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Pelizaeus-Merzbacher Disease: Identification of Xq22 Proteolipid-Protein Duplications and Characterization of Breakpoints by Interphase FISH

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Summary

Pelizaeus-Merzbacher disease (PMD) is an X-linked, dysmyelinating disorder of the CNS. Duplications of the proteolipid protein (PLP) gene have been found in a proportion of patients, suggesting that, in addition to coding-region or splice-site mutations, overdosage of the gene can cause PMD. We show that the duplication can be detected by interphase FISH, using a PLP probe in five patients and their four asymptomatic carrier mothers. The extent of the duplication was analyzed in each family by interphase FISH, with probes from a 1.7-Mb region surrounding the PLP gene between markers DXS83 and DXS94. A large duplication $\geqslant$500 kb was detected, with breakpoints that differed, between families, at the proximal end. Distinct separation of the duplicated PLP signals could be seen only on metaphase chromosomes in one family, providing further evidence that different duplication events are involved. Quantitative fluorescent multiplex PCR was used to confirm the duplication in patients, by the detection of increased copy number of the PLP gene. Multiallelic markers from the duplicated region were analyzed, since the identification of two alleles in an affected boy would indicate a duplication. The majority of boys were homozygous for all four markers, compared with their mothers, who were heterozygous for one to three of the markers. These results suggest that intrachromosomal rearrangements may be a common mechanism by which duplications arise in PMD. One boy was heterozygous for the PLP marker, indicating a duplication and suggesting that interchromosomal rearrangements of maternal origin also can be involved. Since duplications are a major cause of PMD, we propose that interphase FISH is a reliable method for diagnosis and identification of female carriers.

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Introduction

Pelizaeus-Merzbacher disease (PMD [MIM 312080]) is a rare, X-linked, neurological disorder characterized by dysmyelination of the CNS. Mutations in the proteolipid protein (PLP) gene have been detected in patients (reviewed in Hodes et al. 1993) and animal models of PMD, including the jimpy mouse (Nave et al. 1986), myelin-deficient (md) rat (Boison and Stoffel 1989), and shaking pup (Nadon et al. 1990). The PLP gene is located on chromosome Xq22 (Willard and Riordan 1985; Mattei et al. 1986) and encodes two transcripts, known as "PLP" and "DM20" (reviewed in Griffiths et al. 1995). PLP is an integral membrane protein that forms a major structural component of myelin in the CNS (reviewed in Popot et al. 1991); DM20 is a smaller, alternately spliced isoform (Nave et al. 1987) that is expressed earlier than PLP in the CNS and may have a role in oligodendrocyte differentiation and survival. The PLP gene also has been associated with X-linked spastic paraplegia type 2 (SPG2) which has been suggested to be an allelic form of PMD (Saugier-Veber et al. 1994).

A wide spectrum of mutations has been found (reviewed in Hodes et al. 1993), but these only account for 10%–25% of families in which the disease segregates with the PLP locus (Boespflug-Tanguy et al. 1994). Duplications of the PLP gene have been detected in an increasing number of patients, which suggests that overdosage can also be a causative mechanism for PMD (Ellis and Malcolm 1994; Inoue et al. 1996*a;* Wang et al. 1997; Sistermans et al., in press). Animal models support PLP duplications as a molecular basis for the disease, since transgenic mice with extra copies of the wild-type PLP gene and overexpression of the mRNA exhibit a similar phenotype of abnormal CNS myelination and premature death (Kagawa et al. 1994; Readhead et al. 1994; Inoue et al. 1996*b*). Neurological symptoms and severity of the disease in transgenic mice correlates with PLP-gene copy number and with the level of overexpression (Kagawa et al. 1994; Readhead et al. 1994; Inoue et al. 1996*b*).

Techniques used to identify duplications involving the PLP gene in PMD patients include increased dosage on Southern blots (Ellis and Malcolm 1994; Sistermans et

al., in press), comparative multiplex PCR (CM-PCR) (Inoue et al. 1996*a;* Wang et al. 1997), quantitative PCR (Sistermans et al., in press), and densitometric RFLP analysis (Inoue et al. 1996*a;* Wang et al. 1997). In the present study, we have detected PLP-gene duplications by interphase-nuclei FISH and have detected increased gene dosage, by quantitative fluorescent multiplex PCR. We confirm that PLP overdosage is an important genetic abnormality in PMD and show that interphase FISH is a reliable technique that will facilitate diagnosis and carrier detection. We have characterized the duplication breakpoints in four families and suggest origin and mechanisms for the rearrangements. Similarities between PMD and Charcot-Marie-Tooth disease type 1 (CMT1A) are apparent. CMT1A is the most common subtype of the hereditary motor and sensory neuropathies (HMSN) and is also known as "HMSN1." The majority of patients have a partial duplication of chromosome 17p11.2, which includes the peripheral myelin protein 22 (PMP-22) gene (Timmerman et al. 1992). The importance of gene dosage in myelin disorders is highlighted.

Subjects and Methods

Subjects

Four families (DH, NO, DB, and ND) were studied that included five affected boys diagnosed, by referring neurologists, as having PMD. All mothers were asymptomatic. Families DH and NO have been described in a previous study (Ellis and Malcolm 1994), and, on the basis of pedigree information, mother DH is an obligate carrier. Ten normal female controls and 10 normal male controls were used for the fluorescent quantitative multiplex PCR. For each multiplex assay, 6–16 controls were included, with an equal number of males and females. One normal female control was used for the FISH.

Methods

Metaphase-Chromosome and Interphase-Nuclei Preparation

Lymphoblastoid cell lines from families DH, NO, and DB were cultured according to standard methods. Prior to being harvested, the cells were starved for 5–8 d, to block the cells in the G0 phase of the cell cycle. Cultures were then exposed to colcemid and were harvested according to a standard protocol, to produce metaphase chromosomes in addition to interphase nuclei. Preparations from family ND and from mother DB were obtained from peripheral blood lymphocytes by a thymidine-synchronized technique (Wheater and Roberts 1987).

Clones Mapped by FISH

Cosmids and PAC clones in this study were from contigs mapping to Xq22 (E.K., unpublished results), which contained the following loci: DXS83, DXS101c, DXS1106, DXS54, PLP, DXS1191, and DXS94. The cosmids are from the Lawrence Livermore flow-sorted X-chromosome library (gift of P. de Jong) and were isolated by use of whole YACs as hybridization probes, which originated from a complete YAC contig of the region (Kendall et al. 1997). The two PACs were from a whole-genome library (P. de Jong, Lawrence Livermore National Laboratory) (Ioannou et al. 1994) isolated by use of smaller specific probes. Contigs were assembled around the known marker loci, by a combination of hybridization using cosmid ends generated by vectorette PCR (E.K., unpublished results) and fingerprinting (G. Howell, personal communication). The coordinate numbers for clones mapped by FISH are shown in figure 1. All clones were positive for the respective markers, except dJ79p11, dJ198p4, and U65A4. PAC dJ79p11 was deduced to be positive for DXS101c, by contig information. Contig analysis using cosmid length to estimate physical distance suggested that dJ198p4 and U65A4 were ~20–40 kb from DXS83 and DXS101c, respectively.

FISH

Miniprep DNA was prepared by a standard technique and was labeled with either biotin-16-dUTP or digoxigenin-11-dUTP, by nick translation. FISH was performed as described elsewhere (Fitzgibbon et al. 1993), except that PAC probes required a threefold increase in COT^{-1} DNA, for competition of repeat sequences. Routine hybridizations included a biotin-labeled cosmid or PAC probe and a digoxigenin-labeled X chromosome–specific centromeric probe, to confirm hybridization efficiency and X-chromosome number. Biotin-labeled probes were detected by use of FITC conjugated to avidin, and the centromeric probe, labeled with digoxigenin, was detected by use of rhodamine conjugated to anti-digoxigenin. For two-color FISH using two cosmid probes, the X-centromeric probe was not included, and the signal for the digoxigenin-labeled probe was amplified with Texas Red conjugated to anti-sheep IgG. Cells were counterstained with DAPI and were viewed by use of a Zeiss Axiophot fluorescent microscope with a triple-bandpass filter.

Probes were hybridized to slides from patients, carriers, and controls. Metaphase chromosomes were inspected to confirm that there was successful hybridization to Xq22, and then the hybridization patterns of 60–100 nuclei were scored per slide. Nuclei with the expected number of red centromeric signals (i.e., one for

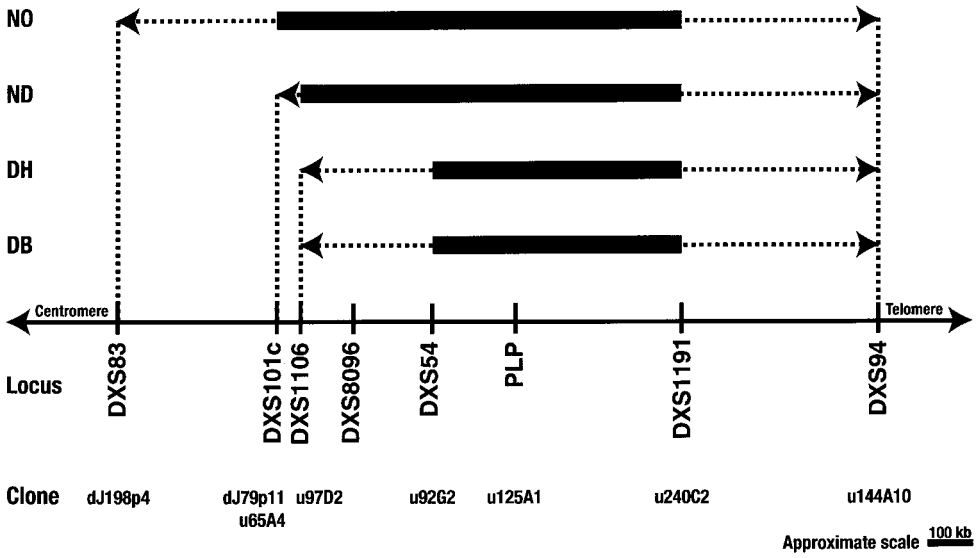

Figure 1 Physical map of Xq22, showing the extent of the duplication involving PLP in four PMD families (NO, ND, DH, and DB), as detected by interphase FISH. The four families were analyzed by two PACS ("dJ" prefix) and six cosmids ("u" prefix) from a 1.7-Mb region containing PLP. The minimum region of duplication in each family is shown by the thicker black line. Probes from this interval that were analyzed were duplicated. For each family, the maximum extent of the duplication is indicated by a dotted line with an arrowhead at both ends; the arrowheads point to probes that were not duplicated. The duplication in family DB differs because it can be resolved on metaphase chromosomes (see fig. 2*D*). All probes were positive for their respective marker locus, except for dJ198p4, dJ79p11, and U65A4. Contig information suggests that dJ79p11 contains DXS101c and that clones dJ198p4 and U65A4 are 20–40 kb from their associated markers. Distances between most markers were obtained from a PFGE map described by Vetrie et al. (1994). The positions of DXS1106 and DXS1191 were deduced on the basis of contig information (data not shown).

males and two for females) and with at least one green signal were analyzed. Each nucleus was scored on the basis of the number of green signals that it contained and on the relative positions of the latter. A result was obtained if >60% of these scores fell into either the duplicated or nonduplicated category. In male nuclei, a single signal indicated a nonduplicated probe, and two adjacent signals indicated a duplication; in female nuclei, two signals with a large distance between them indicated a nonduplicated probe, and two adjacent signals plus another elsewhere in the nucleus indicated a duplication. If DNA replication or poor hybridization caused any question with regard to the classification, the experiment was repeated.

DNA Isolation and Analysis of Multiallelic Markers

Genomic DNA was extracted from either whole blood (in the case of the controls and families ND and DB) or lymphoblastoid cell lines (in the case of families NO and DH), according to standard procedure. Genomic DNA for each individual was analyzed by fluorescent PCR using four polymorphic microsatellite markers from the largest region found to be duplicated by FISH. The markers included the dinucleotide-repeat polymorphism in intron 1 of the PLP gene (Mimault et al. 1995) (heterozygosity .69), DXS1106 (Dib et al. 1996) (heterozygosity .672), DXS8096 (Dib et al. 1996) (heterozygosity .8), and DXS1191 (Dib et al. 1996) (heterozygosity .65). Relative positions of markers on Xq22 are shown in figure 1.

Quantitative Fluorescent Multiplex PCR

The multiplex PCR was adapted from a method described by Inoue et al. (1996*a*). The sensitivity was improved by use of a fluorescent labeling strategy, rather than ethidium bromide staining. An automated DNA sequencer was used to quantify the fluorescently labeled multiplex PCR products. Only a small amount of product is required for analysis, and therefore a low cycle number can be used. Preliminary experiments to determine optimum cycle number showed that at 18 cycles the amplification reactions were exponential and that the amount of PCR product generated was directly proportional to the copy number of the target sequence.

Three pairs of primers were used for the multiplex PCR. Two pairs of primers were from the PLP gene; one of these pairs amplified the dinucleotide-repeat polymorphism (CA-PLP1 and CA-PLP2) (Mimault et al. 1995), and the other amplified exon 7 (PM-7A and PM-7B) (Osaka et al. 1995). The third pair of primers

(PRP44 and PRP200A) amplified exon 2 of the prion protein gene (PRNP), which was used as a control gene (Inoue et al. 1996*a*). The forward primers were labeled with the fluorescent phosphoramidite 6-FAM, at the 5′ end.

Multiplex PCR was performed with 75 ng DNA, 1 × AmpliTaq Gold PCR buffer (Perkin-Elmer), 200 μM each dNTP, 1.5 mM $MgCl_2$, 10 pmol each of primers PRP44 and PRP200, 1.9 pmol each of primers PM-7A and PM-7B, 5 pmol each of primers CA-PLP1 and CA-PLP2, and 0.75 U AmpliTaq Gold enzyme (Perkin-Elmer), in a 10-μl volume. Conditions for amplification were 94°C for 10 min, to activate the AmpliTaq Gold enzyme and to denature; 18 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension for 10 min at 72°C. Products were analyzed on an ABI 377 DNA sequencer using GENESCAN and GENOTYPER software (ABI). Electrophoretograms were produced for each reaction, with three peaks representing the three PCR products. GENOTYPER software was used to calculate both the size, in base pairs, of each product and the area under the peak, which correlates to the quantity of fluorescent signal incorporated by the labeled primers. Dosage estimates for PLP were obtained by comparison of the PLP peak area with the peak area of the PRNP internal control fragment, within each individual fluorescence profile. Two ratio values were thereby obtained for each sample: the ratio PLP CA:PRNP and the ratio PLP exon 7:PRNP. Normal male and female controls were used to obtain a reference range, for samples with one and two PLP alleles, respectively. The variance in control numbers was 6–16 in six experiments. Samples were analyzed two to four times, to allow for experiment variability, and the PLP:PRNP ratios were compared with those for normal controls.

Results

Identification of Xq22 Duplication, by FISH

Figure 2 shows the duplicated signals for a cosmid containing PLP detected by interphase FISH in an affected male (fig. 2*A*) and his mother (fig. 2*B*), who is an obligate carrier of PMD. The affected male has two adjacent yellow signals, which indicate the duplication, and one red signal, which confirms a single X chromosome. Three yellow signals are seen for the female carrier—two adjacent signals indicating the duplication and a third signal elsewhere in the nucleus from the normal chromosome; two red signals confirm the presence of two X chromosomes. The PLP duplication was detected in all five PMD patients and in their four asymptomatic mothers. The hybridization signals could not be resolved on metaphase chromosomes from three families, which suggested that the size of the duplication was <1 Mb (fig. 2*C*) (Trask et al. 1991). In contrast, easily resolvable hybridization signals identified the duplication on metaphase chromosomes in family DB (fig. 2*D*). A greater distance between duplicated PLP hybridization signals was also observed in interphase nuclei from family DB (fig. 2*E*). This suggests that a larger rearrangement is involved and that the duplication causing PMD differs between families.

Characterization of Extent of Xq22 Duplication

Probes proximal and distal to the PLP gene, covering a region of ~1.7 Mb, were mapped, by FISH, onto interphase nuclei and metaphase chromosomes from the four carrier mothers. Cosmid and PAC probes have been assembled into contigs with marker loci (E.K., unpublished data). The order and distance between most markers was determined by construction of a PFGE physical map (Vetrie et al. 1994). This was used to estimate the maximum and minimum sizes of the duplication. Results of the interphase FISH experiments using two PACs and six cosmids are presented in figure 1. The extent of the duplication in each family, with respect to physical distances on Xq22, is shown.

We show that the minimum size of the duplication is ~500 kb in families DB and DH and ~800 kb in families NO and ND. The distal breakpoint mapped between cosmids U240C2 (DXS1191) and U144A10 (DXS94), in all four families. Since these markers are ~400 kb apart, more cosmids from this region will be required in order to refine the position of the distal breakpoints further. The proximal breakpoints for the duplication were found to differ between families. The duplication in family NO was found to extend the most proximal, with the breakpoint mapping between dJ198p4 (contig containing DXS83) and dJ79p11/U65A4 (contig containing DXS101c). The proximal breakpoint in family ND mapped between dJ79p11/U65A4 (contig containing DXS101c) and U97D2 (DXS1106); in families DH and DB, it was positioned between U97D2 (DXS1106) and U92G2 (DXS54). Examples of the differences in proximal breakpoints are presented in figure 2. We show that cosmid U65A4 (DXS101c) is duplicated in family NO (fig. 2*F*) but not in family DH (fig. 1*G*). We also show that cosmid U97D2 (DXS1106) is duplicated in family NO (fig. 2*H* and *I*) but not in family DB (fig. 2*E*).

An unusual rearrangement is thought to have occurred in family DB, because the duplicated signals could be clearly separated on metaphase chromosomes (fig. 2*D*). However, the extent of the duplication appears the same as that for family DH and smaller than that for families NO and DB. Further characterization of the rearrange-

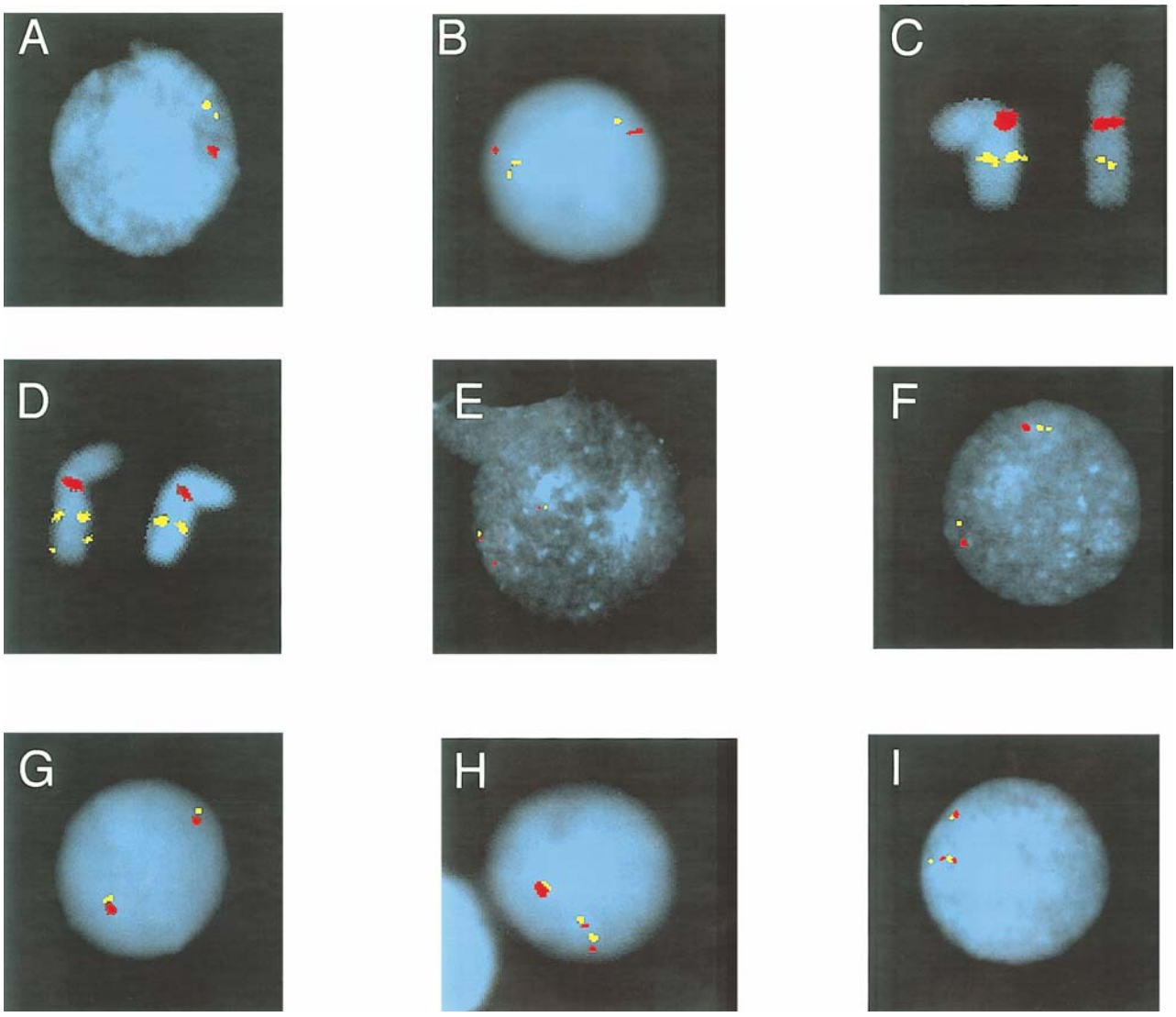

Figure 2 Interphase FISH using cosmids from Xq22. *A,* Interphase nucleus from patient DH, hybridized with a biotin-labeled PLP cosmid (U125A1) (*yellow*) and a digoxigenin-labeled X-centromeric probe (*red*). The two yellow signals indicate a PLP duplication. *B,* Interphase nucleus from mother DH, hybridized with a biotin-labeled PLP cosmid (U125A1) (*yellow*) and a digoxigenin-labeled X-centromeric probe (*red*). The two adjacent yellow signals indicate a PLP duplication, and the single yellow signal indicates the normal X chromosome. *C,* Two X chromosomes from a metaphase spread from mother ND. The red signal indicates the centromere, and the yellow signal indicates the PLP cosmid probe. The larger PLP hybridization signal on the left chromosome suggests that it may carry the duplication. *D,* Two X chromosomes from a metaphase spread from mother DB. The red signal indicates the centromere, and the yellow signal indicates cosmid U92G2 (DXS54), which is ~150 kb proximal to PLP (see fig. 1). An obvious duplication is shown on the left chromsome. *E,* Interphase nucleus from mother DB, hybridized with a digoxigenin-labeled PLP cosmid (U125A1) (*red*) and a biotin-labeled DXS1106 cosmid (U97D2). The duplicated PLP signals (*red*) are farther apart than those found in family HD (see *A* and *B*). Cosmid U97D2 was not duplicated. *F,* Interphase nucleus from mother NO, hybridized with biotin-labeled cosmid U65A4 (*yellow*) and a digoxigenin-labeled X-centromeric probe (*red*). The two adjacent yellow signals indicate that the cosmid is duplicated, and the single yellow signal indicates the normal X chromosome. *G,* Interphase nucleus from mother DH, hybridized with biotin-labeled cosmid U65A4 (*yellow*) and a digoxigenin labeled X-centromeric probe (*red*). The two yellow signals at either side of the nucleus show that the cosmid is not duplicated. *H,* Interphase nucleus from mother NO, hybridized with digoxigenin-labeled U65A4 cosmid (*red*) and biotin-labeled U97D2 cosmid (*yellow*). The yellow-red-yellow-red pattern suggests a duplication in a head-to-tail arrangement. The adjacent red and yellow signals indicate the normal X chromosome. *I,* Interphase nucleus from mother NO, hybridized with digoxigenin-labeled PLP cosmid (*red*) and biotin-labeled U97D2 cosmid (*yellow*). The yellow-red-yellow-red pattern suggests a duplication in a head-to-tail arrangement. The adjacent red and yellow signals indicate the normal X chromosome.

ment is required, and the possibility of a PLP duplication with an insertion event is being investigated.

Orientation of the Duplication

Two-color interphase FISH was used to orient the duplication in a head-to-tail direction in family NO, as shown in figure 2*H* and *I*. Cosmids U65A4 and U97D2 were labeled with digoxigenin and biotin, respectively, and were hybridized onto cells from the carrier mother. Nuclei showed four signals together in a red-yellow-red-yellow order, indicating a tandem duplication, and a pair of signals elsewhere in the nucleus, one red and one yellow, which were from the normal X chromosome. As expected, the signals could not be resolved on metaphase chromosomes, because the probes were too close. Cosmids U125A1 and U97D2 were also hybridized together, and the same pattern was observed after detection by two-color FISH.

Identification of Duplication by Quantitative Fluorescent Multiplex PCR

Two PLP:PRNP-ratio values were generated for each sample, and these were plotted against each other and are shown in figure 3. There was distinct separation of PLP:PRNP ratios for male and female controls (fig. 3), and the mean values were calculated to be 0.4:1 and 0.8:1, respectively (table 1). As expected, the ratio for females was twice that for the male controls; these ratios corresponded to one and two copies of the PLP gene, respectively. These values provided controls for comparison with the ratios obtained for patients and their mothers. Although there was considerable scatter in the ratios, the PLP:PRNP ratios for the affected males were always greater than those for normal males and fell into or above range for normal females (fig. 3*A–D*). These results indicate that the five PMD patients have an increased dose of the PLP gene and confirm the PLP duplication detected by FISH in all four families.

The quantitative PCR was less reliable for detection of female carriers with three copies of the PLP gene (see table 1). The quantitative-PCR result for mother DB showed that the PLP-gene dosage was the same as that for normal females and therefore conflicted with the duplication detected by interphase FISH (fig. 2*D* and *E*). However, karyotyping of metaphase chromosomes from peripheral blood from this individual has identified a more complex mosaic rearrangement, and further characterization is ongoing.

Table 1 shows the differences, in mean PLP:PRNP ratios, that were found between the PMD families. The

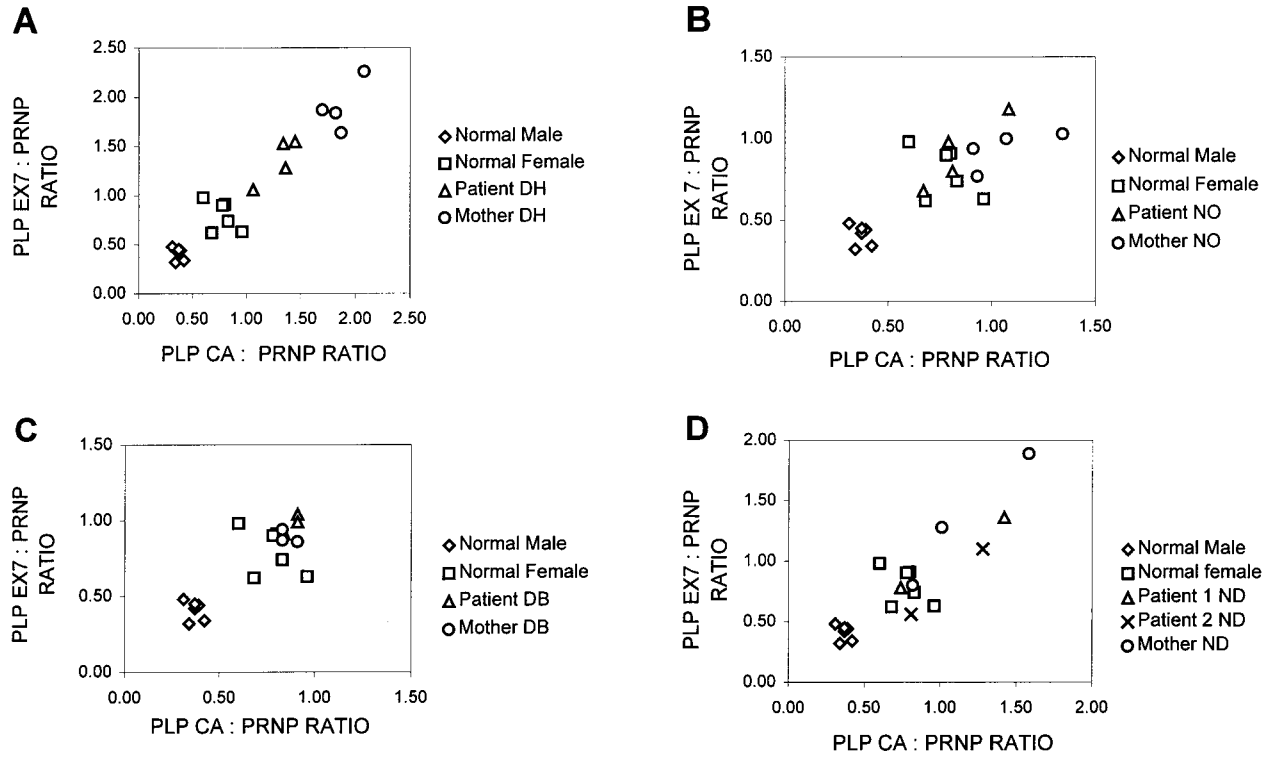

Figure 3 Plots of ratio PLP CA:PRNP against ratio PLP exon 7:PRNP, obtained by quantitative PCR for controls, patients, and mothers. *A*, Family DH. *B*, Family NO. *C*, Family DB. *D*, Family ND.

Table 1

Dosage Estimates Obtained by Quantitative Multiplex PCR, and Comparison with Duplication Detection by Interphase FISH

Sample	PLP:PRNP Ratio		Dosage Estimate, by PCR	Interphase FISH Result
	Mean	Normalized		
Controls:				
Male	.39	.49	One copy PLP (1×)	Normal
Female	.79	1.00	Two copies PLP (2×)	Normal
Patients:				
DH	1.34	1.70	Overdosage (3×)	Duplication
NO	.88	1.11	Overdosage (2×)	Duplication
DB	.94	1.19	Overdosage (2×)	Duplication
ND-1	1.08	1.37	Overdosage (2.8×)	Duplication
ND-2	.94	1.19	Overdosage (2×)	Duplication
Mothers:				
DH	1.89	2.39	Overdosage (4.8×)	Duplication
NO	1.00	1.27	Two or three copies PLP (2.6×)	Duplication
DB	.88	1.11	Normal (2×)	Duplication
ND	1.23	1.56	Overdosage (3×)	Duplication

Note.—PLP:PRNP values were normalized to give a ratio of 1 for female controls and a ratio of ~.5 for male controls. By comparing normalized ratios for controls and patients, dosage estimates for the fluorescent quantitative PCR were obtained (the numbers in parentheses represent the number of copies of the PLP gene, as indicated by PCR). These results are compared with those for interphase FISH (with duplications as indicated).

normalized ratio for patient DH and his mother were much greater than expected for a duplication of the PLP gene. The increased dosage in family DH compared with that in family NO has also been reported by Ellis and Malcolm (1994), who used quantitative Southern analysis. The overdosage suggests that this family may have a triplication of the PLP gene, but this is not supported by interphase FISH. The results for the interphase FISH are shown in figure 2*A* and *B*. The boy clearly shows only two hybridization signals, and the mother has three.

Multiallelic Markers

Polymorphic markers from an intron of the PLP gene and from within the duplicated region (fig. 1) were analyzed to try to identify multiple alleles that would indicate a duplication. The observed heterozgosities for these markers are .65–.8. Only one patient (DB) showed two alleles for any marker. The mothers were heterozygous for one to three of the four markers analyzed (table 2). In accordance with the data on females, eight female controls analyzed were heterozygous for an average of two or three markers (data not shown).

Discusssion

Duplication of the PLP gene is clearly a frequent form of mutation in PMD, and it has been suggested that duplications may be more common than point mutations in PLP (Inoue et al. 1996*a;* Wang et al. 1997; Sistermans et al., in press). We have shown that interphase FISH is a reliable technique for detection of PLP duplications in patients and their carrier mothers.

We have shown that the duplicated segment is large, 500 < 1,650 kb. The proximal breakpoint was shown to differ between families but was ⩾150 kb from the PLP gene. The distal breakpoint was found to be constant, on the basis of the cosmids tested in this study, and was ⩾350 kb from the PLP gene. This finding supports previous work, which had shown that the duplication does not interrupt either the coding sequence of the PLP gene or the immediately flanking regions (Ellis and Malcolm 1994). It suggests that increased dosage of the gene, rather than disruption of PLP coding or regulatory regions, is responsible for the clinical phenotype. The overexpression of the DM20 transcript in two brothers with PMD, which has been reported by Carango et al. (1995), supports this idea. It is also in line with evidence in PLP-transgenic mice (Kagawa et al. 1994; Readhead et al. 1994; Inoue et al. 1996*b*). The duplication was not found to extend to another locus on Xq21, which is reported to map between markers DXS3 and DXS106 in a single family with PMD-like disease (Lazzarini et al. 1997). These markers are proximal to the area that we have shown to be duplicated; and, therefore, a mutation in a second gene within the duplication cannot be the molecular basis for disease in this family.

Quantitative fluorescent PCR could confirm the duplications in all five PMD patients. However, reproducible results confirming increased dosage could not be obtained in all carrier mothers, either in this study or in others (Sistermans et al., in press). The accuracy and sensitivity of the quantitative assay can be affected by a number of factors, including the nonuniform amplification of loci and the quality and source of genomic DNA. This may be the reason why conflicting PCR and FISH results were obtained for mother NO, who already had been known to be a carrier (Ellis and Malcolm 1994). The inconsistency between results obtained by different methods, for mother DB, is probably explained by a further mosaic rearrangement detected during karyotyping of metaphase chromosomes from peripheral blood. More-extensive analysis will be required in order to determine the complex rearrangements in this individual.

One family (DH) consistently gave results suggesting a triplication of the PLP gene. This was true for both the patient and his mother, who was an obligate carrier, whether measured by quantitative PCR (table 1) or the earlier quantitative Southern blot (Ellis and Malcolm 1994). The suggestion of a triplication is not supported by interphase FISH (fig. 2*A* and *B*), in either the mother or the child. If the patient has three copies of the PLP

Table 2

Analysis of Multiallelic Markers from Duplicated Region in PMD

	Allele Size(s) [bp]/ Duplication Status of[a]			
Sample	DXS1106	DXS8096	PLP	DXS1191
Patient DH	178/−	235/?	136/+	243/+
Mother DH	178 and 180/−	235/?	136/+	243/+
Patient NO	180/+	235/+	134/+	243/+
Mother NO	174 and 180/+	235/+	134/+	239 and 243/+
Patient DB	180/−	258/?	134 and 146/+	243/+
Mother DB	180/−	256 and 258/?	134 and 146/+	243/+
Patient ND-1	180/+	258/+	136/+	239/+
Patient ND-2	180/+	258/+	136/+	239/+
Mother ND	178 and 180/+	235 and 258/+	136/+	239 and 243/+

[a] The extent of the duplication in each family is shown, to identify informative markers. Cosmid probes for PLP, DXS1106, and DXS1191, which were found, by FISH, to be duplicated and which therefore are informative are denoted by a plus sign (+), and those probes not duplicated in each family and therefore uninformative are denoted by a minus sign (−). DXS8096 is positioned between DXS1106 and DXS54, but a cosmid probe was unavailable. DXS8096 was assumed to be informative in families NO and ND, because it was contained within the duplication; since we do not know whether it is duplicated in families DH and DB, the status is represented by a question mark (?).

gene on his X chromosome, then two copies must be so close together that they cannot be resolved by interphase FISH. Since the PLP gene is $\geqslant$150 kb from either breakpoint, this would not be predicted in either a head-to-tail or a head-to-head orientation. The interphase FISH did not show consistent evidence of one hybridization signal of greater intensity than the other (fig. 2*A* and *B*). Further analysis of the rearrangement in this family is ongoing. It is noteworthy that patient DH was the most severely affected of those studied. He had the connatal form of the disease and died at age 10 mo.

Another potential method of establishing a duplication is detection of heterozygosity in affected males, after analysis of several multiallelic markers across the region. This approach has been used to detect the 1.5-Mb duplication in autosomal dominant CMT1A, by the presence of three alleles at one or more marker loci (Lupski et al. 1991; Thomas et al. 1997) or by dosage differences between two RFLP alleles (Lupski et al. 1991; Raeymaekers et al. 1991; Thomas et al. 1997). However, despite the use of four markers with heterozygosities of .65–.8, four of five boys with PMD were homozygous for all markers detected. In three boys, the duplication covered all four markers. In the other two boys (DB and DH), two markers were confirmed to be in the duplicated region, and one was outside it. In contrast, each of 12 women analyzed either because they were potentially carrier mothers or as normal controls was heterozygous for an average of two to three markers. Similarly, of four boys with duplications reported by Inoue et al. (1996*a*), none were heterozygous for the less informative *Aha*II polymorphism in exon 4 (heterozygosity .33) (Trofatter et al. 1991), whereas three of the four mothers were heterozygous. The tendency toward homozygosity strongly suggests that both duplicated alleles are usually derived from the same chromosome and that the duplication may have arisen by an intrachromosomal event. Unfortunately, the results show multiallelic marker analysis to be an unsuitable method for detection of duplications in PMD patients.

Heterozygosity of a CA-repeat marker was found in only one patient. Two alleles were detected for the intronic PLP marker (Mimault et al. 1995) in patient DB. There are other unusual features of the duplication in this family. Although it is smaller than the duplication in two other families (NO and ND), the duplicated signals are reproducibly separated by a greater distance in interphase FISH, and this is the only family in which separate signals can be seen on metaphase chromosomes. This suggests that it is not a simple head-to-tail orientation. It is possible that there has been a mutation in the length of the CA repeat, because of slippage during replication, and that the duplication may have originally contained identical alleles. However, these events are rare, and a more likely explanation is that the duplication arose originally in a female, by recombination between two X chromosomes. No other family members are available to allow us to study this point. One other example of a boy heterozygous for a polymorphism within PLP has been reported (Hodes et al. 1993). This finding also suggests that interchromosomal events may be involved in duplications causing PMD.

In all 11 of the families reported thus far, the PLP duplication has been present in the mothers (Inoue et al. 1996*b;* Wang et al. 1997; present study). There have not been any de novo events observed. This is of relevance for genetic counseling and suggests that the rearrangements originate in a male. Confirmation of origin of the

duplication can be obtained only by extension of haplotype analysis to further generations, but, unfortunately, grandparental samples currently are unavailable. Since there was no association between a particular haplotype and the duplication, and because the extent of the duplication varied between families, the origin of the rearrangement in each family is thought to be independent.

There is a growing list of genetic disorders resulting from aneusomies, either duplications or deletions, in the megabase range. In most cases, the chromosomal rearrangement is mediated by unequal crossover, through misalignment of homologous low-copy-number repeats located within and flanking the region. CMT1A and hereditary neuropathy with liability to pressure palsies (HNPP) result from duplications and deletions, respectively, of a 1.5-Mb segment, on chromosome 17p11-12, which is flanked by two homologous sequences, CMT1A-REP (Pentao et al. 1992; Chance et al. 1994; Kiyosawa et al. 1995; Kiyosawa and Chance 1996). Smith-Magenis syndrome (SMS) patients have a common deletion, spanning ~5 Mb, resulting from homologous recombination between low-copy-number repeats found in three copies in the critical region (Chen et al. 1997). In CMT1A, HNPP, and SMS, junction fragments can be detected by pulsed-field gel electrophoresis (Raeymaekers et al. 1992; Chen et al. 1997). In CMT1A and HNPP, there is a recombination hot spot between the CMT1A-REPs, which can be detected by Southern blotting (Kiyosawa and Chance 1996; Reiter et al. 1996). In contrast, limited heterogeneity of the breakpoints has been found at both ends of the Angelman syndrome/Prader-Willi syndrome region (Knoll et al. 1990; Christian et al. 1995; Huang et al. 1997).

PMD can be compared to CMT1A, since both duplications and point mutations in the PMP-22 gene cause the disease. However, in the majority of cases, a common 1.5-Mb duplication is detected (Lupski et al. 1991; Raeymaekers et al. 1991; Nelis et al. 1996), which is generated by a paternal interchromosomal recombination event (Palau et al. 1993; Lopes et al. 1998). There are only a few reports of alternatively sized duplications involving the PMP-22 gene (Ionasescu et al. 1993; Valentijin et al. 1993). Recent analysis of a series of de novo cases of CMT1A and HNPP has shown that, in the four cases of HNPP, there were only one deletion of paternal origin (inter- or intrachromosomal origin unknown) and three deletions of maternal origin (one of unknown origin and two intrachromosomal events). Of the duplications giving rise to CMT1A, 34 of 36 were of paternal origin; 32 of these 34 could be proved to involve both chromosome 17 homologues, and 2 were of maternal origin and were, again, intrachromosomal (Lopes et al. 1998). Thus, the hypothesized deletion that would be the reciprocal of the duplication is actually a very infrequent event, and the mechanism of rearrangement is sex dependent, with most of the de novo maternal events being intrachromosomal.

In the PMD families presented in the present study or in the literature, there has been no example of the duplication arising de novo, so the origin can be tested only indirectly. All mothers tested were carriers, and at least one of the grandmothers was also a carrier, since she had had an affected son. This suggests that the mutation may frequently arise in sperm. If this is the case, then, since males have only one X chromosome, the duplication would have to be intrachromosomal. This is in agreement with the observed tendency toward homozygosity of polymorphic markers in affected boys, which suggests that both duplicated alleles are usually derived from the same chromosome and that the duplication may have arisen by an intrachromosomal event. There has been one report of a complete deletion of the PLP gene on chromosome Xq22, a deletion that also gives rise to PMD (Raskind et al. 1991). A deletion of $\geqslant$29 kb has been reported, which suggests that it is unlikely to be an event reciprocal to the duplication that causes PMD. If, as we suggest, intrachromosomal rearrangements are more common, then a reciprocal deletion may be rare and also could be incompatible with life.

In summary, we have demonstrated that duplications involving the PLP are a cause of PMD. We have shown that the duplications are best detected by interphase FISH, especially in female carriers. Detection of duplications in both patients and female carriers is important for genetic counseling. Unfortunately, analysis of multiallelic markers is not diagnostically useful, since most duplications are intrachromosomal and, therefore, the markers are not heterozygous. Further refinement of the duplication breakpoints will enable us to understand both the mechanism of the rearrangement and the extent of phenotypic difference between families. Analysis of other transcripts from the region is also in progress, since there may be modifier genes that play a role in affecting the severity of the disease.

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Electronic-Database Information

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for PMD [MIM 312080])

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