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Characterization of a Brain-Specific Sp1-Like Activity Interacting with an Unusual Binding Site within the Myelin Proteolipid Protein Promoter

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Summary: The proteolipid protein (PLP) gene encodes the main integral protein of the myelin membrane of the central nervous system. The expression of the gene is regulated in a cell- and development-specific manner.

Comparison of approximately 1.5 kb of the upstream noncoding region from man, mouse, and rat gene revealed an extensive sequence identity of about 95% between −250 and +100 (the most upstream transcription start site is defined as +1) but only about 50% identity further upstream.

To define potential *cis*-acting elements in the promoter of the mouse PLP gene the upstream region was studied by transfection of C6 glioblastoma cells and CHO fibroblasts with various 5′ deletion constructs fused to the reporter gene luciferase. We localized a promoter at position −184 to +90, which is active in both cell lines. Analysis of this region by DNase I footprinting experiments and band shift analysis with nuclear extracts from myelinating brain, liver, C6, and CHO cells shows the binding of several different proteins to the promoter region. One brain-specific and two ubiquitous factors bound to the sequence AAGGGGAGGAG (DR1/2 box). This motif is also present in the upstream region of other myelin-specific genes and in some variants of the glia cell-specific virus JC. The factors bound with similar affinity to a Sp1-binding site. Therefore one of the ubiquitous factors seems to be Sp1 suggesting that Sp1 may play a role in the transcriptional regulation of the PLP gene. It has been shown that the DR1/2 box-binding factors are $Zn^{2\oplus}$-dependent. By Southwestern blotting it has been demonstrated that the DR1/2 box binds a protein of about 66 kDa that is enriched in brain.

Key terms: Gene regulation, glial cells, transcription factor Sp1.

Enzymes:
Chloramphenicol acetyltransferase (EC 2.3.1.28);
DNA polymerase I (EC 2.7.7.7);
DNase I (EC 3.1.21.1);
Luciferase (EC 1.13.12.7);
Restriction endonucleases (EC 3.1.2).

Abbreviations:
BSA, bovine serum albumine; CAT, chloramphenicol acetyl transferase; CHO, chinese hamster ovary; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EDTA, ethylenediamintetraacetic acid; FCS, fetal calf serum; HBS, Hepes and basal salts; Hepes, *N*-hydroxyethylpiperazine-*N*′-2-hydroxypropanesulfonic acid; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; mRNA, messenger ribonucleic acid; OMG, oligodendrocyte myelin glycoprotein; PCR, polymerase chain reaction; PLP, proteolipid protein; PMSF, phenylmethanesulfonyl fluoride; PNS, peripheral nervous system; SDS, sodium dodecyl sulfate; TBE, tris borate EDTA; TLC, thin-layer chromatography.

During myelination, oligodendrocyte precursor cells follow a highly regulated differentiation process which results in the expression of the genes of myelin-specific proteins and lipid-synthesizing enzymes and which leads to the production of large amounts of structural elements of the myelin membrane[1]. The proteolipid protein (PLP) is the most abundant protein of the myelin membrane in the CNS and contributes to about 50% of the myelin proteins[2]. It is essential for the compact and ordered structure of the myelin sheath. The structural importance of PLP is demonstrated best by heritable dysmyelinating diseases in human[3,4], mice[5,6], and rat[7] where mutations in the PLP gene lead to distorted myelin structures and death of oligodendrocytes. The expression of the gene is restricted to oligodendrocytes in CNS[8,9] and to Schwann cells in PNS[10] and correlates with the myelination process[11,12]. Expression of PLP mRNA peaks between postnatal day 20 and 25 and developmental and cell-specific synthesis of PLP seem to be regulated at the transcriptional level.

In an attempt to define the promoter region and oligodendrocyte specific *cis*-elements we report here the sequence analysis and comparison of 1.5 kb of upstream sequences from the human[13], rat[7], and mouse gene. We located a highly homologous region in all three species, extending from position −250 to +100 relative to the most upstream transcription start site which exhibits a similarity in DNA sequence of about 95%. We also found a region with a reduced homology (50%) in the further upstream region. This suggests a possible role of the proximate region in gene regulation.

We used the C6 glioblastoma cell line as a model system to study PLP expression in oligodendrocytes because the immortalized neuronal cells express the PLP gene. Transient expression experiments in C6 glioblastoma and CHO fibroblast cells with different 5′ trunctated promoter fragments were performed.

The promoter region was analysed for trans-acting DNA-binding proteins by DNase I footprint analysis with nuclear extracts from different cells and tissues. Synthetic oligonucleotides corresponding to protected sequences were used for gel retardation assays. DNA-binding factors were further analysed by competition studies and Southwestern analysis.

Materials and Methods

General methods

Unless otherwise specified all molecular biology procedures were essentially performed as described by Ausubel et al.[14]

Cell culture

Cells were grown in DMEM supplemented with 10% FCS. Cells were plated in 6 cm dishes at a density of $1 \times 10^4/cm^2$ 12 h before transfection.

Oligonucleotides

Oligonucleotides corresponding to the promoter sequences were synthesized with the solid phase phosphoamidite method with an automated oligonucleotide synthesizer (Applied Biosystems, Model 380 A) and annealed.

Oligonucleotide sequences:
DR sense,
5′TCGAAGGGGAGGAGAAGGGGAGGAG-3′;
DR antisense,
5′TCGACTCCTCCCCTTCTCCTCCCCT-3′;
UE sense,
5′TCGACTTAAAGAAGGGAGTATCCCTTGTTTAAG-3′;
UE antisense,
5′TCGACCTCCTTTGGGATACTCCCTTGTTTAAG-3′;
DR1/2 sense,
5′TCGAAGGGGAGGAGTCGA-3′;
DR1/2 antisense,
5′TCGACTCCTCCCCTTCGA-3′;
DR-M1 sense,
5′TCGAAGCGGAGGAGTCGA-3′;
DR-M1 antisense,
5′TCGACTCCTCCGCTTCGA-3′;
DR-M2 sense,
5′TCGAAGCGGAGCAGTCGA-3′;
DR-M2 antisense,
5′TCGACTGCTCCGCTTCGA-3′.

DNA-protein binding gel electrophoresis assay (gel shift analysis)

1–5 μg nuclear proteins were preincubated with double-stranded poly d(I-C) (3 μg) for 15 min at 4°C in a 20 μl reaction mixture containing 25mM Hepes pH 7.6, 40mM KCl, 0.1mM EDTA, 1mM DTT, 8% Ficoll, and 5mM $MgCl_2$. In the competition assay a 100-fold molar excess of nonradioactive oligonucleotide was introduced and approximately 100000 cpm or 10 fmol P^{32}-labeled double-stranded oligonucleotide was added and incubated for 30 min at 4°C.

DNA-protein complexes and the unbound probe were separated by polyacrylamide gel electrophoresis (6%) using 0.25 × TBE[15]. Following electrophoretic separation at 10 V/cm the gel was dried on Whatman 3 M paper and exposed to Kodak AR 5 film.

Metal replacement studies were done according to Kadonaga et al.[16]. Nuclear extracts were dialysed against 20mM Hepes (pH 7.9), 150mM KCl, 1mM DTT, and 0.5mM EDTA. Reconstitution was achieved by adding 1mM of $ZnCl_2$, $MnCl_2$ or $NiCl_2$ and incubating for 30 min at room temperature.

Plasmid construction

A series of PLP-LUC hybrid genes was constructed using different *Bam* HI, *Hae* II, *Hae* III, *Nco* I, and *Hind* III fragments and a 200 bp PCR fragment of a mouse genomic cosmid PLP clone were generated. They were ligated as blunt end inserts into the *Sma* I site of the multiple cloning site of pBluescript SK+ (Promega). The orientation of the inserts was determined and the inserts excised with *Sal* I and *Bam* HI for ligation in the correct orientation into the multiple cloning site of p0-LUC (kindly provided by *Dr. Brasier*, Harvard Medical School[17]) yielding plasmids pBB-LUC, pH2B-LUC, pH3B-LUC, pNB-LUC, pHB-LUC, and p200B-LUC, respectively. The clones were verified by restriction enzyme mapping and plasmid sequencing.

Plasmid DNA was prepared by alkaline lysis and purification with Quiagen columns according to the manufacturer's prescription.

Transfection

2.5 μg plasmid DNA, 0.5 μg pSV2-CAT, and 5 μg salmon sperm DNA were precipitated with calcium phosphate and added to the medium following standard procedures[18]. After 4 h of incubation a 2 min glycerol shock (15% glycerol in HBS) was carried out and cells incubated for 48 h at 37°C. They were lysed with Triton buffer[19] and aliquotes of the supernatant used for the luciferase and CAT assay.

Luciferase activity was measured at a 1 mM ATP concentration in a Luminometer (Berthold, Klinluminomat) and after injection of Luciferin (0.1 mM final concentration); light emission was measured for 20 s.

CAT activity was determined according to Gorman et al.[20] and quantitated by radio TLC analysis using the thin layer chromatogram scanner Raytest.

Sequencing

The dideoxy chain termination method[21] was performed on both strands using the Sequenase kit and appropriate oligonucleotides as primers.

Nuclear extracts

Crude nuclear extracts were prepared from brain and liver of 18 to 25-day old rats as described[22] except that 1 mM $MgCl_2$ and 0.25% Triton X-100 were added but EDTA omitted. Nuclear proteins were extracted with a 0.42 M KCl buffer (10 mM Hepes pH 7.9, 1.5 mM spermine, 5 mM spermidine, 1 mM $MgCl_2$, 1 mM DTT, 20% glycerol). The nuclear extract was dialysed against 20 mM Hepes, pH 7.9, 150 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 10% glycerol. The supernatant was shock-frozen and stored at −70°C. All buffers contained protease inhibitors PMSF (0.5 mM), benzamidine (1 mM), leupeptin (0.5 μg/ml), and pepstatin (1.0 μg/ml). Proteins were concentrated to 5 mg/ml in Centricon tubes for DNase I protection experiments.

For the developmental kinetic nuclear extracts were prepared from rats of the corresponding age by the described method.

Protein concentrations were determined by the method of Sedmak et al.[23].

Footprint analysis

The 269 bp *Hind* III/*Bam* HI fragment was labeled asymmetrically with [α-^{32}P]dATP, [α-^{32}P]dGTP and Klenow fragment of polymerase I. 1 ng fragment and 100 μg nuclear proteins (or 100 μg BSA) were incubated in the presence of 5 μg poly d(I-C) in 40 μl binding buffer (20 mM Hepes pH 7.0, 2 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT) for 10 min at room temperature. 2 μl DNase I solution (10–100 μg/ml) was added and the reaction stopped after 1–2 min by the addition of 100 μl stop solution (50 mM EDTA, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast tRNA). The incubation mixture was extracted with phenol/chloroform and precipitated with ethanol.

Samples were analysed electrophoretically in 6% polyacrylamide 7 M urea gels. The fragments were also chemically cleaved at G and A or C and T according to Maxam and Gilbert[24] and applied as size markers.

Southwestern blot

The Southwestern blot analysis was done according to Singh et al.[25] using the DR1/2 oligonucleotide as probe. About 50 μg nuclear protein of brain or liver was used and separated using a 10% SDS polyacrylamide gel. After the protein transfer blotting was controlled by reversible staining of the membrane with Ponceau S.

Results

Sequence homology of the 5′-noncoding region of the human, rat, and mouse proteolipid protein gene

The nucleotide sequences of the 1.5 kb 5′-untranslated region upstream of the translation start codon of man[13], rat[7], and mouse were determined and compared (Fig. 1). The clone of the mouse PLP gene was isolated from a mouse genomic cosmid library. We found the highest sequence similarity in the region from position −250 and +100. About 96% of the 350 bp domain of the mouse and rat PLP gene are identical and 93% of the respective man and mouse sequences. In the further upstream region a 589 bp segment is deleted in the rat gene. Therefore a conserved regulatory function of this sequence is highly unlikely. Well-characterized eukaryotic *cis*-acting transcription elements within the 1.5 kb upstream sequence of the PLP gene are not apparent. A CCAAT box in antisense orientation is located between position −147 and −142. At position −21 to −25 upstream of the main transcription start a degenerated TATA box (TTTAA) is found in the three species. The direct repeat AAGGGGAGGAG between −54 and −75 is also conserved in all three species. This sequence is similar to sequences in the upstream domain of the myelin basic protein (MBP) gene[26–28], the myelin-associated glycoprotein (MAG)[29,30], the oligodendrocyte myelin glycoprotein (OMG)[31], and several variants of polyoma virus JC, a glia-cell-specific virus[32,33]. The homologous sequences are summarized in Fig. 2.

Functional characterization of the PLP promoter

Selected 5′-deleted fragments of the mouse PLP promoter were cloned upstream of the luciferase gene of *Photinus pyralis* in the promoter-deficient p0-LUC vector[17]. The 3′ end of the promoter fragment was positioned in the *Bam* HI site at +90 relative to the proposed main transcription start[12,34]. Deletions between −1392 and −38 were introduced as shown in Fig. 3A. The largest fragment with antisense orientation was used as negative control in transfection experiments. Two cell lines were used for transient expression, C6 glioblastoma cells and CHO cells. Transcription of the PLP gene in C6 glioblastoma cells is active[11], however considerably lower than in oligodendrocytes; CHO cells do not express the PLP gene. The level of luciferase expression of the different constructs is shown in Fig. 3B.

Truncation of the promoter region down to position −184 did not alter the expression, which is 45 to 50% of the pSV2-LUC vector containing the SV40 promoter and enhancer. The constructs p-200B-LUC con-

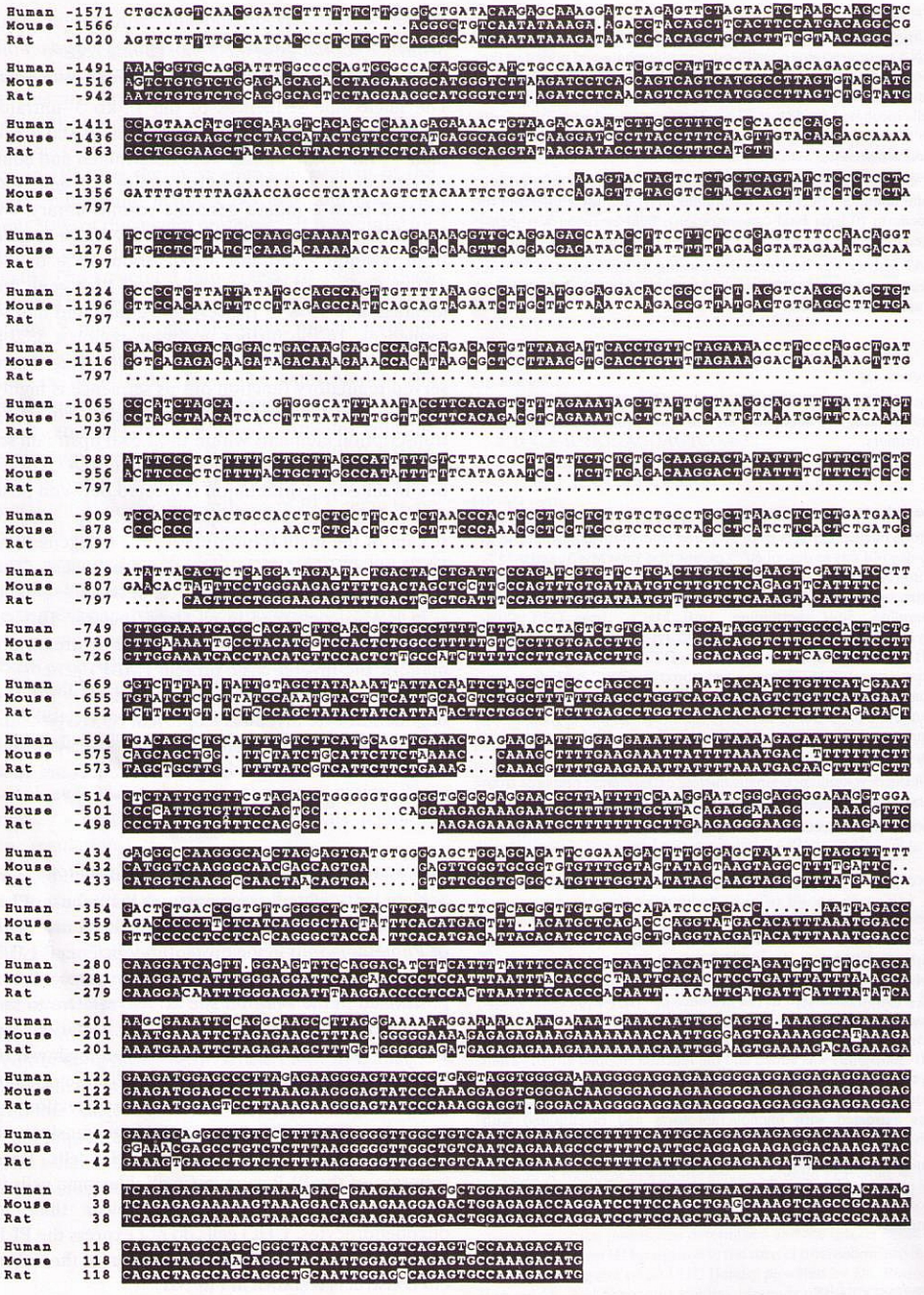


Fig. 1. Aligment of 1.5 kb upstream region from human, mouse, and rat PLP gene.

Comparison of about 1.5 kb upstream noncoding sequence of the human, mouse, and rat PLP gene indicates the presence of a highly homologous region extending from about −250 to +100 in relation to the most upstream transcription start site, with a homology of about 95%. Homology shown in black.

Fig. 2. Homologous sequences of DR1/2 in myelin specific genes.

Homologous sequences of the DR1/2 sequence are found in the upstream region of different myelin specific genes: myelin basic protein (MBP), myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMG), and the promoter region of the glia cell-specific virus JC. The positions refer to the transcription start site and in the OMG to the translation start.

Sequences	Gene	Position
AAGGGGAGGAG	PLP (human, mouse and rat)	-54 und -66
CAGGGGAGGCA	MBP (mouse)	-205
CAGGAGAGGCA	MBP (human)	-79
GCAGGGAGGAG	MAG (mouse and rat)	-31
AAGGGGAGGAG	MAG (rat)	-138
GAAGGGAGGAG	MAG (mouse)	-149
GTAGGGAGGAG	JC-Virus	promoter region
GGGGGGAGGCA	OMG (human)	-258
$NN^{A}_{G}GGGAGG^{AA}_{CG}$	consensus	

taining the fragment between −38 and +90 and the p-BB-LUC with the fragment between −1392 and +90 in the antisense orientation showed an expression comparable with the vector without the promoter (p0-LUC).

In CHO cells the truncated promoter regions exert the same influence on the luciferase gene as in the C6 glioblastoma cells although at a 4 to 5 fold reduced level.

These results indicate a cell-specific expression of the PLP promoter in these cells.

The expression in the two cell types was normalized to pSV2-LUC for comparison of the PLP constructs because we assumed that the SV40 promoter activity was comparable in both cell lines. The ratio of the normalized activity of the promoter-free p0-LUC in the two cell lines corresponds to the ratio of the activities of the constructs. Therefore no cell specificity is apparent when the data of the truncated promoter elements are normalized to the p0-LUC or the longer PLP promoter construct. It is likely that the cell-specific expression of pSV2-LUC is responsible for this effect.

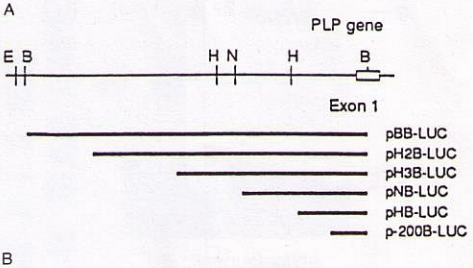
We conclude that a constitutive promoter is located in the 5′ region between position −184 and +90 which is active in C6 and CHO cells. The sequence −184 to −38 is essential for this function, since the smallest promoter sequence of construct p-200B-LUC shows no expression.

DNase I protection of the promoter region

Nuclear extracts of total brain and liver 18- to 22-day old rats and of C6 cells were used for footprint analysis of the 269bp *Hin*d III/*Bam* HI fragment which harbors the promoter domain as determined by the in vitro expression experiments.

Several DNase I-protected sequences on the sense and antisense strand were detected. The length of the protected sequences and the position of the DNase hypersensitive sites with the three different nuclear extracts were similar. The transcription start, the putative TATA box, and several upstream elements are protected (Fig. 4). Thus no tissue-specific protected elements could be detected by this method. Therefore we specified the characterized sequences by gel retardation assays using synthetic oligonucleotides.

A



B

Construct	Extend of the upstream region	Relative luciferase activity [%] C6 cells	CHO cells
pSV2-LUC		100	100
p0-LUC		6.9 ± 3.6	1.6 ± 0.3
pBB-LUC	−1392 bp	45.1 ± 8.6	7.7 ± 1.1
pH2B-LUC	−1078 bp	37.5 ± 11.7	7.3 ± 3.3
pH3B-LUC	−700 bp	43.0 ± 2.5	11.5 ± 4.6
pNB-LUC	−432 bp	58.2 ± 3.6	10.1 ± 3.4
pHB-LUC	−184 bp	52.2 ± 6.4	10.9 ± 9.8
p-200B-LUC	−38 bp	9.9 ± 4.2	2.0 ± 0.7
p-BB-LUC	−1392 bp (antisense)	6.6 ± 2.8	0.7 ± 0.2

Fig. 3. Deletion analysis of the mouse PLP 5′-upstream region in transfected C6 and CHO cells.

A) 5′ Fragments of the truncated mouse PLP promoter were fused to the luciferase gene of the vector p0-LUC. The 5′ end of the PLP gene fragments ranges from −1392 to −38 and the 3′ end was positioned at +90. (E, *Eco* RI; B, *Bam* HI; H, *Hin*d III; N, *Nco* I). B) The constructs were introduced by transfection into C6 glioblastoma cells and CHO fibroblast cells and luciferase activity was determined. The activity was normalized to the expression of the co-transfected pSV2-CAT vector. The results are listed as mean relative luciferase activities derived from at least three different experiments, with standard deviations.

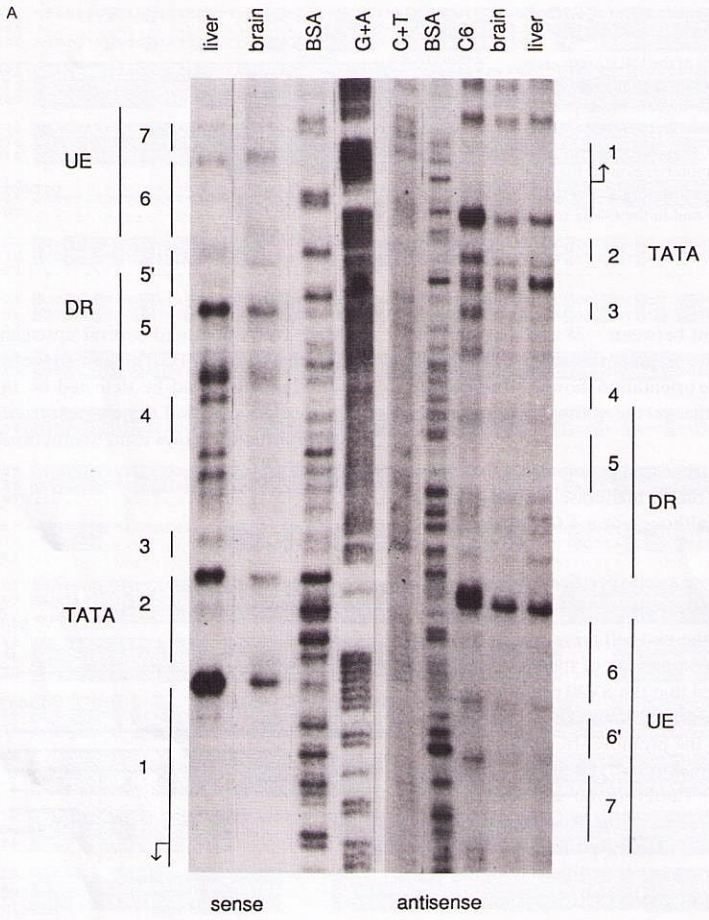


Fig. 4. DNase I footprint analysis of the PLP promoter region. A) Nuclear extracts from brain, liver and C6 cells were used for DNase I footprint assay of a *Hind* III/*Bam* HI promoter fragment extending from −184 to +90 with labeled sense and antisense strand. The position of the transcription start (CAP), of the TATA box and of the oligonucleotides used for the band shift analysis are marked. As negative control the same amount of BSA was added instead of nuclear extract. B) Position of DNase I-protected region within the PLP promoter sequence. The sequences of the oligonucleotides DR and UE are marked.

Gel retardation analysis of the DNase I protected sequence

Two synthetic oligonucleotides within the promoter domain were selected for the analysis of DNA-binding proteins to the protected sequences: the direct repeat (DR) and the nucleotide sequence −82 to −110 (UE). Nuclear extracts of brain, liver, and C6 glioblastoma cells were used. Oligonucleotide UE forms one low molecular and two high molecular complexes of comparable intensity with nuclear proteins of the three different tissues. Oligonucleotide DR also yields two high molecular complexes with nuclear proteins present in the three cell types but in addition a brain-specific complex with slightly lower electrophoretic mobility (Fig. 5). The formation of the protein-oligonucleotide complexes could be inhibited

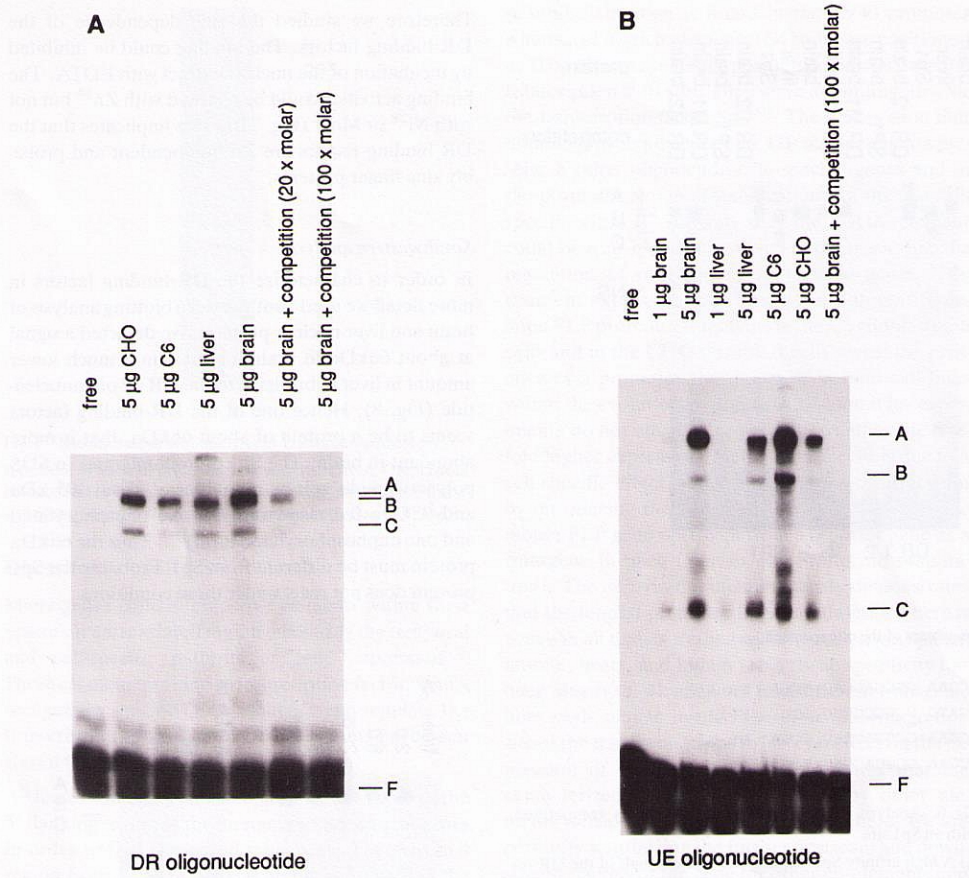


Fig. 5. Bandshift analysis of DR and UE.

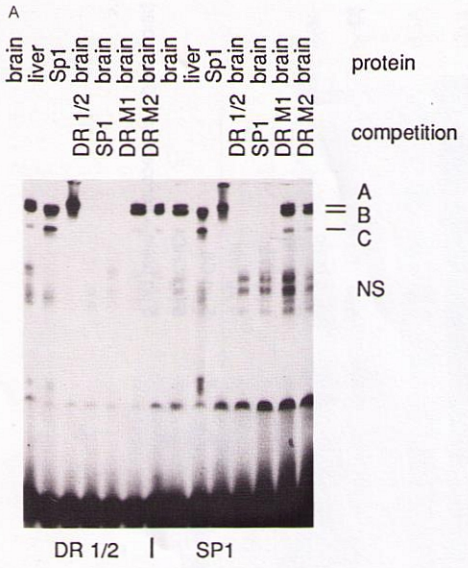
Identification of the tissue distribution of *trans* factors in nuclear extracts from brain, liver, C6, and CHO cells. A) With oligonucleotide DR two ubiquitous *trans* factors (B and C) and one brain-specific (A) factor were detected. B) Oligonucleotide UE forms three ubiquitous complexes with the nuclear extracts (A, B, C). The unlabeled oligonucleotide was used for competition.

by a 100-fold excess of unlabeled oligonucleotide but not with poly d(A-T) and salmon sperm DNA (data not shown).

The DR-binding factors belong to the class of GC box-binding factors

The DR-binding factors were more extensively characterized by an oligonucleotide containing only one copy of the DR repeat (DR1/2) and another oligonucleotide with an GC-rich sequence derived from the SV40 early promoter (SP1). This sequence is a high affinity binding site for the transcription factor Sp1[35]. We used recombinant human Sp1 (Promega) to study binding of the transcription factor to the DR1/2 site. The two oligonucleotides show the same pattern in the gel retardation experiment with brain and liver nuclear extracts as the DR oligonucleotide. Competition with the unlabeled oligonucleotides and two mutated oligonucleotides demonstrated that DR1/2 and SP1 oligonucleotides bind specifically the same factors. The recombinant human Sp1 forms a complex with the DR1/2 and with the SP1 oligonucleotide. The complex migrates between the lower ubiquitous and the brain-specific complex (A, B). This demonstrates clearly that Sp1 binds to the DR1/2 site (Fig. 6).

Therefore we propose that the upper ubiquitous complex in the rat nuclear extract is formed with rat



B

Sequences of the oligonucleotides

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TCGAA GGGGAGGAG TCGA  DR 1/2
 GATC GGGGCGGGG ATC   SP1
TCGAA GCGGAGCAG TCGA  DR M2
TCGAA GCGGAGGAG TCGA  DR M1
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Fig. 6. Analysis of the DR-binding activity by competition with an Sp1 site.

A) A high affinity Sp1-binding site and one half of the DR repeat were used for competition analysis. The binding of the *trans* factors could be inhibited by the unlabeled SP1 and DR oligonucleotide, but not with an oligonucleotide of a mutated sequence (DR M1 and DR M2). Recombinant human SP1 transcription factor was used in lane 3 and 10 for the retardation assay. The transcription factor is bound with equal affinity by the DR1/2 and the SP1 oligonucleotide (NS are nonspecific complexes). B) Sequences of the oligonucleotides used.

Sp1 and that the difference in the mobility is due to differences between rat and recombinant human Sp1.

In order to study the developmental expression of the DR-binding factors we prepared nuclear extracts from the brains of rats of different age and analysed equal amounts of protein by gel shifts with the DR1/2 oligonucleotide (Fig. 7A). The three complexes were present in all extracts and no remarkable difference in expression could be detected from two day old to adult rat brains.

The transcription factor Sp1 belongs to the class of zinc finger proteins[16].

Therefore we studied the zinc dependence of the DR-binding factors. The binding could be inhibited by incubation of the nuclear extract with EDTA. The binding activities could be restored with $Zn^{2\oplus}$ but not with $Ni^{2\oplus}$ or $Mn^{2\oplus}$ (Fig. 7B). This implicates that the DR-binding factors are $Zn^{2\oplus}$-dependent and probably zinc finger proteins.

Southwestern analysis

In order to characterize the DR-binding factors in more detail we used Southwestern blotting analysis of brain and liver nuclear proteins. We detected a signal at about 66 kDa in brain and also in a much lower amount in liver hybridizing to the DR1/2 oligonucleotide (Fig. 8). Hence one of the DR-binding factors seems to be a protein of about 66 kDa, that is more abundant in brain. The Sp1 protein migrates in SDS polyacrylamide gels as a doublet of about 105 kDa and 95 kDa (corresponding to one phosphorylated and one unphosphorylated form)[36]. Thus the 66 kDa protein must be different from Sp1. Probably the Sp1 protein does not react under these conditions.

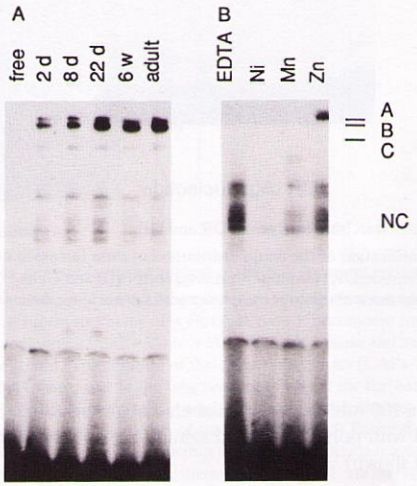

Fig. 7. A) Developmental expression of the DR-binding factors. Equal amounts of nuclear protein of rat brains of different age (2 days, 8 days, 22 days, 6 weeks, and adult) were used for gel shift analysis with the DR1/2 oligonucleotide. The complexes A, B, and C are detectable at comparable amounts in the different extracts.

B) Metall reconstitution of the DR-binding factors. The binding of the complexes is inhibited by EDTA but could be reconstituted by adding $ZnCl_2$. No reconstitution could be seen by adding of $NiCl_2$ or $MnCl_2$. Therefore the binding of the complexes seems to be $Zn^{2\oplus}$-dependent.

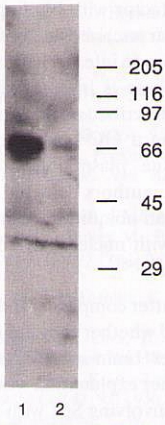

Fig. 8. Southwestern blot analysis of DR-binding proteins.

A protein of about 66 kDa reacting with the DR1/2 Oligo could be detected in brain and in liver nuclear extracts but the intensity of the signal in the brain is much higher. 1, Brain; 2, liver.

Discussion

Many genes contain *cis*-active elements within their upstream untranslated region mediating the temporal and cell-specific patterns of gene expression[15]. These *cis*-elements bind to transcription factors which recognize a specific DNA sequence and regulate the transcription of the gene[37]. *cis*-Elements have been shown to be conserved during evolution.

We have sequenced and compared about 1.5 kb of the 5′ flanking region of the human, mouse, and rat genes in order to find conserved sequences. The proximal region from −250 to +100 is highly conserved in the three species (about 95% identity), whereas the upstream region shows only about 50% identity. This indicates that the evolutionary conserved proximal region contains sequences essential for the functional expression of the gene. Furthermore the longest identical sequences in all three species are in the proximal region, a 53 bp sequence (−26 to +26) and a 34 bp sequence (−75 to −42) as opposed to one decamer (−276 to −295) and three octamers (−490 to −497, −532 to −539 and −581 to −588) within the distal region. (All position numbers are those of the mouse gene). The analysis of the nucleotide sequences revealed a CCAAT element in antisense orientation (−146 to −142) but no sequences homologous to other well characterized *cis*-acting elements. A typical TATA box is missing, but the sequence TTTAA at position −25 could represent a putative TATA element for the most upstream transcription start site. One remarkable element is the direct repeat (DR) of the 11 bp sequence AAGGGGAGGAG (DR1/2) at position −75 to −54. It is conserved in all three species.

A similar situation is found in the SV40 promoter where six GC-rich elements (GC box) are positioned as 11 bp direct repeats at a position equidistal from the transcription start site. They serve as binding sites for the transcription factor Sp1[38]. The observation that homologous sequences of the DR1/2 element are present in other oligodendrocyte-specific genes and in the promoter region of some variants of the glia cell-specific virus JC suggests that the DR1/2 element could be a *cis*-element involved in the tissue-specific regulation of oligodendrocyte-specific genes. The transient expression experiments of different 5′-deleted PLP promoter fragments in the C6 glioblastoma cells and in the CHO fibroblast cells reveal the presence of a promoter that is active in both cell lines within the evolutionary conserved region. Our experiments do not answer the question whether the five-fold higher expression level in the C6 cells is due to a cell-specific effect. We have addressed this question by introducing the 1.5 kb promoter sequence of the mouse PLP gene upstream from a reporter gene as a transgene in mice (Körner and Stoffel, in preparation). The experiment unambiguously demonstrates that the longest promoter fragment described here is active in all tissues studied so far (brain, liver, spleen, muscle, heart, and kidney). No tissue specificity has been observed. We analysed four different transgenic lines with similar results, therefore the integration site of the transgene seems to have no effect on the expression of the construct. This indicates that the characterized promoter is regulated by other elements outside of the region described here. We are currently scrutinizing the further upstream and downstream regions of the gene for other *cis*-elements and their binding proteins which might be responsible for the tissue-specific regulation of the PLP gene.

Recently the PLP promoters of rat[39] and man[42] were examined with transient expression experiments in glial cell lines with the result that the region corresponding to the proximal region of the mouse promoter is sufficient for expression.

This corresponds to our data, but in the distal region the studies further revealed elements with positive and negative influence on the expression. The differences between these studies and our results refer to inter-species variations within the distal region of the PLP promoter. Another study of the mouse PLP promoter using mouse hepatoma cells and hamster glia cells[40] failed to detect any activity below background with a construct containing the proximal region and is therefore contradictory to our results as well as to the results of the two other studies.

Our analysis of the described promoter region for DNA-binding factors in nuclear extracts of the two

cell types, liver and brain, demonstrates that different *trans*-factors interact with the promoter. Most of the elements seem to belong to the class of common transcription factors because they were found in all extracts. These factors could be responsible for the constitutive promoter activity. Surprisingly the sequences of the binding sites are not homologous to *cis*-elements characterized so far.

Gel retardation experiments showed that three complexes are formed with the direct repeat sequence (DR). One of these complexes was formed only with nuclear extracts of brain but not of liver or of C6 and CHO cells.

Competition experiments with a high affinity Sp1-binding site[35] and binding of human recombinant Sp1 proved that the DR1/2 sequence is a Sp1-binding site, thereby we demonstrate for the first time that the DR1/2 site is an Sp1-binding site and a binding site for a brain-specific factor.

A recent study of the DR1/2 homologous sequence within the regulatory region of the JC virus demonstrated that this site also bound to Sp1[41].

Similar patterns of binding sites for *trans*-factors as in our study were found within the corresponding proximal region of the human[42] and rat genes[39].

One of these *trans*-factors has been characterized by expression cloning of a zinc finger protein that binds to a site within the human PLP gene at position −249 to −277[43], but this element is located outside the characterized minimal promoter[42]. Furthermore, in that study it was shown that a mutation within the binding site abolishes the binding of *trans*-factors but does not disturb the activity of the promoter in the expression experiment indicating that this factor is not involved in the expression of the promoter in this system.

The DR1/2-binding factors proved to be dependent on $Zn^{2\oplus}$ ions. Therefore they seem to belong to the class of zinc finger proteins.

Analysis of the developmental expression of the factors shows that the brain-specific complex is expressed at a nearly constant level during postnatal development of the rat. The pattern of expression does not correlate with the myelination period yet this does not exclude the factor's role in the regulation of myelin genes. If the factor were expressed additionally in other neuronal tissues this could conceal a higher overall level of expression during myelination. Another explanation is the involvement of the factor in the regulation of the tissue-specific basal activity of the PLP promoter.

The brain-specific factor which recognizes the DR1/2 and also the SP1 oligonucleotides could be a brain-specific *trans*-factor with binding features similar to Sp1. Some characterized factors different from Sp1 have been shown to interact with a GC box motif[44–47], but none of these is brain-specific. Interestingly, a recent report described a brain-specific DNA-binding factor by using a GC box oligonucleotide derived from the tissue plasminogen activator promoter sequence. The authors detected a complex of lower mobility than an ubiquitous complex in the gel retardation assay with nuclear extracts of brain, but not of liver or kidney[48].

Whether the latter complexes and activities are identical to ours and whether they are involved in the regulation of other brain-specific genes remains to be proven. Another explanation would be the formation of a complex involving Sp1 with an interacting brain-specific protein. It is known, that Sp1 is capable of interacting with coactivators, which serve as adaptors between Sp1 and the general transcription machinery[49]. The protein-protein interaction of Sp1 with an enhancer-binding protein has also been described previously[50]. It is known that Sp1 may also exist in a phosphorylated form, but DNA binding of the phosphorylated form cannot explain the existence of the additional complexes because phosphorylation does not change the electrophoretic mobility in the gel retardation experiment[36].

By Southwestern blotting we detected a protein of about 66 kDa enriched in the brain interacting with the DR site. If this protein corresponded to the brain-enriched factor in the shift assay we would postulate that the factor consists of a brain-specific protein.

The observation that the brain-enriched factor shows approximately the same mobility in the shift assay as Sp1, as opposed to the fact that the Sp1 protein migrates at about 95 kDa and 105 kDa in SDS gels, could be explained by different mobilities of the proteins in the two gel systems, by the dimerization of the 66 kDa protein, or by the interaction with other proteins under non-denaturating conditions.

We are now purifying the brain-specific component for its structural characterization.

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