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Proteolipoprotein Gene Analysis in 82 Patients with Sporadic Pelizaeus-Merzbacher Disease: Duplications, the Major Cause of the Disease, Originate More Frequently in Male Germ Cells, but Point Mutations Do Not

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Summary

Pelizaeus-Merzbacher Disease (PMD) is an X-linked developmental defect of myelination affecting the central nervous system and segregating with the proteolipoprotein (*PLP*) locus. Investigating 82 strictly selected sporadic cases of PMD, we found *PLP* mutations in 77%; complete *PLP*-gene duplications were the most frequent abnormality (62%), whereas point mutations in coding or splice-site regions of the gene were involved less frequently (38%). We analyzed the maternal status of 56 cases to determine the origin of both types of *PLP* mutation, since this is relevant to genetic counseling. In the 22 point mutations, 68% of mothers were heterozygous for the mutation, a value identical to the two-thirds of carrier mothers that would be expected if there were an equal mutation rate in male and female germ cells. In sharp contrast, among the 34 duplicated cases, 91% of mothers were carriers, a value significantly ($\chi^2 = 9.20$, $P < .01$) in favor of a male bias, with an estimation of the male/female mutation frequency (k) of 9.3. Moreover, we observed the occurrence of de novo mutations between parental and grandparental generations in 17 three-generation families, which allowed a direct estimation of the k value ($k = 11$). Again, a significant male mutation imbalance was observed only for the duplications. The mechanism responsible for this strong male bias in the duplications may involve an unequal sister chromatid exchange, since two deletion events, responsible for mild clinical manifestations, have been reported in *PLP*-related diseases.

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Introduction

Pelizaeus-Merzbacher disease (PMD; MIM 312080), is an X-linked defect of myelin formation affecting the CNS. Originally described by Pelizaeus (1885), the clinical syndrome was neuropathologically defined by Merzbacher (1910) as a diffuse hypomyelination of the CNS, associated with an abnormally low number of mature oligodendrocytes. The diagnosis is based on early/impaired motor development (during the first 3 mo of life) characterized by severe hypotonia associated with nystagmus and, later, the development of abnormal movements and progressive spastic paraplegia. The severity of the disease correlates with the level of motor performance acquired between 5 and 10 years of age; intellectual development is generally less affected (Boespflug-Tanguy et al. 1996). Conduction velocity analysis and magnetic resonance imaging (MRI) of the brain confirm that the dysmyelinating process is limited to the CNS (Boulloche et al. 1986).

Several lines of evidence led to the identification of the proteolipoprotein gene (*PLP*) as the causative locus in PMD. This gene maps to the long arm of the human X chromosome at Xq21-q22 (Mattei et al. 1986) and encodes the two major myelin proteins of the CNS, the proteolipoprotein (PLP) and its isoform, DM20. Several point mutations in the coding and noncoding regions of the *PLP* gene (for references, see Nave et al. 1996) have been associated with the PMD phenotype but account for only 10%–25% of the families analyzed. However, linkage analysis established apparent genetic homogeneity of families affected by PMD (Boespflug-Tanguy et al. 1994). In fact, in most families in which point mutations were not found, duplications of the entire *PLP* gene are the causative mutation (Ellis et al. 1994; Inoue et al. 1996; Boespflug-Tanguy et al. 1997; Sistermans et al. 1998).

Investigating 82 patients with sporadic PMD, we found a *PLP* mutation in 63 (77%). Complete *PLP*-gene

duplication was the most frequent abnormality (62% of all mutations), and coding or splice-site point mutations were found in 38% of all mutations. To determine the origin of both types of *PLP* mutation, we analyzed the maternal status in 56 cases. In addition, in the 17 three-generation families in which the mother was a carrier but the grandmother was not, we used haplotype segregation analysis to determine the grandparental origin of the mutations. We found that the de novo *PLP* duplications are of male origin in the large majority of cases, whereas point mutations occur with the same frequency in male and female gametes.

Families, Material, and Methods

Diagnosis and Selection of Families Affected by PMD

Patients with PMD were referred to us by neuropediatricians in several European countries because of our interest in this condition. PMD was diagnosed by the combination of a clinical PMD syndrome (hypotonia and nystagmus of early onset, abnormal trunk and limb movements, development of spastic paraplegia with pyramidal tract signs, and slow progression of the disorder) in a male patient, with neurophysiological and MRI evidence for a dysmyelinating process confined to the CNS (Boespflug-Tanguy et al. 1994). Appropriate informed consent was obtained from all families.

Two groups of subjects were used in this study: one group of individuals, used to determine conditions for the analysis of duplications, and one group of 82 families affected by sporadic PMD, used to evaluate type and origin of *PLP* abnormalities. The first group of subjects comprised 45 normal males, 34 noncarrier females, and 18 obligate carrier mothers. Because of the absence of any clinical signs in the great majority of females who were heterozygous for PMD, the 34 noncarrier mothers were identified by *PLP* haplotype segregation analysis in 24 large families with *PLP*-duplicated probands. In the same families, the 18 obligate carrier mothers were selected if they had both an affected son and an affected brother.

DNA Preparation

Genomic DNA was extracted by the guanidinium chloride technique, as described elsewhere (Jeanpierre 1987).

Detection of PLP *Point Mutations*

DNA amplification using intronic primers flanking each exon of the human *PLP* gene (Gencic et al. 1989) was performed under standard conditions adapted for each set of primers, as described elsewhere (Pham-Dinh et al. 1991). Amplified DNA was sequenced on both strands, with the same primers used in the amplification reaction and the DNA Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequence analysis was performed with an Applied Biosystems model 377 DNA Sequencer.

Detection of PLP *Duplications in Patients with PMD*

A multiplex PCR-based test was performed to coamplify exon IV from the *PLP* gene (Gencic et al. 1989) and exon IV from the *CFTR* gene (Dean et al. 1990). The use of one fluorescently labeled primer in each set allowed the quantification of the multiplex PCR products on an automated sequencer: *PLP* SG50, 5′-ACTCCAGGATCTCCCAGTTT-3′ (labeled with 6FAM); *PLP* 4R, 5′-CGTACCCTAACTCACCATAC-3′; *CFTR* 4A, 5′-AGTCACCAAAGCAGTACAGC-3′ (labeled with 6FAM); and *CFTR* 4B, 5′-GCTATT-CTCATCTGCATTCC-3′.

Preliminary experiments showed that, between 20 and 24 cycles, the amount of PCR product was directly proportional to the copy number of the target sequence. The reactions were performed in a PHC3 Techne thermalcycler in a final volume of 50 μl containing 100 ng of genomic DNA, 0.2 mM each dNTP, 1.5 mM magnesium chloride, 25 pmol of each primer, and 2 U of *Taq* polymerase in the buffer provided by the manufacturer (Gibco BRL). Cycling conditions for each of the 21 cycles were 1 min at 91°C, 45 s at 58°C, and 1.30 min at 70°C, the first cycle being preceded by one step of 5 min at 94°C and the last cycle being followed by an extension program at 70°C for 10 min. One microliter of amplified DNA, mixed with agonized formamide and loading buffer, was heat denatured and then loaded onto a 12-cm 6.25% denaturing polyacrylamide gel. The intensities of the bands corresponding to exons IV of the *CFTR* and *PLP* genes were analyzed by an Applied Biosystems model 377 DNA Sequencer with the GENSCAN and GENOTYPER software (Applied Biosystems). The *PLP*-gene copy number in each individual was estimated by comparison of the areas under the peaks for *PLP* exon IV (*PLP4*) and *CFTR* exon IV (*CFTR4*).

We evaluated the quality of each experiment and the reproducibility of results between experiments by analyzing three distinct control-DNA samples three times in each experiment: one control was a mixture of eight DNA samples from normal males, one was a mixture of eight DNA samples from obligate carrier females, and the third was a mixture of eight DNA samples from noncarrier females. The final result was the ratio of the *PLP*-gene dosage obtained for each PMD patient to the *PLP*-gene dosage of a mix of eight DNA samples from normal males analyzed in the same experiment.

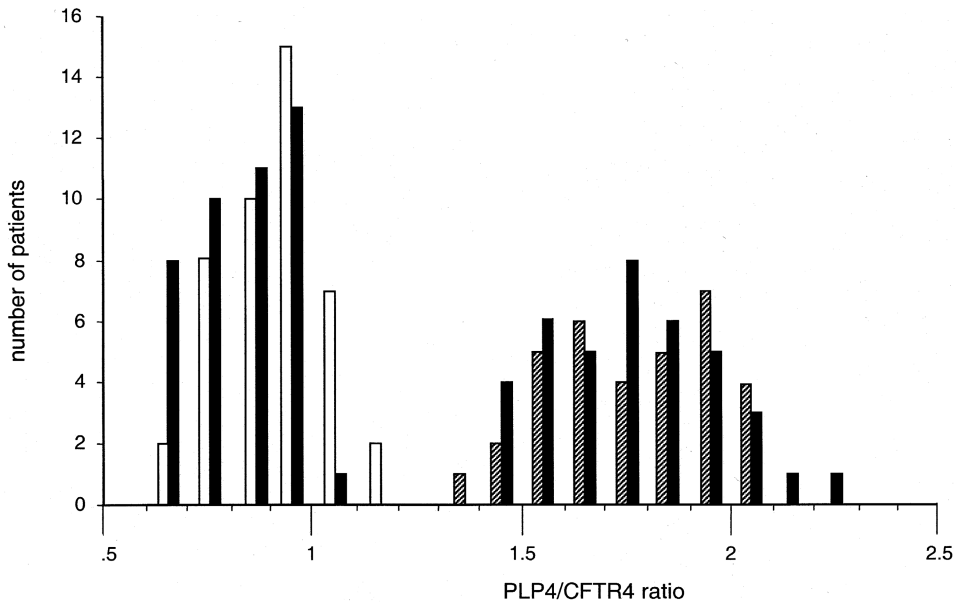

Figure 1 Detection of *PLP* duplication in 82 patients with sporadic PMD. The comparison of the fluorescence intensities of two PCR products obtained by multiplex PCR (*PLP* exon 4 and *CFTR* exon 4) allows a discriminant analysis between one group of 45 normal males (white histograms) and one of 34 noncarrier females (black hatched histograms). The analysis of the *PLP4/CFTR4* ratio in 82 patients with sporadic PMD (black histograms) resulted in two groups: one of 43 subjects that were similar to the normal male population and one of 39 subjects that were similar to the noncarrier female population. Each *PLP4/CFTR4* result corresponds to the ratio of the *PLP* gene signal for one subject to the *PLP* gene signal obtained with the mix of eight DNAs samples from normal males analyzed in the same experiment.

Detection of Heterozygous Carriers for the PLP *Duplication*

To improve discrimination between normal females and females who are heterozygous carriers of the *PLP* duplication, two additional sets of primers were used: one corresponding to exon 2 of the *PLP* gene (*PLP2*), the other to exon 48 of the dystrophin gene (*DMD48*): *PLP2*-SG 47, 5′-ATGAGCTACCTACTGGATGT-3′ (labeled with 6FAM); *PLP2R*, 5′-GGGCAGGTACTTACACATTG-3′; *DMD48MR*, 5′-CCTGAATAAAGTCTTCTTTACCACAC-3′ (labeled with 6FAM); and *DMD48MF*, 5′-AAGCTTGAAGACCTTGAAGAGC-3′. Both pairs, *PLP2-CFTR4* and *PLP4-DMD48*, were coamplified in the same conditions as *PLP4-CFTR4*.

RFLP Markers

The genomic probe pXG-12 (DXS94), which detects three *Pst*I alleles with sizes of 6.5, 7.2, and 7.4 kb, was used on Southern blots as described elsewhere (Boespflug-Tanguy et al. 1994). Three other RFLP markers, *PLP* exon IV/*Aha*II (Trofatter et al. 1990), *DXS17*/*Taq*I (Kornreich et al. 1992), and *PLP* intron V/*Msp*I (Wu et al. 1987), were analyzed after a PCR reaction as described elsewhere (Boespflug-Tanguy et al. 1994).

Multiallelic Markers

A *PLP*-intragenic microsatellite (Mimault et al. 1995) and the two nearest known flanking extragenic microsatellites, DXS1191 and DXS1106 (Dib et al. 1996), were used. The PCR-amplification reactions were performed with one fluorescently labeled primer in each set and a Gene Amp PCR System 9600 (Perkin Elmer) according to standard procedure (Mimault et al. 1995). Fragment analysis was performed on an Applied Biosystems model 377 DNA Sequencer with the GENSCAN and GENOTYPER software (Applied Biosystems).

Estimation of the Male/Female Ratio of Mutation Frequencies

Two different methods were used to estimate the male/female ratio of mutation frequencies (k). (1) Indirect estimation was performed in two-generation families according to the method of Rosendaal et al. (1990), described elsewhere for hemophilia A (Becker et al. 1996). This method determines the proportion of PMD mothers who are carriers of the mutation, this proportion being correlated to the k value. (2) Direct estimation was performed in three-generation families in which the mother was a carrier but the maternal grandmother was not. The origin of the mutation in the maternal grandparental generation was determined by haplotype anal-

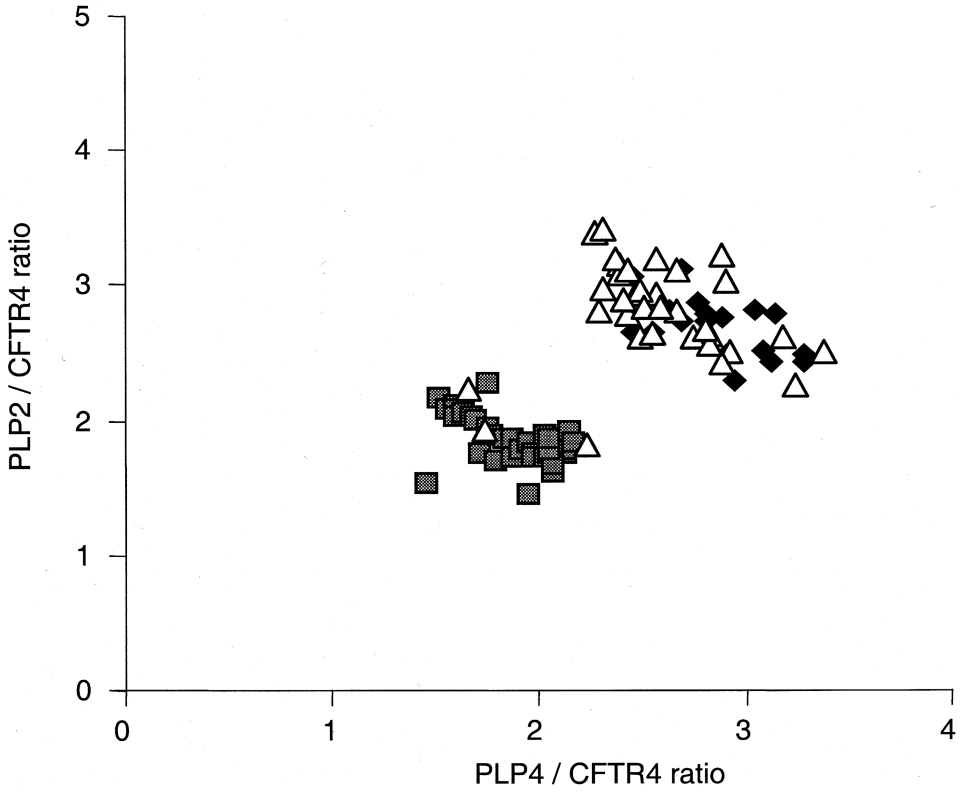

Figure 2 Detection of *PLP* duplication in PMD carrier mothers. Results with two of the three different sets of primers used to perform multiplex PCR (*PLP4 / CFTR4* and *PLP2 / CFTR4*) are represented. Linear discriminant analysis with a nonparametric test revealed a hyperplane that totally separated two classes of females, 18 obligate carriers (blackened diamonds) and 34 noncarrier mothers (black hatched squares), without misclassifying any individual. It allowed testing of the 34 mothers of sporadic *PLP*-duplicated cases with an undetermined status (white triangles): 31 were significantly identified as females with duplication, 3 as noncarriers.

ysis. In this case, k equals the number of cases of grandfather (MGF) origin divided by the number of cases of grandmother (MGM) origin (Haldane 1935). Differences between experimental and theoretical values were evaluated by χ^2 analysis.

Results

Validation of Duplication Detection by Quantitative Fluorescent Multiplex PCR

We first validated our *PLP*-gene duplication analysis with quantitative fluorescent multiplex PCR by proving that the method can distinguish between the presence of one, two, or three copies of the *PLP* gene. First, to assess the ability of the technique to distinguish between one and two *PLP*-gene copies, we analyzed a population of 45 normal males and 34 noncarrier females (fig. 1). A discriminant analysis between the male and female populations, using the *PLP4/CFTR4* ratio as the sole variable, demonstrated a 100% classification of the individuals into their respective groups (Mahalanobis distance^2 = 20.58, F = 405, $P < .0001$). The mean ratio was 0.99 ± 0.12 for the male control population and 1.87 ± 0.24 for the female one.

Second, to assess discrimination between two and three *PLP*-gene copies, we analyzed the 34 noncarrier females and the 18 obligate carrier mothers with three different sets of primers. The mean ± SD for the noncarrier females was 1.87 ± 0.24 for the *PLP4/CFTR4* ratio, 1.84 ± 0.18 for the *PLP2/CFTR4* ratio and 0.94 ± 0.13 for the *PLP4/DMD48* ratio. For the *PLP*-duplicated obligate carriers, the values were, respectively, 2.86 ± 0.26, 2.69 ± 0.18, and 1.39 ± 0.13. Discriminant analysis was used to compare the population of obligate *PLP*-duplicated carriers with the noncarrier females: the two populations were significantly distinct when the *PLP4/CFTR4* ratio was used as the sole variable (Mahalanobis distance^2 = 19.24, F = 266, $P <$ .0001); however, discrimination clearly improved if *PLP2/CFTR4* was introduced as a second variable (Mahalanobis distance^2 = 80.77, F = 466, $P < .0001$) and *PLP4/DMD48* as a third variable (Mahalanobis distance^2 = 140.81, F = 538, $P < .0001$). Therefore, we used all three measures to classify the two groups of

Table 1

***PLP*-Gene Point Mutations**

Family Number	Mutation Site	Type of Mutation[a]
99	Exon 2	C34Y
176	Exon 2	A38T
106	Exon 2	Y59C
191	Exon 2	C168R
107	Exon 3	G73R
21	Exon 4	Y174C
209	Exon 4	W180C
41	Exon 4	T182N
49	Exon 4	D202N
37	Exon 4	D202G
36	Exon 4	D202E
181	Exon 5	L209H
52	Exon 5	W211R
24	Exon 5	P215S
23	Exon 5	C227Y
133	Exon 6	Q233P
143	Exon 6	G245E
217	Exon 6	A247E
147	Exon 1	ATG-ACG
26	Intron 3	asp (G→T)
198	Intron 5	asp (G→A)
149	Intron 5	del 26 nuc
221	Intron 6	dsp (T→C)
182	Exon 6	ins TT fs F239→

[a] asp = acceptor splice site, dsp = donor splice site, ins = insertion, del = deletion, and fs = frameshift.

females for linear discriminant analysis with a nonparametric test (Geoffrey 1992). A hyperplane totally separated the two classes of females without misclassifying any individual (fig. 2). The difference was significant, at risks of .05 and .01. This computed hyperplane allowed reliable classification of females of undetermined status.

Mutation Analysis in Sporadic Probands

Analysis of *PLP4/CFTR4* ratio in the 82 PMD sporadic probands identified two groups (fig. 1). (1) In 39 cases the *PLP4/CFTR4* values (1.87 ± 0.21) were very close to the control female values (1.87 ± 0.24), suggesting the presence of two copies of the *PLP* gene. The duplication event was confirmed by the detection of heterozygosity in two affected males, by analysis of the *PLP* intron 1 dinucleotide-repeat polymorphism (data not shown). (2) In 43 patients, the *PLP4/CFTR4* values (0.95 ± 0.10) were in the same range as those of the control male population. These 43 patients were evaluated by direct sequencing of the seven *PLP* exons and exon/intron junctions. Abnormalities were found in 24 cases (table 1). Of these, 18 (3/4) were missense mutations in coding regions of the *PLP* gene. Therefore, we could demonstrate a *PLP*-gene abnormality in 63 of the 82 cases of sporadic PMD (77%), 62% of the mutations being gene duplications.

Detection of PLP *Mutation in Mothers of Patients with PMD*

To determine the origin of both types of *PLP* mutation, we examined the mothers of the 63 probands in whom mutations were identified (table 2). Only 56 mothers were available for this analysis (table 2, samples 8–63). In the 34 duplicated cases, 31 mothers (91%) were heterozygous for the duplication, as determined by analysis of the ratios of *PLP4/CFTR4*, *PLP2/CFTR4* (fig. 2), and *PLP4/DMD48*. In the 22 point mutation cases, 15 mothers (68%) were carriers of the mutation. No mutation was found in the mothers of 10 (18%) probands (three duplications and seven point mutations), suggesting that a de novo mutation occurred in a germ cell of the patient's mother.

Determination of De Novo PLP *Mutations in the Maternal Grandparental Germ Cells*

To study more precisely the origin of *PLP* mutations, we compared the number of de novo mutations in MGF germ cells with those in the MGM. A blood sample from the MGM was available for 25 (16 duplications and 9 point mutations) of the 46 families, in which the mother was found to be carrier of the mutation (table 2, samples 39–63). In eight families (table 2, samples 56–63), both the mother and the MGM were carriers of the mutation (four duplications and four point mutations) such that the origin of the *PLP* mutation remained undetermined. In the remaining 17 families (table 2, samples 39–55), the mutation was not detected in the MGM's DNA, indicating the occurrence of a de novo mutation between the parental and grandparental generations. Using haplotype segregation analysis in these 17 three-generation families, we determined the grandparental origin of the mutations (table 2). The de novo mutation originated from the MGF germ cells in 11 of the 12 duplicated families and from the MGM only in 1, leading to a sex ratio of k = MGF/MGM = 11/1. Among the five families affected by point mutations, the mutation originated from the grandfather in four and from the grandmother in one, resulting in a sex ratio of k = 4/1.

Discussion

In Pelizaeus-Merzbacher disease, two types of *PLP* mutations, point mutations and duplications of the *PLP* gene, have been identified. Analyzing 82 index cases of sporadic PMD, we found *PLP*-gene abnormalities in 77% of them, with duplication as the most common cause of the disease (62% of those with gene abnormalities). Sistermans et al. (1998), in an analysis of 24 families affected by sporadic PMD, found a *PLP* abnormality in only 25% of cases (one point mutation and

Table 2

Results of the Mutation Analysis in the 63 Families with a *PLP*-Gene Abnormality

Sample	Family Number	Proband	Mother	MGM	Mutation Origin
1	151	Duplication	Not available	Not available	Unknown
2	153	Duplication	Not available	Not available	Unknown
3	164	Duplication	Not available	Not available	Unknown
4	192	Duplication	Not available	Not available	Unknown
5	206	Duplication	Not available	Not available	Unknown
6	147	Point mutation	Not available	Not available	Unknown
7	182	Point mutation	Not available	Not available	Unknown
8	39	Duplication	Duplication/no abnormality	Not available	Unknown
9	40	Duplication	Duplication/no abnormality	Not available	Unknown
10	51	Duplication	Duplication/no abnormality	Not available	Unknown
11	55	Duplication	Duplication/no abnormality	Not available	Unknown
12	60	Duplication	Duplication/no abnormality	Not available	Unknown
13	74	Duplication	Duplication/no abnormality	Not available	Unknown
14	79	Duplication	Duplication/no abnormality	Not available	Unknown
15	118	Duplication	Duplication/no abnormality	Not available	Unknown
16	121	Duplication	Duplication/no abnormality	Not available	Unknown
17	122	Duplication	Duplication/no abnormality	Not available	Unknown
18	136	Duplication	Duplication/no abnormality	Not available	Unknown
29	187	Duplication	Duplication/no abnormality	Not available	Unknown
20	188	Duplication	Duplication/no abnormality	Not available	Unknown
21	189	Duplication	Duplication/no abnormality	Not available	Unknown
22	227	Duplication	Duplication/no abnormality	Not available	Unknown
23	106	Point mutation	Point mutation/no abnormality	Not available	Unknown
24	133	Point mutation	Point mutation/no abnormality	Not available	Unknown
25	143	Point mutation	Point mutation/no abnormality	Not available	Unknown
26	149	Point mutation	Point mutation/no abnormality	Not available	Unknown
27	176	Point mutation	Point mutation/no abnormality	Not available	Unknown
28	191	Point mutation	Point mutation/no abnormality	Not available	Unknown
29	61	Duplication	No abnormality/no abnormality	Unnecessary	Maternal
30	94	Duplication	No abnormality/no abnormality	Unnecessary	Maternal
31	105	Duplication	No abnormality/no abnormality	Unnecessary	Maternal
32	49	Point mutation	No abnormality/no abnormality	Unnecessary	Maternal
33	52	Point mutation	No abnormality/no abnormality	Unnecessary	Maternal
34	181	Point mutation	No abnormality/no abnormality	Unnecessary	Maternal
35	198	Point mutation	No abnormality/no abnormality	Unnecessary	Maternal
36	209	Point mutation	No abnormality/no abnormality	Unnecessary	Maternal
37	217	Point mutation	No abnormality/no abnormality	Unnecessary	Maternal
38	221	Point mutation	No abnormality/no abnormality	Unnecessary	Maternal
39	7	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
40	28	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
41	31	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
42	19	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
43	42	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
44	46	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
45	56	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
46	57	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
47	132	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
48	150	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
49	154	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGF
50	23	Point mutation	Point mutation/no abnormality	No abnormality/no abnormality	MGF
51	24	Point mutation	Point mutation/no abnormality	No abnormality/no abnormality	MGF
52	26	Point mutation	Point mutation/no abnormality	No abnormality/no abnormality	MGF
53	37	Point mutation	Point mutation/no abnormality	No abnormality/no abnormality	MGF
54	96	Duplication	Duplication/no abnormality	No abnormality/no abnormality	MGM
55	21	Point mutation	Point mutation/no abnormality	No abnormality/no abnormality	MGM
56	15	Duplication	Duplication/no abnormality	Duplication/no abnormality	Unknown
57	44	Duplication	Duplication/no abnormality	Duplication/no abnormality	Unknown
58	50	Duplication	Duplication/no abnormality	Duplication/no abnormality	Unknown
59	144	Duplication	Duplication/no abnormality	Duplication/no abnormality	Unknown
60	36	Point mutation	Point mutation/no abnormality	Point mutation/no abnormality	Unknown
61	41	Point mutation	Point mutation/no abnormality	Point mutation/no abnormality	Unknown
62	99	Point mutation	Point mutation/no abnormality	Point mutation/no abnormality	Unknown
63	107	Point mutation	Point mutation/no abnormality	Point mutation/no abnormality	Unknown

Table 3

Comparison Between the Numbers of Carrier and Noncarrier Mothers Observed in the 56 *PLP*-Mutated Families and the Values that Would Be Expected If There Were an Equal Mutation Rate in Females and Males

Mutation Type (No.) and Carrier Status	Experimental Result	Theoretical Value	χ^2 Value
Point mutations (22):			
Carrier	15	15	
Noncarrier	7	7	...
Duplications (34):			
Carrier	31	22.66	9.20
Noncarrier	3	11.33	$P < .01$

six duplications). The low percentage of *PLP* abnormalities found in their study probably reflects the mode of selection of their patients. They relied on the finding of severe hypomyelination on MRI, which is, however, not specific for PMD and can be observed in numerous acquired as well as hereditary diseases. Combination of this MRI abnormality with the clinical features of PMD and severe CNS conduction impairment on electrophysiological investigations (Boespflug-Tanguy et al. 1994, 1996) is necessary for the accurate diagnosis of PMD.

Various techniques have been proposed for detection of *PLP* duplications, including Southern blotting (Ellis et al. 1994; Sistermans et al. 1998), comparative multiplex PCR (CM-PCR) with ethidium bromide staining for quantification (Inoue et al. 1996), and, more recently, quantitative fluorescent multiplex PCR (Sistermans et al. 1998; Woodward et al. 1998) and FISH (Woodward et al. 1998). The three different sets of multiplex PCR primers we used in different amplification reactions (*PLP4*/*CFTR4*, *PLP2*/*CFTR4*, *PLP4*/*DMD48*) allowed clear discrimination between noncarrier mothers and obligate *PLP*-duplicated carriers. Using results with these three primer pairs for linear discriminant analysis with a nonparametric test, we obtained a hyperplane that perfectly separated the two populations of females. This computed hyperplane allows testing of females with undetermined status. The accuracy and sensitivity of our quantitative assay may be a consequence both of the fact that the primers all correspond to nonpolymorphic coding regions of the different genes and of the use of only two pairs of primers per multiplex PCR amplification.

To improve genetic counseling and to understand the mechanism underlying *PLP* mutations, we determined the origin of point mutation and duplication events by two independent methods. In the families of patients with point mutations, the number of carrier and noncarrier mothers observed is not different from the theoretical values (2/3 carrier and 1/3 noncarrier mothers), leading to a k value of 1 (table 3). In sharp contrast, the experimental values for the duplication cases significantly differ from the theoretical values (table 3), indicating that the mutations are more frequent in male-derived than in female-derived chromosomes ($k = 9.30$). This strong male bias in duplication events was confirmed by the analysis of the grandparents (table 4).

As pointed out by A. C. Chandley (1991) in analyzing de novo mutations, for most aneuploids there is a bias toward maternal origin, whereas point mutations and structural rearrangements, whatever their size, seem to occur much more commonly in males. This appears to be due to the large differences between oogenesis and spermatogenesis. Indeed, large structural rearrangements, which seem to be more frequent in males, can be linked to the movements and contractions of the genome observed during spermatid—but not oocyte—morphogenesis. For point mutations and small structural rearrangements, the active proliferation of spermatogonia, which after puberty divide every 16 days throughout life, may result in a large number of mutations that accumulate with age, to produce the paternal age effects (Penrose 1955). Moreover, both the lower overall sensitivity of the female to mutation induction and the fact that many millions of spermatozoa are produced for the fertilization of a single oocyte would both contribute to rendering the male more error-prone than the female (Chandley 1991). These observations are in agreement with our findings only concerning *PLP* duplications, which occur preferentially, but not exclusively, on the paternal chromosome during spermatogenesis. However, no grandpaternal age effect was observed in our series of *PLP*-duplicated patients (unpublished data).

Investigation of the parental origin of the mutations that cause different genetic disorders reveals a remarkably complex picture. For some, the nearly exclusive paternal origin and the underlying mechanism are well established. This is the case for point mutations in Apert syndrome, which involves a CpG dinucleotide and shows an extreme bias in favor of paternal origin, with evidence for increasing incidence with increasing paternal age (Moloney et al. 1996). Because DNA methylation is low in oocytes (Driscoll et al. 1990), mutations

Table 4

Comparison between the Numbers of *PLP* Mutations of MGM and MGF Origin and the Values Would Be Expected If There Were an Equal Mutation Rate in Females and Males

Type (No.) and Grandparental Origin of Mutations	Experimental Result	Theoretical Value	χ^2 Value[a]
Point mutations (5):			
MGM	1	2.5	1.8
MGF	4	2.5	NS
Duplications (12):			
MGM	1	6	8.33
MGF	11	6	$P < .01$

[a] NS = not significant.

of this type may occur less frequently during oogenesis than during spermatogenesis, where replication errors may also accumulate over the life span of the individual. Similarly, a male origin has been described for the inversion process that disrupts the factor VIII gene at the telomeric extremity of the long arm of the X chromosome (Rossiter et al. 1994). Here the proposed mechanism is a flipping of the tip of the X chromosome, which allows homologous pairing between copies of the A genes localized both within and outside of the factor VIII gene (Lakich et al. 1993). This can occur only during male meiosis, when Xq is unpaired from its homolog. Finally, the demyelinating peripheral neuropathy, Charcot-Marie-Tooth disease type 1A (CMT1A), is, in 77% of cases, caused by a large duplication of the *PMP*-22 gene, whereas a milder form, hereditary neuropathy with liability to pressure palsies (HNPP), is caused by a large deletion of *PMP*-22. The nearly exclusive paternal origin of this duplication process has been well documented (Palau et al. 1993). Reiter et al. (1996) have proposed that the CMT1A duplication and HNPP deletion are reciprocal products of an unequal crossing-over event between misaligned flanking CMT1A-REP repeats. In this case, a mariner transposon–like element near the hot spot of recombination may mediate strand-exchange processes through cleavage by a transposase, the transcript of which is apparently produced in human testis but not in ovaries. The recent analysis of 4 de novo HNPP deletions and 36 CMT1A duplications (Lopes et al. 1998) suggest that unequal crossing-over events, which are frequently the cause of duplications, have a paternal origin, whereas intrachromosomal rearrangements, which are more frequently the cause of deletions, are maternal. Thus, the sequences involved in recombination are likely to be sex-dependent. However, the restricted number of cases (four) with a proved maternal origin do not allow this conclusion to be drawn with certainty.

For other genetic disorders the results are less clear, but a paternal bias is frequently—though not always—observed. Moreover, the paternal bias tends to be stronger for point mutations than for deletions, especially when they arise at CpG dinucleotides or involve particular non-CpG transitions (Ketterling et al. 1993; Grimm et al. 1994; Becker et al. 1996; Lazaro et al. 1996; Sommer and Ketterling 1996). The sex ratio seems to be close to one in hemophilia B (Ketterling et al. 1993) and one in Williams syndrome (Pérez-Jurado et al. 1996; Urban et al. 1996) but is less clear for Duchenne muscular dystrophy (Müller et al. 1992; Van Essen et al. 1992; Grimm et al. 1994) and even tends to suggest a maternal origin in hemophilia A (Becker et al. 1996) and neurofibromatosis type 1 (Lazaro et al. 1996).

In conclusion, in PMD patients, *PLP* duplications, which are the most frequent type of causative mutation, are mostly generated in male germ cells. The mechanism responsible for such male bias may involve unequal sister chromatid exchange, as has been proposed in other X-linked genetic disorders in which both duplications and deletions are found (Darras et al. 1987; Hu et al. 1990). Indeed, in *PLP*-mutated patients, two deletion events responsible for moderate clinical manifestations already have been reported (Raskind et al. 1991; Nave et al. 1996). The extent of the duplication differs between families (Woodward et al. 1998). Determination of the duplication endpoints may explain whether flanking sequences mediate the recombination process.

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Electronic-Database Information

The accession number and URLs for data in this article are as follows:

Généthon, http://www.genethon.fr
Genome Database, http://gdbwww.gdb.org
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nim.nih.gov/Omim (for PMD 312080)

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