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Pelizaeus–Merzbacher disease: Tight linkage to proteolipid protein gene exon variant

(X chromosome/polymerase chain reaction/linkage analysis/allele-specific oligonucleotide)

JAMES A. TROFATTER, STEPHEN R. DLOUHY, WILLIAM DEMYER, P. MICHAEL CONNEALLY, AND M. E. HODES*

Department of Medical Genetics, Indiana University Medical Center, Indianapolis, IN 46223

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ABSTRACT **Pelizaeus–Merzbacher disease (PMD) is a human X chromosome-linked dysmyelination disorder of the central nervous system for which the genetic defect has not yet been established. The jimpy mutation *jp* of the mouse is an X chromosome-linked disorder of myelin formation. The mutation is at an intron/exon splice site in the mouse gene for proteolipid protein (PLP). With the jimpy mouse mutation as a precedent, we focused our attention on the human *PLP* gene, which is found at Xq22. The polymerase chain reaction was used to amplify the exons of the *PLP* gene of an affected male from a large Indiana PMD kindred. DNA sequencing showed a C → T transition at nucleotide 40 of the second exon. An affected third cousin also showed this sequence variation, while two unaffected male relatives (sons of an obligate carrier female) had the normal cytidine nucleotide. Allele-specific oligonucleotides were used to generate data for linkage studies on the above mentioned PMD kindred. Our results show tight linkage ($\theta = 0$) of PMD to *PLP* with a lod (logarithm of odds) score of 4.62. In six other unrelated PMD kindreds, only the normal-sequence oligonucleotide hybridized, which indicates genetic heterogeneity. The radical nature of the predicted amino acid change (proline to leucine), suggests that the PMD-causing defect may have been delineated in one kindred.**

The proteolipid protein (PLP) is the major protein present in the myelin sheath of the central nervous system (CNS). PLP is produced by the oligodendrocyte whose plasma membrane is actively involved in myelin formation (1). Though the complete function of PLP is uncertain, it is believed to play a substantial role in the stabilization of the lamellar structure of the myelin sheath (1, 2). Without a myelin sheath, the CNS axons cannot perform normal nerve conduction.

Pelizaeus–Merzbacher disease (PMD) is a human X chromosome-linked recessive disorder characterized by the abnormal formation of myelin in the CNS (3, 4). The lack of myelin presents clinically as a number of characteristic neurologic signs: (*i*) abnormal eye movements; (*ii*) psychomotor retardation; and (*iii*) bilateral pyramidal tract signs, involuntary movements, and ataxia (5). The biochemical defect is not known, although studies of CNS myelin protein from PMD-affected males show a decrease in all myelin proteins, especially PLP (4). Delineation of the jimpy mutation *jp*, loss of the *Plp* gene's fourth intron/fifth exon splice site, has enabled study of PLP dysfunction in the mouse (6, 7). This mouse model exhibits many neurologic signs similar to those of PMD; however, some phenotypic differences have been noted.

Since the genes for PMD and PLP are both located on the X chromosome (8, 9), preliminary studies were performed with known X chromosome-linked restriction fragment length polymorphisms to help localize PMD. These analyses did not permit precise localization of the disease gene. We then focused our attention on the exons of the human *PLP* gene and attempted to find sequence variants that could be used for linkage analysis. We used the polymerase chain reaction to amplify the *PLP* exons and determined DNA sequences of affected and normal males from a large Indiana PMD kindred.

MATERIALS AND METHODS

M13 mp18 and mp19 replicative form DNA, *Eco*RI, phage T4 DNA ligase, phage λ *Hin*dIII marker, and φX174 *Hae* III marker were obtained from Bethesda Research Laboratories. T4 polynucleotide kinase was purchased from New England Biolabs. Sequenase kit was from United States Biochemical, random primed labeling kit was from Boehringer Mannheim, and GeneAmp kit was from Perkin–Elmer/Cetus. X-Omat XAR-5 film was obtained from Kodak. Adenosine 5'-[α-thio]triphosphate, [α-^{32}P]ATP, and [γ-^{32}P]ATP were from New England Nuclear. Probes pRL1 (from H. Willard, University of Toronto), *DXYS1* (from D. Page, Whitehead Institute), *DXS72* (from B. Schmeckpeper, Johns Hopkins), and *DXS94* (from M. Siniscalco, Sloan–Kettering Institute) were kindly provided to us.

Oligonucleotides. Primers and allele-specific oligonucleotides (ASO) were synthesized on an Applied Biosystems DNA synthesizer (model 381A). The 20 base pairs flanking each exon of human *PLP* (10), minus the AG or GT splice signals, served as primers. Either an *Eco*RI or *Hin*dIII restriction enzyme site was added to the 5' end of each primer with an additional TCTC 5' to this site. Primers for exon 2 (intron 1 and intron 2, respectively) were 5'-TCTCGAATTCCCCTTCTTCTTCCCC-3' and 5'-TCTCAAGCTTGTGGGAGGGCAGGTACTT-3'. ASO sequences were derived from the coding strand of exon 2: normal-sequence ASO, 5'-AAGCAAAGGGGGCCCCT-3', and variant-sequence ASO, 5'-AAGCAAAGAGGGCCCCT-3'. (The polymorphic nucleotide is underlined.)

Genomic DNA Isolation. All genomic DNA was isolated as described (11).

Polymerase Chain Reaction. Amplification followed Gene Amp protocols. Each reaction mixture contained 600 ng of genomic DNA and 96 pmol of each primer. Each sample was denatured at 95°C for 10 min and quickly cooled, and 2.5 units of *Thermus aquaticus* (*Taq*) polymerase was added. For exon 2, the thermal amplification profile was as follows: 5 cycles at 95°C for 2 min, 58°C for 2.5 min, and 72°C for 2 min; followed by 26 cycles at 95°C for 2 min, 63°C for 2.5 min, and 72°C for 2.5 min; followed by 7 min at 72°C after the final

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Abbreviations: PMD, Pelizaeus–Merzbacher disease; PLP, proteolipid protein; ASO, allele-specific oligonucleotide; CNS, central nervous system.

*To whom reprint requests should be addressed.

cycle. All amplifications were performed in the Perkin–Elmer/Cetus thermal cycler. Amplified DNA was extracted with phenol/chloroform, 1:1 (vol/vol), and concentrated in TE buffer (1× TE = 10 mM Tris·HCl, pH 8.0/1 mM EDTA) by three centrifugations in a Centricon-30 (Amicon) microconcentrator. One-tenth of the amplified DNA was size-fractionated on a 4% composite agarose gel (1% LE SeaKem agarose/3% NuSieve GTG agarose; FMC) to check exon lengths. ϕX174 *Hae* III DNA was used as size markers.

Cloning and Sequencing. Phage M13 replicative form DNA was digested with restriction enzymes and resuspended in TE buffer (15 ng/μl). Amplified DNA was blunt-ended or cut with restriction enzymes and resuspended in TE (20 ng/μl). M13 replicative form (150 ng) and amplified DNA (20 ng) were ligated overnight at 16°C. JM101 cells were made competent, transformed with ligated DNA, and plated according to protocol (12). White plaques were cultured and screened with the radiolabeled pRL1 insert as described (12). Single-stranded DNA was obtained from PLP-positive clones, and sequencing reactions followed the Sequenase dideoxy protocol with adenosine 5′-[α-thio]triphosphate and 1.5 μg of the single-stranded DNA. Reaction mixtures were separated by electrophoresis on 8% polyacrylamide/8 M urea, and the film was exposed to the gel for 1–3 days at −70°C.

Dot Blot. One piece of 0.2 μM Nytran filter (Schleicher & Schuell) was wetted with 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and placed on a Hybri·Dot manifold (Bethesda Research Laboratories). Approximately 200 ng of denatured, amplified DNA was applied to each well. A duplicate filter was made, and each was baked for 1 hr at 80°C. Filters were rinsed with 2× SSC and prehybridized [5× SSC/1% sodium dodecyl sulfate (SDS)/0.2% Ficoll/0.2% polyvinylpyrrolidine/0.2% bovine serum albumin/25 μg of sheared salmon sperm DNA per ml] for 3–6 hr at 65°C. Each ASO (17 pmol) was 5′ end-labeled (12). The prehybridization solution was discarded, and the normal-sequence ASO (diluted to 15 ml in 5× SSC/1% SDS) was applied to one of the filters. The variant-sequence ASO was applied in a similar manner to the duplicate filter. Both filters were hybridized with probe for 16–24 hr at 49.5°C. Filters were washed for 45 min in 0.5% SDS/0.5× SSC at 49.5°C and exposed to film for 2–4 hr.

Linkage Analysis. Data entry was facilitated by LIPIN (13), and a maximum-likelihood estimate of two-point data was obtained by LIPED (14). The 95% confidence interval (recombination fractions on either side of peak recombination fraction) is obtained by subtracting 1 lod (logarithm of odds) unit from the peak lod score (15).

RESULTS

Restriction Fragment Length Polymorphisms. A number of common restriction fragment length polymorphisms helped to exclude much of the X chromosome but did not establish linkage (J.A.T., S.R.D., and M.E.H., unpublished data). PMD did show weak linkage to the Xq22 marker *DXS94* (θ = 0, lod = 1.42) and the Xq21 markers *DXYS1* (θ = 0.17, lod = 0.88) and *DXS72* (θ = 0.19, lod = 0.87). Although no recombination was seen between the latter two markers in this family, two crossover events were observed with PMD when compared with the *DXYS1-DXS72* block (θ = 0.17, lod = 1.00). The known Xq22 *PLP Msp* I polymorphism (16) was uninformative for this family.

DNA Sequencing. *PLP* exons were amplified from genomic DNA and were the proper size as predicted from published exon lengths (data not shown). Initially, exons from one affected male (Fig. 1, V-8) were sequenced to determine whether any nucleotide variation was present within the exon coding regions of the *PLP* gene. A base transition from cytidine to thymidine was found at nucleotide 40 of exon 2 (Fig. 2, V-8 lanes at arrow). Additional clones from the original plate and clones from a second independent preparation were sequenced to confirm that this change was not caused by a *Taq* polymerase error. The results were consistent with the original finding (data not shown). Exons from a second affected male and two unaffected males (Fig. 1, V-2, III-3, and III-4) were sequenced to determine whether the variant was present in any other family members. The second affected male also showed the variant thymidine (data not shown), while both unaffected males had the normal cytidine at base 40. The sequence of normal male III-3 is also shown in Fig. 2. An affected male from a second large, apparently unrelated, Indiana PMD kindred did not show this variant (Fig. 2, UNR lanes).

ASO Analysis. Exon 2 was amplified from genomic DNA of family members, and two dot blots were prepared. One blot was hybridized to the normal-sequence ASO, and the second, to the variant-sequence ASO. The results of the probings are shown in Fig. 3 *A* and *B*. No affected individuals were available for analysis in generations III and IV. DNA from unaffected males in these two generations hybridized to the normal-sequence ASO (III-3, III-4, IV-4, IV-14, and IV-15), while obligate carrier females showed positive results with both probes (III-1, III-9, III-11, IV-2, IV-8, IV-10, and IV-13). Only six males were available for examination in generation V. DNA from both unaffected males (V-1 and V-3) hybridized to the normal-sequence ASO, while DNA from the four affected males (V-2, V-6, V-7, and V-8) hybridized to the variant-sequence ASO.

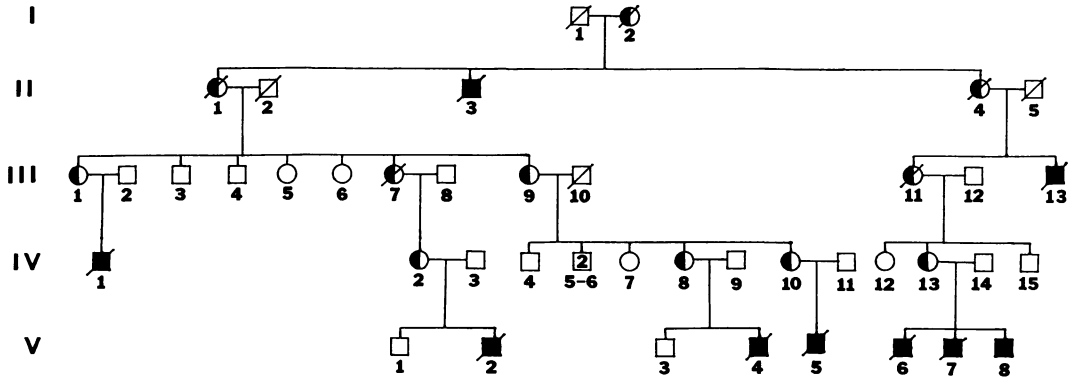

FIG. 1. Linkage pedigree of large Indiana PMD kindred. Roman numerals (I–V) represent generations, while arabic numbers represent individuals. An empty square represents a normal male; a shaded square represents an affected male; an empty circle represents a female with unknown carrier status; a half-shaded circle represents an obligate carrier female (affected descendants); diagonal slash through a circle or square indicates a deceased individual; a value within a circle or square indicates a number of individuals of identical status.

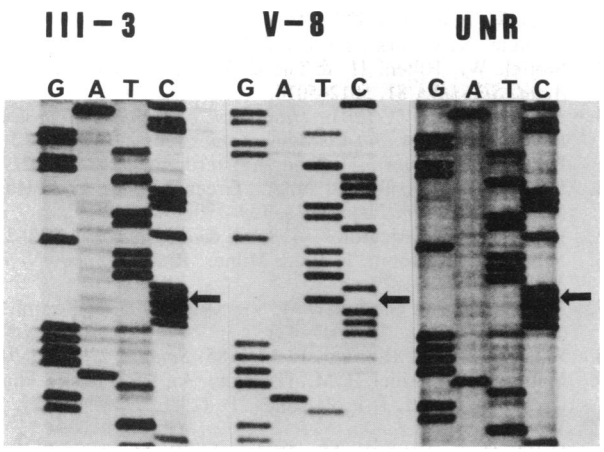

FIG. 2. Dideoxy sequencing reactions run on an 8% polyacrylamide/8 M urea gel of the second exon of the *PLP* gene. III-3 is an unaffected male from the linkage pedigree, while V-8 is his affected first cousin twice removed. UNR is an affected male from a second large Indiana PMD kindred (17). The arrow indicates the site of the variant nucleotide.

Linkage Analysis. Data from the ASO studies were analyzed by the LIPED program to estimate the maximum likelihood of recombination. The pairwise analysis of *PLP* versus the gene for PMD showed perfect linkage ($\theta = 0$) with a lod score of 4.61 and a 95% confidence limit extending out to 12 centimorgans. To obtain a gene frequency for the variant T nucleotide, we examined 60 unrelated X chromosomes by the dot-blot procedure. No DNA from unrelated individuals hybridized to the variant-sequence probe (data not shown). These results also confirmed the sequencing data from the unrelated, affected individual (Fig. 2, UNR lanes). From these results, we have tentatively estimated a gene frequency of 0.02 for this variant allele, although the actual frequency is likely to be much lower.

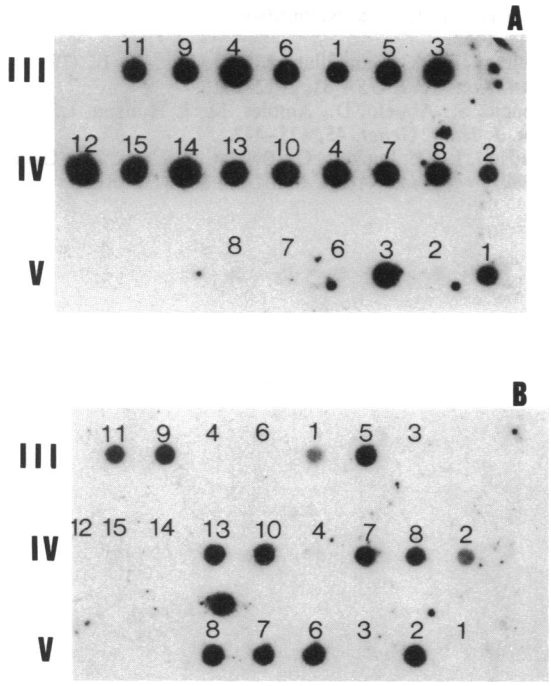

FIG. 3. Dot blot of amplified *PLP* exon 2 from individuals in the linkage pedigree. (*A*) Blot has been probed with the normal-sequence ASO. (*B*) Blot has been probed with the variant-sequence ASO. The weak signals on two individuals (III-1 and IV-2) on *B* represent true carrier females (retested on a second blot, which is not shown). The dot between generations IV and V near individual V-8 is an artifact.

DISCUSSION

PMD is a devastating X chromosome-linked neurologic disease in humans that presents morphologically as a lack of myelin in the central nervous system (3, 4). The disease can occur in several forms: a severe type in which affected males die before or during their second decade of life and a milder type that allows affected males to live into their third decade (3, 5).

We have studied a large Indiana family with the severe form of PMD in which affected males die between the ages of 7 and 13. Clinical and neuropathologic features of some affected males from this family have been described (17). This report suggested that the genetic defect probably involved myelin production as opposed to myelin maintenance (17). More recently, other workers on PMD have implicated a defect in PLP which represents 50% of the total myelin protein (4, 8, 18). However, no conclusive data regarding defective gene expression or regulation are available. Published data show that whenever PLP levels are low or absent, other myelin protein concentrations are also far below normal. Therefore, it is not known if low PLP levels cause a decrease in the production of these other proteins or if the low PLP concentrations are secondary to the true defect.

Because the *PLP* gene had not yet been excluded as the site of the PMD defect, we searched the exon regions of PLP for sequence variants that might be used to generate linkage data. The large Indiana family with severe PMD showed a variant nucleotide at position 40 of the second exon. The C → T transition results in the creation of a *Mnl* I restriction enzyme site ($CCTCN_7$, in which N is any nucleotide). The remaining exons had DNA sequences identical to those published (10). Segregation of the exon 2 *PLP* variant and the PMD gene is completely concordant in this family, thus indicating tight linkage between PMD and *PLP* (lod = 4.61, $\theta = 0$). This places the PMD gene in or very near the Xq22 region. In addition, we have detected recombination between the PMD gene and the Xq21 markers *DXYS1* and *DXS21*, which are therefore most likely proximal to the PMD gene.

We also examined six additional, apparently unrelated PMD families either by DNA sequencing or dot-blot analysis of their second exon and did not find the C → T transition in any individual. One of these (Fig. 2, UNR lanes) represents a large family with a mild form of PMD. Members of that family are much less severely impaired and live longer (into their fourth decade) than the family with the C → T mutation in exon 2 of *PLP* discussed herein. Clinical, ultrastructural, and biochemical features of some affected members of this mild PMD family were reported in 1973 (19).

If the *PLP* gene is involved in PMD, our data indicate genetic heterogeneity for PMD, since the exon 2 variant has been found in only one family thus far. It is conceivable that some structural mutations in PLP significantly disrupt its function and lead to a severe phenotype, whereas others might have less severe complications. Similarly, some cases of PMD might be due to either moderate or severe alterations in the level of expression of structurally normal PLP. PMD may in this respect resemble Duchenne muscular dystrophy, in which unrelated families have unique mutations at the dystrophin locus (20). If this were the case, each PMD family might prove unique with regard to the genetic defect at the *PLP* locus. Also, it is possible that some cases that meet the clinical criteria for PMD may be due to defects in a gene(s) other than *PLP*.

Our linkage results demonstrate that the PMD gene is within the vicinity of the *PLP* gene but do not prove that the PMD gene and *PLP* are identical. However, the presence of

the same nucleotide variant in affected third cousins, coupled with the inability to find this variant in unrelated individuals helps to support the hypothesis that a mutation in the *PLP* gene may cause a form of PMD. Even if a common ancestor did by chance have a rare, neutral variant of *PLP*, the likelihood that third cousins would both inherit that variant by chance is only 1/256. The hypothesis that the mutation that we have discovered represents the causative defect is further supported by the nature of the predicted (proline to leucine) amino acid change, which is not conservative either evolutionarily or structurally. The mouse, rat, and human all possess a proline at this amino acid site, coded for by the identical triplet, CCC (21). Six amino acids on the N-terminal side and 12 amino acids on the C-terminal side of this proline are also identical at both the protein and nucleic acid level in all three species.

Finally, proline is known as an α-helix breaker, and a recent study has shown that proline also occurs with a high frequency at the beginning of α-helices (22). Two models proposing PLP's membrane structure have been described that support one or the other function of proline (2, 23). The first model describes an α-helix with the proline in question situated near its beginning. This helix is transmembrane, starting from the extracellular matrix and extending toward the cytoplasmic space (2). The second model presents a membrane-associated helix-turn-helix motif for this region, with the proline acting as the crucial beginning of the turn after the first helix (23). Both models are based on theoretical predictions and experimental results. Thus, because of the proposed membrane position and the helical region involved, the amino acid change in this PMD family could be expressed as a structural distortion in mature PLP that results in abnormal myelin production or stabilization.

Note Added in Proof. Two additional variants of human *PLP* have been found recently in PMD patients by L. Hudson and coworkers (24, 25).

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