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Cloned Proteolipid Protein and Myelin Basic Protein cDNA

Transcription of the Two Genes During Myelination

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Summary: cDNA clones of rat brain proteolipid protein (PLP), also named lipophilin, the major integral myelin membrane protein, and of myelin basic protein (MBP), the major extrinsic myelin protein, have been isolated from a rat brain cDNA library cloned into the *Pst*I site of pBR322. Poly(A)$^+$ RNA from actively myelinating 18-day-old rats has been reversely transcribed. Oligonucleotides synthesized according to the established amino-acid sequence of lipophilin and the nucleotide sequence of the small myelin basic protein of the N-terminal, the central and C-terminal region of their sequences were used as hybridization probes for screening. The largest insert in one of several lipophilin clones was 2 585 base pairs (bp) in length (pLp1). It contained 521 bp of the C-terminal coding sequence and the complete 2 064 bp long non-coding 3′ sequence. The myelin basic protein cDNA insert of clones pMBP5 and pMBP6 is 2 530 bp long and that of clones pMBP2 and pMBP3 640 bp. These clones were also characterized. pMBP2 was sequenced and used together with the lipophilin cDNA clones as hybridization probes to estimate the lipophilin and myelin basic protein mRNA levels of rat brain during the myelination period. The expression of the lipophilin and myelin basic protein genes during development of the myelin sheath appears to be strictly coordinated.

Klonierung des Proteolipid-Proteins and basischen Myelinproteins. Transkription der beiden Gene während der Myelinisierung

Zusammenfassung: cDNA-Klone von Rattenhirn-Proteolipid-Protein (PLP), auch Lipophilin genannt, sowie von basischem Myelinprotein (MBP), den beiden wichtigsten Myelinproteinen, wurden aus einer Rattenhirn-cDNA-Bank in pBR322 isoliert. Poly(A)$^+$-RNA aus myelinisierenden 18 Tage alten Ratten wurde revers transkribiert. Oligonucleotide, die nach der bekann-

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Enzymes:
DNA Ligase, recommended name:polydeoxyribonucleotide synthase (ATP), poly(deoxyribonucleotide):poly(deoxyribonucleotide) ligase (AMP-forming) (EC 6.5.1.1);
DNA Polymerase I, recomm. name:DNA-directed DNA polymerase, deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (DNA-directed) (EC 2.7.7.7);
Polynucleotide 5′-hydroxyl-kinase, ATP:5′-dephosphopolynucleotide 5′-phosphotransferase (EC 2.7.1.78);
Restriction endonucleases: *Ava*II (EC 3.1.23.4), *Bam*HI (EC 3.1.23.6), *Eco*RI (EC 3.1.23.13), *Hae*III (EC 3.1.23.17), *Hin*dIII (EC 3.1.23.21), *Nco*I (EC 3.1.23.-), *Pst*I (EC 3.1.23.31), *Rsa*I (EC 3.1.23.101), *Sac*I (EC 3.1.23.34);
*Reverse transcriptase recomm. name:*RNA-directed DNA polymerase, deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (RNA-directed) (EC 2.7.7.49);
Terminal transferase, recomm. name:DNA nucleotidylexotransferase, nucleoside-triphosphate:DNA deoxynucleotidylexotransferase (EC 2.7.7.31).

Abbreviations:
AMV = avian myeloblastosis virus, ATP = adenosinetriphosphate, DEAE = diethylaminoethyl-, EDTA = Ethylenediamine tetraacetic acid, MBP = myelin basic protein, $NaDodSO_4$ = sodium dodecyl sulphate, PLP = Lp = proteolipid protein, lipophilin, SSC = 0.15M sodium chloride, 0.015M sodium citrate, Tris = Tris(hydroxymethyl)aminomethane.

ten Aminosäuresequenz des Lipophilins und der Nucleinsäuresequenz des Myelinproteins im N-terminalen, zentralen und C-terminalen Bereich der Sequenz synthetisiert wurden, dienten als Hybridisierungsproben. Das größte Insert in einem von mehreren Lipophilin-Klonen (Klon pLp1) war 2 585 Basenpaare (bp) lang. Es enthielt 521 bp der C-terminalen kodierenden Sequenz und die vollständige 1 064 bp lange nichtkodierende 3′-Sequenz. Die MBP-cDNA-Inserts der Klone pMBP5 und pMBP6 sind 2 530 bp und die der Klone pMBP2 und pMBP3 640 bp lang. Die Klone pLp1 und pMBP2 wurden durch Sequenzierung charakterisiert. Die Inserts beider Klone dienten als Hybridisierungsprobe zur Bestimmung der mRNA-Konzentration von PLP und MBP während der Myelinisierungsperiode. Die Expression beider Gene während der Entwicklung der Myelinscheiden verläuft danach streng koordiniert.

Key words: Proteolipid protein, myelin basic protein, rat brain cDNA library, expression during myelination.

Proteolipid protein (PLP), also named lipophilin, is the main integral membrane protein of myelin in the central nervous system. It has been discovered in the chloroform-methanol extract of brain in 1951[1]. It is synthesized in oligodendrocytes together with myelin basic protein (MBP), an extrinsic membrane protein. MBP is located in the cytosolic cleft between the two opposing inner layers of the myelin bilayers, which are plasma membrane processes of the oligodendrocyte of highly specialized composition and organization.

Lipophilin and myelin basic protein account for approximately 50% and 30–40% respectively of myelin proteins. Owing to the mass distribution of brain white matter they constitute the most significant protein species of brain.

Due to the insolubility of the complete polypeptide chain and its fragments the primary structure of bovine and human lipophilin has not been elucidated until recently[2–5]. The 276 amino-acid residues long polypeptide sequence is characterized by five long hydrophobic sequences separated by four hydrophilic domains. Three of the five hydrophobic sequences are apted to span the myelin lipid bilayer provided that transmembranal segments form α-helical structures in the lipid bilayer, whereas two must be embedded in the lipid bilayer in *cis*-orientation. This implies that the N- and C-termini of the hydrophobic loops enter and leave the same side of the membrane.

Tryptic cleavage experiments on native myelin membranes of bovine brain followed by isolation and sequence analysis of the fragments gave insight into the topography of the proposed arrangement of membrane lipophilin in the myelin membrane[6].

The immunological cross reactivity of antibodies against human PLP with bovine and rat myelin PLP indicated large homologies within these three species. We also completed the amino-acid sequence of human lipophilin with the protein sequencing technology recently elaborated for the bovine myelin membrane protein. In support of the immunological cross reactivity we found complete homology with the bovine polypeptide sequence[5].

PLP is intimately associated with the multilamellar structure of myelin and the organized growth of the plasma membrane myelin sheets around the axon. The proteolipid protein gene is expressed in a close linkage to the developmental state of the organism: in some species the peak height of membrane synthesis in the myelination period is reached during intrauterine life, whereas others, e.g. rodents, develop a burst of myelin membrane synthesis during the second to fourth postnatal week[7].

In this communication we describe the isolation and characterization of several lipophilin and myelin basic protein cDNA clones from a sized rat brain cDNA library. These clones were characterized by restriction analysis and nucleotide sequence determination.

Nick-translated restriction fragments of PLP and MBP clones and synthetic oligonucleotides with homology to their reversely translated sequences were used for Northern blot analysis of mRNA from rat brains at different stages of the myelination period. We found that PLP and MBP transcription rates parallel the well-known expression of the protein components of the myelin membrane during the myelination events.

These clones were also used to isolate the lipophilin gene from a human genomic library and for the mapping of the proteolipid gene to chromosome X as reported in the subsequent publications*.

* Diehl, H.-J., Schaich, M., Budzinski, R.-M. & Stoffel, W., in preparation.

Materials and Methods

Rat brain poly(A)$^+$ RNA isolation

Total RNA of brains of 18-day-old rats was isolated using established procedures[8]. Poly(A)$^+$ RNA was separated from total RNA by repeated chromatography over oligo(dT)$_{12-18}$ cellulose (Type 3, Collaborative research)[9].

Construction of cDNA library

Single- and double-stranded cDNA was synthesized from 15 µg of poly(A)$^+$ RNA with oligo(dT)$_{12-18}$ as primer and AMV reverse transcriptase (Boehringer, Mannheim) according to Gubler and Hoffman[10]. Protruding 3′ and 5′ ends of the double-stranded cDNA were repaired by the Klenow fragment of DNA polymerase I following established procedures[11]. The double-stranded cDNA was subjected to electrophoresis in a 1% agarose gel containing 0.5 µg/ml ethidium bromide. cDNA in the size range above 500 bp was stacked on a NA 45 membrane (Schleicher & Schüll, Düren) by electroelution: a NA 45 strip was inserted in a slit at the distance of the 500 bp marker. The electrophoresis was continued at 75–100 mA until all the double-stranded cDNA > 500 bp was collected on the NA 45 paper. The paper was thoroughly rinsed in 20mM Tris/HCl, pH 8.0, 0.1mM EDTA, 0.15M NaCl buffer (low salt buffer), then covered with 300 µl high salt buffer (1M NaCl) and incubated at 68 °C for 1 h. The NA 45 paper was removed and the DNA precipitated with 2 volumes of ethanol at – 80 °C for 30 min. 15 to 25 dC residues were linked to the 3′ ends of the size fractionated double-stranded cDNA in the tailing reaction with terminal transferase (Boehringer, Mannheim). The dC-tailed double-stranded cDNA was annealed to the dG-tailed *Pst*I site of pBR322 overnight and used for transformation of competent *E. coli* RRI cells. The library contained approximately 25000 tetracycline resistant clones.

Oligonucleotide synthesis and labelling

The oligonucleotides listed in Fig. 1 were synthesized by the solid phase phosphoamidite method[12] on an automated Applied Biosystems Synthesizer, Model 380 A. Each oligonucleotide was purified by filtration over a Sepak cartridge and by polyacrylamide gel electrophoresis (7.5 to 20% acrylamide). The UV-quenching band of the product was eluted with 0.3M NaCl, 1mM EDTA and purified over a DEAE-cellulose column (bed volume 200 µl). The oligonucleotides were 5′-end labelled with [γ-^{32}P]ATP (Amersham, Braunschweig) and T$_4$ polynucleotide kinase (BRL, Eggenstein). The 100^{mer} double-stranded oligonucleotide was synthesized by the ligation of nine overlapping oligonucleotides by T$_4$-DNA ligase (Boehringer, Mannheim) and cloned into the *Eco*RI site of the restricted polylinker sequence of M13-mp8 for the generation of single-stranded probes.

Identification of lipophilin and myelin basic protein cDNA clones

Transformation of competent *E. coli* RRI cells to tetracycline resistance was carried out as described[13]. Colonies were plated at high density on nitrocellulose filters BA 85 (Schleicher & Schüll) and replicas taken as described previously[14,15].

Two replica filters of each master filter were prepared for hybridization, prehybridized and then hybridized overnight in the hybridization solution containing ^{32}P-end-labelled oligonucleotide (5 × 10^6 cpm/pmol at 1 pmol/ml). Hybridization temperatures were selected corresponding to the base composition of the oligonucleotide probes[11,16]. Filters were washed[15] and subjected to autoradiography at – 70 °C using Kodak XAR-5 film and intensifying screens. Positive clones were purified through two subscreening rounds.

DNA Sequence analysis

Nucleotide sequences were determined by the M13 chain-terminating method[17] and double strand sequencing of restriction fragments cloned into the polylinker sequence of pUC13[18]. Specific heptadecameric and hexadecameric oligonucleotides as well as synthetic primers designed according to our sequencing results were used as primers.

Northern blot hybridization analysis

Aliquot portions of formaldehyde treated poly(A)$^+$ RNA samples (10 to 20 µg) were separated by electrophoresis in 1.0% agarose slab gels containing formaldehyde (2.2M) in 0.2M morpholinopropanesulfonic acid, pH 7.0[19]. RNA was transferred to nitrocellulose filter BA 85 by capillary blotting and prehybridized in

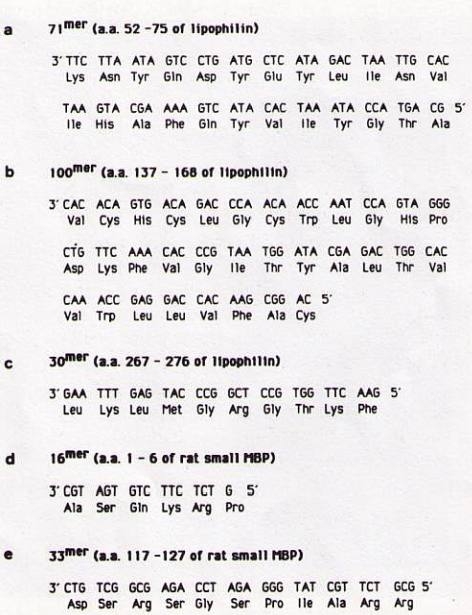

Fig. 1. Synthetic oligonucleotides used in the high density colony screening procedures for proteolipid protein cDNA clones (a–c) and myelin basic protein (d and e) used as 5′-^{32}P-labelled probes.

The sequences represent the anticodon strand for the peptides shown underneath. a.a. = amino acid.

50mM sodium phosphate buffer with 50% formamide, 10% dextrane sulfate, 4 x SSC, 5 x Denhardt's solution and 250 μg/ml sonicated, denatured salmon sperm DNA at 42 °C[20]. After 4 to 6 h the radioactive probe [5 x 10^6 cpm/(pmol x ml)] was added and hybridization was carried out at 42 °C for 12–18 h. Nitrocellulose blots were washed twice with 2 x SSC, 0.1% $NaDodSO_4$ for 15 min at room temperature and once in 0.2 x SSC, 0.1% $NaDodSO_4$ at 40 °C for 15 min. For autoradiography the blots were exposed to a Kodak XAR-5 or Fuji film with intensifying screens at – 70 °C.

Southern blot hybridization analysis[21]

DNA probes were digested with respective restriction enzymes, size fractionated by agarose gel electrophoresis (1% in the buffer: 0.89M Tris/borate, 0.89M boric acid, 2mM EDTA), alkali denatured (0.4M NaOH, 0.6M NaCl 30 min) neutralized (0.5M Tris/HCl pH 7.4, 1.5M NaCl), and blotted to a Gene Screen Plus membrane. The membrane was prehybridized and hybridized with the respective radioactive probes, 5'-^{32}P-labelled oligonucleotides or ^{32}P-nick-translated DNA fragments with specific activities of 5 x 10^6 cpm/pmol at 1 pmol/ml according to the Gene Screen Plus manual.

Results

The amino-acid sequences of bovine and human proteolipid protein[2–7] as well as myelin basic protein of rat and other species[22,23] are known. Their highly conserved structures allow a broad approach with the hybridization and screening techniques using ^{32}P-end-labelled synthetic oligonucleotides. These probes correspond to reversely translated amino-acid sequences of different regions of the polypeptide chains, applying the common codon usage.

Fig. 1 summarizes oligonucleotide sequences derived from the amino-acid sequence of PLP according to the common codon usage and synthesized for our screening procedures. In all cases the homology of these nucleotides was better than 85% as compared with our established PLP nucleotide sequence and those of others[24–26]. The MBP sequence corresponds to those reported recently[27].

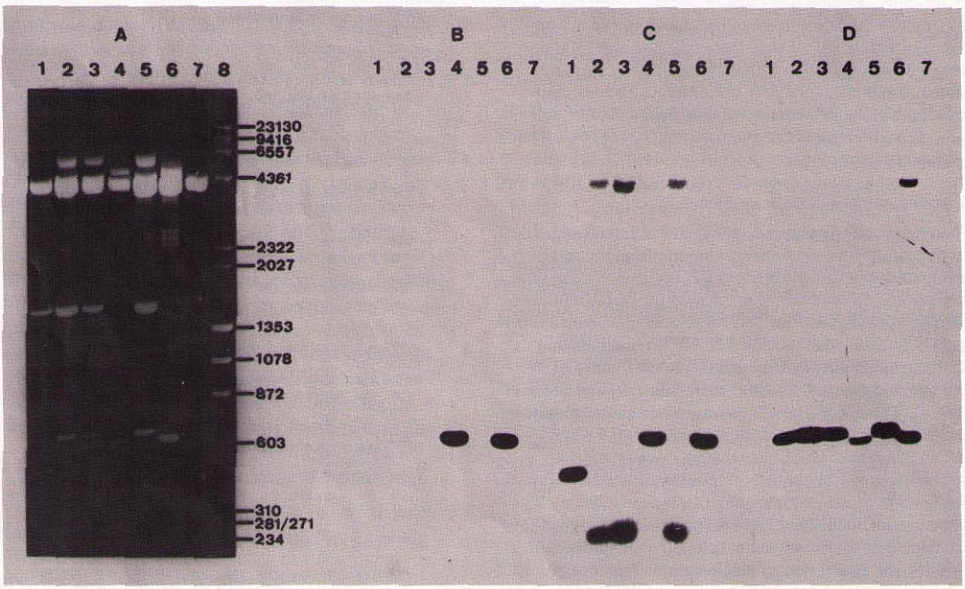

Fig. 2. Southern blot hybridization analysis of *Pst*I-restricted lipophilin clones isolated from a rat brain cDNA library in *Pst*I-site of pBR322.

(A) Electrophoretic separation of *Pst*I-restricted lipophilin clones in agarose gel (1%) containing ethidium bromide (0.5 μg/ml) and hybridization of the corresponding Southern blot with (B) 71^{mer} oligonucleotide (a.a. 52–75) (C) 100^{mer}-oligonucleotide (a.a. 137–168) (D) 30^{mer}-oligonucleotide (a.a. 267–276). Numbers to the right represent λ/*Hin*dIII and ΦX 174/*Hae*III fragments as size markers. Small DNA fragments (< 500 bp) are not visible under the photographic conditions applied.

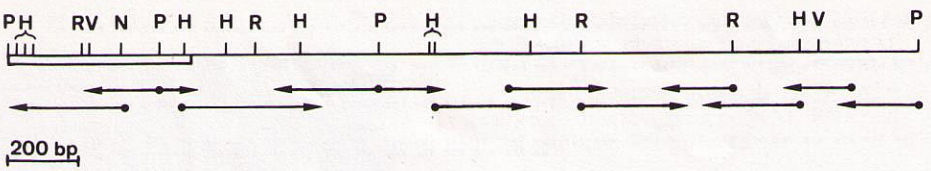

Fig. 3. Restriction map of the lipophilin cDNA and strategy for nucleotide sequence analyses.

Abbreviations: P, *Pst*I; H, *Hae*III; R, *Rsa*I; N, *Nco*I; V, *Ava*II. The box corresponds to the C-terminal 172 a.a. of rat lipophilin. Horizontal arrows refer to the direction and extent of sequence determination. Nucleotide sequence analyses of cDNA inserts were performed by the M13 chain-terminating method[17] and direct double-strand sequencing[18] of *Pst*I, *Hae*III and *Rsa*I restriction fragments cloned into the polylinker region of pUC13.

Isolation and characterization of lipophilin cDNA clones

Screening of the rat brain cDNA library for lipophilin clones: The 100^{mer} double-stranded oligonucleotide corresponding to Val^{137} to Cys^{168} was cloned into the *Eco*RI site of the RF-form of M13mp8. The single-stranded phage was used for the nucleotide sequence analysis of the probe and then as a template for the synthesis of highly labelled probes. Six lipophilin cDNA clones were obtained with this 100^{mer} and the 30^{mer} oligonucleotide as probes.

The *Pst*I-restriction enzyme analysis of the PLP-clones is presented in Fig. 2A. Clone pLp1 released fragments of 1 500, 650 and 450 bp, pLp2 fragments of 1 500, 650 and 250 bp, whereas both pLp4 and pLp6 yielded a 610 bp *Pst*I fragment. Clones pLp3 and pLp5 are apparently identical with pLp2. 5′-^{32}P-labelled oligonucleotides corresponding to different amino-acid sequences of lipophilin sequence as listed in Fig. 1 were then applied to the Southern blot hybridization analysis, Fig. 2B, 2C and 2D.

The 71^{mer} oligonucleotide (Lys^{52}–Ala^{75}) hybridized only to the 610 bp *Pst*I fragments of pLp4 and pLp6. The 100^{mer} oligonucleotide coding for Val^{137}–Cys^{168} hybridized with these fragments, too, but in addition visualized a 450 bp *Pst*I fragment of pLp1 and a 250 bp *Pst*I fragment in clones pLp2, pLp3 and pLp5. The 30^{mer} oligonucleotide corresponding to the C-terminal sequence Leu^{267}–Phe^{276} hybridized to the 610 bp *Pst*I fragment of pLp4 and pLp6 and a 650 bp fragment of clones pLp1, pLp2, pLp3 and pLp5.

pLp1, containing the longest insert of approximately 2 600 bp, was characterized in detail. The restriction map of pLp1 and the sequencing strategy is given in Fig. 3. The nucleotide sequence of the 2 585 bp insert in lipophilin clone pLp1 is shown in Fig. 4. The deduced amino-acid sequence of rat lipophilin is aligned to the coding region of the insert. Compared to the rat amino-acid sequence the bovine PLP sequence differs in the amino acids: 143 (cysteine), 188 (alanine), 190 (cysteine) and 198 (threonine).

Isolation and Characterization of myelin basic protein (MBP) cDNA clones

Screening the rat brain cDNA library with a 33^{mer} oligonucleotide corresponding to amino-acid residues Ala^{117}–Gly^{127} and a 16^{mer} oligonucleotide which encodes the N-terminus (Ala^{1}–Arg^{6}) of rat small MBP[27] yielded four MBP-clones pMBP2, pMBP3, pMBP5, pMBP6. pMBP2 is identical with pMBP3 and pMBP5 with pMBP6 as documented in Fig. 5.

The four clones were digested with *Pst*I, which released a 640 bp insert from pMBP2 and pMBP3. Three fragments of 1 500 bp, 630 bp and 400 bp were released from clones pMBP2 and pMBP3, which add up to a 2 530 bp insert. In Southern blot analysis the two different MBP clones hybridized with the 33^{mer} oligonucleotide probing the C-terminal translated 3′ region. The 5′-^{32}P-labelled 16^{mer} oligonucleotide probing the N-terminal region corresponding to amino-acid residue Ala^{1}–Pro^{6}, however, hybridized only with the 640 bp insert of pMBP2 and pMBP3. Therefore the insert of pMBP5 and pMBP6 must encode to its largest extent the 3′-non-coding region.

Nucleotide sequencing confirmed the hybridization analysis. The nucleotide sequence of clone pMBP2 is depicted in Fig. 6. Confirming the rat small MBP amino-acid sequence of Mårtenson et al.[23,28] our nucleotide sequence data show that amino acid 124 is methionine rather than isoleucine as determined by Roach et al.[27].

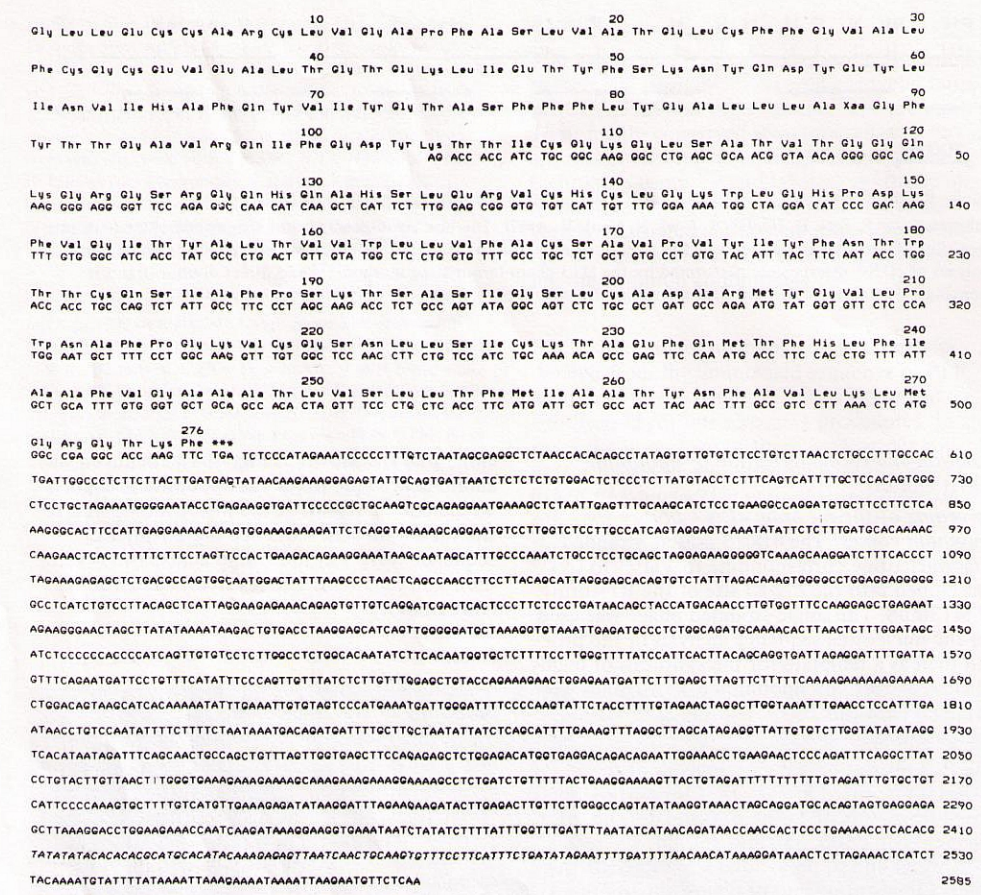

Fig. 4. Nucleotide sequence of rat lipophilin clone pLp1.

The deduced amino acid sequence is aligned to the cDNA sequence. The N-terminal 104 amino acids were supplemented by our amino-acid sequence data of bovine PLP[4].

Northern blot hybridization analysis

Rat brain, fetal calf brain (5–7 months), human liver poly(A)$^+$ RNA and total calf brain RNA were separated by electrophoresis. Hybridization was performed with the labelled 100^{mer} oligonucleotide, the ^{32}P-nick-translated pLp1 insert (2 585 bp) and the 1 500 bp *Pst*I fragment of the pLp1 clone beyond the 3′ end of the lipophilin coding region (Fig. 7). The two probes mentioned first hybridized to two brain specific mRNAs with 3 200 and 1 600 nucleotides in length. The 1 500 bp *Pst*I fragment, however, targeted only the large 3.2 kilobase (kb) mRNA. This indicates that the two mRNAs differ in their 3′-non-coding region. The longer mRNA has a 3′-non-coding region of 2 062 nucleotides, not considering the poly(A)$^+$ tail.

The relative abundance of the 3.2 kb mRNA and 1.6 kb mRNA isolated from 18-day-old rat brains is approximately 2:1. This ratio was determined densitometrically by scanning the hybridization intensity.

Myelin basic protein is encoded only in one mRNA

In Northern blot hybridization of 18-day-old rat brain poly(A)$^+$ RNA the ^{32}P-labelled 33^{mer}

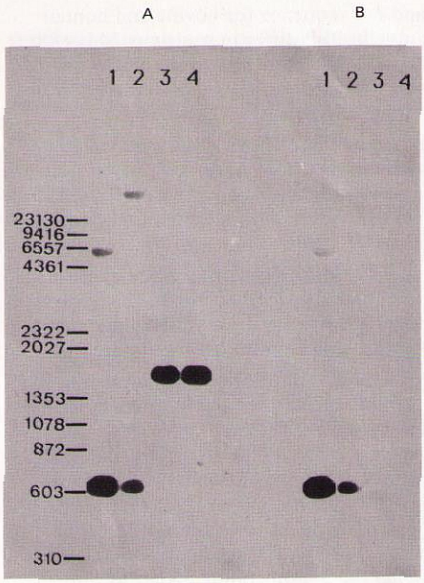

Fig. 5. Southern blot hybridization analysis of *Pst*I restricted pMBP clones isolated from rat brain cDNA library cloned into the *Pst*I site of pBR322.

DNA (1 μg) of pMBP2 (lane 1), pMBP3 (lane 2), pMBP5 (lane 3) and pMBP6 (lane 4) was digested with *Pst*I and separated by electrophoresis in an agarose gel (1%) containing ethidium bromide (0.5 μg/ml). DNA fragments were transferred to a Gene Screen Plus membrane, denaturated, neutralized and probed with ^{32}P-labelled oligonucleotides as outlined under Materials and Methods. *Hybridization with (A) 33^{mer}-oligonucleotide* (a.a. 117–127) (B) 16^{mer}-oligonucleotide (a.a. 1–6). The sequences of the two MBP specific oligonucleotides are presented in Fig. 1. Numbers on the left indicate size in bp of marker fragments of λ/*Hin*dIII and ΦX 174/*Hae*III.

oligonucleotide (amino acids 117–127 of rat small MBP) recognized a single size poly(A)$^+$ RNA of 2.1 kb length (Fig. 8, lane 1). This RNA was absent in human liver poly(A)$^+$ RNA, fetal calf (5–7 months) brain poly(A)$^+$ RNA and total calf brain RNA, lanes 2–4.

PLP and MBP gene transcription during myelination

Lipophilin and myelin basic protein are transcribed coordinately during myelination in rat brain. This was demonstrated by Northern blot hybridization shown in Fig. 9.

At the time of birth and even five days after birth none of the mRNAs are detectable by Northern blot hybridization analysis with ^{32}P-nick-labelled insert of pLp1 and the 33^{mer} oligonucleotide corresponding to the C-terminal sequence of MBP. The PLP and MBP specific mRNAs become visible between days 5–8 and increase coordinately in their abundance with a peak intensity during the third week (day 15–20). The densitometric analysis (data not shown) of the autoradiograms (Fig. 9) quantitatively expresses the hybridization intensities. The transcription of both genes occurs in a coordinate fashion. It should be mentioned that the large PLP specific poly(A)$^+$ RNA of 3.2 kb length predominates from day 5 on and is still the main transcript even after day 30. Maximal transcription rate is observed between days 15 to 20, during this period the small 1.6 kb poly-(A)$^+$ RNA PLP transcript is detected. In addition a rather strong hybridization with a 2.4 kb transcript occurs, which again looses its intensity towards the end of the myelination period.

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                                        1                                  10
                                  Met Ala Ser Gln Lys Arg Pro Ser Gln Arg His Gly Ser Lys Tyr Leu Ala Thr Ala Ser
CCAAGAAGATCCCACAGCAGCTTCCGAAGGCCTGGATGTGATG GCA TCA CAG AAG AGA CCC TCA CAG CGA CAC GGA TCC AAG TAC TTG GCC ACA GCA AGT   100

 20                                 30                                 40
Thr Met Asp His Ala Arg His Gly Phe Leu Pro Arg His Arg Asp Thr Gly Ile Leu Asp Ser Ile Gly Arg Phe Phe Ser Gly Asp Arg
ACC ATG GAC CAT GCC CGG CAT GGC TTC CTC CCA AGG CAC AGA GAC ACG GGC ATC CTT GAC TCC ATC GGG CGC TTC TTT AGC GGT GAC AGG   190

 50                                 60                                 70
Gly Ala Pro Lys Arg Gly Ser Gly Lys Asp Ser His Thr Arg Thr Thr His Tyr Gly Ser Leu Pro Gln Lys Ser Gln Arg Thr Gln Asp
GGT GCG CCC AAG CGG GGC TCT GGC AAG GAC TCA CAC ACA AGA ACT ACC CAC TAC GGC TCC CTG CCC CAG AAG TCG CAG AGG ACC CAA GAT   280

 80                                 90                                100
Glu Asn Pro Val Val His Phe Phe Lys Asn Ile Val Thr Pro Arg Thr Pro Pro Pro Ser Gln Gly Lys Gly Arg Gly Leu Ser Leu Ser
GAA AAC CCA GTA GTC CAC TTC TTC AAG AAC ATT GTG ACA CCT CGT ACA CCC CCT CCA TCC CAA GGA AAG GGG AGA GGC CTG TCC CTC AGC   370

110                                120                                130
Arg Phe Ser Trp Gly Gly Arg Asp Ser Arg Ser Gly Ser Pro Met Ala Arg Arg ***
AGA TTT AGC TGG GGA GGA AGA GAC AGC CGC TCT GGA TCT CCC ATG GCA AGA CGC TGA GAGCCTCCCTGCTCAGCCTTCCCGAATCCTGCCCTCGGCTTCT   470

TAATATAACTGCCTTAAACGTTTAATTCTACTTGCACCAAATAGCTAGTTAGAGCAGACCCTCTCTTAATCCCGTGGGGCTGTGAACGCGGCGGGCCAGCCCACGGCACCCTGACTGGC   589

TAAAACTGTTTGTCCCTTTTTAT  612
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Fig. 6. Nucleotide sequence of myelin basic protein clone pMBP2.
The deduced amino-acid sequence is aligned to the cDNA sequence.

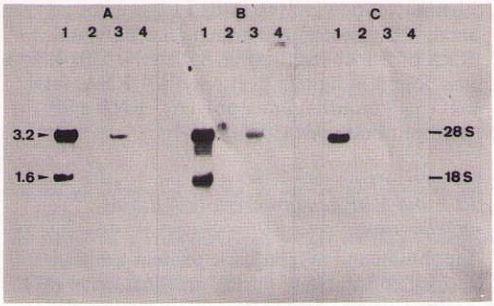

Fig. 7. Northern blot hybridization analysis of 1) rat brain; 2) human liver; 3) fetal calf brain (5–7 months) poly(A)$^+$ RNA and 4) calf brain total RNA. RNA and poly(A)$^+$ RNA (20 µg per lane) were electrophoresed in a 1% agarose gel containing formaldehyde.

RNA was transferred to a nitrocellulose filter BA 85 (Schleicher & Schüll), prehybridized in 50mM sodium phosphate buffer pH 7.0 with 50% formamide, 10% dextrane sulfate, 4 × SSC, 250 µg/ml sonicated, denatured salmon sperm DNA, 5 × Denhardt's solution[29] and hybridized in the same buffer with the following probes: (A) 100^{mer} oligonucleotide; (B) nick-translated lipophilin clone pLp1; (C) 1500 bp *Pst*I fragment of clone pLp1, external to 3′ end of lipophilin coding region. Specific activities of probes: 5 × 10^6 cpm/pmol at 1 pmol/ml. Membranes were washed twice with 2 × SSC, 0.1% $NaDodSO_4$ at room temperature, once in 0.2 × SSC, 0.1% $NaDodSO_4$ at 40 °C and autoradiographed using Kodak XAR-5 film with intensifying screens at − 70 °C. Markers at the right show positions of 28S and 18S ribosomal RNA standards. Approximate lengths of hybridizing RNAs are indicated in kilobases at the left.

Discussion

Recently we elaborated the amino-acid sequence of the extremely hydrophobic main integral protein of the myelin membrane, proteolipid protein (lipophilin), of bovine and human brain and demonstrated the strong homology of the primary structures in these two species. We also suggested that the protein band in electrophoretic analysis named DM 20, which migrates ahead of PLP, is a conformational isomer of PLP, most likely stabilized by disulfide linkages different from that of PLP, because oxidation of the sulfhydryl groups and disulfide bonds transforms the DM 20 into the PLP species of the same molecular mass of 31 kDa[2].

The cDNA data reported here allow a comparison of the amino-acid sequences of rat PLP deduced from the nucleotide sequence with the amino-acid sequence of proteolipid protein of bovine and human myelin which we established with classical protein sequencing methods. The DNA-deduced amino-acid sequence of rat PLP and our sequence for bovine and human brain myelin PLP differ in positions 143, 188 and 190 and 198, which corresponds to a homology of 98% in the comparable region of 172 amino-acid residues.

Several lipophilin cDNA clones were isolated from a rat brain cDNA library with synthetic oligonucleotides corresponding to different sequences of the lipophilin polypeptide chain as hybridization probes.

Our largest insert in lipophilin clone pLp1 is 2585 bp long. 2064 bp belong to the 3′-non-coding region and 521 bp to the coding region up to amino-acid residue 104 of mature PLP.

In Northern blot hybridization analysis the PLP specific oligonucleotides and ^{32}P-nick-labelled PLP inserts clearly detected at least two rat brain specific poly(A)$^+$ RNAs 3200 and 1600 nucleotides in length, but only one in bovine brain of 3.2 kb length. The 1600 nucleotide poly(A)$^+$ RNA of rat brain did not hybridize with the 1500 bp *Pst*I fragment of pLp1, which represents the 3′-non-coding region to the poly(A)$^+$ sequence. Therefore the small PLP transcript misses the 3′-non-coding region with the polyadenylation site 2062 bp downstream the coding region but instead possesses only a 450 bp non-coding 3′ region with a second polyadenylation site, which must be located up-

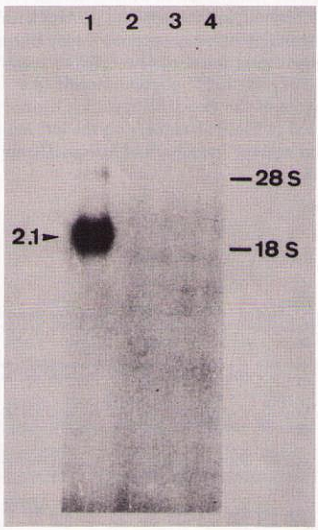

Fig. 8. Northern blot hybridization of 1) rat brain; 2) human liver; 3) fetal calf brain poly(A)$^+$ RNA and 4) calf brain total RNA with 5′-labelled 33^{mer} oligonucleotide corresponding to amino acids 117–127 of rat small MBP.

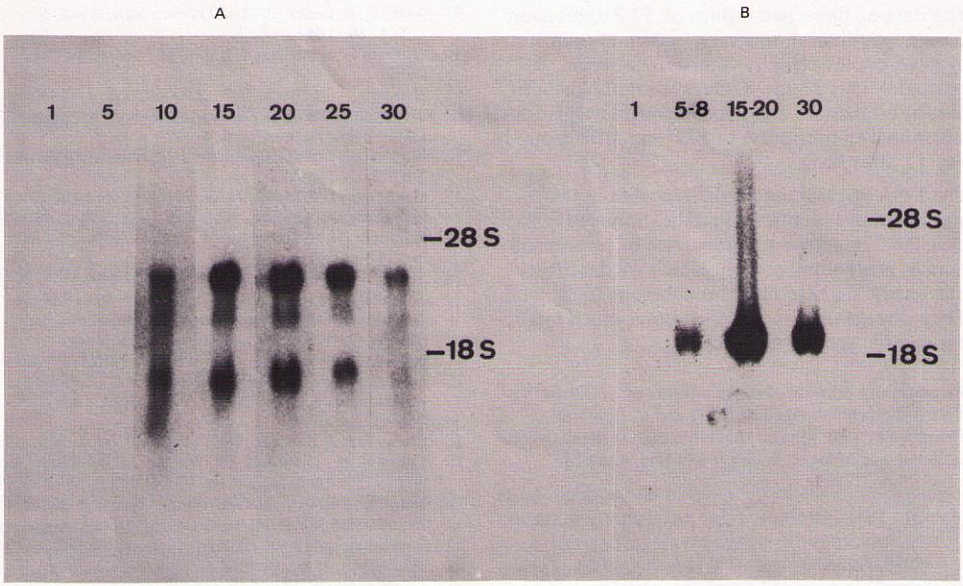

Fig. 9. Northern blot hybridization analysis of PLP (A) and MBP transcripts (B) at various stages of rat brain development.

Poly(A)$^+$ RNAs (10 μg aliquot portions) isolated from rat brains at days after birth indicated at the top of each lane were separated on 1% agarose gels containing formaldehyde, transferred to a nitrocellulose membrane, prehybridized, subsequently probed with nick-translated pLp1 (left) and pMBP2 (right), washed and autoradiographed as described under Fig. 7.

stream of the 1 500 bp *Pst*I fragment but within the 650 bp *Pst*I fragment (Fig. 3). The two PLP transcripts differ in length by approximately 1 600 bases. Assuming identical 5′ ends of both a second polyadenylation site about 1 000 bp downstream the 5′ end of our cDNA clone must be assumed. This is in agreement with the data of Milner et al.[26]. Southern blot analysis of human placental and genomic leucocyte DNA restricted with *Eco*RI, *Bam*HI, *Pst*I, *Sac*I, *Hind*III and combinations thereof as well as the genomic mapping of the proteolipid protein gene near the phosphoglycerate kinase locus on chromosome X led to the conclusion that there is only one PLP gene with at least two spliced transcripts which differ in the length of their 3′-non-coding regions*.

At the N-terminus the amino-acid sequence of mature PLP is extended only by methionine[24,26]. This is obviously released during co- or post-translational processing. In vitro translation studies[30] suggest that PLP is synthesized on polysomes without a signal sequence. No co-translational processing had been observed. Since radioactive microsequencing of the primary translation product was omitted the N-terminal methionine has escaped these observations. The internal hydrophobic sequences are bordered by charged residues. They are apparently responsible for the membrane insertion of the *trans*- or *cis*-membrane spanning sequences of PLP 27, 22, 40, 12 and 30 residues long, the charged residues serving as stop signals in the translocation[6].

The availability of the cloned lipophilin specific DNA probes renders studies possible which examine the genomic DNA sequence of PLP, demyelinating diseases possibly based on genetic defects and functional aspects of this integral membrane protein.

Independent of our work there are reports of a 1 334 bp cDNA sequence encoding the 276 amino-acid sequence of rat brain PLP with a 450 bp 3′-non-coding region[24], a 930 bp bovine brain PLP cDNA clone[25] and a 3 037 bp rat brain specific PLP clone hybridizing to two mRNAs, the size of which was given with 3 200 and 1 600 nucleotides[26]. The latter two groups also used the cDNA probes to study the expression of PLP during myelination.

* Diehl, H.-J., Schaich, M., Budzinski, R.M. & Stoffel, W., in preparation.

The data of these two groups on PLP expression during myelination suggest a maximum number of RNA copies being synthesized between postnatal days 22 to 28. Our repeated Northern blot hybridization analyses, however, indicate a maximal transcription of PLP and MBP around day 18 after birth.

The DNA sequencing results reported in this and the above mentioned publications prove that the 276 amino-acid sequence of bovine and human proteolipid protein which we described previously are extremely homologous to rat PLP. They diverge only in four positions which might express the species specificity.

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