlapping the deletion site on a polyacrylamide gel. The remaining mutations were confirmed by direct sequencing of a genomic PCR product, using primer combinations as indicated in table 2.

For the kindreds carrying the missense mutations G874A and C2235T, and for one of the kindreds with a dinucleotide deletion at positions 1801–1802, the cDNA fragments were derived from fibroblasts of female carriers. It was established, by analysis of genomic DNA from mothers (carriers on the basis of elevated levels of VLCFA) and unaffected sisters, that these alterations were indeed present in the affected alleles, except for the G874A alteration. No other material was available from the kindred in which the latter mutation was detected, and thus definitive proof that this alteration is indeed present in the mutated chromosome is not available. All other mutations were detected in male patient material.

Mutations located in exon 8, intron 8, and exon 9 were difficult to detect in PCR products derived from genomic DNA, as a result of highly homologous sequences with exactly the same length amplified from unknown regions

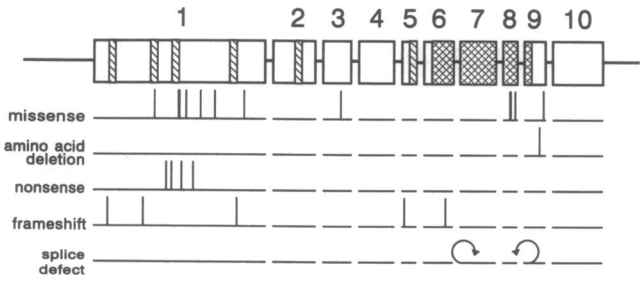

Figure 2 Distribution of mutations in the ALD gene. The boxes represent coding regions distributed over the 10 exons (Sarde et al. 1994); the lines between these boxes represent introns (not in scale), and those before exon 1 and behind exon 10 represent UTRs of the transcribed sequence. Hatched boxes represent regions encoding the six putative membrane-spanning domains (Klein et al. 1985); and the cross-hatched boxes represent the region encompassing the ATP-binding folds and their interjacent region. Each vertical bar represents the location of a mutation detected in this study. For the frameshift mutations, the last amino acid that is not altered by the mutation is indicated. The arrows represent the alteration of the splice acceptor site used, an alteration due either to mutation of the authentic splice acceptor site, resulting in the use of a cryptic acceptor site farther downstream, or to the generation of a novel splice acceptor site upstream of the actual splice site.

outside Xq28 (see also Sarde et al. 1994). With a primer in intron 7 and in the UTR, DNA from a human control and from a somatic cell hybrid containing Xq28 as the only source of human DNA was amplified. Comparison of sequences derived from either PCR product revealed differences between the ALD gene and the homologous sequences outside Xq28, which were used to design primers with preference for the ALD gene. These primers were applied under stringent conditions to generate PCR products and sequences that were more Xq28 specific. Although amplification of other copies was not completely avoided, it was possible to confirm the mutations previously observed in the cDNA, by comparison of sequences derived from control and patient DNAs.

The most drastic alteration of the gene structure found led to a frameshift at amino acid position P534. In the cDNA, the region between nucleotide 1988 in exon 6 and nucleotide 2378, the start of exon 10, was absent, while 88 nucleotides of unknown origin were inserted. Analysis of the mutation at the genomic level revealed a replacement of a region between nucleotide 1988 in exon 6 and nucleotide 2368 in exon 9, by a segment of ~574 nucleotides, which apparently harbors a cryptic splice donor site 89 nucleotides downstream of nucleotide 1988. Sequence comparison revealed that this segment is derived from an internal part of intron 7, which is ~2 kb long (Sarde et al. 1994).

Discussion

We have detected a mutation in the coding region of the ALD gene in all 28 investigated ALD kindreds. No indications of drastic promoter mutations were obtained, as RNA could be detected in all patients. A single partial (and complex) deletion was observed, in agreement with the low (6%) frequency of such mutations reported by Mosser et al. (1993). No deletion of the entire gene has been reported to date.

The ALD gene product shows all characteristics of an ABC transporter, with only a single domain containing putative membrane-spanning segments and one ATP-binding domain. The functional entity of a typical ABC transporter consists of two sets of these domains. In some transporters, both sets are assembled in a single polypeptide chain (like P-glycoprotein), while others are formed by dimerization of two polypeptides with a structure analogous to that of ALDP (like the peptide transporter, which is encoded by two closely linked genes on chromosome 6) (for review see Higgins 1992). This suggests that ALDP functions as a homodimer or forms a heterodimer with a homologous protein. Mutations affecting this putative partner would most likely also lead to an ALD phenotype. We can now practically exclude the presence of such a putative gene on the X chromosome, as we found mutations in the known ALD gene in all kindreds tested. Formally, we cannot exclude the possibility that mutations in an autosomal gene encoding a presumed partner of ALDP are responsible for the disease in some rare patients. It has been proposed that PMP70 might be such a partner (Valle and Gärtner 1993).

In 14 (50%) of 28 kindreds, the mutation affected a single amino acid residue. Since no direct functional assay is available, definitive proof is lacking that these mutations cause ALD. However, it is unlikely that these alterations are merely polymorphisms, since in all these cases only one alteration was found, which was different for 13 of 14 kindreds. None of these alterations were detected in kindreds with a mutation resulting in a truncated product. Moreover, although ALDP and PMP70 share only 30% amino acid identity, 10 of 13 different mutations affect amino acids conserved between both proteins, and 2 mutations affect residues with very conservative changes between ALDP and PMP70 (see table 2), suggesting that they indeed affect functionally or structurally important residues of the protein. In the remaining mutation, S149N, the ALDP residue is replaced by the corresponding PMP70 residue. By analogy with mutations detected in the cystic fibrosis transmembrane conductance–regulator gene, another member of the family of ABC transporters involved in a genetic disease, one might expect to find a high frequency of alterations in the ATP-binding domain, especially in its two ATP-binding–site motifs (for review see Tsui 1992). However, only three different mutations, affecting two different amino acid residues, were found in one of the ATP-binding–site motifs. These three mutations affect amino acid residues that are strongly conserved among different ABC transporters (Mosser et al. 1993). Although the missense mutation R660W and the deletion of an isoleucine at position 657 are located 30 and 27 amino acids downstream of the second ATP-binding site motif,

respectively, they are situated within a strongly conserved region. Possibly, these latter two mutations affect the structure of the ATP-binding domain, leading to diminished ATP binding or hydrolysis. Recently, a search for mutations in exons 6 and 8 that encode the 2 ATP-binding-site motifs in 50 French patients uncovered four missense mutations (including the R617C mutation also found by us [Fanen et al. 1994]). In our series, the majority of missense mutations were found far upstream from the ATP-binding domain. Remarkably, the relative number of missense mutations is high in exon 1, especially between the third and fourth putative transmembrane regions, which can only be partially attributed to the high CpG richness in this region (see table 2). Apparently, these mutations affect ALDP function, suggesting that this is a functionally or structurally important region that might be involved in substrate selection or transport or in homo- or heterodimerization.

Mutations leading to a truncated and possibly labile protein are detected in 50% of the kindreds. These truncations are caused by nonsense mutations, deletions of one or a couple of nucleotides, a deletion affecting multiple exons, or splice acceptor-site mutations. In all these cases, the predicted translation products are truncated upstream or within the ATP-binding domain, suggesting that the mutations result in complete absence of ALDP function.

The majority of mutations were found only once in the kindreds investigated. Exceptions are one of the ATP-binding-site motif mutations (E609K), which was detected in 2 kindreds, and a dinucleotide deletion resulting in a frameshift immediately downstream of E471, which was observed in 4 of the above kindreds and 1 of 10 different kindreds screened for this mutation on genomic DNA only (Kemp et al. 1994*b*). The five kindreds with the dinucleotide deletion had different haplotypes in the ALD region, suggesting that this mutation occurred independently in these kindreds. In the two kindreds with the mutation G2211A, the haplotype of the polymorphic markers close to the ALD gene was different (results not shown), making it likely that these mutations also result from two independent events.

The observed distribution of the mutations may be used to develop a strategy to detect mutations in other ALD kindreds. Screening for the mutation is the most reliable genetic assay to establish the carrier status of females in ALD kindreds. This is especially important, since in 5%–10% of female carriers no elevated level of VLCFA is detected (for review see Moser and Moser 1989). Starting with the screening of exon 5 for the presence of the recurrent dinucleotide deletion at positions 1801–1802, followed by analysis of the large coding region of exon 1, in which mutations were found frequently (see table 2 and fig. 2), a majority of mutations are expected to be discovered (18 of 28 mutations in our series). The dinucleotide deletion is easily testable on genomic DNA, by using primers flanking the putative hot spot and by scoring for the size difference (Kemp et al. 1994*b*). Mutations in the first exon can easily be detected by analysis of genomic PCR products, e.g., by SSCP or direct sequencing. When no mutations are found, the remaining exons should be tested with emphasis on exons 8 and 9. These exons are difficult to examine at genomic level, because of the presence of very homologous sequences elsewhere in the genome (also see Sarde et al. 1994), and should preferentially be investigated on PCR products derived from cDNA. Therefore, collecting fibroblasts of patients is advisable when the mutation has not been previously defined in the kindred.

Considerable phenotypic variation can be found within a single X-linked ALD kindred. This indicates that, apart from the mutation in the ALD gene, other genetic or environmental factors may modulate the phenotype (Maestri and Beaty 1992). However, a correlation between genotype and phenotype might still exist: a certain type of ALD mutation might, for instance, be present in kindreds with AMN patients only. The identification of ALD mutations will allow further investigation of the relationship between genotype and phenotype.

In conclusion, the systematic analysis of X-linked ALD mutations demonstrates that the identified ALD gene is most likely the only gene responsible for ALD on the X chromosome, limiting mutation detection to the analysis of only a single gene. Moreover, the distribution of the mutations in this gene suggests an efficient screening strategy, which is of special relevance for the identification of carriers in ALD kindreds.

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