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Aberrant splicing of proteolipid protein mRNA in the dysmyelinating jimpy mutant mouse

(oligodendrocyte maturation/myelin/DM-20)

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ABSTRACT **cDNA clones encoding proteolipid protein (PLP) were isolated from a mouse brain library and sequenced. We describe two transcripts arising from the PLP locus by alternative splicing: the major one encodes the 277-amino acid PLP protein and the minor one corresponds to the DM-20 protein, a PLP-like protein of 20,000 M_r that shares both amino and carboxyl regions with PLP. These two transcripts lack ≈70 bases in PLP mRNA from the dysmyelinating jimpy mutant. The deletion spans amino acids 208–232; however, this region is present in the jimpy PLP-encoding gene. We propose that the jimpy mutant suffers a point mutation or the deletion of a few bases in the PLP gene that alters the normal splicing pattern and generates partially deleted PLP transcripts.**

The most abundant protein of the myelin sheath is proteolipid protein (PLP), which is elaborated by oligodendrocytes in the central nervous system. PLP plays a structural role in myelin and probably promotes the apposition of extracellular surfaces of the myelin lamellae. PLP appears to be encoded by a single gene because (*i*) a simple pattern of bands is observed on Southern blots probed with rat (1) or bovine (2) PLP cDNA, (*ii*) *in situ* hybridization occurs at a single chromosomal location (3), and (*iii*) analysis of overlapping human PLP genomic clones supports this conclusion (L.D.H. and J.A.B., unpublished data). Another proteolipid closely related to PLP is also found in reduced amounts in the myelin sheaths of oligodendrocytes. This proteolipid (DM-20) has identical amino acid sequences as PLP at the amino and carboxyl termini, but DM-20 is missing an internal domain of ≈40 amino acids (4–6).

Our laboratory has isolated genomic and cDNA clones for both human and mouse PLP, and we report here the sequence of the highly conserved mouse PLP cDNA. RNA probes were synthesized from mouse cDNA to characterize RNA processing from the PLP locus and identify putative mutations of the PLP-encoding gene, employing methods based on the ability of ribonuclease A to recognize and cleave single base mismatches in RNA heteroduplexes (7, 8). Two mouse dysmyelinating disorders, jimpy (*jp*) (9) and that associated with the *jp* allele (10) jimpy^{msd}, were of particular interest as candidates for PLP mutations because of the absence of PLP protein in jimpy mice (11), the identification of the oligodendrocyte as the affected cell (for review, see ref. 12), and the mapping of the PLP (this work; refs. 3 and 13) and jimpy (9, 10) locus to the X chromosome. We report that both PLP and DM-20 transcripts are partially deleted in jimpy mice, which accounts for the dysmyelinating phenotype of these mutant mice. Moreover, the existence of a single mutation that affects PLP and DM-20 transcripts suggests that one gene encodes both PLP and DM-20 by alternative splicing.

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MATERIALS AND METHODS

Chromosome Mapping. DNA from Chinese hamster–mouse hybrid cells (14) was blotted onto nylon membranes and probed with a 2-kilobase (kb) *Eco*RI fragment (subcloned from phage λ PLP1) containing both coding and noncoding regions of mouse PLP cDNA. The hamster form of PLP was evident as a 10-kb *Hin*dIII band, readily distinguishable from the 5-kb major murine band.

RNA Isolation and Mapping. Wild-type mice were (C57BL/6J × C3H/HeJ)F_1, and all mutant mice were maintained on this background. RNA was extracted from mouse brain with guanidinium thiocyanate, selected with oligo(dT)-cellulose and either subjected to electrophoresis on formaldehyde gels (1 μg per lane) and transferred to nylon membranes or directly dotted onto nylon membranes (20 ng to 10 μg per spot). Filters were probed with either nick-translated mouse PLP cDNA or mouse myelin basic protein (MBP) cDNA that was a 600-base-pair (bp) *Pst* insert from plasmid pdF191 (15) or ^{32}P-phosphorylated 25-mer nucleotides. Several RNA and dot blots were scanned on a Shimadzu densitometer, and the results were averaged.

To prepare RNA probes, the 980-bp *Eco*RI–*Sac* I fragment from phage λ PLP 2 was subcloned into plasmid pGEM3 and digested with either *Nco* I, *Bgl* II or *Eco*RI. Single-stranded, antisense RNA probes were synthesized from the three templates using the SP6 RNA polymerase as recommended by Promega Biotec (Madison, WI) with 50 μCi of [α-^{32}P]CTP (400 Ci/mmol; 1 Ci = 37 GBq; Amersham) and purified on denaturing gels. RNA probes (2×10^5 cpm) were hybridized overnight at 45°C with 0.1–1 μg of poly(A)$^+$ RNA in 80% formamide/40 mM Pipes, pH 6.7/0.4 M NaCl/1 mM EDTA as described by Melton *et al.* (7). Single-stranded regions were eliminated with either RNase A (12 μg at 16°C for 1 hr; Boehringer Mannheim) or RNase T1 (166 units at 16°C for 1 hr; Bethesda Research Laboratories) or a combination, and the protected fragments were resolved on 8 M urea/5% acrylamide gels.

RESULTS AND DISCUSSION

Conservation of PLP Sequence. PLP is remarkably well conserved among mouse (this report), rat (1, 16), cow (2), and human (C.P., L.D.H., and R.A.L., unpublished work) at the amino acid and nucleotide level. The protein encoded by the mouse PLP cDNA (Fig. 1) is identical to the rat (16) and human (C.P., L.D.H., and R.A.L., unpublished work) PLP and displays only two conservative amino acid differences from the bovine sequence (2). The striking degree of conser-

Abbreviations: PLP, proteolipid protein; MBP, myelin basic protein.

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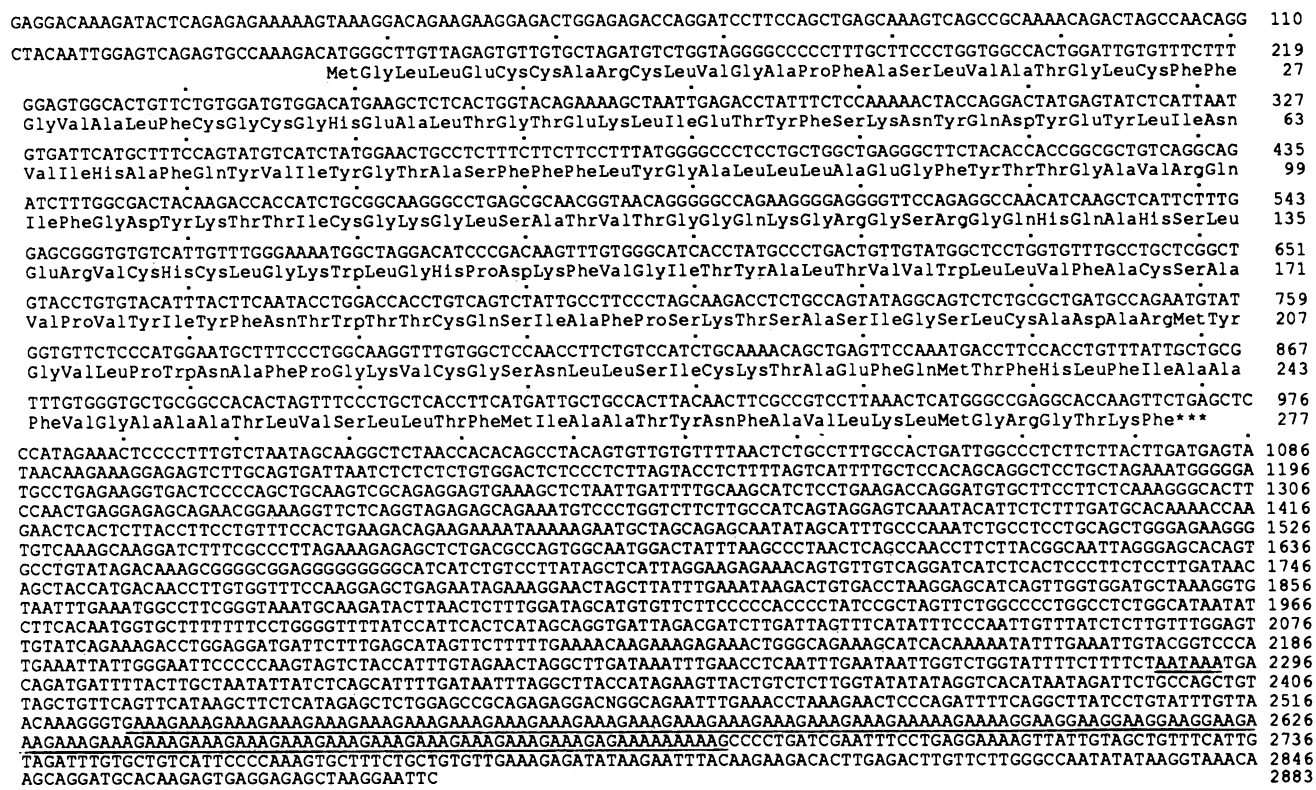


FIG. 1. Sequence of the murine PLP cDNA. Two overlapping cDNA clones were selected from a mouse brain library (15) with oligonucleotide probes (I and II of Fig. 3) complementary to rat PLP. Inserts subcloned from phage λ PLP1 (position 199–2883) were completely sequenced by chemical (18) or dideoxy chain termination (19) methods. The most 5′ 300 bases of phage λ PLP2, which extends from position one to the secondary polyadenylylation site at 2288, were also sequenced. The nearly full-length cDNA sequence lacks 24 bases of 5′ untranslated sequence (from results of primer extension experiments) and the last hundred bases of 3′ untranslated sequence, including the major polyadenylylation site. The purine repeat region is underlined.

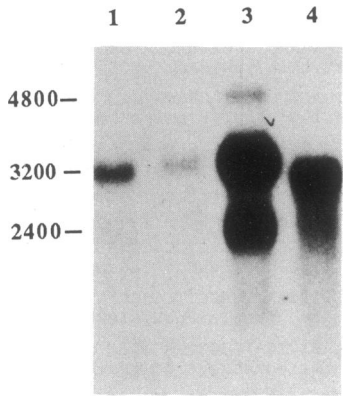
vation among PLP genes is not unexpected for a structural protein whose critical function tolerates little diversity and is similar to that observed for the other major structural protein in myelin, MBP (20). A distinguishing feature of the 3′ untranslated region of the mouse PLP cDNA is the 172-base purine sequence (underlined in Fig. 1) composed chiefly of the sequence GAAA repeated 33 times. This region is reminiscent of satellite DNA, which contains short purine-rich repeats (21), and it has been also observed by M. Wilson and colleagues in PLP cDNA clones from a number of different mouse strains (M. Wilson, personal communication). A remnant of this region is present in rat PLP (1) at the corresponding site. The rat sequence has a 33-base stretch in which the GAAA motif is repeated only six times and possibly represents a partial deletion of an ancestral, highly duplicated, purine-rich region.

Another distinction among PLP genes is the use of alternative polyadenylylation sites. Mouse PLP has two functional polyadenylylation sites: the major one that produces the 3200-base transcript and a minor site (underlined in Fig. 1) that yields a 2400-base transcript (Fig. 2, lane 3). The 2400-base transcript was characterized by its hybridization to coding region probes and inability to hybridize to the terminal 700 bases of PLP cDNA (Fig. 2); moreover, the λ PLP 2 clone (Fig. 1) appears to be a full-length copy of the 2400-base mRNA. Despite the conservation of polyadenylylation sites in the 3′ untranslated region of mouse, rat (1), and, to a lesser extent, human (C.P., L.D.H., and R.A.L., unpublished work) PLP mRNAs, each species uses different secondary polyadenylylation sites in addition to the major site at ≈3000 bases. The rat uses a secondary polyadenylylation site corresponding to position-1480 of Fig. 1 (N. Zeller, unpublished results; ref. 1) that is present but not utilized significantly in mice. The rat mRNA also shares the polyadenylylation site at ≈2290 (Fig. 1) with mouse, but rat mRNA displays only trace amounts of the 2400-base transcript (1). Both the rat (N. Zeller, unpublished results) and the mouse (Fig. 2, lane 3) also have small amounts of a 4800-base PLP mRNA whose termini are unknown. Unlike rodents, human PLP mRNA has a single transcript of ≈3000 bases (Fig. 2, lane 4; C.P., L.D.H., and R.A.L., unpublished work). Secondary polyadenylylation sites are used in <1% of human PLP transcripts, suggesting that the species variation in the choice of polyadenylylation sites does not critically affect PLP production.

PLP Maps to the X Chromosome. As a first step in determining whether the X-linked *jp* mutation (9, 10) carried by jimpy mice maps to the PLP-encoding gene, we tested a panel of hamster–mouse hybrids for the presence of PLP. Table 1 shows that PLP also resides on the murine X chromosome. No discordancy was present for the X chromosome/PLP association, and PLP was not associated with any other chromosomes. This result has been extended by Dautigny *et al.* (3), who report that the PLP gene in mouse is located at XF1, the vicinity of the *jp* mutation.

PLP mRNA Is Partially Deleted in Jimpy Mice. Jimpy mice have reduced levels of PLP mRNA, approximately 10% of control mice, at the peak period of myelination (18 days postnatal) as shown in Fig. 2. However, this effect is not specific for PLP mRNA: jimpy mice show commensurately reduced mRNA levels for the other major myelin protein, MBP. Jimpy mutant is unusual among the dysmyelinating disorders in its drastic effects on both PLP and MBP mRNA. Shiverer mutant mice have a genome partially deleted for the MBP-encoding gene (15, 22, 23) and, consequently, synthesize only small amounts of abnormal myelin, but PLP mRNA

A



B

GENOTYPE	AGE (Days)	PLP mRNA (% of +/+)	MBP mRNA (% of +/+)
+/+	4	3	ND
	6-8	8	ND
	18	100	100
jp/Y	18	10	12
shi/shi	18	112	0
qk/qk	18	102	97

FIG. 2. PLP message in normal and dysmyelinating mouse brain. (*A*) PLP transcripts detected in poly(A)$^+$ mRNA, 1 μg per lane, with a 2-kb *Eco*RI probe subcloned from phage λ PLP 1: lane 1, 18-day jimpy brain; lane 2, 6–8-day wild-type brain; lane 3, 18-day wild-type brain; and lane 4, adult human (Alzheimer) brain. When this blot was reprobed with the most 3′ end of PLP cDNA (679-bp *Eco*RI fragment subcloned from phage λ PLP 1), the 2400-base transcript did not hybridize. ^{14}C-labeled rRNA (not shown) was used to mark size (in bases). (*B*) The total amount of PLP or MBP mRNA for each sample is expressed relative to the amount present at the peak of PLP or MBP synthesis in 18-day-old wild-type (+/+) mouse brain. Dysmyelinating mice included jimpy (*jp*/Y), shiverer (*shi/shi*), and quaking (*qk/qk*). ND, not determined.

levels are unaffected. Similarly, quaking mutant mice have a reduced capacity to myelinate (12), but PLP and MBP mRNA levels are unaffected.

Jimpy mice produce all three PLP mRNA transcripts seen in wild-type mice (in overexposed autoradiograms of Fig. 2)—the 4800-, 3200-, and 2400-base transcripts. To determine whether the PLP message is altered in these mice as well as being quantitatively affected, we analyzed the message with a series of overlapping RNA probes complementary to the coding region of PLP. The results of these experiments, shown in Fig. 3, identify and map a deletion in the jimpy PLP mRNA and provide evidence for alternative splicing from the wild-type PLP locus. When poly(A)$^+$ mRNA was hybridized with ^{32}P-labeled RNA probes, then RNase-digested, and electrophoresed on denaturing gels, two classes of protected fragments were observed. In wild-type mice, transcripts corresponding to the full-length probe were evident—207 bases for the *Sac* I–*Nco* I probe I, 519 bases for the *Sac* I–*Bgl* II probe II, and 917 bases for the *Sac* I–*Eco*RI probe III. A second class of minor bands was also seen in wild-type mice. The sizes and position of these bands suggest they are derived from a mRNA for the DM-20 protein, a PLP-like protein missing an internal domain of PLP (4–6). The domain absent in DM-20 has been characterized by Trifilieff *et al.* (6) using an antibody directed against a peptide from amino acid 117 to 129 of the PLP sequence. Their results, along with our preliminary data, mapping the intron–exon junctions of the human PLP gene (L.H., unpublished results) position the region corresponding to the DM-20 deletion as shown in Fig. 3*C*. A full-length probe to the mouse PLP-coding region protects two minor transcripts of 481 and 382 bases, whereas the probe that contains the 3′ half of mouse PLP-coding region only protects the 382-base band. These results place the missing region of the minor transcript in the same position (Fig. 3) as that predicted by antibody and mapping data and suggest that DM-20 may represent an alternatively spliced form of PLP. The relatively small amounts of the putative DM-20 transcripts are consistent with this hypothesis, because DM-20 protein is present at only 10% of the levels of PLP protein in rat brain (24).

Table 1. PLP maps to the X chromosome

Mouse chromosomes	Hybrid clones, no. PLP presence/chromosome retention				Percent discordant
	+/+	−/−	+/−	−/+	
1	9	2	3	3	35
2	7	3	4	2	38
3	6	3	1	1	18
4	5	3	6	2	50
5	2	5	10	0	59
6	7	3	5	2	41
7	9	2	2	3	31
8	4	4	7	1	50
9	4	5	7	0	44
10	3	5	8	0	50
11	0	4	9	0	69
12	4	1	3	3	55
13	4	3	3	1	36
14	4	5	7	0	44
15	7	0	0	4	36
16	4	3	3	1	36
17	8	3	1	1	15
18	3	4	4	0	36
19	8	5	3	0	19
X	10	5	0	0	0

Jimpy mRNA displayed a dramatically different pattern of protected fragments. All RNA probes generated a small 144-base transcript not detected in wild-type mice (Fig. 3*A*). RNA probes II (*Sac* I–*Bgl* II 540) and III (*Sac* I–*Eco*RI 980) also generated bands of 325 and 744 bases, respectively. Comparison of the summed sizes of jimpy fragments for each RNA probe with the full-length transcripts found in wild-type mice indicates that jimpy PLP mRNA is deleted for ≈70 bases at the *Nco* I site (Fig. 3*C*). The existence of a deletion at this site in jimpy PLP mRNA was confirmed by another approach. An oligonucleotide corresponding to the presumptive deleted region in jimpy PLP mRNA was synthesized and tested for hybridization to jimpy mRNA. As apparent in Fig. 3*B* and summarized in 3*C*, of the five oligonucleotide probes used, only the probe corresponding to the deleted region (probe 7) failed to hybridize to jimpy mRNA. Altered PLP transcripts in jimpy mice were also identified in an S1 nuclease experiment with a rat PLP probe (3), which differed from the mouse sequence (Fig. 1) at 19 bases; due to the ability of S1 nuclease to detect a single base mismatch, interpretation of these results is not straightforward.

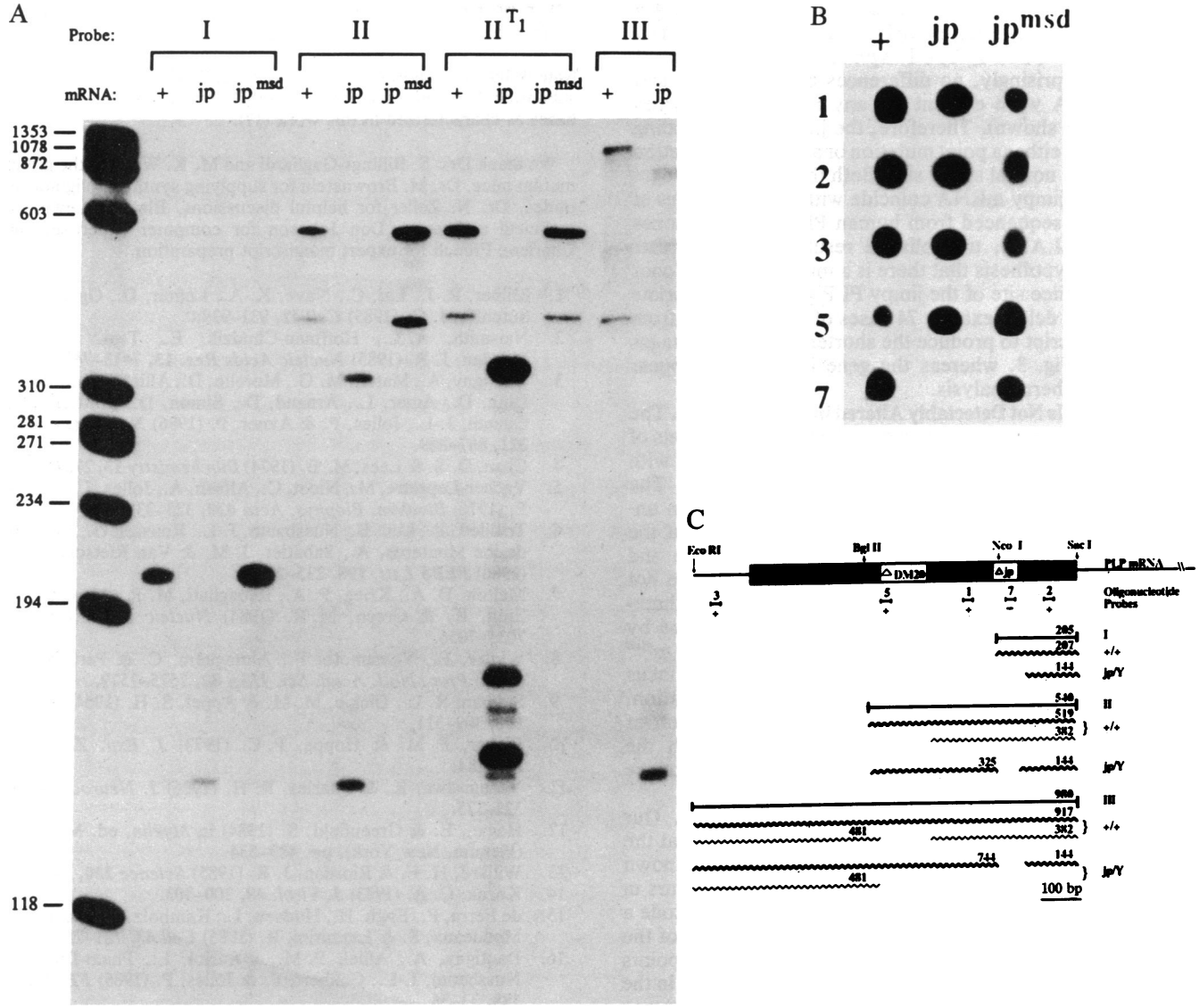


FIG. 3. The coding region of PLP mRNA is partially deleted in jimpy mutant mice. (*A*) PLP transcripts detected with RNA probes I (205-base *Sac* I–*Nco* I), II (540-base *Sac* I–*Bgl* II), and III (980-base *Sac* I–*Eco*RI) in (C57BL/6J × C3H/HeJ)F_1 mouse brain (+), jimpy (*jp*) or jimpy^{msd} (*jp^{msd}*). No differences were observed between several wild-type mouse strains [(C57BL/6J × C3H/HeJ)F_1, DBA, and NIH]. All samples were treated with RNase A before electrophoresis except for the three samples marked II^{T1}, which were incubated with RNase T1. RNase T1 and RNase A yielded similar patterns, although RNase T1, which cleaves only after guanine residues, was not always as complete (compare II and II^{T1}). Each lane corresponds to 20 ng of wild-type poly(A)$^+$ mRNA or 200 ng of mutant poly(A)$^+$ mRNA. The 2400- and 3200-base PLP mRNAs shown in Fig. 2 should generate identical patterns of protected fragments, as the two mRNAs differ only in the length of their 3′ untranslated region. Phage ϕX174 treated with *Hae* III was used as a size marker: RNA transcripts of known sequence (RNA probes I, II, and III) appear 1–5% smaller than single-stranded DNA on these gels. (*B*) Deletion in PLP mRNA of jimpy detected by oligonucleotide probes (see Fig. 3*C* for positions of probes 1, 2, 3, 5, and 7) on dot blots containing 1 μg of poly(A)$^+$ RNA from wild-type (+) mouse brain or 10 μg of poly(A)$^+$ RNA from jimpy (*jp*) or jimpy^{msd} (*jp^{msd}*) mouse brain. (*C*) The schematic shows PLP mRNA with the coding region boxed, and the regions corresponding to sequences deleted from DM-20 (ΔDM-20) or jimpy (Δ*jp*) mRNA marked with open boxes. Restriction sites used to generate RNA probes are indicated above the PLP mRNA. The positions of RNA probes I–III are indicated with a heavy line; the precise size of each probe in bases is shown above the line. Beneath each RNA probe are the mRNA fragments that protected the probe from RNase digestion (wavy lines) in wild-type (+/+) or jimpy (*jp*/Y) mouse brain. The size of each transcript as estimated from the DNA standard is indicated above the transcript. The summary of the *jp^{msd}*-protected fragments is not included, because the pattern observed for *jp^{msd}* was identical to that found for wild type (*A*). The transcripts corresponding to a DM-20 mRNA (382 and 481 bases) are shown as thin wavy lines. The oligonucleotide probes used in *B* are shown directly below the PLP mRNA in bracketed lines with the results of hybridization to jimpy mRNA summarized below the brackets: + indicates hybridization of the 25-mer to jimpy poly(A)$^+$ mRNA, − indicates absence of hybridization.

Jimpy mRNA also contained minor DM-20 transcripts but contained only the 481-base-protected fragment corresponding to the 5′ end of the coding region. The other half of DM-20 mRNA (382-base-protected fragment) was not apparent, indicating that DM-20 transcripts in jimpy mutants, like the PLP transcripts, are also deleted for the 70 bases around the *Nco* I site. These results provide further support for the existence of a single gene that encodes both PLP and DM-20 by alternative splicing. The other possibility, the existence of one gene for PLP and a closely linked one for DM-20, is unlikely because jimpy mice would have to have mutations at both gene loci to produce the observed pattern (Fig. 3).

The structure of the PLP gene in jimpy mice was further characterized in a series of genomic Southern blots with a battery of enzymes that flanked or encompassed the mRNA deletion (*Bsm* I, *Bst*NI, *Nco* I, *Nla* IV, *Pvu* II, and *Scr* FI) or

enzymes that might cut within adjacent introns (*Sau* 3a, *Alu* I, *Rsa* I, *Bam*HI, *Hin*dIII, *Eco*RI, *Pst* I, *Bgl* II, *Pvu* II, *Xba* I, *Xho* I, *Eco*RV, *Dra* I, *Hpa* I, *Kpn* I, *Nde* I, *Nru* I, *Sma* I or *Sph* I). Surprisingly, no differences between jimpy and wild-type DNA were evident for any restriction enzymes used (data not shown). Therefore, the jimpy PLP-encoding gene may have either a point mutation or a very small deletion that destroys a normal splice site. Both the 5′ and 3′ borders of the deleted jimpy mRNA coincide with the boundaries of the fifth exon sequenced from human PLP genomic clones (L.D.H. and J.A.B., unpublished results), which further supports the hypothesis that there is a mutation in the donor or acceptor splice site of the jimpy PLP gene. Inappropriate splicing would delete exactly 74 bases of coding region from the PLP transcript to produce the shortened jimpy messages observed in Fig. 3, whereas the gene itself would appear intact by Southern analysis.

PLP mRNA Is Not Detectably Altered in Jimpy^{msd} Mice. The allelic form of *jp*, *jp^{msd}*, produced similarly reduced levels of PLP mRNA in brain but displayed a wild-type profile with RNA or oligonucleotide probes (Fig. 3 *A* and *B*). The jimpy^{msd} phenotype could, nonetheless, result from an undetected point mutation in the PLP coding region. Of the eight possible single base pair mismatches, Perucho and coworkers found that T·U and U·U mismatches were not readily cleaved by RNase A, indicating that only three-fourths of the single base mutations would be detected by these methods (8). An alternative possibility for the *jp^{msd}* mutation is that it is a regulatory mutation at the PLP locus that sharply reduces PLP mRNA synthesis. Either mutation, the identification of which awaits the characterization of jimpy^{msd} genomic clones, would be consistent with the jimpy^{msd} phenotype in which the abnormalities present are similar to jimpy but much less severe (25).

PLP Is Required for Oligodendrocyte Maturation. Our results demonstrate that a mutation affecting splicing at the PLP locus results in the jimpy phenotype. It is not known whether translation of the altered PLP transcripts occurs in jimpy mutant mice. PLP mRNA from jimpy could encode a protein that would preserve the first 207 amino acids of the PLP sequence. The 74-base deletion, whose end points correspond to the boundaries of the fifth exon for PLP in the human genome (L.D.H., unpublished data), would destroy the proper reading frame and create a shortened protein (243 amino acids) with a unique carboxyl terminus. No evidence of PLP in jimpy mice has been reported by immunologic techniques (11), although the mutant protein may have an altered conformation that circumvents immunologic detection, or the abnormal protein may be rapidly degraded.

Like the other dysmyelinating mutants, jimpy and its human counterpart, Pelizaeus–Merzbacher disease (26), fail to synthesize myelin. Yet the absence of PLP creates more devastating effects than those effects observed by halting myelination in the other dysmyelinating disorders. A curious aspect of the lack of PLP is the block in oligodendrocyte maturation evident in jimpy mutant mice: mature oligodendrocytes are absent from both jimpy (27, 28) and Pelizaeus–Merzbacher (26) brains, observations consistent with the markedly reduced levels of oligodendrocyte-specific mRNA (Fig. 2), proteins, and lipids in affected brains (for review, see ref. 12). Conceivably, a mutant PLP protein is produced in jimpy mice that is toxic to the oligodendrocyte at the initial stages of myelination. More likely is the possibility that the absence of PLP precludes further differentiation of jimpy oligodendrocytes. In addition to its structural role in the myelin sheath, PLP may function at a critical stage of oligodendrocyte development that precedes myelin assembly. It is of note that PLP acts as an ionophore *in vitro* (29, 30), a property that may contribute to the essential role of PLP in oligodendrocyte maturation.

Note. While this report was being reviewed, R. Milner and colleagues submitted their sequence of jimpy cDNA, which was deleted for 74 bases as characterized in this work (17).

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