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Splice site selection in the proteolipid protein (PLP) gene transcript and primary structure of the DM-20 protein of central nervous system myelin

(myelination/membrane protein/jimpy mutant mouse/alternative splicing/X-chromosome-linked gene)

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ABSTRACT **Proteolipid protein (PLP) is the major myelin membrane protein of the central nervous system. We have isolated a copy of an alternatively spliced PLP gene transcript from a mouse brain cDNA library that was screened for PLP-related sequences. The encoded 241-amino acid protein differs from PLP by an internal deletion of 35-amino acid residues (116–150) from the major hydrophilic domain. This PLP variant is identical with the DM-20 protein of myelin, previously described as a brain-specific myelin component and known to be related to PLP. We determined the corresponding nucleotide sequence of the rat PLP gene and found that DM-20 mRNA results when a second 5′ splice site, located 105 nucleotides within the third exon of the primary PLP transcript, is utilized in precursor mRNA (pre-mRNA) splicing. This demonstrates that alternative 5′ splice site selection can determine the protein product of a cellular gene. DM-20 mRNA is expressed in rat brain with approximately 50% abundance relative to PLP mRNA and appears to be developmentally coregulated.**

Myelination in the mammalian central nervous system (CNS) is the specialized function of differentiated oligodendrocytes and involves the coordinated expression of myelin-specific gene products. Proteolipid protein (PLP) is the most abundant myelin protein of the CNS and is a highly hydrophobic integral membrane protein (reviewed in ref. 1). Its primary structure has been established by protein sequencing (2–4) and more recently from the cloning of PLP cDNAs from several species (5–10). A mutation in the mouse PLP gene is the primary genetic defect of the dysmyelinating mutant mouse jimpy (8–12), which demonstrates the critical role of PLP in CNS myelin assembly.

Another CNS specific myelin protein of lower abundance has been termed "intermediate protein" or DM-20 (13), which reflects its apparent molecular mass. Depending on the proteolipid extraction method (14), DM-20 copurifies with PLP, but the two proteins can easily be separated by $NaDodSO_4$ gel electrophoresis. PLP and the DM-20 protein are related by amino- and carboxyl-terminal amino acid sequence homology and immunological crossreactivity (1). The exact structure of DM-20 and its relationship to PLP had been controversial, but recent evidence has suggested that DM-20 differs from PLP by an internal deletion of residues 100–140 (plus or minus a few amino acids) (15, 16). To determine the exact relationship between PLP and DM-20 and to test the possibility that the DM-20 protein is a result of alternative PLP precursor mRNA (pre-mRNA) splicing, we screened a mouse brain cDNA library for PLP-related sequences. A full-length copy from such an alternatively spliced PLP mRNA was identified and shown to encode a variant PLP form. All evidence suggests that this variant proteolipid is the DM-20 protein of myelin. To analyze the mechanism of alternative splicing, we determined the sequence of the corresponding part of the rat PLP gene[§] and found that RNA processing involves alternative 5′ splice site selection in the protein coding region of the primary PLP gene transcript, a splicing mechanism previously seen in the gene expression of DNA tumor viruses and a simple means of generating protein diversity.

MATERIALS AND METHODS

Animals. Timed-pregnant Sprague–Dawley rats (Charles River Breeding Laboratories) provided a source of postnatal 11- and 20-day and adult (over 50-day-old) brains. B6CBA mice were obtained from The Jackson Laboratory. Male 21-day-old mice were used for brain RNA extraction.

Isolation of RNA. Cytoplasmic RNA was isolated from fresh brains by extraction with phenol/chloroform/isoamyl alcohol (50:50:1) (17) and was enriched for poly(A)$^+$ mRNA by passage over oligo(dT)-cellulose (18). The RNA concentration of each preparation was determined by measuring the absorbance at 260 nm: a solution of 38 μg of RNA per ml was assumed to have an absorbance of 1.0.

cDNA and Genomic Cloning. A mouse brain cDNA library was prepared from cytoplasmic poly(A)$^+$ RNA of 21-day-old mice according to the cloning procedure of Okayama and Berg (19, 20). The library was amplified prior to transformation in *Escherichia coli* MC1061 and had a complexity of approximately 10^5 independent transformants. Colony hybridization screening (21) was carried out using the ^{32}P-labeled cDNA insert of the rat PLP clone p27 (5). A genomic library made from rat liver DNA (22) was screened using a radioactively labeled cDNA fragment corresponding to the PLP coding region of clone p27. Positive isolates were mapped by restriction mapping and subcloned into pUC18 plasmid vectors (Pharmacia).

DNA Sequencing. Sequence analysis of 5′ end-labeled fragments was accomplished using a modified version (23) of the partial chemical degradation method (24).

RNA Protection Analysis. An 821-base-pair (bp) *Bam*HI–*Pst* I fragment of the PLP coding region in p27 (nucleotides 45–866) was subcloned into the transcription vector pGEM-4 (Promega Biotec, Madison, WI). The plasmid was linearized with *Bgl* II (nucleotide 418 in p27) and, by using T7 RNA polymerase, antisense RNA transcripts were generated *in vitro* under the conditions suggested by the manufacturer.

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Abbreviations: PLP, proteolipid protein; CNS, central nervous system.

[§]This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J02945).

Hybridization with 1.0 μg of total cytoplasmic RNA from rat brain was carried out as described (25). Only RNase A (Boehringer Mannheim) was used in the subsequent digestion step (40 μg/ml, 37°C, 30 min). Protected fragments were separated on 5% polyacrylamide gels and exposed to Kodak XRP-5 film. The autoradiographic intensity of the bands was determined with an LKB UltroScan XL laser densitometer and was corrected for fragment length.

RESULTS

Identification of an Alternatively Spliced PLP mRNA. The known structural homology and size difference between PLP and the DM-20 protein suggested the possibility that both proteolipids are derived from alternatively spliced mRNAs of the single X-chromosome-linked PLP gene. To identify PLP-positive clones that might encode the DM-20 protein, a cDNA library (8) derived from cytoplasmic poly(A)$^+$ RNA of 20-day-old mouse brain was screened using a full-length cDNA copy (clone p27 of ref. 5) of the 3.2 kilobase rat PLP mRNA as a probe. Following colony hybridization screening of 10^5 transformants, 35 PLP positive clones were isolated and analyzed for restriction fragment length differences. Plasmid DNA was digested with the restriction enzyme *Rsa* I, and fragments were separated on an agarose gel and were blotted to nitrocellulose (26). In one experiment, the blot was hybridized to a radioactively labeled 396-bp *Rsa* I fragment from clone p27 that spans nucleotides 249–645 in the protein-coding region of PLP mRNAs (5). This fragment, which is preserved between rat and mouse, detects the corresponding *Rsa* I fragment in the mouse PLP cDNAs. Four of the 35 PLP-positive clones contained an *Rsa* I fragment that was approximately 100 bp shorter than was predicted from the nucleotide sequence of p27 or pC4, a previously characterized mouse PLP cDNA (8). Clone p11, one of the four variant isolates and a candidate to encode the DM-20 protein, was chosen for sequence analysis.

Primary Structure of the DM-20 Protein of Myelin. Restriction mapping confirmed the general homology and colinearity between p11 and other PLP cDNAs that we had characterized previously. Clone p11 contains a nearly full-length copy of a 2.4-kilobase mRNA, the second most abundant PLP mRNA size class in mice. Determination of the nucleotide sequence of the open reading frame revealed only one difference from other mouse PLP cDNAs—the absence of 105 bp from nucleotides 471 to 575 (Fig. 1). This difference, which does not disrupt the reading frame, results in the loss of 35 amino acid residues from Val-116 to Lys-150 in the 276-residue PLP sequence. No amino acids are changed at the boundaries of the deletion. The predicted size of the 241-amino acid PLP variant is 26.5 kDa and matches the observed 4 kDa difference in the apparent molecular mass of DM-20 and PLP on NaDodSO$_4$ gel electrophoresis (13). The deleted sequence comprises a large part of the major hydrophilic domain of PLP and is located between two putative membrane-embedded domains. Seven out of the 35 deleted amino acids carry net charges, six of which are positive. The size and location of the deletion are in good agreement with the findings of Trifilieff *et al.* (15, 16), and we conclude that the alternatively spliced mRNA encodes DM-20.

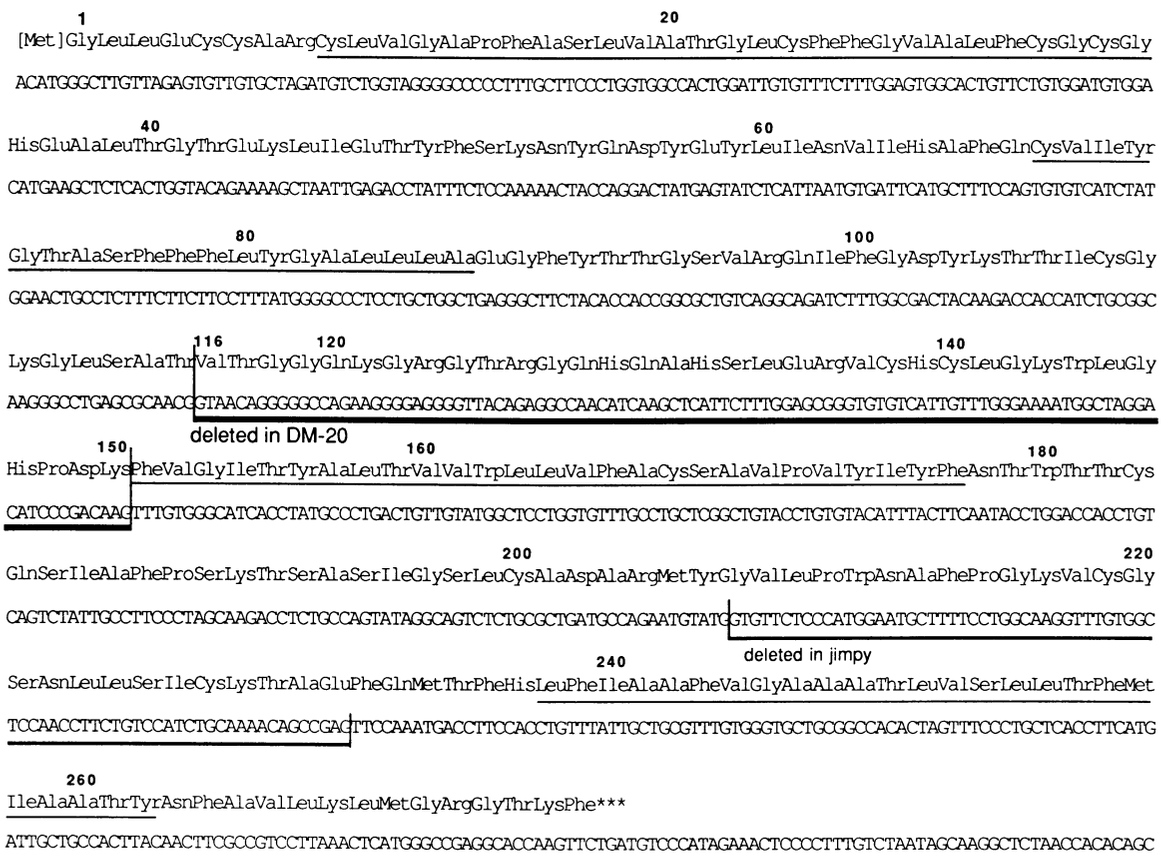

FIG. 1. Primary structure of the DM-20 protein of myelin deduced from mouse brain cDNA sequences. The nucleotide sequence is compiled from the protein coding region of DM-20 mRNA (deduced from the cDNA insert of clone p11) and PLP mRNA (deduced from clone pC4, ref. 8). A single open reading frame of 831 bp is translated into the amino acid sequence above the nucleotide sequence and is numbered for the 276-residue PLP starting with the amino-terminal glycine of the mature protein. A sequence of 105 nucleotides that is absent from DM-20 mRNA and corresponds to amino acid residues 116–150 is shown with a thick bar. Four hydrophobic and putative membrane-embedded domains of PLP are underlined. The position of 74 nucleotides (exon V) deleted in jimpy mice and affecting both the PLP and DM-20 structure is indicated with a thin bar.

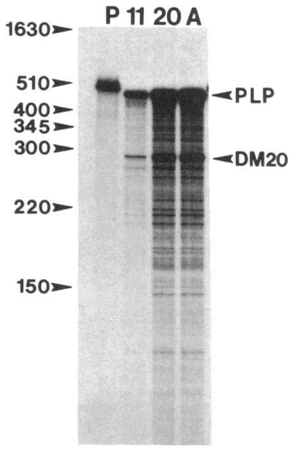

FIG. 2. Developmental coexpression of rat brain PLP and DM-20 mRNA. A radioactively labeled antisense RNA probe (lane P) derived from nucleotides 45 to 866 (*Bam*HI–*Pst* I fragment) of cDNA clone p27 was hybridized to total rat brain RNA of three different ages [postnatal days 11 and 20 and adult (A)]. After digestion with single-strand-specific RNase A and separation on a 5′ polyacrylamide gel (lanes 11, 20, and A), two hybrids were predominantly protected from RNase digestion (indicated by arrows): the 445-nucleotide band corresponds to PLP mRNA, and a fragment of 292 nucleotides is protected by DM-20 mRNA. The autoradiograph is overexposed to show the DM-20 derived fragment at postnatal day 11. The ratio of PLP to DM-20 mRNA shows no significant developmental change, suggesting a coregulation of the two mRNAs.

Coexpression of PLP and DM-20 mRNA. Since the expression of myelin genes in the rodent brain is developmentally regulated, we confirmed the postnatal expression of DM-20 mRNA relative to PLP mRNA by RNase A protection experiments (25). An 821-bp *Bam*HI–*Pst* I fragment from the coding region of clone p27 was cloned into the transcription vector pGEM-4. After linearization with *Bgl* II (447 bp upstream of the *Pst* I site), uniformly labeled ^{32}P-labeled antisense RNA transcripts were generated with T7 RNA polymerase. This probe was hybridized with total cytoplasmic RNA from rat brain of ages 11 and 20 days postnatal and adult and was digested with single-strand-specific RNase A, and the protected labeled fragments were separated by gel electrophoresis. We predicted that the 467-nucleotide probe should be nearly completely protected by PLP mRNA to generate a 445-nucleotide product or partially protected by DM-20 mRNA to produce fragments of 292 and 48 nucleotides.

These fragment sizes were obtained (Fig. 2); their relative ratio (after correction for fragment length) indicates that DM-20 mRNA is expressed with nearly 50% abundance relative to PLP mRNA. Only a minor change in the ratio is observed between postnatal day 11 (46%) and adult (49%) brains, suggesting that both forms are coordinately expressed during early and late stages of myelination. The relative abundance of DM-20 mRNAs is slightly higher in normal mouse (60%) than in the jimpy mutant mouse (40%), which carries a mutation in the PLP gene that affects RNA splicing (8) and leads to the exclusion of exon V from both PLP and DM-20 mRNAs (data not shown).

Alternative 5′ Splice Site Selection in the Rat PLP Gene. The results of genomic Southern blot experiments (5, 8) and gene mapping studies demonstrating linkage of the PLP gene to the X chromosome in human (27) and mouse (11) strongly suggest that there is only one PLP gene in rat and mouse. The observation that jimpy mutant mice have the same deletion in the mRNA for PLP and DM-20 (refs. 10 and 12 and unpublished results) is further evidence for a single PLP gene. The complete homology of all mouse PLP cDNA sequences upstream and downstream of the region absent in DM-20 mRNA as shown here, therefore, strongly suggests that both PLP and DM-20 must be derived by alternative RNA splicing of a single gene transcript.

To test possible models of alternative splicing, we determined the nucleotide sequence of the corresponding part of the rat PLP gene (Fig. 3). A genomic library made from rat liver DNA in λ Charon 4A was screened for PLP-positive clones using labeled p27 cDNA as a probe. Positive isolates were mapped by restriction analysis, subcloned into the plasmid vector pUC18, and partly sequenced. The sequence of subclone p214, which contained the region of interest, was compared with PLP and p11 cDNA sequences to provide the exon–intron organization around the alternative splice sites in the rat PLP gene (Fig. 3). The 105-bp sequence, absent from the DM-20 cDNA, forms the 3′ end of a 262-bp exon that spans nucleotides 194–455 in the p11 cDNA. These 262 nucleotides are exactly equivalent to exon III of the human PLP gene (28); assuming an evolutionarily conserved exon–intron structure, we refer to it as exon III of the rat PLP gene. It contains both the major hydrophilic domain, largely deleted in DM-20, and the flanking membrane-embedded domain (amino acid residues 64–87) on its amino-terminal side. On the 3′ side, the protein coding region is interrupted by an intron that exactly separates the hydrophilic domain from the next membrane-embedded domain on the carboxyl-terminal side (residues 151–190). Thus, in the formation of DM-20, RNA splicing involves the removal of intron III and an additional 105-nucleotide PLP exon sequence (labeled exon III B in Fig. 3). The 5′ end of the deleted sequence, therefore, constitutes an alternative 5′ donor splice site, which is located within the open reading frame (exon III A) and is utilized with approximately 50% relative efficiency.

DISCUSSION

Structure of the DM-20 Protein of Myelin. We have shown that rat and mouse brain PLP gene transcripts exist in two

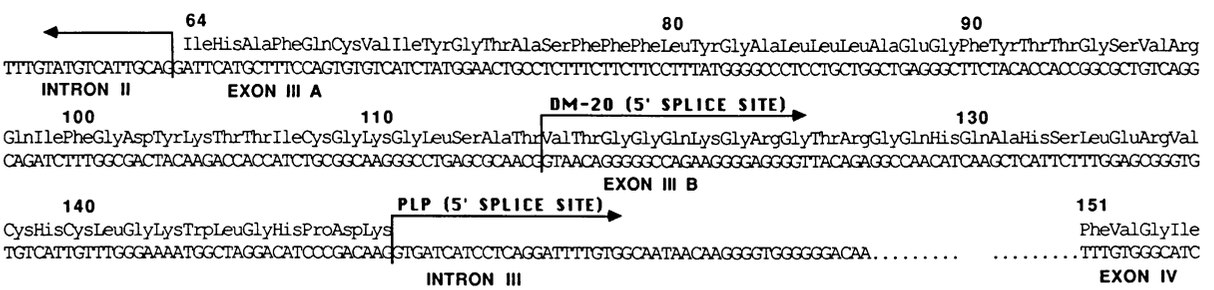

FIG. 3. Genomic organization of the PLP exon sequences involved in alternative splicing. The nucleotide sequence, derived from subclone p214, shows the exon–intron organization of the rat PLP gene surrounding the region of alternative splice site selection. Exon III (262 bp) contains two functional 5′ splice sites: the upstream site is utilized in the formation of DM-20 mRNA (exon III A), and utilization of the downstream splice site generates PLP mRNA (exons III A and B). Numbering of amino acids is according to the 276-residue PLP.

alternatively spliced forms. Inclusion of the complete third exon of the PLP gene after splicing results in PLP mRNA, which encodes the 276-amino acid major PLP. The alternatively spliced mRNA, which is expressed with about 50% abundance relative to PLP mRNA, lacks the 3′ part of exon III and encodes the sequence of a related 241-amino acid proteolipid. We conclude that this PLP variant is identical to the previously described DM-20 protein for the following reasons: (*i*) PLP and DM-20 are two structurally (29) and immunologically (30) related CNS proteolipids that are found in myelin in a ratio similar to the relative abundances of the two alternatively spliced PLP mRNAs and are expressed with the same developmental time course. (*ii*) The deletion of 35 amino acids from the sequence of PLP would exactly account for the observed size difference between PLP and DM-20 of approximately 4 kDa on $NaDodSO_4$/polyacrylamide gels (13). (*iii*) The location of this deletion in the PLP sequence (residues 116–150) is consistent with the findings of Trifilieff *et al.* (16), who have shown that DM-20 is not detectable with anti-peptide antibodies directed against residues 117–129 of PLP, whereas site-specific antibodies directed against other regions of PLP do crossreact with both proteolipids. From this and a protein chemical analysis (15), it was proposed that DM-20 differs from PLP by the deletion of residues 100–140 (plus or minus a few amino acids).

PLP contains multiple membrane-spanning domains, and two models have been proposed for its orientation in the lipid bilayer (3, 31). The part of PLP that is shown to be absent in DM-20 (residues 116–150) comprises a large portion of the major hydrophilic domain of the protein and exactly precedes the third membrane-embedded domain (Fig. 4). Trifilieff *et al.* have shown with site-specific antibodies that this domain is not detectable on the surface of cultured oligodendrocytes but is accessible after fixation of the cells (16), which is in agreement with the PLP model of Laursen *et al.* (31). The positively charged residues in PLP might contribute to the interaction of this domain with the opposing membrane surface during intracellular myelin compaction; perhaps their function is similar to that presumed for myelin basic proteins. DM-20, however, lacks most of these charged residues and presumably contains its functional site in the second extracellular domain of the molecule as proposed by Laursen *et al.* (31). This part of the molecule, which is identical in PLP and DM-20, is exposed on the cell surface, is acylated, and most

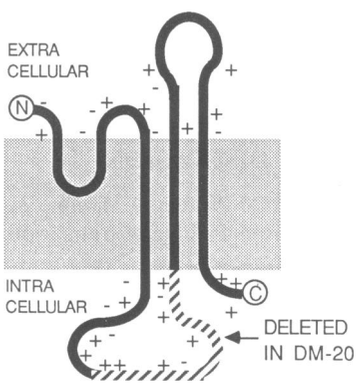

FIG. 4. Schematic representation of PLP and the DM-20 protein of myelin. PLP is the predominant integral membrane protein of CNS myelin and has multiple membrane-spanning domains. DM-20 differs from PLP by the internal deletion of amino acid residues 116–150 from the major hydrophilic domain, which precedes the third membrane-embedded domain of the 276-amino acid PLP. The extracellular domain at the carboxyl-terminal side is the same in both proteins, is acylated *in vivo*, and is likely to be involved in the extracellular compaction of the myelin sheath. [This schematic representation was adapted from a model of Laursen *et al.* (31); an alternative model for PLP has been proposed by Stoffel *et al.* (3).]

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                exon        intron
5'..... TCCCGACAAG GTGATCATCC .....3'   PLP
5'..... GAGCGCAACG GTAACAGGGG .....3'   DM-20
5'..... AG GTAAGT .....3'               CONSENSUS
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FIG. 5. Comparison of the PLP and DM-20 specific 5′ splice sites in the rat PLP gene with the consensus sequence. The consensus 5′ splice site was taken from Padgett *et al.* (40).

likely contributes to the extracellular compaction of the myelin sheath.

Splice Site Selection in the PLP Gene. We have determined the complete primary structure of DM-20 and demonstrated that it is encoded by a separate mRNA and is not simply a posttranslationally modified form of PLP. Two other classes of myelin proteins are also derived from complex transcription units: five forms of myelin basic protein (32–34) and two forms of the myelin-associated glycoprotein (35). In both cases, alternative splicing involves the differential exclusion of separate exons, a mechanism observed in a number of eukaryotic genes (for review, see ref. 36). Generating two proteins by selection of alternative 5′ splice sites, to our knowledge, has been described only for SV40 gene transcripts and in related viral systems. The expression of both the large and small tumor antigens of SV40, which share the amino terminus of the protein, results from the use of two alternative splice sites in the early gene transcript (37). We, here, describe an apparently similar 5′ splice site selection in the protein coding region of a cellular gene, a simple mechanism to change the protein product of a gene and to generate protein diversity.

Assuming that both mRNA forms are equally stable *in vivo*, PLP mRNA results from an about 2-fold higher splicing efficiency of the downstream splice site. Several reports have shown that *in vitro* a "strong" alternative splice site contains a higher degree of homology to the consensus sequences (38, 39). By comparing the nucleotide sequences of the two alternative donor sites of the PLP gene, we find no obvious difference in the match between the consensus 5′ splice site (40) and both the PLP and the DM-20 donor splice site of exon III (Fig. 5). In *in vitro* systems, upstream 5′ donor sites are more frequently used than identical alternative splice sites downstream (38, 41). However, such a position effect is known to be sequence and distance dependent (42). There is, so far, no evidence for a differential expression of PLP and DM-20 mRNAs. Whether a cis-acting splice site "signal strength" and "position effect" alone can sufficiently explain the *in vivo* observed ratio of splice site selection awaits further characterization. We noticed that both alternative splice sites of exon III show a decreased homology to the consensus sequence as compared to splice junctions of all the remaining exons in the PLP gene (28).

The functional difference between PLP and DM-20 for the development of compact myelin is unknown. Most likely, PLP was the primary proteolipid and the DM-20 form developed later in evolution (43), following the introduction of an alternative splice site into the protein coding region. In more primitive vertebrates, such as lungfish, Waehnelt and coworkers detected a single myelin protein that immunologically crossreacts with antibodies against mammalian PLP (44). This protein is glycosylated and has an apparent molecular mass very similar to that of rat brain PLP. It may, therefore, represent the evolutionary precursor of both PLP and DM-20.

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