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Individual exons encode the integral membrane domains of human myelin proteolipid protein

(human proteolipid protein genomic clones/exon–intron organization/homology of bovine, rat and human proteolipid protein/mapping to X chromosome)

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ABSTRACT **The gene encoding human proteolipid protein (PLP) was isolated from a human genomic library by hybridization with labeled DNA of a PLP-specific cDNA clone. The entire PLP gene spans approximately 17 kilobases. Restriction and sequence analysis revealed seven exons and six introns. The entire nucleotide sequences of the exons and of the exon–intron transitions were determined, and the intron lengths were measured. Exon I includes only ATGG of the translated region, the N-terminal methionine codon and G of glycine, the first amino acid of mature PLP. Each hydrophobic *trans*- and *cis*-membrane domain of PLP together with its adjacent hydrophilic sequence correlates closely with one exon of the gene except for the C-terminal transmembrane helix that is encoded by two exons. The amino acid sequence of human PLP derived from the nucleic acid sequence is highly conserved. Human and rat PLP are completely homologous, whereas only four amino acid residues are exchanged in bovine PLP sequence derived from protein sequencing and a partial cDNA clone. Homology search on the nucleic acid level among human, bovine, and rat brain PLPs indicates an unusually high homology in the coding regions. Hybridization analysis with DNA of human–rodent hybrid clones revealed that the gene encoding PLP segregates with human X chromosome in the region q13–q22.**

Proteolipid protein (PLP; lipophilin, Lp) is the primary constituent protein of myelin in the central nervous system. Protein sequence studies on bovine and human PLP have revealed a 276-amino acid polypeptide with five strongly hydrophobic domains (1–3) that interact with the lipid bilayer as *trans*- and *cis*-membrane segments (3). Rodent PLP cDNA was recently cloned in our laboratory (4) as well as in two other laboratories (5, 6), and bovine PLP cDNA was cloned by a fourth group (7).

Here we report on the molecular organization of the human gene encoding PLP. The gene is about 17 kilobases (kb) in length and consists of seven exons and six introns. Exon I encodes only methionine, the N-terminal amino acid residue of the unprocessed primary translation product of PLP. Exons II, III, IV, and V all correlate with one hydrophobic domain and the adjacent hydrophilic connecting sequence defined at the protein level. Only the C-terminal transmembrane sequence is encoded by exons VI and VII. The latter also contains the hydrophilic C-terminal sequence of PLP and the 3′ untranslated region.

Southern blot hybridization analysis of restricted DNA from human–rodent cell hybrids led to the mapping of the PLP gene onto the X chromosome. The shortest overlap suggests that the gene location is in the region q13–q22 near the locus for 3-phosphoglycerate kinase.

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MATERIALS AND METHODS

The λ bacteriophage EMBL3 library and *Escherichia coli* strain NM539 were provided by A. M. Frischauf (European Molecular Biology Laboratory, Heidelberg, F.R.G.) (8).

Hybridization Probes. Rat PLP cDNA clone pLP1 contains a 2585-basepair (bp) insert in the *Pst* I restriction site of plasmid pBR322 (4). The complete clone pLP1 and its 1500-, 640-, and 480-bp *Pst* I fragments were nick-labeled (9). PLP-specific synthetic oligonucleotides were 5′ end-labeled with [γ-^{32}P]ATP (>185 TBq/mmol, Amersham) in the presence of T4 polynucleotide kinase (Bethesda Research Laboratories) (10).

Isolation of Genomic PLP Clones. Plaques (7.5×10^5) of the λ phage EMBL3 library were screened with ^{32}P-labeled cDNA clone pLP1. Nitrocellulose filters (BA 85, Schleicher & Schüll) were prehybridized for 6 hr at 42°C in 50% (vol/vol) formamide/5× Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin)/2× SSPE (1× SSPE = 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA)/100 μg of sonified salmon sperm DNA per ml and subsequently hybridized with the cDNA probe at 10^5 cpm/ml. Filters were washed in 0.2× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0)/0.1% $NaDodSO_4$ at 65°C for 30 min and autoradiographed at −70°C overnight with intensifying screens.

Mapping of the Gene Encoding Human PLP by Southern Blot Hybridization Analysis. Phage DNA was digested with the respective restriction enzymes and size-fractionated by gel electrophoresis [0.7% agarose and 5% (wt/vol) polyacrylamide in TBE buffer (0.089 M Tris borate/2 mM EDTA)]. The agarose gels were alkali-denatured (0.5 N NaOH, 1.5 M NaCl for 45 min), neutralized (0.5 M Tris·HCl at pH 7.5, 1.5 M NaCl for 45 min), and blotted onto a GeneScreen*Plus* membrane (New England Nuclear) with 10× SSC. The membrane was hybridized with the respective radioactive probes, either the ^{32}P-nick-translated *Pst* I fragments of pLP1 or 5′-^{32}P-labeled oligonucleotides with specific activities of about 5×10^6 cpm/pmol at 1 pmol/ml according to the GeneScreen*Plus* manual.

DNA Sequence Analysis. Restriction fragments were size-fractionated by agarose gel electrophoresis (0.7% in TBE buffer), transferred onto DEAE filter membrane (NA 45, Schleicher & Schüll) by electrophoresis, and eluted with 1 M NaCl in the presence of 1% arginine. Nucleotide sequences were determined by plasmid sequencing of restriction fragments cloned into the polylinker sequence of pUC13 with the M13 sequencing and M13 reverse-sequencing primer (New England Biolabs) (11). PLP-specific heptadecameric primers were synthesized by the solid-phase phosphoramidite method on an automated Applied Biosystems synthesizer (Model

Abbreviations: PLP, proteolipid protein, kb, kilobase(s); bp, base pair(s).

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Table 1. Sequences and relative positions of hybridization probes

Probe	Hybridization position	Amino acids encoded	Sequence*	Length, bp
a	−21 to +1	—	ACCCATGTCTTTGGCACTCTGGCT†	24
b	1 to 30	Gly^{1}–Leu^{10}	CAGACATCTAGCACAGCACTCTAACAAACC	30
c	310 to 726	Lys^{104}–Ala^{242}	*Pst* I-fragment of pLP1‡	450
d	727 to 1366	Phe^{243}–Phe^{276}	*Pst* I-fragment of pLP1	640
e	1367 to 2262	—	*Pst* I-fragment of pLP1	1500

*Oligonucleotide sequences are complementary to rat mRNA.
†The first two bases belong to the intron between exon I and II.
‡From Schaich *et al.* (4).

380A; Foster City, CA). If necessary, large fragments were further digested with the restriction enzymes *Hae* III and *Rsa* I. These subfragments were inserted in plasmid pUC13 and sequenced as described.

Hybridization Analysis of Human Leucocyte DNA. Human leucocyte DNA of healthy donors was isolated (12), digested with *Eco*RI, *Hin*dIII, and *Pst* I (5 μg/lane), separated by a 0.7% agarose gel in TBE, and blotted onto a GeneScreen*Plus* membrane. The membrane was incubated in 50% formamide/10% (wt/vol) dextran sulfate/1% $NaDodSO_4$/1 M NaCl for 6 hr at 42°C. One hundred micrograms of sonified salmon sperm DNA per ml and nick-labeled DNA (1×10^5 cpm/ml at ≈2 ng/ml) were added, and the membrane was further incubated for 24 hr at 42°C. The blots were washed in 0.2× SSC/1% $NaDodSO_4$ at 65°C for 30 min and exposed to Kodak XAR film for 5–14 days with an intensifying screen.

Chromosomal Mapping of the PLP gene. *Bam*HI and *Eco*RI-restricted DNAs of human-rodent hybrid cells blotted onto nylon membranes (PALL, Glen Cove, NY) were hybridized with the nick-labeled 1200-bp *Eco*RI fragment of clone EMBL3-LP6. The hybridization conditions were as described.

RESULTS

Human PLP Genomic Clones. Two genomic PLP clones, EMBL3-LP6 and EMBL3-LP7, were isolated from a genomic library cloned into phage λ EMBL3 (8). Approximately 7.5 $\times 10^5$ plaques were screened with nick-labeled DNA derived from the PLP cDNA clone pLP1 (4).

Characterization of Genomic Clones EMBL3-LP6 and EMBL3-LP7. The strategy for analyzing the gene encoding PLP consisted of the restriction endonuclease digestion of the two clones. Fragments of single and double digestions were separated by agarose and polyacrylamide gel electrophoresis. Southern blot analysis with *Pst* I fragments of pLP1 and synthetic oligonucleotides as probes for the 5′ end of the PLP gene (Table 1) established the restriction map shown in Fig. 1*a*.

Hybridization analysis with probes b and e (Table 1) revealed that clone EMBL3-LP7 is the N-terminal and clone EMBL3-LP6 is the C-terminal genomic clone. Their restriction analysis proved that the clones have a length of ≈15 kb each and are independent but carry an overlapping region of 2.5 kb.

The human gene encoding PLP spans a region of ≈17 kb, estimated from hybridization data of the 5′ oligonucleotide (probe a) and the 1500-bp *Pst* I fragment of pLP1 (probe e).

Restriction analysis and hybridization with different probes (a–e) yielded seven independent fragments indicating that there were at least seven exons. EMBL3-LP7 contains exons I–VI; EMBL3-LP6 contains exons IV–VII.

Sequence Determination of the Seven Exons. The exon-bearing fragments were inserted into pUC13 for plasmid sequencing. Fig. 1*b* summarizes the sequencing strategy applied to the human gene encoding PLP. Exons III–VII could be identified with cDNA fragments of pLP1 (probes c, d, e). Because these fragments did not contain the N-terminal sequence up to base 310, an oligonucleotide derived from the

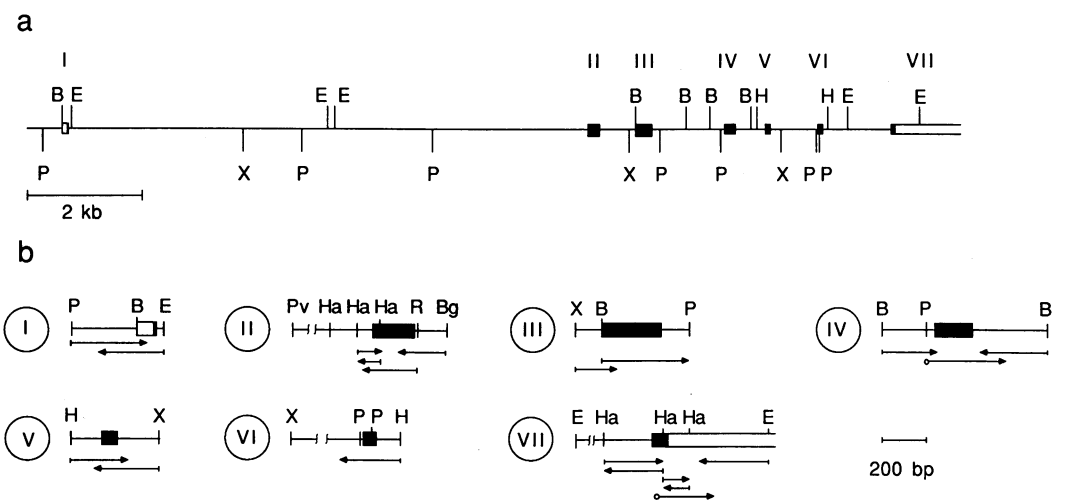

FIG. 1. (*a*) Restriction map and organization of exons and introns of the human gene encoding PLP. The structure of the gene is represented schematically by a line with the seven exons (roman numerals) as bars. Solid bars represent coding sequences, and open bars represent 5′ and 3′ untranslated sequences. (*b*) DNA sequencing strategy of the seven exons. Genomic fragments were inserted into pUC13 and analyzed by plasmid sequencing. All exons were subcloned with enzymes shown in *a* except for exon II, which was subcloned by restriction with *Pvu* II and *Bgl* II. Genomic fragments of exons II and VII were further digested with *Hae* III and *Rsa* I. The obtained subfragments were also inserted in pUC13 for sequencing. Arrows starting with a vertical line represent sequences obtained with M13 sequencing or M13 reverse sequencing primer. Arrows with a circle at the beginning indicate that oligonucleotides synthesized according to PLP genomic DNA were used as sequencing primers. B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; Ha, *Hae* III; P, *Pst* I; Pv, *Pvu* II; R, *Rsa* I; X, *Xba* I.

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I     AAGAAAATGA AACAATTGGC AGTGAAAGGC AGAAAGAGAA GATGGAGCCC TTAGAGAAGG GAGTATCCCT GAGTAGGTGG GGAAAAGGGG AGGAGAAGGG GAGGAGGAGA GGAGGAGGAA         -205
                                          ①                                                                           ②
      AGCAGGCCTG TCCCTTTAAG GGGGTTGGCT GTCAATCAGA AAGCCCTTTT CATTGCAGGA GAAGAGGACA AAGATACTCA GAGAGAAAAA GTAAAAGACC GAAGAAGGAG GCTGGAGAGA         -85

           ③                                                                                            Met G(ly)                                 1
      CCAGGATCCT TCCAGCTGAA CAAAGTCAGC CACAAAGCAG ACTAGCCAGC CGGCTACAAT TGGAGTCAGA GTCCCAAAGA C ATG G gtaagtttcaaaaactttag...                    -1

II                         (G)ly Leu Leu Glu Cys Cys Ala Arg Cys Leu Val Gly Ala Pro Phe Ala Ser Leu Val Ala Thr Gly Leu Cys Phe Phe Gly Val Ala   29
      ...ttccccttcttcttccccag  GC TTG TTA GAG TGC TGT GCA AGA TGT CTG GTA GGG GCC CCC TTT GCT TCC CTG GTG GCC ACT GGA TTG TGT TTC TTT GGG GTG GCA  +87

      Leu Phe Cys Gly Cys Gly His Glu Ala Leu Thr Gly Thr Glu Lys Leu Ile Glu Thr Tyr Phe Ser Lys Asn Tyr Gln Asp Tyr Glu Tyr Leu Ile Asn Va(l)       63
      CTG TTC TGT GGC TGT GGA CAT GAA GCC CTC ACT GGC ACA GAA AAG CTA ATT GAG ACC TAT TTC TCC AAA AAC TAC CAA GAC TAT GAG TAT CTC ATC AAT GT gtaa  +188
      gtacctgccctcccac...
III                        (Va)l Ile His Ala Phe Gln Tyr Val Ile Tyr Gly Thr Ala Ser Phe Phe Phe Leu Tyr Gly Ala Leu Leu Leu Ala Glu Gly Phe Tyr     81
      ...ttgtctacctgttaatgcag  G ATC CAT GCC TTC CAG TAT GTC ATC TAT GGA ACT GCC TCT TTC TTC TTC CTT TAT GGG GCC CTC CTG CTG GCT GAG GGC TTC TAC  +243

      Thr Thr Gly Ala Val Arg Gln Ile Phe Gly Asp Tyr Lys Thr Thr Ile Cys Gly Lys Gly Leu Ser Ala Thr Val Thr Gly Gly Gln Lys Gly Arg Gly Ser Arg   116
      ACC ACC GGC GCA GTC AGG CAG ATC TTT GGC GAC TAC AAG ACC ACC ATC TGC GGC AAG GGC CTG AGC GCA ACG GTA ACA GGG GGC CAG AAG GGG AGG GGT TCC AGA  +348

      Gly Gln His Gln Ala His Ser Leu Glu Arg Val Cys His Cys Leu Gly Lys Trp Leu Gly His Pro Asp Lys                                                150
      GGC CAA CAT CAA GCT CAT TCT TTG GAG CGG GTG TGT CAT TGT TTG GGA AAA TGG CTA GGA CAT CCC GAC AAG gtgatcatcctcaggatttt...                      +450

IV                            Phe Val Gly Ile Thr Tyr Ala Leu Thr Val Val Trp Leu Leu Val Phe Ala Cys Ser Ala Val Pro Val Tyr Ile Tyr Phe Asn Thr   179
      ...acccatgtcaatcattttag TTT GTG GGC ATC ACC TAT GCC CTG ACC GTT GTG TGG CTC CTG GTG TTT GCC TGC TCT GCT GTG CCT GTG TAC ATT TAC TTC AAC ACC  +537

      Trp Thr Thr Cys Gln Ser Ile Ala Phe Pro Ser Lys Thr Ser Ala Ser Ile Gly Ser Leu Cys Ala Asp Ala Arg Met Tyr G(ly)                              207
      TGG ACC ACC TGC CAG TCT ATT GCC TTC CCC AGC AAG ACC TCT GCC AGT ATA GGC AGT CTC TGT GCT GAT GCC AGA ATG TAT G  gtgagttagggtacgggtgc...      +619

V                          (G)ly Val Leu Pro Trp Asn Ala Phe Pro Gly Lys Val Cys Gly Ser Asn Leu Leu Ser Ile Cys Lys Thr Ala Glu                       231
      ...gctttttgtgtcttacttag  GT GTT CTC CCA TGG AAT GCT TTC CCT GGC AAG GTT TGT GGC TCC AAC CTT CTG TCC ATC TGC AAA ACA GCT GAG gtgagtgggttattt  +693
      gggtt...

VI                            Phe Gln Met Thr Phe His Leu Phe Ile Ala Ala Phe Val Gly Ala Ala Ala Thr Leu Val Ser Leu                                 253
      ...ctcttttcattttcctgcag TTC CAA ATG ACC TTC CAC CTG TTT ATT GCT GCA TTT GTG GGG GCT GCA GCT ACA CTG GTT TCC CTG gtgagttgactttgaatgat...     +759

VII                           Leu Thr Phe Met Ile Ala Ala Thr Tyr Asn Phe Ala Val Leu Lys Leu Met Gly Arg Gly Thr Lys Phe Stop                         276
      ...ctctcttctgttccctacag CTC ACC TTC ATG ATT GCT GCC ACT TAC AAC TTT GCC GTC CTT AAA CTC ATG GGC CGA GGC ACC AAG TTC TGA TCCCCCGTAG           +841

      AAATCCCCCT TTCTCTAATA GCGAGGCTCT AACCACACAG CCTACAATGC TGCGTCTCCC ATCTTAACTC TTTGCCTTTG CCACCAACTG GCCCTCTTCT TACTTGATGA GTGTAACAAG...     +961
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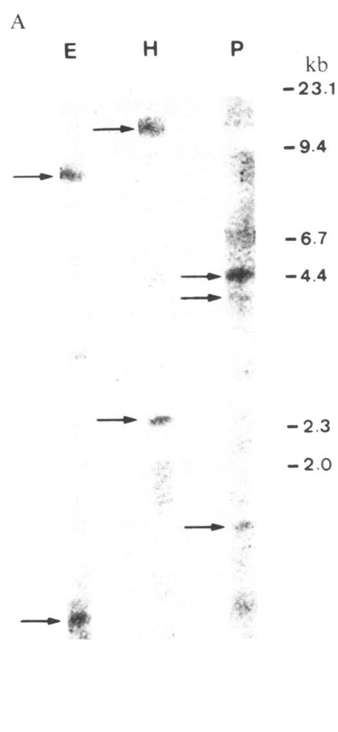
FIG. 2. Nucleotide sequence of human PLP encoding gene. The complete sequence of the protein-encoding region of human PLP derived from genomic clones EMBL3-LP6 and EMBL3-LP7 and the nucleotide-derived amino acid sequence are shown. Nucleotide 1 is the G of glycine, the first amino acid residue in mature PLP. The proposed CAAT (①) and TATA (②) boxes and the possible cap site (③) are underlined. At position −42 the first nonsense codon (TAG) is marked that appears in frame upstream from the translated sequence. Underlined coding sequences represent the hydrophobic domains of the protein. Only 202 bases of exon VII are shown.

N-terminal part of rat cDNA (5, 6) was synthesized (probe b). After sequencing, it became obvious that exon II does not cover the complete coding 5′ end of the gene but begins within the codon for the first amino acid of mature PLP. An additional oligonucleotide had to be synthesized for identifying exon I (probe a), which could be found in a *Pst* I/*Eco*RI fragment 8.8 kb upstream of exon II. Fig. 2 shows the combined exon sequences with joining intron borders and the untranslated regions of the 5′ and 3′ end.

The Human Genome Contains Only One Gene Encoding PLP. The existence of an additional PLP gene is unlikely: when human leucocyte DNA is digested with the restriction enzymes *Eco*RI, *Pst* I and *Hin*dIII, no fragment can be found in Southern blot hybridization analysis (Fig. 3*A*) that does not also appear in the PLP gene integrated into the two clones EMBL3-LP6 and EMBL3-LP7 (Fig. 3*B*). It is improbable that a second PLP gene carries exactly the same restriction sites.

Human Gene Encoding PLP Segregates with the X Chromosome. The gene for human myelin PLP was mapped to the X chromosome by hybridization of cloned PLP to DNA of a panel of hybrid clones from human fibroblasts and mouse (RAG19, A9) or Chinese hamster cells (V79), all deficient in hypoxanthine phosphoribosyltransferase (14–16). Some hybrid clones carried fragments of the human X chromosome originating from reciprocal translocations in the respective human parental cells. The DNA of the hybrid clones was digested with *Eco*RI and in a second experiment with *Bam*HI, their fragments were separated by agarose gel electrophoresis, blotted onto nylon membranes, and hybrid-



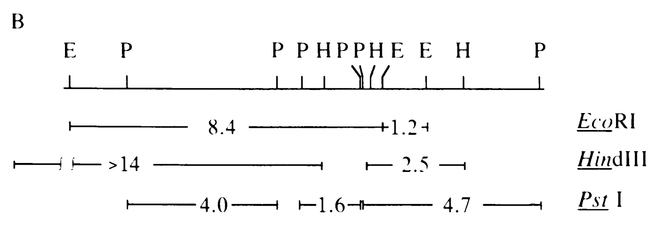


FIG. 3. Comparison of restriction sites of human leucocyte DNA and human gene encoding PLP derived from the human genomic clones EMBL3-LP6 and EMBL3-LP7. (*A*) Leucocyte DNA was digested with *Eco*RI (lane E), *Hin*dIII (lane H), and *Pst* I (lane P), size-separated by agarose gel electrophoresis, and blotted onto GeneScreen*Plus* membrane. Southern blot hybridization was performed with nick-labeled cDNA fragments c and d (Table 1). Arrows point to the following fragments: *Eco*RI; 1.1 kb and 8.3 kb; *Hin*dIII, 2.5 kb and ≈15 kb; *Pst* I, 1.5 kb, 4.1 kb, and 4.5 kb. (*B*) Part of the restriction map derived from the genomic clones. Fragments corresponding to those appearing in *A* are listed together with their approximate size in kb. The large N-terminal *Hin*dIII fragment (>14 kb) extends over the 5′ end of the genomic clone EMBL3-LP7.

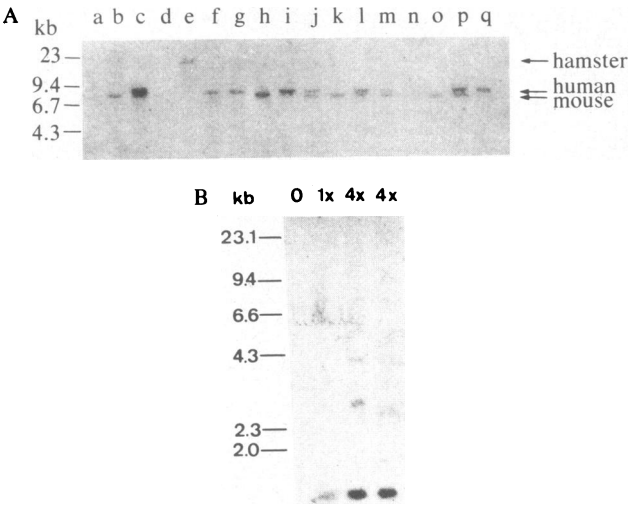


FIG. 4. (*A*) *Bam*HI-digested DNA of human–rodent somatic cell hybrids were hybridized with the nick-labeled C-terminal 1200-bp *Eco*RI-fragment of EMBL3-LP6. Lane a, mouse genomic DNA (RAG) clones; lane e, hamster genomic DNA (P3) clones; and lane q, human genomic DNA. Other lanes are identified to the left of Fig. 5*A*. A blot of the same DNAs but *Eco*RI-digested was taken as control for the missing signals in lanes a, d, and n. (*B*) Dose-dependent hybridization (0, 1×, 4×, 4×) of the human X chromosome with the nick-labeled 1200-bp *Eco*RI fragment DNA was restricted with *Eco*RI.

ized with the gel-purified and nick-labeled 1.2-kb C-terminal *Eco*RI fragment. The 9.3-kb *Bam*HI and the 1.2-kb *Eco*RI fragments were taken as test markers for human PLP. The result of the Southern blot hybridization analysis (Fig. 4*A*) is given in Fig. 5. The human PLP gene clearly segregates with the X chromosome in the panel of human–rodent somatic cell hybrids. The smallest overlapping region is at Xq13–q22 near the 3-phosphoglycerate kinase locus. This agrees with a recent report (13).

DISCUSSION

The data presented here demonstrate that human PLP is encoded in a single 17-kb gene. The coding region is contained in seven exons separated by six introns. We obtained the complete nucleotide sequence of the human PLP mRNA by combining two genomic clones of a λ phage EMBL3 library. The two clones overlap in exons IV, V, VI, and VII. The 5′ end of the gene, the coding sequences spread over the seven exons, and the 3′ stop codon as well as the 3′ untranslated region could be determined unambiguously by comparison with the cDNA nucleotide sequences elaborated previously (4–6). A striking feature is the complete homology of the amino acid sequence of rat myelin PLP derived from the cDNA sequence and the human primary protein structure derived from the exon sequences in the human genomic clones. The homology of the human and rat PLP coding nucleotide sequences is >97%.

Exon I contains the 5′ untranslated region of human PLP mRNA and the 5′-G of the triplet for glycine, the N-terminus of mature PLP, that is preceded by a methionine codon in the primary translation product. As far as rat PLP is concerned, there is no evidence for a signal peptide (6, 17). For human PLP the sequence of exon I led to the same conclusion: following the ATG of methionine upstream according to the reading frame, a nonsense codon (TAG) is found at position −42 (Fig. 2). Between these two codons there is no further ATG. This suggests that the primary translation product contains only methionine in addition to the mature sequence.

The only possible promoter element can be found at position −113 (TAAAA), which is 61 bases downstream from a sequence with a high homology to the classical CAAT box (18). The highly homologous 5′ end of rat PLP mRNA extends over the "TATA" box-like element (TAAAA) (6). If it is assumed that the cap site (18) of the human gene encoding PLP is about 30 bp downstream from this postulated promotor element, then the 5′ untranslated region of the human PLP mRNA is about 40 bp shorter than that of rat. The relationship of these facts to regulation of the PLP gene requires further investigation.

Exon–intron junction sequences of eukaryotes (18) are well conserved in the PLP gene. Among those species investigated so far PLP is the most strongly conserved protein of all proteins sequenced. The coding of each *cis*- and *trans*-membrane domain in a separate exon is another striking observation. Four of the five hydrophobic domains are encoded in individual exons. The fifth *trans*-membrane domain is divided in two exons (Fig. 6), like the C-terminal *trans*-membrane domain of the low density lipoprotein receptor (19).

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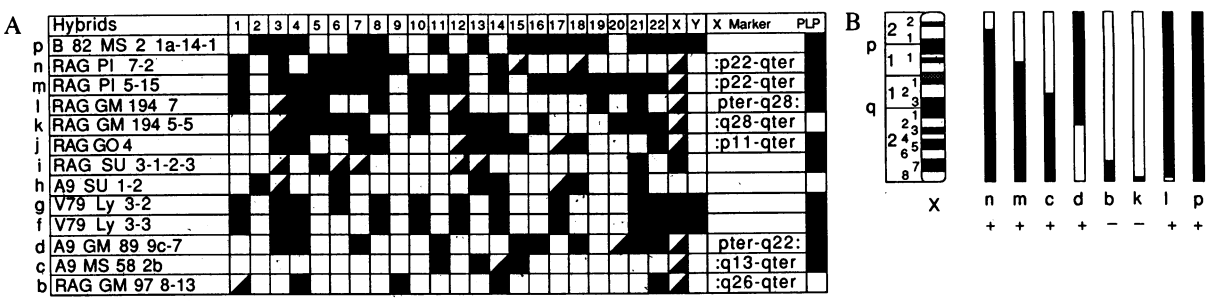

FIG. 5. (*A*) Segregation of human PLP in a panel of human-rodent somatic cell hybrids. A genomic 9.3-kb *Bam*HI fragment and a 1.2-kb *Eco*RI fragment were detected in human cells but not in mouse or hamster control samples taken as test markers for human PLP. The fragments segregated with human Xq13–q22 as indicated by summary in *B*. The presence of the entire chromosome is represented by filled spaces; the presence of chromosomal parts is represented by triangles. Empty spaces indicate that the chromosome or parts of it cannot be detected by biochemical and cytogenetic analysis. The letters on the left refer to lanes in Fig. 4*A*. (*B*) The solid parts of the eight bars to the right indicate the portion of chromosome X contained within the respective hybrid clone of *A*. Presence or absence of PLP is indicated by + or −. The smallest overlapping region is at q13–q22.

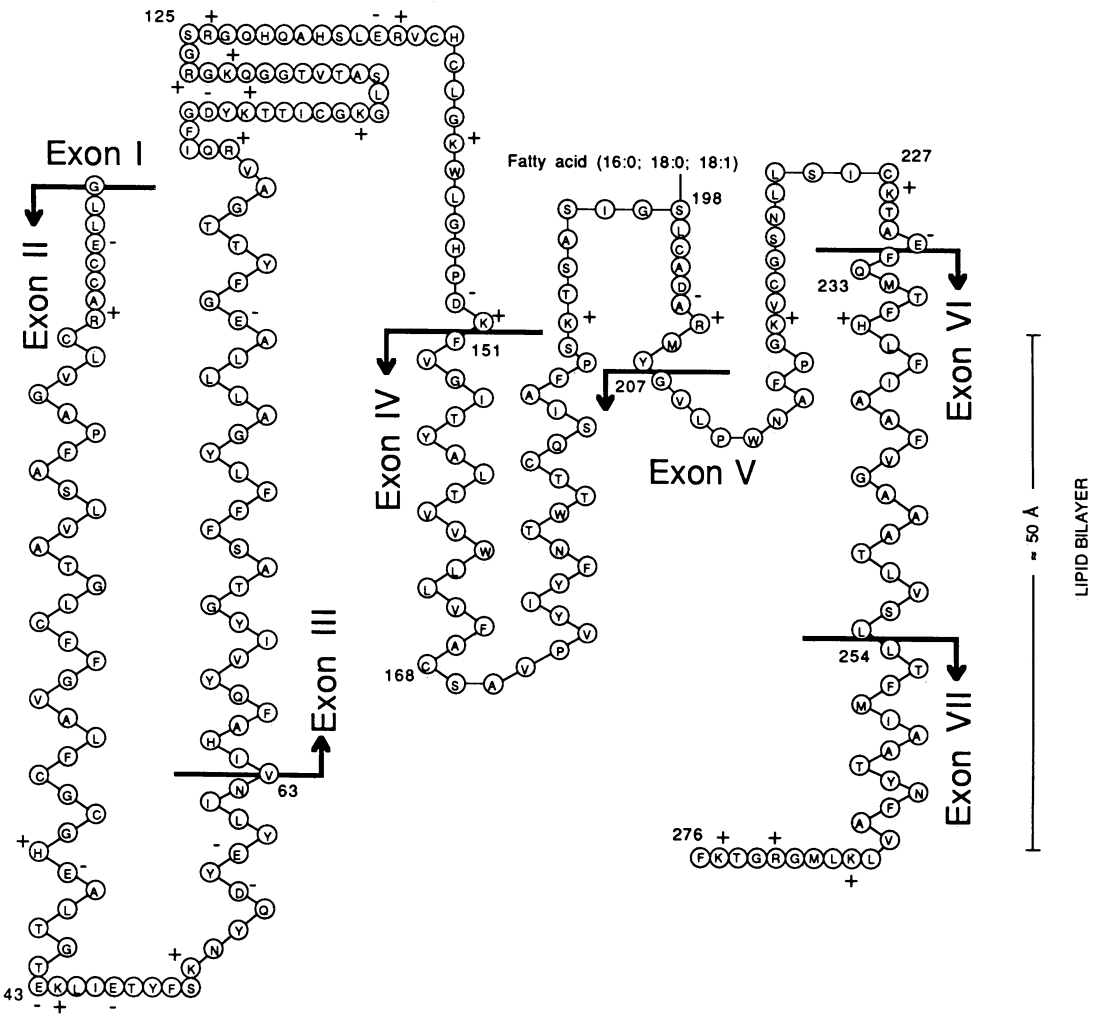

FIG. 6. Amino acid sequence of mature human PLP is folded as suggested by biochemical analysis (3); the hydrophobic α-helical *trans*- and *cis*-membrane sequences are integrated in or span the 50-Å lipid bilayer of the central nervous system myelin membrane. Within the protein, positions are marked that correspond to exon borders of the gene.

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