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Jimpy mutant mouse: A 74-base deletion in the mRNA for myelin proteolipid protein and evidence for a primary defect in RNA splicing

(brain-specific genes/lipophilin/neurological mutation/Pelizaeus–Merzbacher disease/X-linked disease)

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ABSTRACT **The mouse mutant jimpy carries an X chromosome-linked recessive gene defect that affects the formation of myelin in the central nervous system. To understand the molecular basis of the jimpy mutation, we have examined the expression of mRNAs encoding myelin proteolipid protein (PLP). PLP mRNAs were detectable in jimpy brain RNA at 21 days after birth but were severely reduced in abundance compared to wild-type littermates. Nucleotide sequence analysis of cDNA clones for PLP mRNA, isolated from a cDNA library of jimpy brain poly(A)$^+$ RNA, revealed that the PLP mRNA expressed in jimpy contained a deletion of 74 nucleotides with respect to the wild-type sequence. This deletion causes a frameshift in the open reading frame resulting in an altered carboxyl terminus for jimpy PLP. Probes specific for the deleted sequence, however, hybridize with equal efficiency to genomic DNA from jimpy and wild-type littermates, suggesting that the defect in the jimpy PLP mRNA is generated by aberrant RNA processing rather than by deletion of genomic sequences. We conclude that a mutation in the gene for PLP that leads to an incorrectly spliced RNA transcript is the primary defect of this genetic disorder.**

Neurological mutants (1) are an important tool in developmental neurobiology as they may indirectly demonstrate the function of a single gene product in the development of the nervous system. In many mutant phenotypes, however, the primary defect is difficult to distinguish from secondary symptoms of the disease, and the affected gene product has been identified for only a few of the neurological mutations of the mouse. Identification of the nature of the mutation is essential for a full understanding of the molecular basis of the defect and of the role of the affected gene and its products in normal brain development.

The mutant mouse jimpy (2, 3) carries an X-linked recessive genetic disorder (*jp*) that exclusively affects the formation of myelin in the central, but not in the peripheral, nervous system. Its first symptom, a characteristic body tremor preceding locomotor activity, appears around postnatal day 11, after the onset of myelination in the normal mouse brain. Later, convulsions develop and death occurs in the fifth week. Histological studies of jimpy mice reveal an almost complete absence of white matter in the central nervous system, and only some axons show more than a few sheaths of uncompacted myelin (4). Various biochemical abnormalities have been reported in the mutant, in particular a drastic decrease of structural myelin proteins and enzymes involved in myelin lipid metabolism (summarized in ref. 4).

Myelin proteolipid protein (PLP), or lipophilin, is of particular relevance to the jimpy mutation because it is expressed specifically in the central nervous system (5) and is one of the most severely reduced myelin constituents of jimpy brains (6–9). Furthermore, the gene for PLP has been mapped to the X chromosome in both man and mouse (10). We have cloned and determined the full-length sequences of two mRNAs encoding rat brain PLP (11): these studies provided the opportunity to characterize PLP mRNAs in the jimpy mouse. We demonstrate here that PLP mRNA is expressed at considerably reduced abundance in the brains of jimpy mice and that cDNA clones encoding jimpy PLP contain a 74-base deletion within the coding region. The sequence deleted from the RNA, however, is detectable in genomic DNA from both jimpy and wild-type animals. These data suggest that the jimpy phenotype results from a primary genetic lesion in the PLP gene, probably resulting in aberrant RNA splicing of PLP mRNAs.

METHODS

Jimpy Mice. Heterozygous B6CBA-A$^{w\text{-}J}$/A-*Ta jp* female mice, obtained from The Jackson Laboratory, were crossed with wild-type males of the same strain. Jimpy offspring (*Ta jp*/Y) were identified by clinical symptoms and the expression of the genetically linked tabby (*Ta*) coat color marker (12). Healthy age-matched wild-type littermates (++/Y) served as controls.

RNA Analysis. Cytoplasmic poly(A)$^+$ RNA was isolated from the brains of 21-day-old jimpy and wild-type mice as described (11). The poly(A)$^+$ RNA fractions of four animals each were pooled for all subsequent experiments. For RNA gel blotting, aliquots (2 μg) of each poly(A)$^+$ mRNA sample were fractionated by electrophoresis on 1.5% agarose gels in 1 M formaldehyde and transferred to nitrocellulose (13). Blots were prehybridized at 42°C in 50% (vol/vol) formamide/0.75 M NaCl/25 mM Pipes, pH 6.8/0.2% $NaDodSO_4$/25 mM EDTA/salmon sperm DNA at 100 μg/ml/yeast tRNA at 100 μg/ml/5× Denhardt's solution for at least 4 hr and hybridized with ^{32}P-labeled probes for 20 hr in the same solution but with 1× Denhardt's. The radioactive probes were generated by labeling restriction fragments derived from cDNA clones of PLP (11) with ^{32}P by nick-translation (14) to specific activities of 2–7 × 10^8 cpm/μg. Blots were washed twice in 2× SSC/0.2% $NaDodSO_4$ at 42°C for 1 hr and once in 0.4× SSC/0.2% $NaDodSO_4$ for 45 min at 65°C (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.) They were exposed to Kodak XRP-5 film at −70°C with Cronex Lightning Plus intensifying screens.

cDNA Cloning. cDNA libraries were constructed from poly(A)$^+$ RNA prepared from jimpy and wild-type brains according to the method of Okayama and Berg (15, 16). Before amplification both libraries had a complexity of 10^5

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Abbreviation: PLP, proteolipid protein.

independent transformants. Clones of mouse PLP mRNAs were selected by colony hybridization screening using the ^{32}P-labeled insert of the rat PLP cDNA clone p27 (11).

DNA Sequencing. Sequence analysis of 5'-end-labeled fragments was accomplished using a modified version (17) of the partial chemical degradation method (18).

Southern Blot Analysis. DNA was extracted from individual livers of the same jimpy and wild-type mice used for RNA extraction. After treatment with $NaDodSO_4$ and proteinase K, the DNA was digested with restriction endonucleases, fractionated on agarose gels, transferred to nitrocellulose (19) and hybridized with ^{32}P-labeled probes as described for RNA gel blots.

Oligonucleotide Probes. Two 40-base oligonucleotides were synthesized: one corresponding to the sense-strand sequence between positions 630 and 669 and the second to the antisense-strand sequence between positions 655 and 694. For probe generation the two oligonucleotides were annealed by their 15-nucleotide complementary sequences, and the single-stranded regions were filled in using the Klenow fragment of DNA polymerase I in the presence of ^{32}P-labeled nucleotide triphosphates, under conditions specified for nick-translation (14). This procedure generates a 65-nucleotide probe that is labeled on both strands.

RESULTS

Expression of PLP mRNAs in Jimpy Mice. Although there have been numerous studies of myelin preparations (8) or brain homogenates (6, 7, 9) from jimpy mice, none were able to identify the 30-kDa PLP molecule unambiguously. Because PLP mRNAs are highly abundant in brain, we began our investigation by testing for the expression of PLP mRNA in the mutant, as a potentially more sensitive assay. Cytoplasmic poly(A)$^+$ RNA was isolated from the brains of 21-day-old jimpy and wild-type mice and hybridized on RNA gel blots with the radioactively labeled insert of p27, a full-length cDNA clone of rat PLP mRNA (11). The major PLP mRNA of rats and mice is 3200 nucleotides long (11); it is clearly present in jimpy brain RNA but is reduced in abundance by a factor of ≈20 (Fig. 1). A second abundant 2400-nucleotide transcript is expressed in normal mouse brain and is detectable in jimpy brain RNA gel blots on longer exposure of the blots (data not shown). A third transcript of 1600 nucleotides, which is highly abundant in rat brain (11), has a very low abundance in mice (Fig. 1) and is undetectable in jimpy. Comparing different exposures of the same blot, it is possible to detect a slight reduction in the size of the 3200-nucleotide jimpy PLP mRNA.

Jimpy PLP mRNA Contains a 74-Base Deletion. To analyze jimpy PLP transcription products in more detail, a cDNA library was constructed from jimpy brain poly(A)$^+$ RNA. We identified 35 PLP-positive clones in 10^5 plated colonies of this library by colony hybridization with the rat PLP cDNA. Two cDNA clones were chosen for further study, pJ-31 and pJ-81. A control clone, pC-4, was also selected from the cDNA library constructed from wild-type mouse brain poly(A)$^+$ RNA. Restriction mapping of the mouse cDNAs confirmed the general homology with the rat PLP mRNA sequences. These experiments also indicated that clone pJ-31 contained a nearly full-length cDNA complementary to the 3200-nucleotide PLP mRNA and that clones pJ-81 and pC-4 were derived from the 2400-nucleotide mRNA.

The nucleotide sequences corresponding to the coding regions of each of these clones were determined and compared to the sequences of rat PLP mRNA (11). Generally, the sequences were identical, confirming that the selected mouse cDNA clones corresponded to PLP mRNAs. The nucleotide sequence of jimpy clone pJ-31, however, revealed a significant difference from wild-type mouse and rat brain PLP

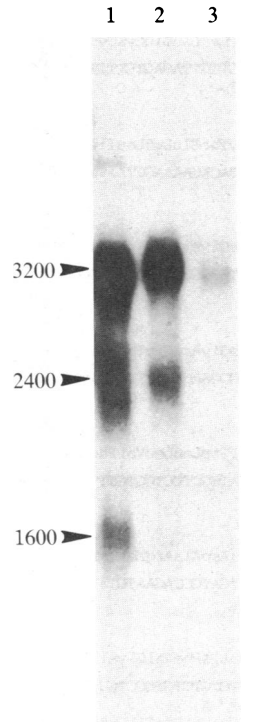

FIG. 1. RNA gel blot analysis of PLP mRNAs. Aliquots (2 μg) of poly(A)$^+$ cytoplasmic RNA were separated by electrophoresis on 1.5% agarose gels, blotted to nitrocellulose, and hybridized with ^{32}P-labeled p27 cDNA insert (11). Lanes: 1, rat brain; 2, control mouse brain; and 3, jimpy mouse brain. The sizes of the major PLP mRNA bands in nucleotides are indicated at the left. Hybridization of this and other blots with the plasmid 1B15, which hybridizes to a ubiquitous rodent mRNA (20), showed no difference in the hybridization signal between jimpy and wild-type RNAs, indicating that the decrease in PLP mRNA in jimpy brain was not due to RNA degradation or an insufficient amount of the sample (data not shown).

mRNAs (Fig. 2). Beginning at position 625, 74 nucleotides are deleted from jimpy PLP mRNA. Sequences immediately surrounding the deletion site are completely conserved and identical to both wild-type mouse and rat PLP mRNA. Sequencing of the second jimpy cDNA isolate, pJ-81, independently confirmed the 74-nucleotide deletion and excluded the possibility of a cloning artifact. The 74-nucleotide sequence is completely present in the control cDNA clone pC-4 and is, in fact, identical in sequence to the corresponding region of rat PLP mRNA. The size of the deletion is consistent with the small size reduction observed for jimpy PLP mRNA (Fig. 1).

The deletion in the jimpy PLP mRNA interrupts the protein coding region and causes a shift in the protein reading frame (Fig. 2). The new open reading frame ends 28 nucleotides upstream of the normal stop codon, is 34 codons shorter than that encoding rat PLP, and specifies a 242-amino acid protein with an altered carboxyl terminus. A second, single-base deletion was also observed in the sequence of clone pJ-31 at nucleotide position 78 (Fig. 2), which would also introduce a frame shift in the PLP coding region. Unfortunately, the cDNA clone pJ-31 was the only characterized jimpy clone that included this region, and we have no independent confirmation of the second deletion. As discussed below we think that it is unlikely that this base change reflects the jimpy mutation.

Apart from the deletion in the jimpy sequence, rat and mouse PLP show 99% homology in their amino acid sequences (Fig. 2): 2 base changes replace a tyrosine by cysteine (residue 69) and a serine by threonine (residue 125);

FIG. 2. Nucleotide sequences of cDNAs encoding jimpy and wild-type mouse brain PLP mRNA. The sequence shown was determined for clone pJ-31 and is numbered from the second nucleotide preceding the ATG initiation codon. Nucleotides that are different in the corresponding sequence of rat brain PLP cDNA (11) are indicated below the line. The single open reading frame is translated into amino acid sequence and numbered starting with the amino-terminal glycine of the mature protein: residues that are different in the protein sequence of rat PLP are indicated above the line. The nucleotides that are absent from the jimpy PLP cDNA sequence are underlined: the nucleotide sequence shown was determined for the wild-type mouse PLP clone pC-4. The sequence of jimpy PLP cDNA between nucleotides 605 and 840 was confirmed with clone pJ-81. The variant carboxyl-terminal amino acid sequence that would be generated by the deletion is shown below the line following the site of the deletion. The site of the second, single nucleotide deletion at position 78 in clone pJ-31 is indicated by ★; the thymidine residue at this point appears in the rat cDNA sequence. In most cases, particularly around the deletions, the sequence shown was confirmed on both DNA strands.

15 further base differences between mouse and rat remain silent. These data further demonstrate the high evolutionary conservation of PLP sequences, reported for the rat (11, 21, 22), cow (23–25), and human (26) proteins.

The 74-Nucleotide Sequence Is Not Deleted in Jimpy Genomic DNA. To understand the mechanism of generation of the deletion in jimpy PLP mRNA, it was essential to determine whether the deleted sequence was present in the PLP gene in jimpy mice. We noted that the deleted sequence contained the recognition sites for the restriction enzymes *Nco* I (CCATGG) and *Bsm* I (GAATGC). Restriction mapping and sequence analysis of the wild-type and jimpy cDNA clones suggests that these are the only sites for these enzymes. Genomic DNA was prepared from the individual livers of the same jimpy and wild-type mice used for RNA preparation, digested with these and other restriction enzymes and analyzed by Southern blotting. No differences were found between jimpy and wild-type mice in the patterns of restriction fragments that hybridized with the insert of rat PLP clone p27 (Fig. 3*A*). In particular, the digests with *Nco* I and *Bsm* I each produced two hybridizing fragments of apparently similar mobility from both wild-type and jimpy DNA. Although one cannot rule out the possibility that there are additional sites for both of these enzymes in nearby intron regions, the simplest interpretation of this experiment is that the sites within the 74-nucleotide deletion are present in jimpy DNA and, hence, that the PLP gene in jimpy mice does not contain a deletion.

To provide more definitive evidence for the presence of the 74-nucleotide sequence in jimpy DNA, we synthesized an oligonucleotide corresponding to 65 nucleotides of this sequence and used it to probe the same Southern blot. This probe hybridized with a single restriction fragment in each of the digests, and there was no difference in the patterns observed for jimpy or wild-type mouse DNA (Fig. 3*B*). The 74-nucleotide sequence that is deleted from jimpy PLP mRNA is, therefore, at least partially preserved in the jimpy gene for PLP. These experiments have been repeated for DNA preparations from different individual mutant mice with no change in result.

DISCUSSION

The observation of a 74-nucleotide deletion in jimpy PLP cDNA clones, together with the evidence that the gene for PLP maps to the X chromosome (10), strongly suggests that the PLP gene is the site of the genetic lesion in jimpy mice. Differences between jimpy and wild-type PLP mRNA demonstrated by S_1-nuclease protection experiments (27) are also approximately consistent with the size and position of the deletion described here. A primary defect in the PLP gene in jimpy mice is consistent with the severe reduction in PLP concentration in jimpy brains (6–9) and with ultrastructural (28, 29), biochemical (30), and *in vitro* studies (31) that indicate that the primary genetic defect is expressed in oligodendrocytes. The significantly reduced mRNA levels in the mutant (Fig. 1) might be attributed to a decreased number

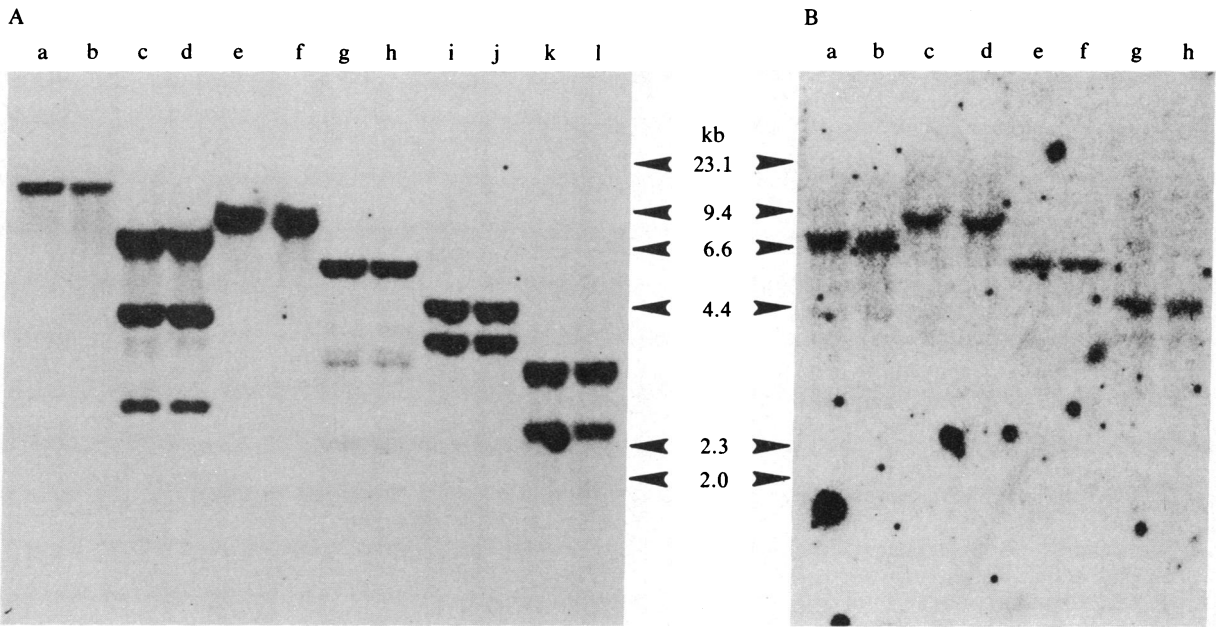

FIG. 3. Southern blots of jimpy and wild-type genomic DNAs. Aliquots (10 μg) of jimpy and wild-type liver DNA were digested with the restriction endonucleases, separated by electrophoresis on 0.6% agarose, blotted to nitrocellulose, and hybridized with ^{32}P-labeled probes. (*A*) The blot was hybridized with a full-length rat PLP cDNA: wild-type DNA (lanes a, c, e, g, i, and k) or jimpy DNA (lanes b, d, f, h, j, and l) were digested with *Kpn* I (lanes a and b), *Eco*RI (lanes c and d), *Bam*HI (lanes e and f), *Hin*dIII (lanes g and h), *Nco* I (lanes i and j), *Bsm* I (lanes k and l). (*B*) The same blot was washed and rehybridized with an oligonucleotide probe corresponding to 65 nucleotides of the 74-nucleotide deletion: wild-type DNA (lanes a, c, e, and g) or jimpy DNA (lanes b, d, f, and h) digested with *Eco*RI (lanes a and b), *Bam*HI (lanes c and d), *Hin*dIII (lanes e and f), or *Nco* I (lanes g and h). kb, Kilobases.

of oligodendrocytes (32), reduced mRNA stability, or unknown transcriptional feedback mechanisms. Similar deficiencies in the mRNAs for other myelin components in jimpy have been described (33, 34).

An Altered Carboxyl Terminus for Jimpy PLP. The single-base deletion observed at the 5′ end of the sequence of clone pJ-31 would result in the synthesis of a severely truncated PLP molecule only 45 amino acids long. Although this change could explain dysmyelination in the mutant, studies have shown (35) that jimpy brain poly(A)$^+$ RNA can be translated *in vitro* to produce immunoreactive PLP of approximately normal size. These data are more consistent with an effect of the 74-nucleotide deletion alone, which would result in the synthesis of a PLP molecule with a molecule weight of 26,400 and containing 88% of the wild-type PLP sequence, sufficient to be recognized by antibodies directed against normal PLP. The single-base deletion may, therefore, not be the primary mutation but may have been generated by a transcription error in the generation of clone pJ-31. Alternatively, this change could have arisen secondarily in some mutant stocks in the absence of selective pressure.

The evolutionary conservation of PLP sequences suggests a strong structure–function relationship for this protein and models have been proposed for the integration and orientation of PLP in the membrane and its possible role in myelin compaction (36, 37). For example, there are four long stretches of hydrophobic amino acids that have been proposed as transmembrane domains (11, 36, 37). The 74-nucleotide deletion in the coding region of jimpy PLP mRNA plus the resulting frameshift together predict a protein of 242 amino acids, 34 amino acids shorter than PLP, but including a variant carboxyl-terminal sequence of 36 amino acids (Fig. 4). Although the amino-terminal 206 amino acids of this molecule are identical to wild-type PLP, it lacks the fourth membrane crossing region (residues 238–267) and a short hydrophobic region (residues 205–218) that is thought to mediate contact with the opposing membrane (36). Hence the mutant protein may not be inserted properly in the myelin membrane or participate effectively in myelin compaction, resulting in incomplete myelination. Interestingly, the variant carboxyl terminus of jimpy PLP contains a high proportion of cysteine residues (8 of 36 residues): these might further disrupt the ability of this protein to form functional myelin.

The 74-Nucleotide Deletion Results from Aberrant RNA Splicing. Southern blot experiments comparing jimpy and wild-type genomic DNA showed no restriction fragment length differences using a full-length rat PLP cDNA probe (Fig. 3*A*). In particular there were no differences in the hybridization patterns of digests with enzymes having recognition sites within the deletion. An oligonucleotide probe directed against the deleted sequence also gave the same pattern for jimpy and wild type (Fig. 3*B*). These experiments indicate that there is no corresponding deletion in jimpy genomic DNA and, therefore, that the 74-nucleotide sequence is most likely to be deleted in jimpy PLP mRNA by aberrant splicing of PLP transcripts. This is in marked contrast, for example, to the dysmyelinating mouse mutant shiverer, which also displays deficient myelination in the central nervous system but which has been shown to possess a large deletion in the gene for myelin basic protein (38).

The nucleotide sequence surrounding the 5′ site of the deletion in the jimpy cDNA clones and the wild-type clone pC-4 has homologies to the consensus sequence at the 5′- or donor splice site (39). For example, the sequence ATG/GTG is identical to a 5′-splice site in the mouse α-amylase gene (40)

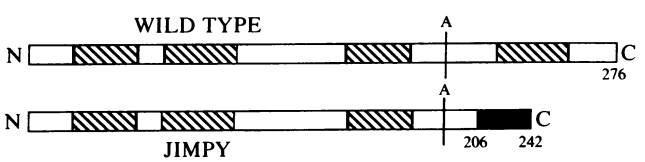

FIG. 4. Comparison of the structures of wild-type and jimpy PLP. The hydrophobic regions in the normal and mutant proteins are shown by the hatched areas; the filled bar indicates the altered carboxyl terminus of the putative jimpy PLP. A, fatty acylation site.

and is similar to other donor sites found in mouse and rat gene exon/intron boundaries. Our results are compatible with a model in which a point mutation or a small deletion or insertion in the gene for PLP leads to the activation of a cryptic splice site in an exon, 74 nucleotides upstream of an exon/intron boundary. Other models are possible—elimination of an exon corresponding to the 74-nucleotide sequence or activation of a cryptic 3'-acceptor site—but we believe the latter to be less likely. Determination of mutant and wild-type genomic sequences will be required to define exactly the nucleotides involved in the mutation and the precise mechanism of aberrant splicing.

Incorrectly spliced transcripts of the human β globin gene are a well-characterized defect in several cases of β-thalassemias: RNA splicing defects result from point mutations, which, in most cases, cause the utilization of alternate 5'-donor sites (41). Many of the aberrant mRNAs are found to be less stable *in vivo* (42). Congenital Pelizaeus–Merzbacher disease is a X-linked recessive leukodystrophy in human (43) and is referred to as a genetic disorder analogous to the jimpy mutation in mouse. Unrelated cases of Pelizaeus–Merzbacher disease that differ in the onset of disease and clinical severity might similarly comprise a group of mutations that vary in their manifestation like homozygote thalassemias.

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