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Molecular Diagnostics for Myelin Proteolipid Protein Gene Mutations in Pelizaeus-Merzbacher Disease

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Summary

Pelizaeus-Merzbacher disease (PMD) is a clinically heterogeneous, slowly progressive leukodystrophy. The recent detection of mutations in the myelin proteolipid protein (PLP) gene in several PMD patients offers the opportunity both to design DNA-based tests that would be useful in diagnosing a proportion of PMD cases and, in particular, to evaluate the diagnostic utility of single-strand conformation polymorphism (SSCP) analysis for this disease. A combination of SSCP analysis and direct sequencing of PCR-amplified DNA was used to screen for PLP mutations in 24 patients affected with leukodystrophies of unknown etiology. Two heretofore undescribed mutations in the PLP gene were identified, Asp202His in exon 4 and Gly73Arg in exon 3. The ease and efficiency of SSCP analysis in detecting new mutations support the utilization of this technique in screening for PLP mutations in patients with unexplained leukodystrophies.

Introduction

Pelizaeus-Merzbacher disease (PMD) is a rare neurodegenerative disorder that primarily affects the white matter of the central nervous system (CNS). Definition of the disorder is problematic. Some investigators define PMD solely on neuropathologic criteria, regarding PMD as a sudanophilic leukodystrophy with relative preservation of axons and persistence of perivascular islands of myelin (Norman et al. 1966). Boulloche and Aicardi (1986) proposed a definition based on both genetic and clinical criteria, including (1) involvement of several males in a pedigree, consistent with X-linked recessive inheritance; (2) nystagmus within the first months of life; (3) psychomotor deterioration before 2 years of age; and (4) progressive pyramidal, dystonic, and cerebellar signs during the first years of life. These criteria delineated a clinically homogeneous group of patients; however, their neuropathologic features varied from cases with a complete lack of myelin to cases with mild myelin abnormalities. Other investigators define PMD on the basis of both clinical and neuroimaging or neuropathological findings. For example, Baraitser (1990) classifies PMD into six subtypes. However, the multiple modes of inheritance of these PMD subtypes indicate that PMD is, by that classification system, genetically heterogeneous.

As the various genes involved in this group of leukodystrophies are identified, reclassification of the various subtypes into distinct diseases will become possible. Recently, defects in the myelin proteolipid protein (PLP) gene, which is located on the X chromosome, have been found in several PMD patients (Gencic et al. 1989; Hudson et al. 1989*b;* Trofatter et al. 1989; Weimbs et al. 1990; Pham-Dinh et al. 1991; Pratt et al. 1991), indicating that PLP defects may account for many cases of X-linked PMD. However, sequencing of the entire coding region of the PLP gene failed to reveal mutations in several other PMD patients who showed X-linked inheritance of the disease (Hudson et al. 1989*b;* Pham-Dinh et al. 1991), suggesting either that defects in regions other than the coding re-

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gion are responsible for PMD or that genetic heterogeneity exists even within PMD subtypes that show X-linked inheritance.

Because of the severity of PMD symptoms and the inability to treat this disease, reliable prenatal diagnosis and carrier detection for at least some PMD families would be valuable. Linkage-based diagnosis for carrier testing and prenatal diagnosis would be possible in some families, by using RFLPs within or closely linked to the PLP gene. However, as in other X-linked lethal disorders, a high proportion of new mutations among affected individuals is expected (Haldane 1935). An important question in families with an affected individual but with no other history of PMD is whether the mother is a carrier. Direct detection of the mutation responsible for the disease would solve the diagnostic difficulties in such families. Even in apparent de novo cases, prenatal diagnosis by direct detection of the mutation would be useful, because of the possibility of gonadal mosaicism in one of the parents.

PCR amplification followed by sequencing of the seven exons of the PLP gene in PMD patients was the method used to detect the previously characterized mutations. All mutations were different single-base substitutions, suggesting that many different mutations in the PLP gene can cause PMD. Therefore, any screening test must be designed to detect previously unknown mutations. Because of the genetic heterogeneity of PMD, some and perhaps many PMD patients will contain normal PLP gene sequences, even when rigorous clinical and genetic criteria for the diagnosis of PMD are used. Thus, a diagnostic test for PLP mutations might be expected to yield a high percentage of negative results. As it is expensive and time-consuming to screen for mutations in the PLP gene by sequencing all seven exons, a rapid screening method must be designed that can detect, with high efficiency, the presence of mutations.

We have evaluated the diagnostic utility of single-strand conformation polymorphism (SSCP) analysis (Orita et al. 1989) for this disease. SSCP involves the simultaneous labeling and amplification of a target DNA sequence by PCR. The PCR product is then denatured and resolved by PAGE, and mutations are detected by the altered mobility of the separated single strands. A combination of SSCP analysis and direct sequencing of PCR-amplified DNA was used to screen for PLP mutations in 24 patients affected with leukodystrophies of unknown etiology. Two heretofore undescribed mutations were identified. We suggest that the ease and efficiency of SSCP analysis is detecting new mutations support the utilization of this technique in the diagnosis of PMD patients.

Patients and Methods

Patients

Genomic DNA was extracted from leukocytes, fibroblasts, and lymphoblasts according to a procedure described elsewhere (Theophilus et al. 1989). Control DNA was obtained from healthy adults lacking signs or symptoms of a CNS degenerative process. Lymphoblast cultures from PMD patient GM7157 and from his mother, GM7156, were obtained from the NIGMS Human Genetic Cell Repository (Camden, NJ). These cell lines carry the Trp-to-Arg substitution at position 162 (Trp162Arg) in exon 4 of the PLP gene (Hudson et al. 1989*b*). Genomic DNA also was obtained from leukocytes or fibroblasts from 24 pediatric male patients affected with severe CNS white-matter disease of unknown etiology. Extensive evaluations for causes, including metabolic-genetic causes, of CNS white-matter disease, were negative, and these patients were therefore considered candidates for having defects in the PLP gene. Four of these patients—PMD1, PMD2, PMD3, and 388—had a clinical diagnosis of PMD. Three of these patients (PMD1, PMD2, and 388) were from families with a history of X-linked dysmyelinating disease.

Preparation of Probes and Primers

Some of the oligonucleotides used as primers in the PCR, as well as all of the sequencing primers, were a gift from Dr. Lynne Hudson (Hudson et al. 1989*b*). The remaining oligonucleotides (designated the Smith primers) were synthesized with an Applied Biosystems 381A DNA synthesizer (see the Appendix). Oligonucleotide probes were labeled using gamma-[^{32}P]-ATP and polynucleotide kinase (Maniatis et al. 1982). Unincorporated nucleotides were removed by centrifugation through Quick Spin G-25 Sephadex columns (Boehringer-Mannheim).

PCR Amplification

Double-stranded PCR products were generated in an Ericomp Single Block System thermocycler. Each 100-μl reaction for unlabeled PCR contained 1 μl of genomic DNA, 0.2 mM each dNTP, 0.05 M KCl, 0.01 M Tris-HCl (pH 8.0), 2.5 mM MgCl, 0.17 mg BSA/ml, and 600 ng of each primer. With the Hudson PCR primers, cycling conditions for each of 35 cycles

were 1 min at 95°C, 30 s at 55°C, and 1 min at 72°C, followed by one cycle of 4 min at 72°C. The same reaction mix was used for the Smith PCR primers, except that the annealing temperature was 65°C. To generate radiolabeled PCR products, the concentration of dATP was lowered to 20 μM, and 1 μCi of alpha-^{32}P-dATP was added. Reactions were heated for 7 min at 95°C prior to addition of 2.5 units of *Taq* polymerase (Cetus) and then were overlaid with 100 μl mineral oil. Single-stranded PCR products used for sequencing were made with 3.4 μl of double-stranded product in a 100-μl reaction, using the same nucleotide stock and buffer and only one primer.

Direct Sequencing of PCR Products

We used the same primers and followed the same protocol as did Hudson et al. (1989*b*). In brief, single-strand PCR products were purified on Centricon 30-columns (Amicon, Beverly, MA), were extracted with an equal volume of phenol:chloroform (50:50 [v/v]), and were ethanol precipitated and resuspended in 30 μl of ddH_2O, an amount adequate for four sequencing reactions. DNA was sequenced by the dideoxy-chain termination method, with Sequenase™ kit (USB) reagents and protocols.

Dot Blots

PCR products were denatured in 10 mM Tris (pH 7.0) and 0.3 M NaOH at 65°C for 30 min. Samples were neutralized in an equal volume 2 M NH_4OAc and immediately were loaded on dot blot apparatus (Millidot-D; Millipore, Bedford, MA), using a Zeta-probe™ membrane. DNA was cross-linked to the filter for 30 s at 120,000 μJ/cm UV light (Stratalinker 1800; Stratagene, La Jolla, CA). Prehybridization and hybridization were carried out at 45°C in 5 × Denhardt's, 1% SDS, 1 mg yeast RNA/ml, 0.9 M NaCl, 180 mM Tris (pH 8.0), and 6 mM EDTA. The probe used was 1 × 10^6–2 × 10^6 cpm/ml. Filters were rinsed at room temperature and were washed for 30 min at 52°C in 4 × SSC.

SSCP Analysis

SSCP analysis of PCR products was carried out on 0.2-mm, 10%-glycerol, 6%-polyacrylamide, 1 × TBE gels at 4°C, for 15–27 h at 200 V (Orita et al. 1989). Radiolabeled PCR products were first diluted 1:8 in 7 M urea, then 1.5 μl of this dilution was mixed with 1.5 μl 95% formamide containing 0.5% bromophenol blue and 0.5% xylene cyanol. Samples to be denatured were boiled for 2 min and then were chilled on ice immediately prior to being loaded.

Restriction-Enzyme Digestion of PCR Products

Radiolabeled PCR products were digested with *Nla*III, according to the supplier's instructions (New England Biolabs, Beverly, MA). Digests (10 μl) were incubated for 3 h at 37°C and were analyzed on a 6% polyacrylamide gel.

Results

Direct Sequencing of PCR-amplified Coding Regions of the PLP Gene

Regions of the PLP gene of four clinically diagnosed PMD patients (PMD1, PMD2, PMD3, and 388) were amplified by PCR and screened, by direct sequencing, for the presence of mutations. For all patients and controls, we sequenced all coding regions, at least 15 bp of intron at each intron/exon boundary, 350 bp upstream of the translation initiation codon (containing the putative cap site, TATA, and CAAT boxes), and 200 bp of 3′ untranslated region. Only

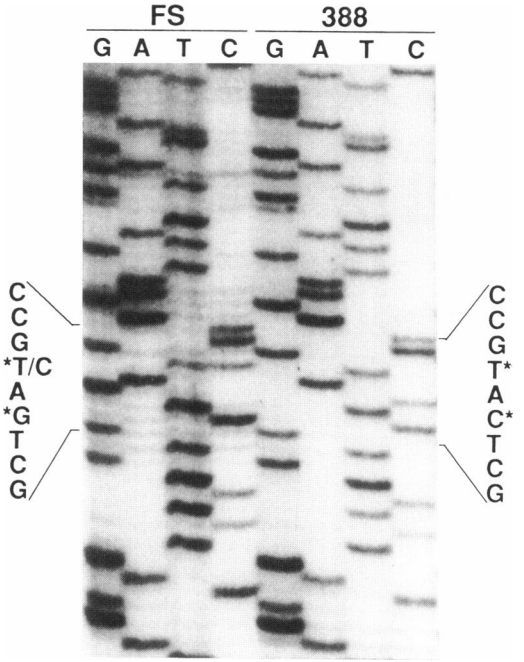

Figure 1 Direct sequencing of PCR products. The relevant portions of the sequencing gels are shown, both for control DNA FS and for PMD patient 388 DNA. Asterisks denote base differences between the two samples.

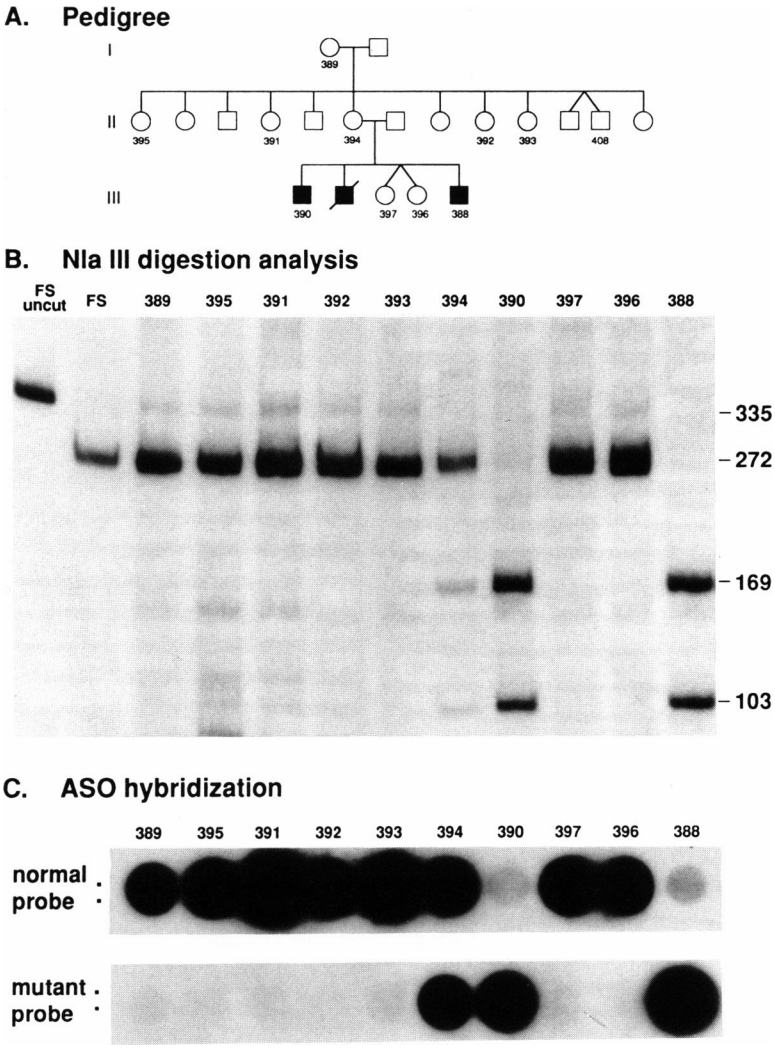

Figure 2 Screening for the mutation Asp202His in PMD patient 388's family. *A*, Pedigree with affected family members represented as blackened symbols. Patient 388 has 9 normal female and 11 normal male maternal cousins who are not shown. *B*, Restriction enzyme *Nla*III cutting normal alleles once and the mutant allele twice. Family members 388 and 390 are hemizygous, and 394 is heterozygous for the mutant allele. *C*, Dot-blot analysis confirming restriction-digestion results.

two differences from the normal sequence (Diehl et al. 1986) were found. The previously reported frequent polymorphism, a T-to-C change in the third position of codon 202 (Pham-Dinh et al. 1991; Trofatter et al. 1991), was present in the heterozygous state in one normal control (fig. 1). Also, a G-to-C change in the first position of codon 202 was found in patient 388 (fig. 1). This mutation predicts a substitution of aspartic acid by histidine (Asp202His), which could conceivably be the mutation causing PMD in this patient. This result was confirmed with an independently amplified PCR product.

The presence of the Asp202His mutation in patient 388's family was also investigated (fig. 2). Exon 4 of the PLP gene of the designated family members was PCR-amplified and analyzed by restriction-enzyme digestion and allele-specific oligonucleotide (ASO) hybridization. The mutation causes the creation of a second *Nla*III site in the PCR fragment, so that digestion by *Nla*III is a rapid way to distinguish the normal allele (expected fragment sizes 272 bp and 63 cp) from the mutant allele (expected sizes 169 bp, 103 bp and 63 bp). The mutant allele is detected only in affected males and their mother, an observation that is consis-

tent with a causative role for this allele for PMD in this family (fig. 2*B*). These results were confirmed by ASO hybridization of independent PCR products, in which, with use of the mutant probe, positive signals were detected only in the affected males and their mother (fig. 2*C*). Unaffected male 408 lacked this mutation, according to *Nla*III digestion and SSCP analysis (data not shown). The Asp202His mutation was not found in 50 PLP alleles from individuals with apparently normal CNS myelin (data not shown).

SSCP Analysis of Known Mutations

The three PLP alleles described above (the two normal PLP alleles and the Asp202His mutant allele), as well as the previously characterized Trp162Arg mutation found hemizygously in cell line GM7157 and heterozygously in GM7156 (Hudson et al. 1989*b*), were used to evaluate SSCP as a screening method for detecting mutations in the PLP gene. The ability of SSCP to detect mutations depends on the secondary structure of the single-strand DNAs; thus, electrophoretic mobility with SSCP is sequence dependent. Therefore, two different sets of PCR primers were compared—(1) the Hudson primers that amplified 60 bp of intron 3, all of exon 4, and 87 bp of intron 4 (total length 335 bp) and (2) the Smith primers that amplified 22 bp of intron 3, all of exon 4, and 22 bp of intron 4 (total length 212 bp). The results are shown in figure 3. All alleles were detected by using either type of PCR product, but the silent polymorphism in control FS was much more difficult to distinguish in the longer strands. It is interesting that the differences in migration of the mutant fragments in patient 388 are more dramatic than that of the fragments containing the silent polymorphism, which is only 2 bp upstream.

SSCP Screening of Patients Affected with Leukodystrophies of Unknown Etiology

Because SSCP analysis could distinguish all four PLP alleles tested and is an inexpensive and technically easy method, we decided to use SSCP analysis to screen an additional group of 20 male patients affected with CNS white-matter disease of unknown etiology, to determine whether any had PLP gene mutations. Since SSCP analysis works best for fragments of 200 bases or less (Orita et al. 1989; also see above), PCR products longer than this were digested by a restriction enzyme, to generate two shorter pieces before analysis. For example, *Bgl*II digestion of the 322-bp PCR product spanning exon 3 of the PLP gene allows the analysis of two fragments, one of 135 bp and one of 187 bp.

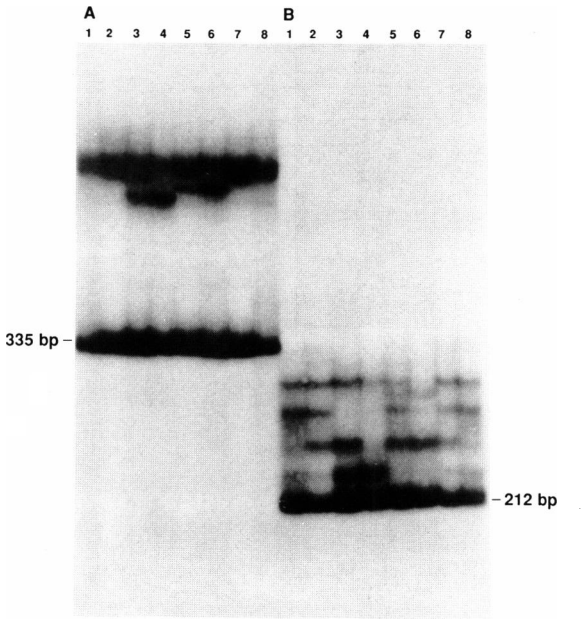

Figure 3 SSCP analysis of PCR-amplified exon 4 of the PLP gene. Panels *A* and *B* use primers located at different intronic positions. All mutant alleles are detected by this SSCP analysis. The following DNAs are shown: Lanes 1 and 8, Control RD (homozygous for the control normal alleles). Lanes 2 and 7, Control FS (heterozygous for both common and less common normal alleles). Lanes 3, GM7156 (heterozygous for both less common normal allele and Trp162Arg mutant allele). Lanes 4, GM7157 (hemizygous for Trp162Arg mutant allele). Lanes 5, DNA 394 (heterozygous for both common normal allele and Asp202His mutant allele). Lanes 6, DNA 388 (hemizygous for Asp202His mutant allele).

Likewise, *Pvu*II digestion of exon 1 gives fragments of 114 bp and 136 bp; *Alu*I digestion of exon 2 gives 77-bp and 151-bp fragments; and *Rsa*I digestion of exon 4 gives fragments of 81 bp and 120 bp. As a positive control, all gels included a sample heterozygous for the polymorphism in exon 4 of the PLP gene, which was the most difficult known mutation to detect. Gels also included an undenatured control lane, to allow identification of bands due to residual undenatured sample. It should be noted that, as a result of the denaturation of each DNA duplex, more than two bands may appear on a gel presumably because of the presence of different conformers of the same sequence (Orita et al. 1989) and that the presence of these bands is reproducible between gels.

A mutation was detected in exon 3 of two brothers (fig. 4). Sequencing of this exon in both brothers revealed a G-to-A mutation in codon 73 (data not shown), predicting a Gly-to-Arg substitution (Gly73-

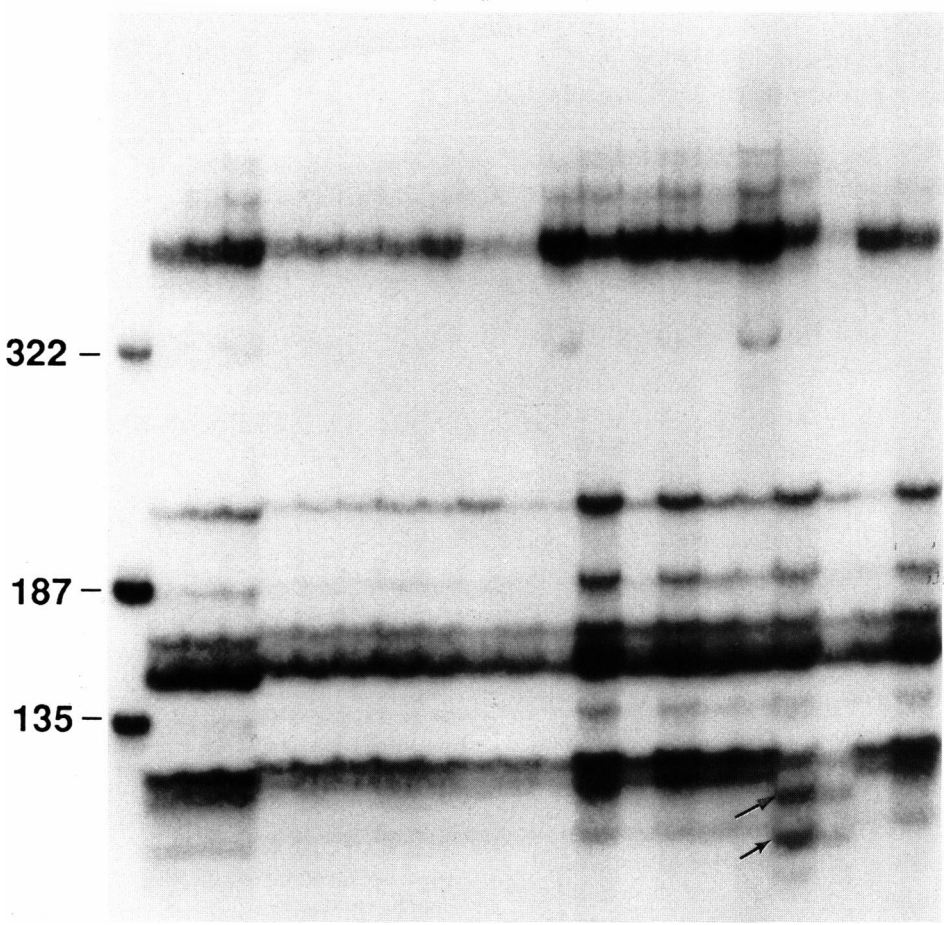

Figure 4 SSCP analysis of PCR-amplified exon 3 of the PLP gene of 20 patients suffering from leukodystrophies of unknown etiology. Lane 1, Undenatured, *Bgl*III-digested PCR products. Lanes 2–21, Denatured, *Bgl*II-digested PCR products of 20 patients. Arrows indicate anomalously migrating bands seen in lanes 18 and 19. (Note that lanes 1–4 were loaded 5 min before lanes 5–21 and that they therefore have migrated slightly farther in the gel.)

Arg). ASO hybridization confirmed both the presence of this mutation in the genome of both brothers and its absence from 50 PLP alleles from individuals with apparently normal CNS myelination (data not shown).

Discussion

We identified two new mutations in the PLP genes of PMD patients. The PLP gene encodes the myelin PLPs, called "PLP" and "DM20," which are very hydrophobic integral membrane proteins (reviewed in Hudson et al. 1989*a*). One mutation is a single G-to-C change causing an amino acid substitution, from aspartic acid to histidine, in residue 202. This substitution results in a change of charge. Because of the close proximity of a positively charged arginine at position 204, this mutation would result in a marked change in charge distribution in this region of the molecule, which could be important for intramolecular and/or intermolecular interactions in the extracellular matrix. In addition, residue 202 is close to the cysteine at position 200, which is in disulfide linkage (Shaw et al. 1989). There are several models for PLP topology (Laursen et al. 1984; Stoffel et al. 1984; Hudson et al. 1989*a*; Popot et al. 1991). Most of the models place residue 202 in a loop between hydrophobic regions C and D in the extracellular matrix. Two of the previously reported PLP mutations (Pro215Ser [Gencic et al. 1989] and Val218Phe [Pham-Dinh et al. 1991]) in PMD patients are also located in this loop.

The observation that the Asp202His mutation was

present in the mother and two affected sons of a family with no prior history of PMD makes it likely that this mutation is the mutation causing PMD in this family. The PLP gene is highly conserved across the species—there is no difference in amino acid sequence between human, rat, and mouse PLP (Nadon et al. 1990). In addition, several previously identified mutations, some leading to quite small predicted changes in the PLP protein (e.g., Ala to Val in jimpy^{msd} mice; Gencic and Hudson 1990), have been associated with dysmyelinating disease. These results suggest that there is little tolerance for amino acid substitutions in the PLP proteins without compromising their functional integrity.

The second mutation identified was a G-to-A change in codon 73, predicting a Gly-to-Arg substitution. This substitution is located within one of the putative transmembrane helices (Popot et al. 1991) and may perturb helix geometry, thereby affecting the overall structure or stability of the protein. Three of the previously characterized PLP mutations (Pro14-Leu [Trofatter et al. 1989], Thr155Ile [Pratt et al. 1991], and Trp162Arg [Hudson et al. 1989*b*]) in PMD patients have been located in putative transmembrane helices. The presence of the Gly73Arg mutation in both affected brothers of a family with no other history of PMD, the absence of this mutation in 50 normal PLP alleles, and the highly conserved nature of PLP make it likely that this is the mutation causing PMD in this family. However, it should be noted that neither the mutations identified in this paper nor any of the PLP gene mutations reported elsewhere have been demonstrated to perturb the function of their PLP or DM-20 products.

The failure to detect mutations in the coding regions, in splice-acceptor and splice-donor sites, and in the putative promoter region of the PLP gene in three of the four clinically diagnosed PMD patients analyzed by direct sequencing adds to the growing number of reports of PMD patients who do not have mutations in these regions (e.g., see Hudson et al. 1989*b*; Pham-Dinh et al. 1991). These results suggest either that these patients have defects in other regions of the PLP gene or that PMD, even when stringently defined (Boulloche and Aicardi 1986), is a genetically heterogeneous disease. More data need to be accumulated before this issue can be resolved. Hudson et al. (1989*b*) suggested that only patients with a demonstrated defect in the PLP gene should be classified as having PMD. It may be premature to jettison all clinical criteria, and at the present time we would suggest making the diagnosis of PMD for leukodystrophy patients who have a demonstrated defect in the PLP gene and/or meet the clinical criteria defined by Boulloche and Aicardi (1986). Regardless of the nomenclature used, we would suggest that male patients who have an unexplained leukodystrophy, who show preservation of peripheral nerve myelin, and who test negative for the usual battery of metabolic-genetic causes should be screened for the presence of mutations in the PLP gene.

Of course, the preceding discussion presupposes that an assay for detecting PLP mutations is available to clinicians. Because PLP is expressed at high levels only in CNS myelin, DNA-based tests are the obvious choice as diagnostic tool, both for identification of the basic defect in the patient and for the detection of carrier status and prenatal diagnosis for the patient's family. Although direct sequencing of PCR products has 100% efficiency in detecting mutations in the PLP gene, it is expensive and time consuming, especially in view of the large percentage of leukodystrophy patients who will not have detectable mutations in the PLP gene. Alternatively, SSCP analysis is an inexpensive and technically simple technique, requiring only electrophoretic analysis of PCR fragments, and therefore it is well suited for the rapid analysis of multiple samples. Although SSCP analysis may not be capable of detecting all mutations (in our laboratory it detected 11 of 12 in a panel of mutations; data not shown), it could distinguish the five alleles examined in this paper and has proved to be an excellent diagnostic tool for other human genetic disorders (reviewed in Hayashi 1991). Also, the ability of SSCP analysis to detect novel PLP mutations in patients affected with leukodystrophies of unknown etiology suggests its usefulness in the diagnostic evaluation of such patients.

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Appendix

Oligonucleotides Used for PCR Amplification of Coding Regions of the PLP Gene

Exon 1	
Forward:	5′-ACTCTAGAAAGCAGGCCTGTCCCTTTAA-3′
Reverse:	5′-CCGAATTCGAATCTTCAATGCTAAAGTT-3′
Exon 2	
Forward:	5′-CCGAATCGGTTTCCCCTTCTTCTTCCCC-3′
Reverse:	5′-ACTCTAGAGTGGGAGGGCAGGTACTTAC-3′
Exon 3	
Forward:	5′-ACTCTAGAGTTTGTCTACCTGTTAATGC-3′
Reverse:	5′-CCGAATTCACAAAATCCTGAGGATGATC-3′
Exon 4	
Forward:	5′-ACTCTAGACAACCCATGTCAATCATTTT-3′
Reverse:	5′-CCGAATTCAAGCACCCGTACCCTAACTC-3′
Exon 5	
Forward:	5′-ACTCTAGACCCTGCTTGCTTTTTGTGTC-3′
Reverse:	5′-CCGAATTCATAACCCAAATAACCCACTC-3′
Exon 6	
Forward:	5′-ACTCTAGATCCTCTTTTCATTTTCCTGC-3′
Reverse:	5′-CCGAATTCAGATCATTCAAAGTCAACTC-3′
Exon 7	
Forward:	5′-ACTCTAGATTCTCTCTTCTGTTCCCTAC-3′
Reverse:	5′-CCGAATTCAGGGGGATTTCTACGGGGGA-3′

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