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Two New Arylsulfatase A (ARSA) Mutations in a Juvenile Metachromatic Leukodystrophy (MLD) Patient

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Summary

Fragments of the arylsulfatase A (ARSA) gene from a patient with juvenile-onset metachromatic leukodystrophy (MLD) were amplified by PCR and ligated into MP13 cloning vectors. Clones hybridizing with cDNA for human ARSA were selected, examined for appropriate size inserts, and used to prepare single-stranded phage DNA. Examination of the entire coding and most of the intronic sequence revealed two putative disease-related mutations. One, a point mutation in exon 3, resulted in the substitution of isoleucine by serine. Introduction of this alteration into the normal ARSA cDNA sequence resulted in a substantial decrease in ARSA activity on transient expression in cultured baby hamster kidney cells. About 5% of the control expression was observed, suggesting a small residual activity in the mutated ARSA. The second mutation, a G-to-A transition, occurred in the other allele and resulted in an altered splice-recognition sequence between exon 7 and the following intron. The mutation also resulted in the loss of a restriction site. Apparently normal levels of mRNA were generated from this allele, but no ARSA activity or immuno-cross-reactive material could be detected. A collection of DNA samples from known or suspected MLD patients, members of their families, and normal controls was screened for these mutations. Four additional individuals carrying each of the mutations were found among the nearly 100 MLD patients in the sample. Gene segregation in the original patient's family was consistent with available clinical and biochemical data. No individuals homozygous for either of these two mutations were identified. However, combinations with other MLD mutations suggest that the point mutation in exon 3 does result in some residual enzyme activity and is associated with late-onset forms of the disease. The splice-site mutation following exon 7 produces late-infantile MLD when combined with other enzyme-null mutations, implying that it is completely silent enzymatically.

Introduction

Metachromatic leukodystrophy (MLD) is a human genetic disease most commonly associated with a deficiency of the lysosomal enzyme arylsulfatase A (ARSA) (Kolodny 1989). MLD is usually subdivided into three clinical subtypes based on the age at onset. Typically, the late-infantile form is first noted sometime before the age of 2 years, the juvenile form at 5–10 years of age, and the adult form after sexual maturity. There is also an apparently benign low-ARSA phenotype, which is difficult to differentiate from MLD on the basis of enzyme measurements (Dubois et al. 1975; Lott et al. 1976). This is commonly referred to as ARSA pseudodeficiency (PD) (Kihara et al. 1980). Differences in functional enzyme activity between PD and the MLD subtypes can be shown in intact cells by loading with a putative physiological substrate, cerebroside sulfate (Fluharty et al. 1978). All of the aforementioned ARSA defects appear to be due to allelic alterations in the ARSA gene, and their molecular bases are only now beginning to be understood.

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Stein et al. (1989) were the first to isolate a cDNA for human ARSA. Alterations associated with PD and two MLD-related mutations have recently been reported (Gieselmann et al. 1989; Polten et al. 1991). The latter two mutations together account for 50%–60% of the MLD genes which have been evaluated. The mutations show a simple genotype-phenotype relationship such that homozygosity for one is always associated with late-infantile disease, whereas homozygosity for the other is most often found in individuals with adult-onset disease. Mixed heterozygotes for the two mutations manifest the juvenile form of MLD.

We report here on the characterization of two additional ARSA mutations in a patient with juvenile MLD. The new mutations appear to be much rarer than those previously reported, and neither has yet been found in homozygous combination. However, their heterozygous combinations with other MLD-causing mutations suggest that they fit into the same general genotype-phenotype pattern as do the mutations reported elsewhere.

Patient and Methods

The Patient

The patient was one of two children in a family with no history of neurological disorders. He fits into the late-juvenile-onset category delineated by Kolodny (1989). He developed normally through age 9 years. His early academic performance was superior, and he played both flute and piano. After an operation at 9.5 years of age, social and scholastic difficulties were noted. He was often moody, eventually showing problems in short-term memory. In his 11th year he began psychiatric treatment, and by age 13 years was institutionalized. The progressive nature of the condition and the development of ataxic symptoms led to a clinical reevaluation. MLD was diagnosed on the basis of deficient ARSA, urinary sulfatide excretion, and a white-matter defect. Both the father and older sister had low enzyme levels and were shown to be carriers of the ARSA PD allele.

DNA Preparation and PCR Amplification of ARSA Gene Fragments

Genomic DNA was prepared from cultured fibroblasts derived from a Swiss patient with juvenile MLD and from members of his family. Coding regions of the ARSA gene were amplified by the PCR in two segments, C and D, and were examined as described in a communication recently published elsewhere (Polten et al. 1991). The PCR fragments were purified and ligated into M13 mp18 and mp19 cloning vectors. Viral plaques containing ARSA gene fragments were identified by hybridization to a radiolabeled human ARSA cDNA. Ten to 12 plaques of each type and orientation were selected and grown as minipreps. Double-stranded phage DNA was isolated and evaluated for inserts of appropriate size, by restriction-enzyme excision and agarose-gel electrophoresis. In some cases it was necessary to reclone virus isolates to obtain clones of acceptable homogeneity. Several independent clones of each fragment and each orientation were used to prepare single-stranded DNA for sequencing.

Sequencing

Sequencing was carried out by the dideoxy chain-termination method (Sanger et al. 1977). Multiple (usually six or more) clones were used for initial sequence screening. Sequencing primers included the universal M13 primers, various intron primers, and an occasional exon primer. Much of the human ARSA coding region was evaluated in both directions, and all exons were examined in at least one direction. The overall sequencing strategy is summarized in figure 1.

Whenever a possible sequence alteration was detected, additional clones covering the same region were investigated. Sequence differences found in more than one clone and confirmed in both sequencing di-

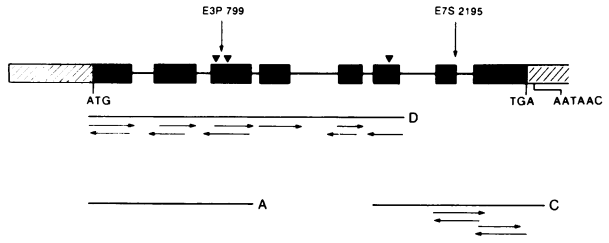

Figure 1 Schematic representation of human ARSA gene structure and sequencing strategy employed. Bars represent exons, and lines represent introns. Hatched segments represent 5′ and 3′ untranslated regions, while blackened segments indicate coding regions. ATG and TGA mark the initiation and termination codons, respectively, while AATAAC is the normal polyadenylation signal. The black triangles mark the three potentially glycosylated asparagine residues, while the vertical arrowheads above the gene indicate the sites of the two mutations described in the present paper. The lines labeled C and D indicate the regions amplified by PCR and cloned into M13 mp18 and mp19. The horizontal arrows under the amplified gene fragments approximate the segments examined for sequence variations in coding (→) or noncoding (←) clones.

rections were considered to be bona fide mutations. Once a putative mutant sequence had been identified, two clones of each genotype (with and without the mutation) were selected for each strand orientation. These were then used to survey any remaining sequence in that particular gene subfragment (C or D). A small number of sequence alterations were detected only in single clones and were considered to be errors introduced during PCR amplification. Sequence data were compared with the human ARSA genomic sequence recently reported by Kreysing et al. (1990).

Oligonucleotides

Oligonucleotides employed as PCR primers, sequencing primers, and sequence-specific probes and for site-directed mutagenesis were synthesized and purified in the laboratory by Dr. Bernnard Schmidt. An Applied Biosystems DNA synthesizer was employed. A listing of the various oligonucleotides used in the present study is presented in table 1.

PCR

PCR was carried out using *Taq* polymerase (AmpliTaq) from Cetus, by the procedure outlined elsewhere (Gieselmann et al. 1989; Polten et al. 1991). A PC-controlled robot and three regulated waterbaths were used for thermocycling. The A fragment, encompassing exons 1–3 and including introns, was obtained by using primers ARSA 1c or ARSA 1 ER1c and ARSA 14nc. Fragment C, spanning exons 6–8, came from primers ARSA 19 ER1c and ARSA 20 HI3nc. Fragment D, spanning exons 1–6, was obtained from primers ARSA 1 ER1c and ARSA 17 BH1nc. For fragments A and C (approximately 1 kb each) a PCR program of 1 min denaturation at 97°C, 1 min annealing at 50°C, and 4 min extension at 70°C was used. For fragment D (approximately 2 kb) the extension time was increased to 5 min. The buffer, which differs somewhat from that suggested by Cetus, has been described elsewhere (Gieselmann et al. 1989).

Table 1

Oligonucleotides Employed in Present Study

Designation Structure[a] (5′→3′)	Use
ARSA1c (TGCTGGAGCCAAGTAGCCCT) or ARSA1ER1c (TCGAATTCTGCTGGAGCCAAGTAGCCCT)	PCR primer, fragments A and D; sequencing
ARSA14nc (CAAAGACTGGAGTTAGCACT)	PCR primer, fragment A; sequencing
ARSA19ER1c (CGGAATTCTTGATGGCGAACTGAGTGAC)	PCR primer, fragment C
ARSA20HI3nc (GCGAAGCTTCCTCATTCGTACCACAGG)	PCR primer, fragment C
ARSA17nc (CAGTGCAGGAGGCACTGAGG) or ARSA17BH1nc (GAGGATCCCAGTGCAGGAGGCACTGAGG)	PCR primer, fragment D
ARSA44nc (CAACAGTGGGATGGGGAC)	Site-specific probe
ARSA45nc (CAACAGTGGGCTGGGGAC)	Site-specific probe; mutagenesis
ARSA49nc (AGGGGTTACCCTGGGTGA)	Site-specific probe
ARSA50nc (AGGGGTTATCCTGGGTGA)	Site-specific probe
ARSA35nc (ACTTTCCAGTCCGCACAGC)	RFP probe
ARSA24nc (TGCAATCCATTGGGAGGAAA)	Sequencing
ARSA30c (TGTCTCAGGGACTCTGTG)	Sequencing
ARSA39nc (TGGTTCCTACCTGGTCGT)	Sequencing
ARSA15c (ACCTGCCAGCCCAGCCCTCA)	Sequencing
ARSA27c (ATGACCTCATGGCCGACGCCCAGCGCCAGG)	Sequencing
ARSA23c (GCTCATGAGCGCCTCCTGTG)	Sequencing
ARSA26nc (AGGGTTCCAAGGAGAGGGCCTGCGGACTGA)	Sequencing
ARSA34c (TATGTGCAGTGCTTG)	Sequencing
ARSA8nc (GTCAGAGGCTGGAGGCGTG)	Sequencing
ARSA2nc (ACCCAGGCTCTGCCCACAGT)	Sequencing
P + (nc) (CTGGTGTTATTACGTTATCA)	Sequencing

[a] The list includes all of the synthetic oligonucleotides utilized in the present study. They are designated by (1) either "ARSA" or "P + " and an arbitrary laboratory code number, (2) type of restriction (e.g., ER1), when present, and (3) whether they represent coding (c) or noncoding (nc) sequences. Some of these oligonucleotides have been reported in previous publications.

Site-specific Oligonucleotide Probe (SOP) Screening

Total DNA was prepared from either cultured MLD fibroblasts or blood leukocytes. The sample population included nearly 150 individuals and was drawn from western Europe and North America. It was composed of MLD patients, their family members, and various controls. The MLD sample of approximately 100 individuals included all of the common subtypes at roughly the frequency at which they occur in the population. There were also some individuals of uncertain diagnosis.

For SOP analysis, A (or occasionally D) and C fragments were PCR amplified as described above. Filters were prepared by first subjecting aliquots of the PCR reaction mixtures to alkaline denaturation. Neutralized samples were then heated to 95°C for 5 min and immediately were applied to the HyBond™ nylon membranes by using a Schleicher and Schuell slot-blot apparatus. The usual blot represented 2.5 μl of the PCR reaction mixture but was adjusted upward when the PCR yield was low. Filters were air-dried and were fixed to the membrane by UV illumination (30 s, face-down on the transilluminator) followed by heating at 80°C for 1–2 h. Filters were prehybridized for 1–2 h. at 42°C in a mixture containing 6 × SSC, 0.1% SDS, 0.1% sodium pyrophosphate, and 100 μg denatured salmon sperm DNA/ml. A [^{32}P]-labeled oligonucleotide probe was then added to the mixture, and hybridization continued for 6–18 h at the same temperature. The hybridization mixture was recovered and frozen and could be reused over a period of 2–3 half-lives (4–6 wk).

Filters were rinsed three times in 6 × SSC and 0.1% SDS at room temperature to remove adhering radioactive material and then were washed two times in the same solution for 10 min at the test temperature. Temperature in these washes was carefully monitored using a thermometer directly in the wash solution. To minimize test-to-test variation the same thermometer was always employed. After the final wash filters were drained, mounted wet between sheets of thin plastic film, and sealed to prevent drying. The filters were exposed to X-ray film with intensifying screens at −70°C for 3 h to several days, depending on the radioactivity of the bound probe.

Where possible, differential test temperatures were established using test filters having known heterozygotes and homozygotes for each genotype. When no homozygote for a particular mutant gene was available, as was the case for the mutations reported here, the best temperature was inferred from the behavior of heterozygote samples. When practical, one set of filters was always washed at 42°C, to monitor possible differences in the amount of DNA on spots of the same filter. These low-temperature filters could then be rewashed at a different test temperature if the initial results were unclear.

Restriction-Site Analysis

A reaction mixture containing 3 μl React 2 buffer (10 ×) (BRL), 2 μl of a fragment C PCR reaction mixture, and 1 μl of the *Bst*E2 stock was taken to a total of 30 μl with water. The digestion mixture was incubated at 60°C for 1 h. The reaction was stopped with 5 μl of gel-loading buffer IV (Sambrook et al. 1989, p. B24) and electrophoresed on a 1.2% agarose gel with TAE buffer at 100 V until the dye front had moved 7–8 cm. Fluorescent bands were photographically recorded under UV illumination, and DNA fragments were transferred to Hybond nylon membranes by Southern blotting. After fixation of the nucleotide fragments to the membrane by UV illumination and heating, the membrane was hybridized with a labeled exon 7 oligonuclèotide (ARSA 35nc) and was washed at 42°C and radioautographed.

mRNA Analysis

Total RNA was prepared from confluent fibroblasts. After the medium was removed, cells were rinsed with PBS and were harvested directly into 4 M guanidinium isothiocyanate by scraping. Cellular debris were removed by centrifugation, and the DNA was fragmented by shearing. Extracts were then purified by centrifugation through a cesium chloride cushion (Stein et al. 1989).

Nucleic acid content was initially estimated by measurement of the 260-nm absorbance, and apparently equal amounts were spotted on a 1% agarose/0.7% formaldehyde gel and were examined by electrophoresis. Ethidium bromide fluorescence intensity on preliminary gels was sometimes used to adjust sample volumes to equivalent RNA levels on analytical gels. The gels were blotted onto Hybond nylon membranes by standard northern blot methodology. After fixation the filter was hybridized overnight with a freshly labeled human ARSA cDNA probe. Hybridization was at 42°C in 48% formamide, 10% dextran sulfate, 4.8 × SSC, 1 × Denhardt's solution, and 100 μg salmon sperm DNA/ml. After hybridization, filters were washed twice with 2 × SSC containing 0.1% SDS for

15 min at room temperature and twice with 0.2 × SSC at 60°C for 30 min. Filters were drained, sealed in thin plastic, and put up with X-ray film with intensifying screens for 1 to several days.

Site-directed Mutagenesis and Transient ARSA Expression

Site-directed mutagenesis was carried out by the Eckstein method (Nakamaye and Eckstein 1986), in which α S dCTP is used to decrease nuclease susceptibility of the newly synthesized mutant strand and thereby improve the yield of mutant clones. BHK cells at a density of about 5×10^5/6-cm Petri dish were transfected with the expression vector pBEH (Artelt et al. 1988) containing either the normal or the mutant ARSA cDNA sequence. A calcium phosphate/glycerol shock procedure was employed (Kingston 1987). After growth for 48 h to allow expression of the human ARSA, cells were harvested in the presence of detergent. The ARSA was measured by a modified Baum assay (Baum et al. 1959) and β hexosaminidase with *p*-nitrophenyl N-acetylglucosamine (Fluharty et al. 1970). Total protein was estimated by the Lowry procedure (Lowry et al. 1951).

Results

Mutations

Two putative mutations were identified in the cloned PCR fragments of this juvenile MLD patient. Both were found in multiple clones of the same fragment and were confirmed in both coding and noncoding segments. One mutation was a T-to-G transversion at nucleotide 799, resulting in a change from isoleucine to serine in exon 3 (designated E3P799). The other mutation was a G-to-A transition at nucleotide 2195 (E7S2195). This occurs at the splice junction following exon 7 and is the first nucleotide of the subsequent intron. The mutation results in an alteration in the splice-initiation signal. (Gene alterations are designated with regard to the following: [*a*] location in gene structure, e.g., E3 = exon 3 or its immediately adjacent splice-recognition sequence; [*b*] type of alteration, e.g., P = point mutation leading to amino acid substitution; or S = mutation in splice-recognition sequence; and [*c*] number of initial nucleotide in altered sequence, e.g., 799 = 799th nucleotide beyond start of initiation codon.) Sequencing-gel segments showing these mutations are presented in figure 2*a* and *b*.

The splice-site mutation (E7S2195) also results in the loss of a restriction-endonuclease cleavage site. *Bst*E2 and its isoschizomers recognize the sequence G-G-T-N-A-C-C. The sequence CAG GGTAACC CCT, which occurs in the normal sequence, is changed to CAG GTTAACC CCT in the mutant.

Three *Bst*E2 sites, two of which are in PCR fragment C, occur in the ARSA gene. Treatment of fragment C with *Bst*E2 generates three subfragments which are well resolved on agarose-gel electrophoresis. A new, larger subfragment was present when the C fragment from this patient was evaluated. A similar higher-molecular-weight band was seen on *Bst*E2 treatment of the C fragment from the patient's father but not on treatment of those from his mother and sister. Three additional MLD patients showing the larger restriction fragment were detected among 40 patients evaluated by this procedure. It was also possible to show the presence of the larger restriction fragment on Southern blots of these gels by using [^{32}P]-labeled oligonucleotide ARSA 35, which is specific for a normal exon 7 sequence near the site of the mutation. Only a single labeled fragment is generated from each genotype. This latter method of detection should be applicable to unamplified genomic DNA samples. The results of these tests are presented in figure 3.

Specific Oligonucleotide Probes

Oligonucleotide probes specific for the two mutations were synthesized and used to survey appropriate PCR fragments. Five additional individuals positive for the point mutation in exon 3 (E3P799) were detected among the nearly 100 MLD patients. Two of these five individuals were members of the patient's family—i.e., his sister and mother. The father was not positive. Two were individuals with adult MLD, but neither was homozygous for this mutation. The fifth individual suffered from juvenile MLD. One of the adult patients also carried another recognized MLD mutation (E2S609, termed "I" by Polten et al. [1991]), which previously had been found only in late-infantile and juvenile MLD patients. The putative second mutations in the other adult MLD and juvenile MLD patients remain unknown.

Other than those identified by the restriction-enzyme survey, no individuals positive for the E7S2195 mutation were detected. Again, only four MLD patients in our sample carried this mutation. All occurred in heterozygous combination with another characterized MLD mutation. Except for the juvenile-onset proband, all others are late-infantile MLD patients. Two of these also carried the E2S609 mu-

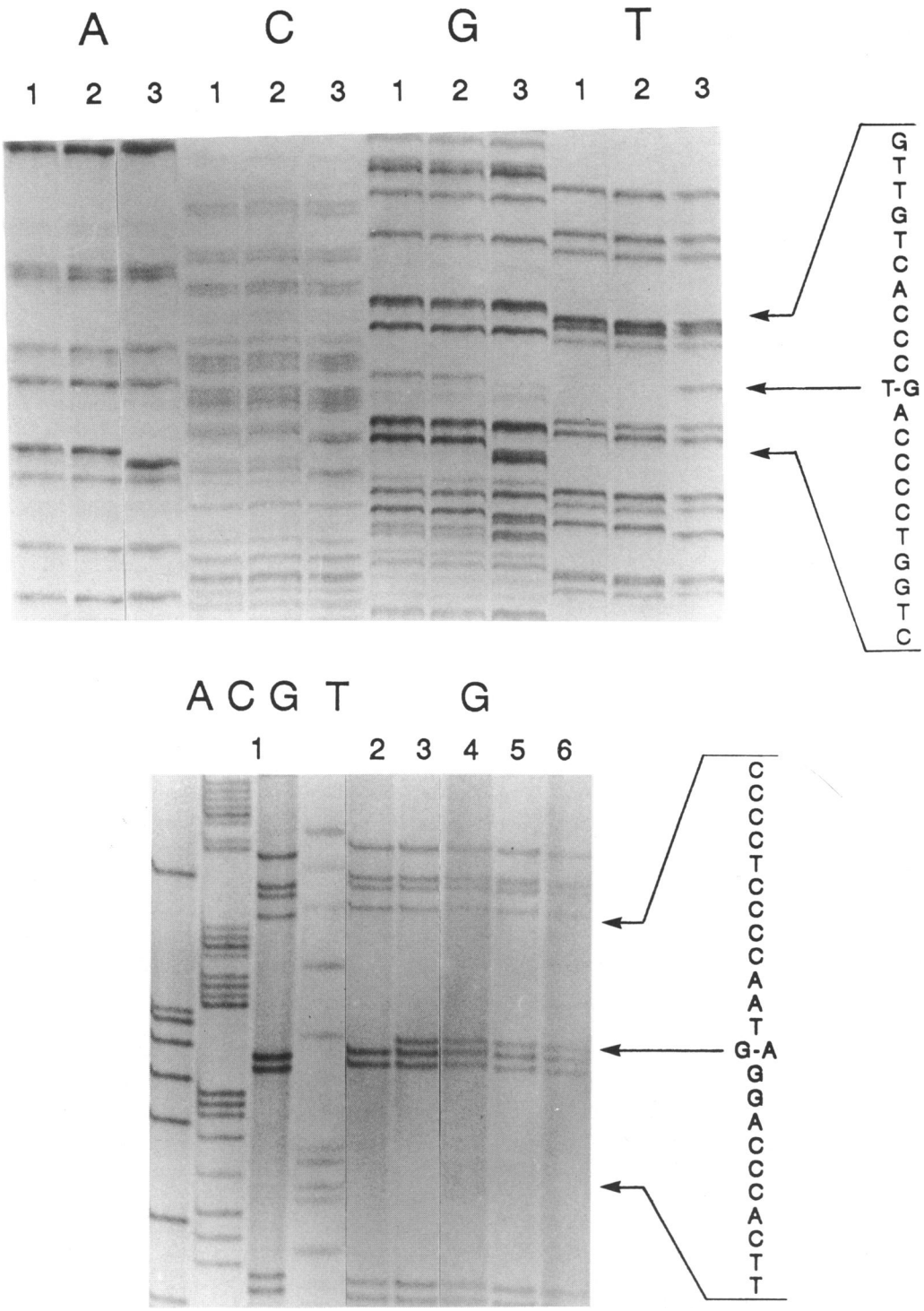

Figure 2 Sequencing gel segments showing mutations at nucleotides 799 and 2195. *Top,* T-to-G transversion at nucleotide 799 in clones 1 and 2, with normal sequence in clone 3. These are noncoding mp19 clones and are presented in a reversed direction of migration so that coding sequences can be read directly. It should be noted that the mutation has produced in the migration of adjacent oligonucleotides a distortion which persists for about 15 bases. The sequence in this region is unaltered, and such a permutation is not encountered when the same region is sequenced in the other direction. *Bottom,* G-to-A transversion at nucleotide 2195 in clones 1 and 2. All four bases are shown for clone 1, and only G is shown for clones 2–6. These were coding mp18 clones.

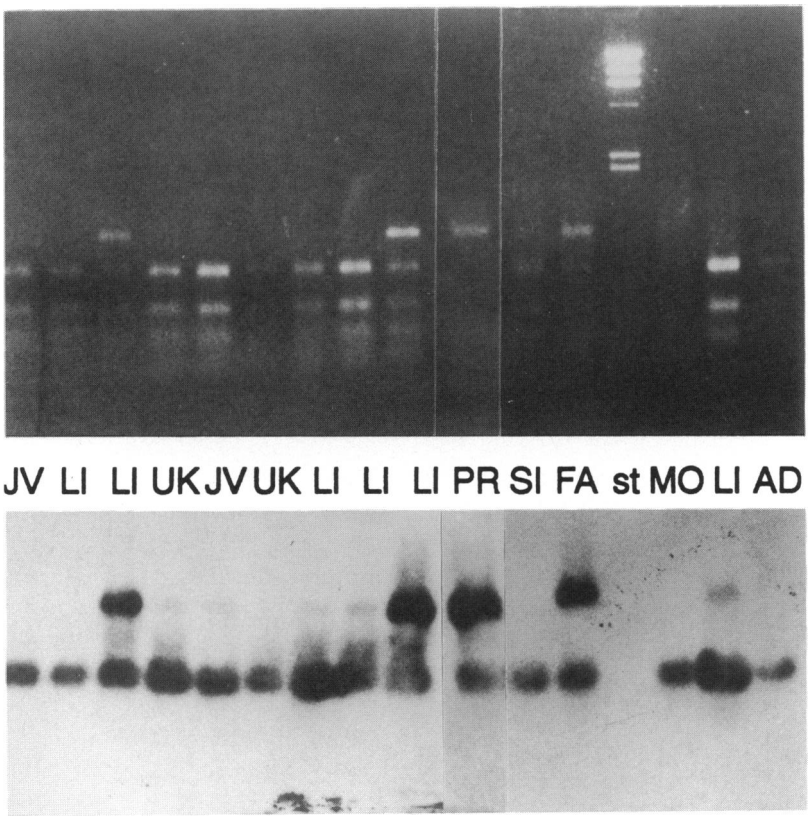

Figure 3 *Bst*E2 endonuclease polymorphism due to E7S2195 mutation. Portions of PCR-amplified C fragments were digested with *Bst*E2, and the fragments were separated by agarose-gel electrophoresis as described in the text. The upper panel is a composite of bands visualized by direct UV fluorescence in the presence of ethidium bromide. Gels were then blotted to nylon membranes and hybridized to a [^{32}P] oligonucleotide probe specific for nucleotides 2152–2170. The C fragment of the normal ARSA gene contains two *Bst*E2 sites generating fragments of roughly 180, 340, and 470 bp. The mutant provides only two fragments, one of roughly 180 bp and one of roughly 810 bp. The oligonucleotide probe binds to the normal 340-bp and the mutant 810-bp fragments. The propositus (PR) and his father (FA), mother (MO), and sister (SI) are included in the composite, along with a variety of MLD patients of late-infantile (LI), juvenile (JV), adult (AD), or unknown (UK) age at onset. Molecular-weight standards are labeled (st). The patient, his father, and two late-infantile-onset MLD patients have the larger fragment indicative of the mutation, in addition to a normal 340-bp restriction fragment.

tation, while the third had a deletion in exon 8 (E8D2506; W. Bohne, K. von Figura, and V. Gieselmann, unpublished data). In addition, three normal heterozygotes for this mutation were identified in the families of two of the patients. Examples of SOP analyses for these two mutations are shown in figure 4*a* and *b*. For a summary of the relationship of these mutations to MLD type, see table 2.

mRNA Studies in Cells with the Splice-Site Mutation

Splice-site mutations often result in little or no mRNA. While no cell line homozygous for the E7S2195 mutation was available to test this issue directly, there were lines where this mutation was in heterozygous combination with the E2S609 mutation. The latter had been shown not to produce any 2.1-kb mRNA for ARSA. Therefore, any 2.1-kb mRNA observed in the heterozygous E2S609/E7S2195 cell lines would have to have come from the gene with the E7S2195 mutation. Northern blots of RNA isolated from normal fibroblasts, from patients with the E2S609/E2S609, and from E2S609/E7S2195 genotypes were hybridized first with the ARSA cDNA and subsequently with a lysosomal acid phosphatase (LAP) cDNA. Autoradiographic films were quantified densitometrically to compare ARSA and LAP mRNA levels in the mutant cells (figure 5). This experiment confirms that alleles carrying the E2S609 splice-site mutation do not produce detectable levels of ARSA mRNA. It is surprising that the presence of the E7S2195

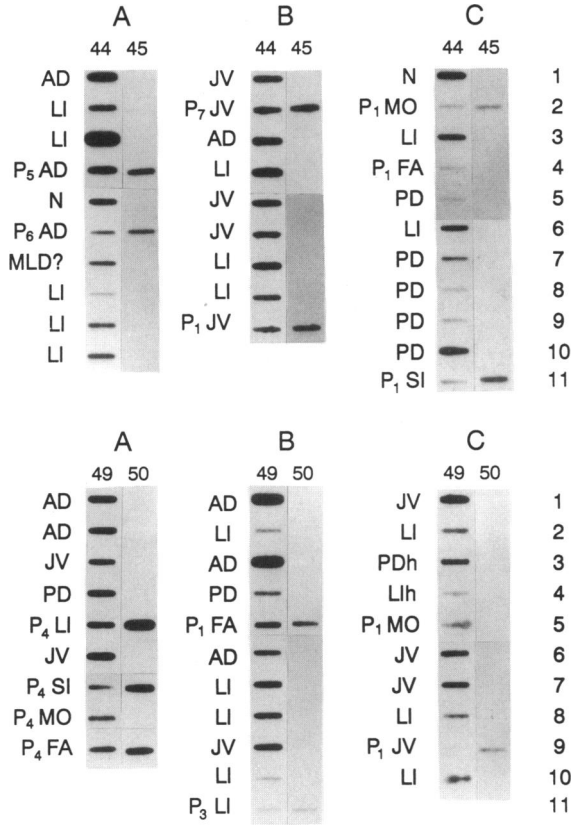

Figure 4 Specific oligonucleotide probe analyses for E3P799 and E7S2195 mutations. Appropriate PCR fragments (A or C) were denatured and directly slot blotted onto nylon membranes and were fixed, and hybridization with [^{32}P]-labeled oligonucleotides was evaluated. The composite figures have been assembled so that normal and mutant probes are adjacent. A representative selection of informative filter segments has been assembled from the nearly 150 individuals surveyed in this manner. MLD patients are labeled either according to type (LI, JU, or AD) or as unknown (MLD?). Patients carrying mutations identified in the present study are labeled (P_1–P_7). Members of patients' families are indicated (P_nFA = father; P_nMO = mother; and P_nSI = sibling). Other genotype indicators shown are are normal (N) and PD. An "h" following the designator indicates a heterozygous carrier. *Top,* Filters probed with oligonucleotides ARSA 44nc and ARSA 45nc corresponding, respectively, to normal and mutant sequences around E3P799 mutation. *Bottom,* Filters probed with oligonucleotides ARSA 49nc and ARSA 50nc corresponding, respectively, to normal and mutant sequences around E7S2195.

splice-site mutation does not appear to depress synthesis or stability of the 2.1-kb transcript believed to be the template for the bulk of the ARSA synthesis.

Given the apparently normal level of mRNA from the E7S2195 allele, we tested for anti-ARSA cross-reactive material (CRM) in fibroblasts of a patient with the genotype E2S609/E7S2195. No ARSA polypeptides could be detected either in metabolic labeling experiments or by an ELISA which could detect less than 3% of normal ARSA CRM values (data not shown).

Table 2

Genotype and MLD Clinical Form in Patients with E3P799 and E7S2195 Mutations

Genotype[a]	Age at Onset		
	0–2 Years (late infantile)	3–16 Years (juvenile)	>16 Years (adult)
E3P799/E7S2195.....	...	1	...
E3P799/E2S609	...	...	1
E3P799/?	...	1	1
E7S2195/E2S609.....	2	...	...
E7S2195/E8D2506 ...	1	...	...

[a] The E2S609 mutation is that designated "I" by Polten et al. (1991). The E8D2506 mutation is a 10-bp deletion in exon 8 (W. Bohne, K. von Figura, and V. Gieselmann, unpublished data).

Site-directed Mutagenesis

The point mutation in exon 3 (E3P799) was introduced into a human ARSA cDNA clone (Stein et al. 1989) and was inserted into the expression vector pBEH (Artelt et al. 1988). This was used to transfect BHK cells under conditions where the unmodified gene construct was strongly expressed. Cultures transfected with the vector containing the E3P799 mutation produced only about 5% of the ARSA activity increase obtained with an unaltered ARSA cDNA insert. Cell protein and β hexosaminidase activity were unaltered. These data are summarized in table 3. No

Table 3

ARSA Transient-Expression Studies

Vector Type[a]	Activity of		
	Protein (mg/ml)	β Hexosaminidase (mUnit/mg)	ARSA (mUnit/mg)
Control	3.05	18.7	2.4
Control + normal ARSA.....	3.20	18.8	13.8
Control + ARSA/E3P799 ...	3.15	17.5	3.0

[a] The E3P799 mutant sequence was placed into the normal ARSA cDNA by site-directed mutagenesis, and both normal and mutant sequences were subcloned into the expression vector pBEH. These vectors were then used to transfect nearly confluent cultures of BHK cells. After 48 h cells were harvested and assayed for ARSA and β-N-acetyl hexosaminidase activities. Enzyme levels are compared with those in a BHK culture transfected with an insert-free vector.

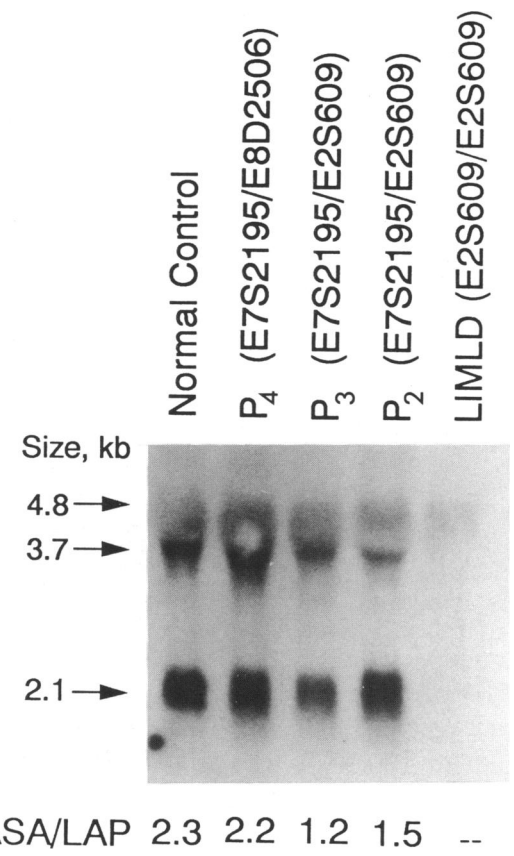

Figure 5 ARSA messenger RNA in cells from late-infantile-onset MLD patients carrying E7S2195 mutation. Total RNA from cultured fibroblasts was isolated, electrophoretically fractionated, and blotted onto a nylon membrane (see text). The blot was hybridized with [^{32}P]-labeled ARSA cDNA and was evaluated autoradiographically. Subsequently, the membrane was washed free of the ARSA cDNA and rehybridized with a LAP cDNA probe and again was autoradiographed. Autoradiographic signals were evaluated densitometrically for comparison of signal ratios. Sizes of the RNA species hybridizing with ARSA cDNA are indicated. Cell lines from a normal control, from two patients with mixed heterozygosity for the E7S2195 and E2S609 mutations, and from one patient with mixed heterozygosity for the E7S2195 and a deletion mutation (E8D2506) are compared with those from a cell line homozygous for the E2S609 mutation. Only the latter is deficient in the ARSA 2.1-kb mRNA product. ARSA/LAP ratios for cDNA densitometric signals are presented below the corresponding electrophoretic lanes.

alteration other than the expected T-to-G transversion was observed when the sequence of the expression-vector construct was examined over the region of the mutation.

Discussion

Two new mutations in the human ARSA gene have been recognized in a juvenile MLD patient. One, designated E3P799, was a point mutation resulting in the replacement of an isoleucine by serine in exon 3. This alteration appears to substantially reduce, but not completely eliminate, enzyme activity. When the E3P799 mutation was introduced into the cDNA for normal human ARSA and was transiently expressed in BHK cells, about 5% of the control activity was induced. This low residual expression is consistent with this mutation occurring in individuals with late-onset (juvenile or adult) forms of MLD.

E7S2195, the second mutation in this patient, altered an intron-exon splice-site recognition sequence. This change resulted in a complete absence of functional ARSA. There was also no evidence for any immuno-CRM. However, there did not appear to be a decrease in mRNA production. Since this mutation leads to a complete absence of enzyme in combination with other ARSA-null mutations, it would be expected to produce late-infantile MLD in homozygotes as well.

Both of the newly described mutations were relatively rare in the MLD population evaluated, but neither was unique to the family in which it was discovered. The E3P799 mutation occurred in four of the nearly 100 MLD patients surveyed. Thus, this mutant allele accounts for roughly 2% of this MLD gene pool. The E7S2195 mutation was also detected in four MLD patients, accounting for another 2%. These mutations are, therefore, more than 10-fold less frequent than those described elsewhere (Polten et al. 1991). Homozygotes for the new mutations would be expected to be quite rare in a randomly breeding population and might only be encountered in cases of consanguinity. The phenotypic characteristics of these mutations must, at present, be inferred either from the properties of heterozygote combinations with other MLD mutations or from site-directed mutagenesis studies.

The gene carrying the E3P799 mutation was otherwise identical to the reference human ARSA gene sequence (Kreysing et al. 1990). The E7S2195 mutation always occurred in combination with the E7P2162 polymorphism. The latter is a common polymorphic variant of the human ARSA gene, occurring in nearly one-half of the total gene pool evaluated. It is unlikely that any further gene alterations are associated with either of the two new MLD alleles. The entire coding sequence and most of the intervening intron sequences were examined, and no other reproducible sequence variations were encountered (other than previously noted corrections to the originally reported cDNA sequence [Kreysing et al. 1990]). Additional alterations

in control regions either preceding the coding sequences or following the polyadenylation signal cannot be excluded.

Both the independence of the E3P799 and E7S2195 mutations and their segregation with respect to the exon 7 polymorphism were confirmed by the investigation of other family members. The patient's father carried the E7S2195 mutation and the E7P2162 polymorphism, in addition to an ARSA PD allele. The E3P799 alteration was found in the mother, while the exon 7 polymorphism was absent. The patient's sister had the E3P799 mutation, lacked the exon 7 polymorphism, and carried the ARSA PD gene as well. This is summarized as a pedigree in figure 6.

It appears likely that the partial enzyme deficiency associated with the E3P799 mutation results from a combination of a lowered activity in the mutant enzyme and a decreased stability of that enzyme. The site-directed mutagenesis results suggest that the mutant protein may have about 5% of the activity of the normal enzyme in the synthetic substrate assay. The residual activity toward physiological substrate in intact juvenile and adult MLD cell lines, however, is less than 1% of that in the typical control. Moreover, cells from an adult MLD patient heterozygous for this mutation and the null-activity E2S609 mutation have been shown in an extensive series of studies to be responsive both to leupeptin and to E64 stimulation of functional cerebroside sulfatase activity (A. L. Fluharty, K. K. Tsay, and R. Fisher, unpublished data). Since thiol protease inhibitors enhance enzyme activity, it can be inferred that a portion of the overall enzyme deficit is due to protease susceptibility of the mutant enzyme.

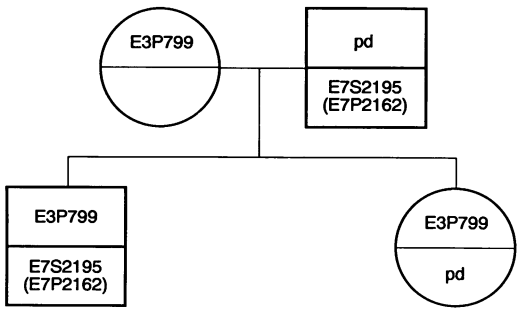

Figure 6 Pedigree of patient P_1's family. The ARSA genotypes established for this family are shown. The father carries the PD alterations on one allele and the E7S2195 mutation on the other. This latter allele also has a neutral polymorphism, E7P2162, in exon 7. The mother carries the E3P799 mutation on one allele; the other allele has the normal reference sequence. The patient and his sister both received the E3P799 allele from the mother. The patient received the E7S2195 allele from the father, while his phenotypically normal sister inherited the PD gene.

Thus, the allele containing the E3P799 mutation gives rise to a low but finite ARSA activity, and this activity is sufficient to delay the onset of demyelination and clinical symptoms. While we have no examples in which two copies of this gene are present in the same individual, we would expect this to result in adult-onset disease. Two of the individuals heterozygous for this mutation are adult MLDs (onset later than 16 years of age). In the index case the combination of this mutation with one leading to a complete absence of functional enzyme resulted in juvenile MLD. However, the other example of a combination of the E3P799 mutation with a null-enzyme allele occurred in an adult MLD patient who has survived into his fifth decade. It is therefor possible that this mutation results in a somewhat less severe enzyme deficit than does the adult MLD–associated E8P2382 mutation reported elsewhere (as "A" in a paper by Polten et al. [1991]).

In two cells line where both the E2S609 and the E7S2195 mutations were present, no immuno-CRM or functional ARSA activity could be documented. Therefore, the E7S2195 splice-site mutation appears to result in a complete absence of enzyme protein, even though it supports a normal level of ARSA mRNA synthesis. Reading through the intron between exons 7 and 8 would produce an altered reading frame in exon 8 and a premature chain termination. Such a transcript might still be processed to a poly A–tailed mRNA, even though it would code for a severely altered and presumably unstable protein. Alternatively, the mutation might lead to the elimination of the preceding exon (7) from the message. Further investigation of the ARSA mRNA produced by these cell lines will be needed to evaluate these postulates.

This E7S2195 mutation would be expected to produce late-infantile MLD in a homozygote. Although no such individual was encountered in our extensive sample of MLD patients, this expectation was supported by the finding that, when this gene was in heterozygous combination with other null-ARSA mutations, late-infantile MLD resulted.

The two mutations described here fit, for the most part, the simple genotype-phenotype pattern noted by Polten et al. (1991). One results in a complete absence of functional enzyme and thus gives rise to late-infantile or juvenile MLD, depending on the nature of the other allele. The other mutation causes an alter-

ation in the ARSA sequence, which leads to both a decreased enzyme activity and an enhanced susceptibility to proteolytic digestion. The mutant enzyme from the E3P799-containing allele may be somewhat more metabolically effective than that derived from the "adult" mutation described by Polten et al. (1991). However, this apparent difference could as well reflect variations in the subcellular environments of individual cell lines. Future studies on such partially active mutant enzymes, biosynthesized in permanently transfected expression systems, will allow detailed investigation of their properties and could aid in the design of rational therapies for MLD patients carrying these genes.

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