Spectrum of Mutations in the Gene Encoding the Adrenoleukodystrophy Protein

Marjolijn J. L. Ligtenberg,1 Stephan Kemp,2 Claude-Olivier Sarde,3 Björn M. van Geel,2 Wim J. Kleijer,4 Peter G. Barth,2 Jean-Louis Mandel,3 Bernard A. van Oost,1 and Pieter A. Bolhuis2

1Department of Human Genetics, University Hospital Nijmegen, HB, Nijmegen; 2Department of Neurology, Academic Medical Center, Amsterdam; 3Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de l'INSERM, Strasbourg; and 4Department of Clinical Genetics, Erasmus University, Rotterdam

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...
in introns, and the numbers refer to their position in a partial genomic DNA sequence encompassing part of intron 7 through exon 10 (Sarre 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, 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), and 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 MgCl₂, 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 MgCl₂, 16.6 mM (NH₄)₂SO₄, 10 mM 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. (1994b). 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. (1994b).
Confirmation of Mutations on Genomic DNA

Chromosomal DNA was isolated from EDTA-anticoagulated blood or fibroblasts as described by Kemp et al. (1994b) 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. (1994b). The remaining PCRs were performed as described for fragment 2 and 4 of the cDNA. Mutations previously detected on cDNA were confirmed by HpaII 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. 1994b) 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 3 and table 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. 1994b). 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 HpaII. The mutation C929A destroys a HpaII recognition site. Complete digestion was monitored by using a PCR product containing an additional HpaII site (primers 702F and 1145R). The mutation T1045C created a novel HpaII site, which was confirmed
Table 2

Mutations in the Putative ALD Gene in Patients Studied

<table>
<thead>
<tr>
<th>Type of Mutation and cDNA Alteration</th>
<th>Amino Acid Alteration</th>
<th>Exon&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genomic-PCR Primers</th>
<th>Genomic-Mutation Detection&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Phenotype&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Kindred Reference Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missense:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6978&lt;sup&gt;T&lt;/sup&gt;</td>
<td>R104C (R)</td>
<td>1</td>
<td>303F + 821R</td>
<td>303F, 821R</td>
<td>AMN</td>
<td>17</td>
</tr>
<tr>
<td>G832&lt;sup&gt;A&lt;/sup&gt;</td>
<td>S149N (N)</td>
<td>1</td>
<td>702F + 1145R</td>
<td>702F, 931R</td>
<td>AMN</td>
<td>8</td>
</tr>
<tr>
<td>G841&lt;sup&gt;C&lt;/sup&gt;</td>
<td>R152Q (K)</td>
<td>1</td>
<td>702F + 1145R</td>
<td>702F, 931R</td>
<td>ChALD</td>
<td>27</td>
</tr>
<tr>
<td>G874&lt;sup&gt;A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>R163H (R)</td>
<td>1</td>
<td>702F + 931R</td>
<td>702F, 931R</td>
<td>ChALD</td>
<td>14</td>
</tr>
<tr>
<td>G966&lt;sup&gt;C&lt;/sup&gt;</td>
<td>D194H (D)</td>
<td>1</td>
<td>685F + 1145R</td>
<td>914F, 1145R</td>
<td>ChALD</td>
<td>12</td>
</tr>
<tr>
<td>T1045&lt;sup&gt;C&lt;/sup&gt;</td>
<td>L220P (L)</td>
<td>1</td>
<td>914F + 1145R</td>
<td>HpaII</td>
<td>AMN</td>
<td>7</td>
</tr>
<tr>
<td>G118&lt;sup&gt;b&lt;/sup&gt;</td>
<td>G266R (G)</td>
<td>1</td>
<td>702F + 1231R</td>
<td>714F, 1231R</td>
<td>AMN</td>
<td>24</td>
</tr>
<tr>
<td>G1552&lt;sup&gt;A&lt;/sup&gt;</td>
<td>R389L (R)</td>
<td>3</td>
<td>1479F + 1861R</td>
<td>1479F, 1752R</td>
<td>AMN</td>
<td>20</td>
</tr>
<tr>
<td>Amino acid deletion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>del 2355–2357</td>
<td>del I657 (V)</td>
<td>9</td>
<td>849F* + 2478R&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2312F, 1078R*</td>
<td>ChALD</td>
<td>6</td>
</tr>
<tr>
<td>Nonsense:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C737&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Q133&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>702F + 931R</td>
<td>702F, 931R</td>
<td>ChALD</td>
<td>26</td>
</tr>
<tr>
<td>G797&lt;sup&gt;A&lt;/sup&gt;</td>
<td>W137&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>685F + 1145R</td>
<td>702F, 931R</td>
<td>AMN</td>
<td>9</td>
</tr>
<tr>
<td>C855&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Q157&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>702F + 1145R</td>
<td>HpaII</td>
<td>ChALD</td>
<td>15</td>
</tr>
<tr>
<td>C929&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Y181&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>702F + 1145R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frameshift:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>del C442</td>
<td>A19&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1</td>
<td>303F + 821R</td>
<td>303F, 593R</td>
<td>ChALD</td>
<td>2</td>
</tr>
<tr>
<td>del C663</td>
<td>G2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1</td>
<td>303F + 840R</td>
<td>576F, 821R</td>
<td>ChALD</td>
<td>22</td>
</tr>
<tr>
<td>del 1171–1178</td>
<td>F261&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1</td>
<td>702F + 1231R</td>
<td>914F, 1231R</td>
<td>ChALD</td>
<td>28</td>
</tr>
<tr>
<td>(4X):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>del 1801–1802</td>
<td>E471&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5</td>
<td>1781F + 1861R</td>
<td>Polyacrylamide gel</td>
<td>ChALD, AMN</td>
<td>3, 4, 16, 25</td>
</tr>
<tr>
<td>alt 1989–2377</td>
<td>P534&lt;sup&gt;+&lt;/sup&gt;</td>
<td>6–9</td>
<td>1890F + 2669R</td>
<td>1890F, 1078R*</td>
<td>AMN</td>
<td>11</td>
</tr>
<tr>
<td>Splice defect:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>del 2021–2054</td>
<td>R545&lt;sup&gt;+&lt;/sup&gt;</td>
<td>SA 7</td>
<td>1880F + 2132R</td>
<td>1880F, 2114R</td>
<td>ChALD</td>
<td>1</td>
</tr>
<tr>
<td>ins 8 bp 2251&lt;sup&gt;f&lt;/sup&gt;</td>
<td>R622&lt;sup&gt;+&lt;/sup&gt;</td>
<td>SA 9</td>
<td>849F* + 1078R&lt;sup&gt;b&lt;/sup&gt;</td>
<td>849F*, 1078R*</td>
<td>AMN</td>
<td>19</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotide numbers refer to Mosser et al. (1993), EMBL database Z21876.

<sup>b</sup> 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 PM70 protein is indicated in parenthesis.

<sup>c</sup> Exon number affected by the mutation. SA = alteration of the splice acceptor site (for details see Kemp et al, 1994a).

<sup>d</sup> Numbers indicate primers used for sequencing. HpaII = mutation confirmed by HpaII digestion; and polyacrylamide gel = deletion monitored as a decrease in size of the PCR product.

<sup>e</sup> Phenotype of the patient tested in this family—ChALD = childhood ALD; and SympCar = a symptomatic carrier.

<sup>f</sup> Mutation that might be the result of deamination of methylated CpG.

<sup>g</sup> Mutation detected in a female carrier and for which no other family material was available.

<sup>h</sup> 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.
RNA could be detected in all patients. A single partial (and acids downstream of the second ATP-binding site motif,

mutations of drastic promoter mutations were obtained, as an isoleucine at position 657 are located 30 and 27 amino

ally harbors a cryptic splice donor site 89 nucleotides

downstream of nucleotide 1988. Sequence comparison re-

ally 'n ** ATP-bmding-site motifs (for review see

leaved that this segment is derived from an internal part of ^ ally

apparently harbors a cryptic splice donor site 89 nucleotides

downstream of nucleotide 1988. Sequence comparison re-

ally 'n ** ATP-bmding-site motifs (for review see

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 splice 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 se-

quences derived from either PCR product revealed differ-

ences between the ALD gene and the homologous se-

quences outside Xq28, which were used to design primers

with preference for the ALD gene. These primers were ap-

plied 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 ob-

served 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 re-

vealed 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 indica-

tions 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 re-

ported to date.

The ALD gene product shows all characteristics of an

ABC transporter, with only a single domain containing pu-
tative 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 transport-
ers, both sets are assembled in a single polypeptide chain

(like P-glycoprotein), while others are formed by dimeriza-
tion of two polypeptides with a structure analogous to

that of ALDP (like the peptide transporter, which is en-
coded by two closely linked genes on chromosome 6) (for

review see Higgins 1992). This suggests that ALDP func-
tions as a homodimer or forms a heterodimer with a ho-
mologous protein. Mutations affecting this putative part-
ner would most likely also lead to an ALD phenotype. We
can now practically exclude the presence of such a puta-
tive 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 autosom-
al gene encoding a presumed partner of ALDP are re-

ponsible for the disease in some rare patients. It has been

proposed that PMP70 might be such a partner (Valle and

Gärntner 1993).

In 14 (50%) of 28 kindreds, the mutation affected a sin-
gle 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 kin-
dreds. None of these alterations were detected in kindreds

with a mutation resulting in a truncated product. More-

over, 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 in-

deed 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, an-

other member of the family of ABC transporters involved

in a genetic disease, one might expect to find a high fre-
quency of alterations in the ATP-binding domain, espe-
cially in its two ATP-binding–site motifs (for review see

Tsui 1992). However, only three different mutations, af-
tecting two different amino acid residues, were found in

one of the ATP-binding–site motifs. These three muta-
tions affect amino acid residues that are strongly conserved

among different ABC transporters (Mosser et al. 1993). Al-

though 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. 1994b). 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 G221A, 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 strategy, which is of special relevance for the identification of carriers in ALD kindreds.

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 strategy, which is of special relevance for the identification of carriers in ALD kindreds.

Acknowledgments

We thank S. D. van der Velde-Visser, E. M. T. A. Boender-van Rossum, M. E. Meijboom, P. Mooyer, and R. A. Wolterman, for cell culturing; Dr. J. Bakkeren, clinical chemist, Dr. R. J. A. Wanders, biochemist, and Dr. R. B. H. Schutgens, clinical chemist, for the generous gift of fibroblast lines; C. J. M. Dekker and S. W. Deni, for assays of VLCFA; and the clinical geneticists, Drs. P. D. Maaswinkel-Mooy, C. J. van Asperen, L. ten Kate, H. Brunner, A. J. M. Hoogeboom, and B. Hanel, for providing us with clinical material. This work was supported in part by NWO grant 900-716-821 to B.A.V.O. and by support from Stichting NKGG, Utrecht, the Netherlands, to P.G.B. and P.A.B.

References