Studies on a Functional Role of Myelin Proteolipid Protein (PLP) in Glial Differentiation

(グリア細胞分化におけるミエリンプロテオリピド蛋白質の機能解析)

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STUDIES ON A FUNCTIONAL ROLE OF MYELIN PROTEOLIPID PROTEIN (PLP) IN GLIAL DIFFERENTIATION.

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SUMMARY

Myelin proteolipid protein (PLP) is known to be a highly conserved major myelin structural protein. It is synthesized in oligodendrocytes which form myelin of the central nervous system (CNS). Mutations within the PLP gene cause glial cell degeneration and hypomyelination, such as *jimpy* (*jp*) in mouse and Pelizaeus-Merzbacher disease (PMD) in human. This suggests that the PLP gene products have bifunctional role, a functional role in glial cell development in addition to that in myelin assembly. However, the PLP gene expression in the stage for glial cell differentiation had been unknown.

Transcripts from the PLP gene were analyzed in the developing mouse brain by a sensitive method using the polymerase chain reaction (PCR). The mRNA for DM-20, an alternatively spliced transcript from the PLP gene, was detected in the embryonic mouse brain as early as 11th embryonic day, long before the appearance of oligodendrocytes, which were considered to be responsible for the PLP production. Moreover, the DM-20-mRNA was produced in various cell lines, not restricted to glial cells (Part I).

Next, I produced transgenic mice carrying extra wild type mouse PLP genes accompanied by its overproduction. Surprisingly, transgenic mice bearing 4 more PLP genes exhibited "*jimpy* phenotype", including degeneration of oligodendrocytes and hypomyelination in the CNS, while those with 2 more PLP genes showed normal myelination at earlier

stage (3 weeks after birth), but later developed demyelination (Part II). These results strongly suggest that DM-20 is not only one of the structural proteins of myelin, but plays a crucial functional role in glial cell differentiation and/or its survival.

Part I : Selective Expression of DM-20, an Alternatively Spliced Myelin Proteolipid Protein Gene Product, in Developing Nervous System and in Non-Glial Cells.

INTRODUCTION

During development of the central nervous system (CNS), neuroepithelial cells proliferate and differentiate into neurons and glial cells. Neurons extend many neurites to form complex neural network and process information. Glial cells support neuronal functions in various way; e.g. making myelin, separating neural cell bodies from each other, and buffering many ions effluxed from excited neurons. Thus, their functions are quite different, eventhough they are produced differentiated from the same precursor cells, neuroepithelial cells. Therefore, unveiling the molecular machinery determining the fate of a neuroepithelial cell is the first and quite important step toward understanding the mechanism underlying the formation of the complex and yet well organized architecture of the CNS. One approach to identify a molecule that is important for the development of nervous system is to study the molecular defect in mutant animals showing abnormal development in the nervous system.

The *jimpy* mouse (*jp*) is an X-linked recessive mutant, characterized by abnormal myelin formation in the CNS. It is considered to be an animal model of human Pelizaeus-Merzbacher disease (PMD)(Hogan and Greenfield, 1984). In this mutant, all myelin components in the brain are drastically reduced (Hogan and Greenfield, 1984). These reductions are apparently caused by severe degeneration of immature oligodendrocytes, which forms myelin in the CNS (Knapp *et*

al., 1986). It also shows hypertrophy of astrocytes (Skoff, 1976; Knapp *et al.*, 1986), and thus, the *jimpy* mutation causes abnormal glial cell development (Knapp *et al.*, 1987; Vermeesch *et al.*, 1990). A 74 bp deletion in *jimpy* PLP-mRNA was detected by Nave *et al.* (1986)(Fig. 1). This deleted sequence was identical to that of the fifth exon of PLP-mRNA, and thus fifth exon of the PLP gene was not utilized in *jimpy* (Moriguchi *et al.*, 1987). An A to G conversion at the conserved "AG" residues of the 3'-splice site was found (Macklin *et al.*, 1987; Nave at al., 1987), which apparently resulted in the fifth exon deletion of PLP-mRNA (Fig. 1).

Proteolipid protein is most abundant in CNS myelin, constituting 50% of the myelin membrane proteins of the CNS. It is called "proteolipid" because of its unusual property to dissolve in organic solvents (Folch and Lees, 1951). The two isoforms of the proteolipid protein are generally referred to as the myelin proteolipid protein (PLP) [mol mass = 30 kdalton] and the DM-20 protein [mol mass = 25 kdalton] which shows immunocrossreactivity with PLP (Lees and Brostoff, 1984). Both of them are synthesized in mature oligodendrocyte, and thought to play an important role in myelination in the CNS, probably by promoting the apposition of extracellular surfaces of the myelin lamellae (the intraperiod line, Fig. 2). The structural relationship between PLP and DM-20 has been established only recently through cDNA analysis. PLP-cDNA was first isolated from rat (Milner *et al.*, 1985), and later from mouse (Nave *et al.*, 1986), bovine

Fig. 1. PLP gene expression in *jimpy* and normal mice.

The genetic defect of *jimpy* (*jp*) is a point mutation in the splice acceptor signal (AG to GG) before the fifth exon of X-chromosome-linked PLP gene, resulting in a 74 bp (fifth exon) deletion in *jimpy* PLP-mRNA. The protein encoded by this mRNA has an abnormal C-terminal structure owing to the reading frame shift, which is expected to be extraordinary rich in cysteine residues.

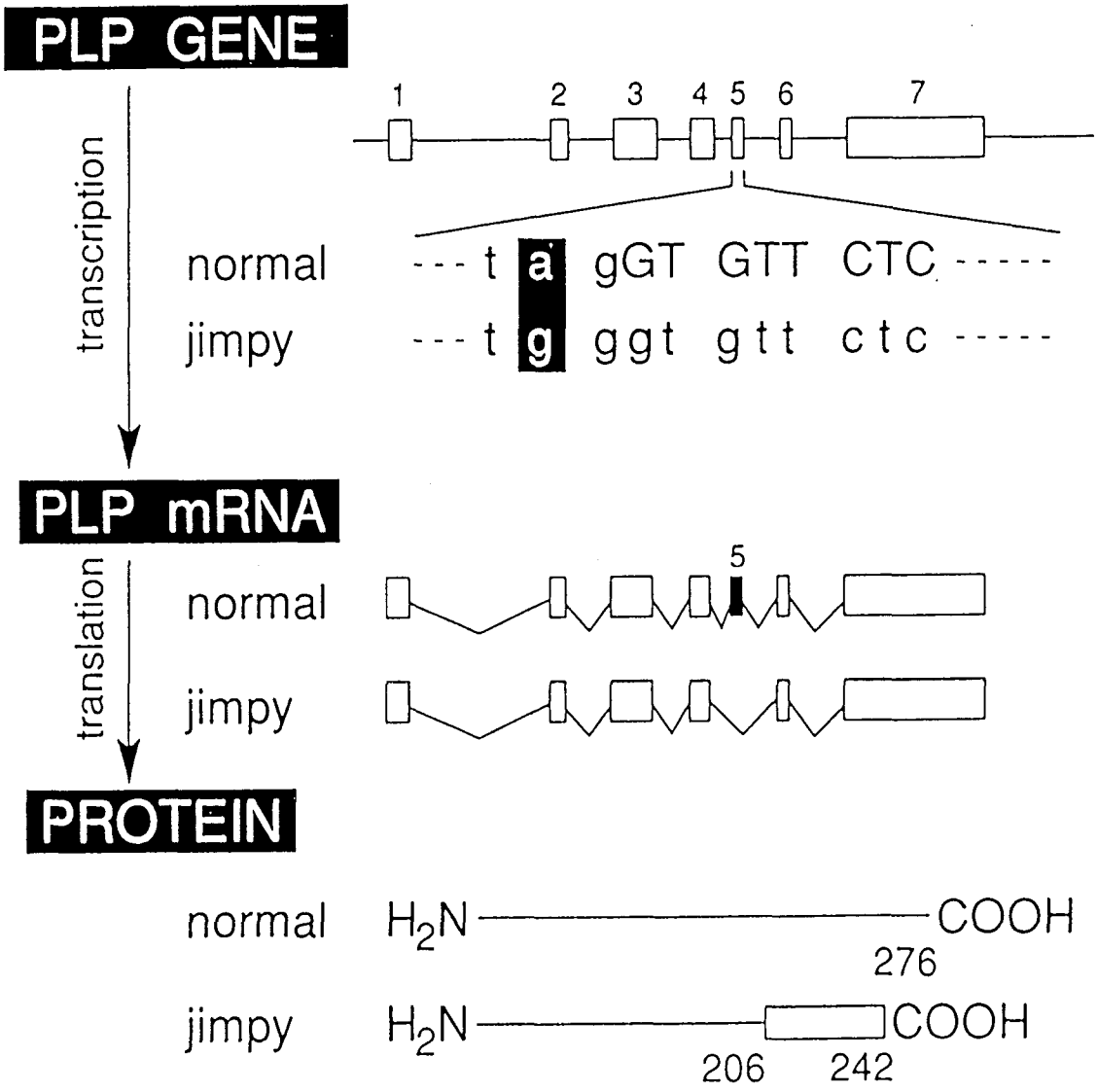
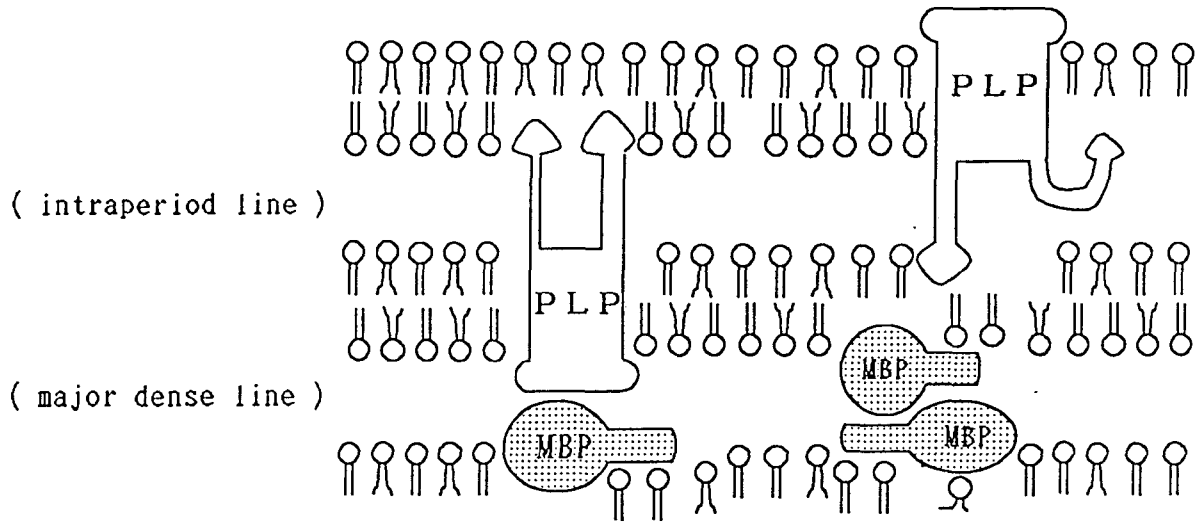

PLP GENE
transcription
1
2
3
4
5
6
7
normal
--- t a gGT GTT CTC -----
jimpy
--- t g ggt gtt ctc -----
PLP mRNA
translation
5
normal
jimpy
PROTEIN
normal
H2N
COOH
276
jimpy
H2N
COOH
206
242

Fig. 2. PLP in the central nervous system (CNS) myelin.

The major CNS myelin proteins, myelin basic protein (MBP) and the proteolipid protein (PLP), represent 70-80% of the total myelin protein content of the membrane. PLP is thought to promote the apposition of extra cellular surfaces of the myelin lamellae. MBP is thought to make myelin lamellae compact by fusing the cytoplasmic surfaces of cell membrane into major dense lines.


(intraperiod line)
(major dense line)
PLP
PLP
MBP
MBP
MBP

(Naismith *et al.*, 1985), and human (Kronquist *et al.*, 1987). These amino acid sequences are highly conserved (100% except 2 amino acids replacements in bovine PLP).

DM-20-mRNA is produced from the PLP gene by alternative splicing, and 105 nucleotides were deleted from PLP-mRNA to form DM-20-mRNA (Nave *et al.*, 1987a). Thus the primary structure of DM-20 is identical to PLP except for an internal deletion of 35-amino acid residues (116-150) from the major hydrophilic domain. Recent studies on the developmental profile of PLP and DM-20 level demonstrated that the DM-20 protein appears prior to the PLP protein at the early stage of myelination (Gardinier and Macklin, 1988; Schindler *et al.* 1990).

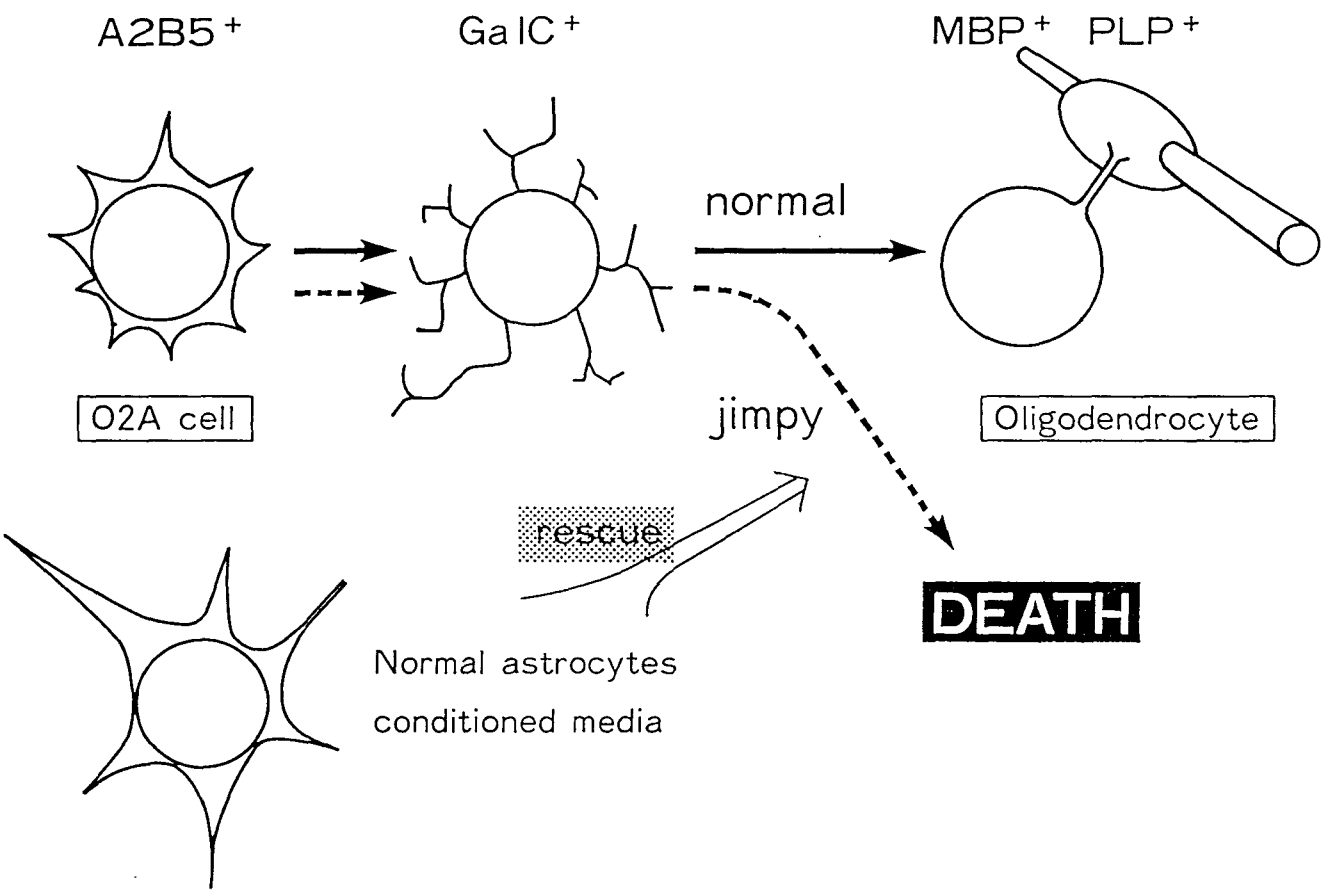
Many mutations within the PLP gene have been identified recently. All of them, except for *rumpshaker* (*rsh*) (Schneider *et al.*, 1992) in mouse, cause abnormal glial cell differentiation and hypomyelination in the CNS (Hogan and Greenfield, 1984; Campagnoni and Macklin, 1988), such as *jimpy* (Macklin *et al.*, 1987; Nave *et al.*, 1986 and 1987b; Moriguchi *et al.*, 1987) and *jimpy*^{md} (Gencic and Hudson, 1990) in mouse, myelin synthesis-deficient (*md*) in rat (Boison *et al.*, 1989), shaking pup (Nadon *et al.*, 1990), and Pelizaeus-Merzbacher disease (PMD) (Gencic *et al.*, 1989; Hudson *et al.*, 1989) in human. They commonly show ataxia, intentional tremor, and tonic convulsions, which finally lead them to death at young age. However, degeneration of immature oligodendrocytes in the PLP mutants is observed

even before the myelinating stage when the PLP gene expression is not detectable (Knapp *et al.*, 1986)(Fig. 3). Hypertrophy of astrocytes is also observed in embryonic *jimpy* brain before oligodendrocytes appear (Knapp *et al.*, 1986). Moreover, it has been shown that conditioned media of normal astrocytes enabled *jimpy* oligodendrocytes to survive in vitro (Bartlett *et al.*, 1988)(Fig. 3). These observations suggest that the PLP gene is expressed at a very low level, below the detection limit, before the myelinating stage, and its production may not be restricted to oligodendrocyte. PLP or DM-20 produced at this early stage might function as a differentiation factor in addition to functioning as a myelin protein. This hypothesis is supported by the recent study for newly discovered PLP mutant, *rumpshaker* (Schneider *et al.*, 1992). In contrast to all other PLP mutants, in *rumpshaker* oligodendrocyte differentiate normally, but they cannot form normal myelin. Therefore, degeneration of immature oligodendrocytes and dysmyelination are uncoupled, supporting the idea that the PLP gene products have bifunctional role, a vital function in glial cell development in addition to that in myelin assembly (Knapp *et al.*, 1986, Schneider *et al.*, 1992).

In this study, I applied the PCR (polymerase chain reaction) technic to detect small amounts of PLP gene transcripts, and found that the PLP gene is transcribed in the mouse brain as early as the 11th embryonic day, long before oligodendrocytes or their direct progenitor cells, O2A

Fig. 3. Oligodendroglial cell fate in normal and *jimpy* mice.

Oligodendrocyte is derived from O-2A progenitor (A2B5 positive) cell and expresses GalC from its immature stage. The PLP gene expression is observed after final maturation of oligodendrocyte. Oligodendrocytes in *jimpy* brain, as well as that in normal mouse brain, can differentiate to immature oligodendrocytes (GalC positive cells). However, most of the *jimpy* oligodendrocytes degenerate before final maturation. Bartlett *et al.*, (1987) demonstrated that normal astrocytes produce soluble factors that rescue the *jimpy* oligodendrocytes from degeneration in culture, and that these factors are not present in the cultured medium of *jimpy* astrocytes.


A2B5+
GalC+
MBP+ PLP+
normal
O2A cell
jimpy
Oligodendrocyte
rescue
DEATH
Normal astrocytes
conditioned media

cells, appear. However, the transcribed product was solely DM-20-mRNA, an alternatively spliced product of the PLP gene (Nave *et al.* 1987a). DM-20-mRNA was also produced in various neural cell lines and in sciatic nerve, suggesting that DM-20, but not PLP, can function as a differentiating factor for oligodendrocytes and possibly for all glial cells (Ikenaka *et al.*, 1992).

RESULTS

A sensitive method for detecting the PLP gene transcripts.

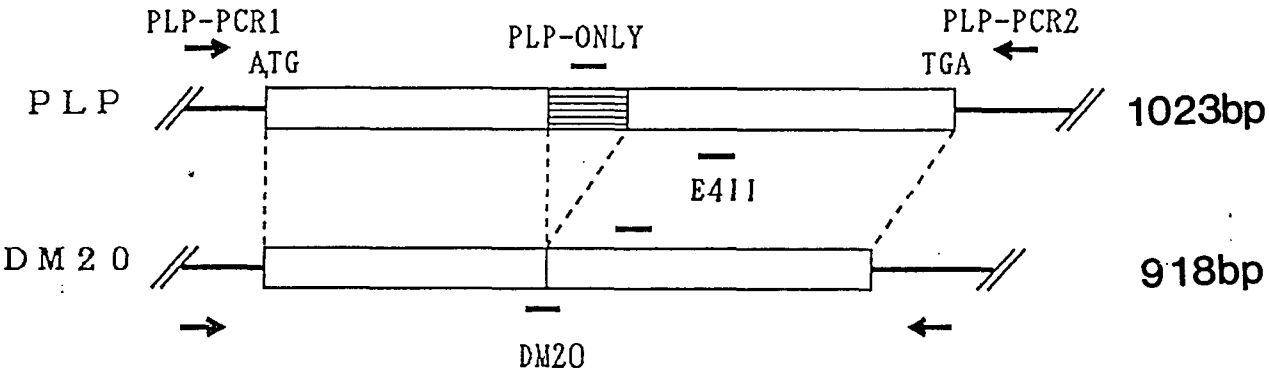
Since many abnormalities in glial system were observed in the PLP mutants before PLP-production had been detected (Campagnoni and Macklin, 1989), I developed a highly sensitive method for detecting the PLP gene transcripts. The method used to detect small amounts of PLP gene products is outlined in Fig. 4. First, cDNA is synthesized by reverse transcriptase using PLP-PCR2 primer, which hybridizes to the PLP gene transcripts just downstream from the translation termination codon. Then the second primer, PLP-PCR1, which hybridizes to the cDNA just upstream from the translation initiation codon, is added, and the polymerase chain reaction (PCR)(Saiki *et al.*, 1985) is performed for 45 cycles. Thus, the amplified region contains the entire coding sequence, and the amplified products of PLP-mRNA and DM-20-mRNA can be separated on agarose or polyacrylamide gel. I also used various probes to specifically detect PLP-mRNA (PLP-ONLY), DM-20-mRNA (DM-20) or both (E4II).

DM-20 is selectively expressed in embryonic mouse brains and in sciatic nerve.

Total RNA was isolated from embryonic mouse brains at E16 and from brains on the 8th and 18th day after birth (P8 and P18), and subjected to the PLP-detection method described above. Amplified products from cloned PLP-cDNA and

Fig. 4. PCR method for detecting the PLP gene transcripts.

After synthesizing cDNA by reverse transcriptase, the PCR reaction is performed for 45 cycles. Oligonucleotide primers, PLP-PCR1 and PLP-PCR2, are represented by arrows to their annealing sites in the target cDNA sequence. Using these primers a 1023-bp fragment is amplified from PLP-mRNA and a 918-bp fragment from DM-20-mRNA. White boxes represent open reading frames and short thin lines on the upper white boxes correspond to the region spliced out in DM-20-mRNA. Bars show the hybridizing sites of the oligonucleotide probes for detecting the PCR products.


PLP-PCR1
ATG
PLP-ONLY
PLP-PCR2
TGA
PLP
1023bp
E411
DM20
918bp
DM20

DM-20-cDNA served as markers. Strong hybridization signals were observed at lanes P8 and P18 using ^{32}P-labeled E4II as a probe (Fig. 5A). The upper band with the same mobility as that of amplified PLP-cDNA clone corresponds to the amplified product from PLP-mRNA, and the lower band to that from DM-20-mRNA. Although no bands were seen in lane E16 in Fig. 5A, a band of the same mobility as that of the DM-20-amplified product was detected upon longer exposure (Fig. 5B). The filter was rehybridized with the DM-20 probe followed by the PLP-ONLY probe and I confirmed that this band was indeed amplified product from DM-20-mRNA (Fig. 5C and 5D). I also detected other minor bands in lanes P8 and P18 (Fig. 5B-D), which may be unidentified alternative splicing products of the PLP gene. However, even when the filter was rehybridized with the PLP-cDNA probe, there were no other detectable hybridizing signals than that obtained with the E4II probe in E16 lane (data not shown). Therefore, DM-20-mRNA is selectively produced in a very small amount in the early stage of neural development.

Although the PCR technic is a highly sensitive method for detecting gene expression, it is not suitable for quantitative analysis. The concentration of primers, the *Taq* polymerase used, purity of the RNAs used, and many other factors influence the extent of amplification. The dose response of my PCR system is shown in the same gel. Reducing the amount of P18 mouse brain RNA by 1/10 (2, 0.2, 0.02, and 0.002 μg of total brain RNA with total liver RNA to make 2

Fig. 5. Detection of PLP gene transcripts in various tissues.

Total RNA extracted from mouse brain (E16, P8, P18), rat sciatic nerve, mouse liver and mouse fibroblast NIH3T3 cells was analyzed using the PCR method described above. Positive controls, which were amplified from cloned PLP-cDNA (closed triangle) and DM-20-cDNA (open triangle), are shown in the left and right lanes (cDNA). Each lane is equivalent to the amplified products from 0.2 μg of total RNA. Both PLP-mRNA and DM-20-mRNA were expressed in P8 and P18 brain, but only DM-20-mRNA was expressed in E16 brain and sciatic nerve. Both PLP and DM-20-bands were undetectable in liver and NIH3T3 cell. NIH3T3 cell harboring PLP-SPUD produced only PLP-mRNA (though it also produced β-galactosidase. see Fig. 6). The dose response of the PCR system was also investigated. 2 (P18), 0.2 (x1/10), 0.02 (x1/100) or 0.002 (x1/1000) μg of total RNA from P18 mouse brain with total mouse liver RNA to make 2 μg RNA was reacted with reverse transcriptase and *taq* polymerase. 45 cycles of PCR were carried out and hybridized with ^{32}P-labeled oligonucleotide probes. A: Hybridization analysis using ^{32}P-labeled E4ll oligonucleotide probe. (short exposure) B: Longer exposure of A. C: ^{32}P-labeled DM-20 oligonucleotide as a probe. D: ^{32}P-labeled PLP-ONLY oligonucleotide as a probe.

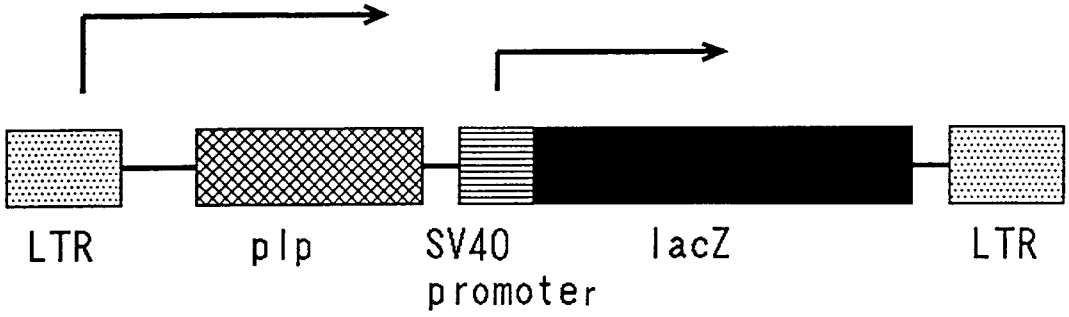
cDNA
E16
P8
P18
x1/10
x1/100
x1/1000
Liver
Sciatic nerve
NIH3T3
NIH/PLP
cDNA
A
B
C
D

μg final RNA sample: note that liver RNA does not contain detectable amount of DM-20-mRNA, see lane Liver) resulted in a reduction of the radioactivity on the filter by approximately one-fifth for each dilution. The radioactivity of the amplified product from the DM-20-mRNA in E16 brain was similar to that from total PLP gene transcripts detected in 1/1000 diluted P18 mouse brain RNA or similar to that from DM-20-mRNA detected in 1/100 diluted sample. Therefore, the amount of DM-20-mRNA detected in the embryonic mouse brain was approximately the 1/1000 of the PLP gene transcripts found in P18 mouse brain (PLP- + DM-20-mRNA). However, I will not discuss the exact quantity of DM-20-mRNA expressed at present.

RNA extracted from NIH-3T3 cells and mouse liver did not contain detectable levels of PLP gene products (Fig. 5), whereas NIH-3T3 cells harboring the plasmid PLP-SPUD produced only PLP-mRNA (Fig. 5, lane NIH/PLP). The structure of PLP-SPUD is shown in Fig. 6. PLP-cDNA inserted in this vector is transcribed from the Moloney leukemia virus (MoLV) LTR promoter. Stable transformant was produced by co-transfection of the plasmid PLP-SPUD and pSV2neo by the calcium phosphate precipitation method. These served as excellent negative controls and demonstrated that the DM-20-mRNA detected in this study was not an artifact. Another interesting finding was that the PLP gene product detected in the Schwann cells, which form peripheral myelin, seems to be mostly DM-20, because amplified products of total RNA ex-

Fig. 6. Construction of PLP-SPUD.

PLP-SPUD produces both PLP and β-galactosidase. PLP-cDNA expression is under control of the Moloney murine leukemia virus (MoLV) LTR. E.Coli. lacZ gene is under control of SV40-promoter.


LTR
plp
SV40
promoter
lacZ
LTR

tracted from rat sciatic nerve hybridized strongly to the DM-20 probe (Fig. 5C, lane Sciatic nerve), while very weakly to the PLP-ONLY probe (Fig. 5D). Therefore, DM-20 is distributed in both the peripheral and central nervous systems, at least in a small amount.

DM-20 is expressed in mouse brain as early as E11.

Total RNA was isolated from embryonic mouse brains at various stages (E11-E18) and from brains on the 2nd, 8th and 18th day after birth (P2, P8 and P18), and subjected to the PLP-detection method to study developmental profile of DM-20 expression. The DM-20-amplified product were detected throughout the developmental stages investigated in this study (E11-P2) when probed with the E411 probe (Fig. 7A). The filter was rehybridized with the DM-20 probe and I confirmed that these bands were indeed amplified products from DM-20-mRNA (Fig. 7B) and not from PLP-mRNA (Fig. 7C). Level of DM-20-mRNA peaked at E14 before birth. PLP-mRNA became detectable at P2 brain (not seen in Fig. 7C, but becomes detectable upon much longer exposure). Therefore, DM-20-mRNA is selectively produced in a very small amount from the very beginning of neural development. These results are schematically drown in Fig. 8.

PLP gene expression in *jimpy* mutant mice at early stages.

Since it is known that the *jimpy* PLP gene is abnormally regulated, I next investigated the PLP gene expression in

Fig. 7. Developmental change of PLP gene expression in mouse brains.

Total RNA extracted from mouse brain (E11 to E18, P2, P8 and P18) was analyzed using the PCR method described above. Positive controls, which were amplified from cloned PLP-cDNA (closed triangle) and DM-20-cDNA (open triangle), are shown in the left lanes (cDNA). A: Hybridization analysis using the ^{32}P-labeled E4II oligonucleotide as a probe. B: Hybridization analysis using ^{32}P-labeled DM-20 oligonucleotide as a probe. C: ^{32}P-labeled PLP-ONLY oligonucleotide as a probe.

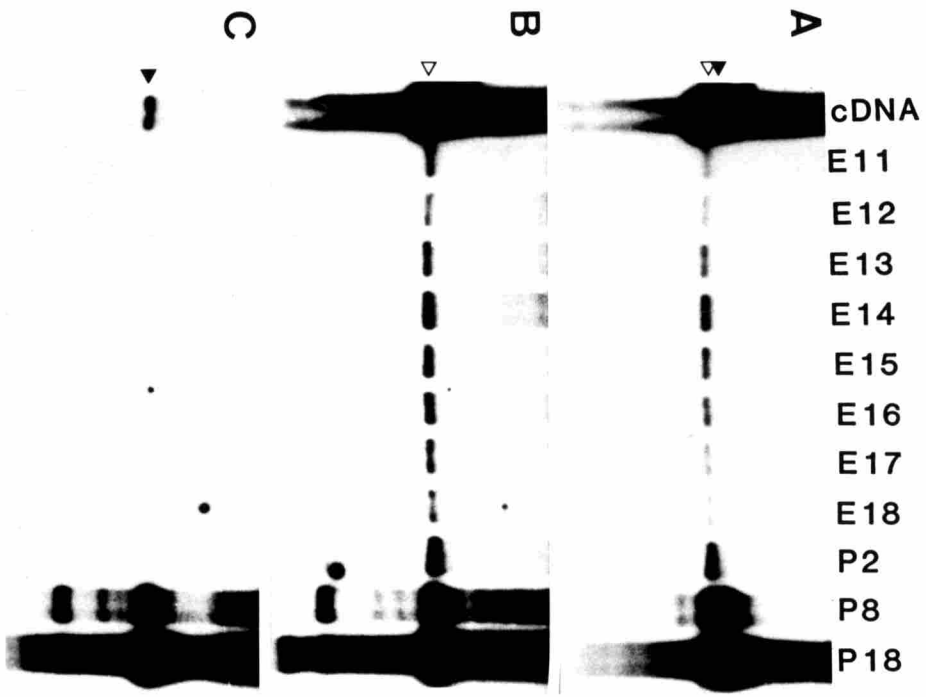
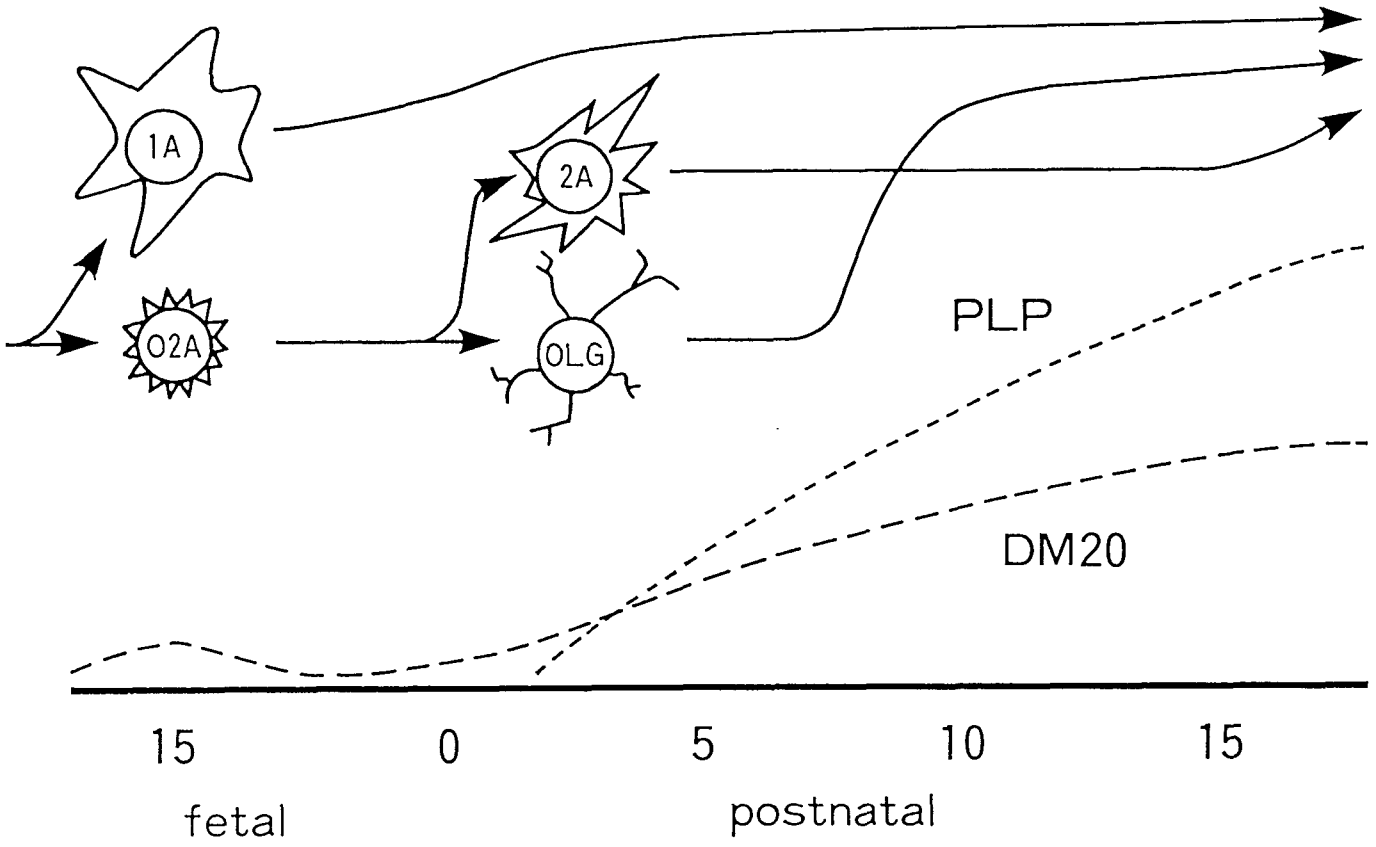

C
B
A
cDNA
E11
E12
E13
E14
E15
E16
E17
E18
P2
P8
P18

Fig. 8. Schematic presentation of the relationship between glial cell differentiation and PLP gene expression.

Only DM-20-mRNA is produced during major gliogenesis and its synthesis continues thereafter. PLP-mRNA appears postnatally and becomes a major transcript of the PLP gene


1A
2A
O2A
OLG
PLP
DM20
15
0
5
10
15
fetal
postnatal

embryonic *jimpy* mouse brain.

All *jimpy* and *jimpy* carrier mice used in this experiment were proven to have *jp*/Y and *jp*/X genotype, respectively, by hybridization with probes, which specifically detect either wild type or *jimpy* type PLP gene (data not shown). Total brain RNAs from E16 and P2 *jimpy* mice and their littermates were subjected to the reverse transcription-polymerase chain reaction (RT-PCR) method (Fig. 9). The amplified products were separated on polyacrylamide gel and electrotransferred to a nylon membrane. As previously described, the size of the amplified products from PLP-mRNA and DM-20-mRNA, were 1023 bp and 918 bp, respectively. Amplified products from *jimpy* brain RNA were expected to have faster mobility than the wild type counterpart because of the absence of the sequence encoded by the fifth exon. The expected sizes of *jimpy* PLP-mRNA and *jimpy*-DM-20-mRNA are 949 bp and 844 bp, respectively. To see the overall amplified pattern the membrane filter was first hybridized with E4II probe, which can detect all of the amplified products; i.e. wild type PLP-mRNA and DM-20-mRNA, and *jimpy* type PLP-mRNA and DM-20-mRNA. Then it was rehybridized with two other probes one by one to clearly light up the molecular species of interest. DM20 probe detect DM-20-mRNA of both type, and E5del probe detect *jimpy* type PLP gene transcripts. DM-20-mRNA was detected in E16 wild type mouse brain as previously described (Fig. 10, lane E16 wild). The amplified product from E16 *jimpy* brain RNA also gave a

Fig. 9. RT-PCR method for detecting the wild type and *jimpy* type PLP gene transcripts.

Annealing sites of the PCR primers, PLP-PCR1 and PLP-PCR2, are shown by arrows. Using these primers a 1023-bp fragment is amplified from PLP-mRNA, a 918-bp fragment from DM-20-mRNA, a 949-bp fragment from *jimpy* PLP-mRNA, and a 844-bp fragment from *jimpy* DM-20-mRNA. White boxes represent open reading frames, and the region spliced out in DM-20-mRNA and *jimpy* type mRNA were indicated. Bars show the hybridizing sites of the oligonucleotide probes for detecting the PCR products. Especially, E5ll probe can only detect wild type mRNAs, whereas E5del probe can only detect *jimpy* type mRNAs.

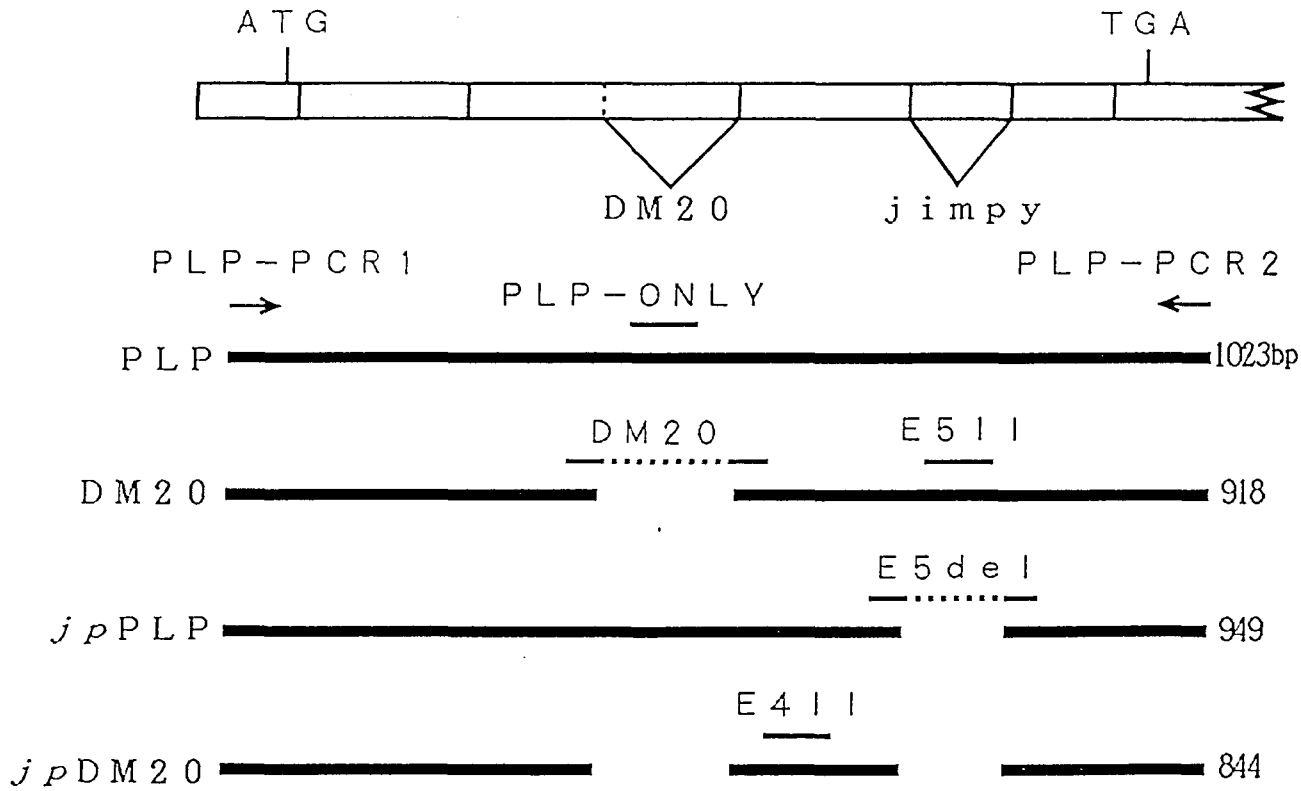
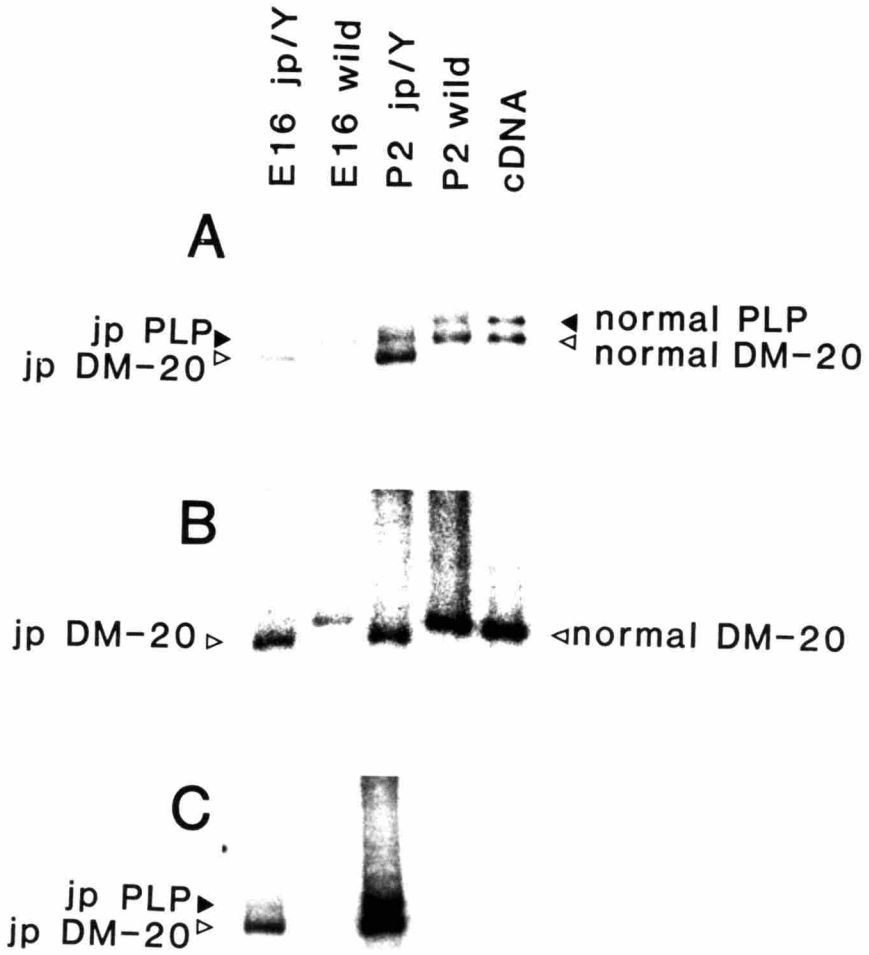

ATG
TGA
DM20
jimpy
PLP-PCR1
PLP-PCR2
PLP-ONLY
PLP
1023bp
DM20
E511
DM20
918
E5del
jpPLP
949
E411
jpDM20
844

Fig. 10. PLP gene expression in *jimpy* brain at early stages

Total brain RNAs from E16 and P2 *jimpy* mice and their littermates were subjected to the reverse transcription-polymerase chain reaction (RT-PCR) method. The size of the amplified products from PLP-mRNA and DM-20-mRNA, were 1023 bp and 918 bp, respectively. The sizes of *jimpy* PLP-mRNA and *jimpy* DM-20-mRNA are 949 bp and 844 bp, respectively. Membrane filter was first hybridized with E4II probe recognizing sequence within exon 4 of the PLP gene, which can detect all of the amplified products (A). DM20 probe can detect DM-20-mRNA of both type (B), and E5del probe can detect *jimpy* type PLP gene transcripts (C).


E16 jp/Y
E16 wild
P2 jp/Y
P2 wild
cDNA
A
jp PLP
jp DM-20
normal PLP
normal DM-20
B
jp DM-20
normal DM-20
C
jp PLP
jp DM-20

single band (Fig. 10, lane E16 *jp*/Y). It had faster mobility than the DM-20-mRNA-amplified product (lane E16 wild), and hybridized to E4II and DM20 probes, as well as E5del probe, which detects *jimpy* type splicing (deletion of the fifth exon) in the transcript. Thus, this band corresponds to the amplified product from *jimpy* type DM-20-mRNA. The amount of DM-20-mRNA in *jimpy* mouse brain was similar to that in wild type mouse brain (lane E16 wild and *jp*/Y).

At P2 another band derived from PLP-mRNA became detectable (Fig. 10, lane P2 wild). This was not observed using E16 or E18 brain (see Fig. 7). Therefore, onset of PLP-mRNA production is at P2 in mouse brain, which is in good agreement with the earlier studies (Gardinier and Macklin, 1988; LeVine *et al.*, 1990). In *jimpy* mouse similar pattern as that in wild type mouse was obtained, although two bands had faster mobility (Fig. 10, lane P2 *jp*/Y). This is caused by skipping of exon 5 as can be proven by hybridization with the E5del probe. The level of PLP- and DM-20-mRNAs in *jimpy* brain were similar to that in wild type brain. Therefore PLP gene expression is regulated normally in *jimpy* mouse brain until the age of P2, including the timing of appearance of PLP-mRNA, although the fifth exon is not utilized.

Expression of DM-20 is not restricted in the glial cell lineage.

I then attempted to identify the types of cells that synthesized DM-20 in these early stages. Astrocytes were the

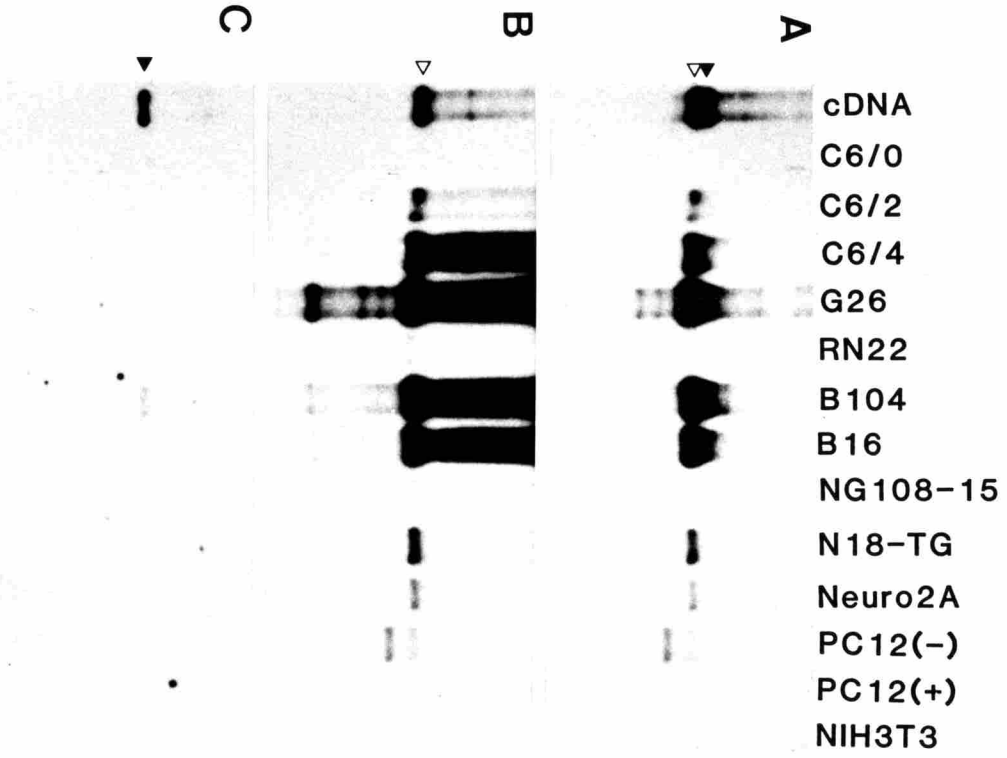
first candidate because hypertrophy had been observed in these cells (Skoff, 1976) and it had been shown that the *jimpy* (*jp*)oligodendrocyte death was prevented by the astrocyte conditioned medium (Bartlett *et al.*, 1988). I analyzed the expression of the PLP gene in primary and secondary astrocyte-enriched culture by the present PCR technic, and detected both PLP- and DM-20-mRNA (data not shown). However, the presence of oligodendrocytes cannot be excluded, and thus, I could not definitely conclude that the PLP gene is expressed in astrocytes.

I therefore analyzed PLP gene expression in C6 glioma, which has been shown to produce PLP-mRNA (Milner *et al.*, 1985). I observed that its expression was induced after removal of the serum from the culture medium by traditional Northern blot analysis (data not shown). Cytoplasmic RNA extracted from C6 cells cultured in serum-deprived medium for 0, 2 and 4 days was analyzed by the new method using the E411 probe. Figure 11A clearly shows that PLP gene expression is induced by serum deprivation (lanes C6/0-4). However, the PLP gene product was not PLP, but was DM-20 (Fig. 11B,C).

Various cell lines of neuronal and glial origin were examined for the production of PLP- and DM-20-mRNA. Figure 11 shows oligodendroglioma G-26 produced quite a large amount of DM-20-mRNA, followed by B104 cell line. In B104 cell small amount of PLP-mRNA was also detected (Fig. 11C, lane B104). However, all of the cell lines tested, including

Fig. 11. PLP gene expression in various neural cell lines.

PLP gene expression in rat glioma C6 cells, 0, 2 and 4 days after removal of the serum from the culture medium (C6/0, C6/2, C6/4, respectively), mouse G-26 oligodendroglioma cells, rat RN-22 Schwannoma cells, rat B104 neuroblastoma cells, mouse B16 melanoma cells, NG108-15 cells, mouse N18TG neuroblastoma cells, mouse Neuro 2A neuroblastoma cells, rat PC12 pheochromocytoma cells with or without NGF-treatment (50 ng/ml for 2 days) (PC12(+) and PC12(-), respectively) and mouse NIH3T3 fibroblast was examined as described above. A: Hybridization analysis using the ^{32}P-labeled E4II oligonucleotide as a probe. B: Hybridization analysis using ^{32}P-labeled DM-20 oligonucleotide as a probe. C: ^{32}P-labeled PLP-ONLY oligonucleotide as a probe.


C
B
A
cDNA
C6/0
C6/2
C6/4
G26
RN22
B104
B16
NG108-15
N18-TG
Neuro2A
PC12(-)
PC12(+)
NIH3T3

NG108-15, N18-TG, Neuro 2A and PC12 cells, all of which are commonly used as models for neurons, also produced DM-20-mRNA (Fig. 11). When nerve growth factor (NGF) was added to the PC12 cell and cultured for 2 days, the amount of DM-20-mRNA was apparently reduced (lane PC12(+)).

There were many unidentified bands in cells producing large amount of DM-20-mRNA. These may also present alternative splicing products of the PLP gene as in P8 and P18 mouse brains.

DISCUSSION

DM-20 production at embryonic stages.

In this study, I showed that DM-20 is detected in the E11 mouse brain, when oligodendrocytes or even their direct precursor cells, O2A cells, are believed to be absent. Based on the following criteria, I concluded that the PCR-amplified product is DM-20. First, the size of the amplified product from embryonic brain RNA was found to be identical to that from the DM-20-cDNA clone, using primers hybridizing to the first and the 7th exons of the PLP gene. Second, the amplified product hybridized to the PLP-cDNA (data not shown) and oligonucleotide probes, including DM-20 (Fig. 5C), and E4II (Fig. 5A,B), but not to the PLP-ONLY probe (Fig. 5D, see Fig. 4 for the probes). Finally, it was not detected in the total RNA extracted from mouse liver, NIH-3T3 cells, or NIH-3T3 cells transfected with the PLP expression vector (Fig. 5). Recently, Timsit *et al.* (1992) also showed that DM-20-mRNA is produced at embryonic stage, and that its localization was restricted to the diencephalic basal plate, which at later stage forms nervous system, by in situ hybridization of 10-day embryos. These results support the idea that DM-20 plays functional roles in glial cell differentiation.

Recently, Schneider, *et al.* showed that in *rumpshaker*, another PLP mutant, degeneration of immature oligodendrocytes and dysmyelination are uncoupled, suggesting that the

PLP gene products have bifunctional role, a vital function in glial cell development in addition to that in myelin assembly.

In E16 and P2 *jimpy* (*jp*)mouse brain, the *jimpy* type DM-20-mRNA was produced and its amount in *jimpy* mouse brain was similar to that in wild type mouse brain (lane E16 and P2; wild and *jp*/Y). This provides the first direct evidence to show that the mutated PLP gene is expressed in embryonic *jimpy* brain, which gives molecular basis for the abnormalities in glial cells found in embryonic *jimpy* brain.

Production of DM-20 in non-glial cells.

DM-20 was found as a proteolipid in the myelin membrane, which shows immunocrossreactivity with the major proteolipid protein (PLP) (Lees and Brostoff, 1984). Recently, it has been shown that DM-20-mRNA is produced from the PLP gene by alternative splicing, and 105 nucleotides were deleted from PLP-mRNA to form DM-20-mRNA (Nave *et al.*, 1987a). Thus the primary structure of DM-20 is identical to PLP except for an internal deletion of 35-amino acid residues (116-150) from the major hydrophilic domain. Recent studies on the developmental profile of PLP and DM-20 level demonstrated that the DM-20 protein appears prior to the PLP protein at the early stage of myelination (Gardinier and Macklin, 1988; Levine *et al.*, 1990; Schindler *et al.* 1990). My results confirm and extend the conclusion obtained from these investigations, because it is now clear that DM-20

production is not restricted to the glial cell lineage and does not have to be linked to myelination. At present, I do not know whether mature neurons contain DM-20-mRNA or not, because it is very difficult to obtain pure neuronal populations completely devoid of glial cells. However, it is interesting that the amount of DM-20-mRNA decreased when the PC12 cells were induced to differentiate into neuron-like cells by NGF addition (Fig. 11).

Several unidentified products were detected in this study. Some of these bands can also be observed in NIH/PLP lane in Fig. 5D and these are possibly artifacts during amplification. However, there are several discrete bands other than those bands, as can be seen in lane P8 (Fig. 5C,D) and in lanes G26 and PC12(-) (Fig. 11A,B), which suggest the presence of other alternatively spliced products from the PLP gene. I am currently cloning and analyzing the structures of these products. However, I could not detect such products in embryonic brains, at least under my detection sensitivity (Fig. 7). Thus, it seemed to be more important to clarify the specific function of DM-20 in glial cell differentiation.

Part II : Glial Cell Degeneration and Hypomyelination caused by Over-expression of the Myelin Proteolipid Protein Gene.

INTRODUCTION

Myelin proteolipid protein (PLP) is known to be one of the major myelin structural protein, whose sequences are highly conserved (Lees and Brostoff, 1984; Campagnoni and Macklin, 1988). Its production has been considered to be restricted in oligodendrocytes which form myelin of the central nervous system (CNS). Many point mutations within the X-chromosome-linked PLP gene have been identified. All of them except for the *rumpshaker* mouse (*rsh*)(Schneider *et al.*, 1992), cause dysmyelinating genetic disease in the CNS caused by abnormal oligodendrocyte differentiation/maturation, such as *jimpy* (*jp*)(Hogan and Greenfield, 1984; Macklin *et al.*, 1987; Nave *et al.*, 1986 and 1987b; Moriguchi *et al.*, 1987) and *jimpy^{med}* (Gencic and Hudson, 1990) in mouse and Pelizaeus-Merzbacher disease (PMD)(Gencic *et al.*, 1989; Hudson *et al.*, 1989) in human. In the "Part I" of this study, I showed that DM-20, an alternatively spliced transcript of the PLP gene (Nave *et al.*, 1987a), was produced from brain at embryonic day (E) 11 long before oligodendrocytes or their direct progenitor cells appear in the CNS of mouse. DM-20-mRNA was also produced in various neural cell lines and in sciatic nerve, suggesting that DM-20, but not PLP, can function as a differentiating factor for oligodendrocytes and possibly for all glial cells.

To investigate specific function of DM-20 in vivo, it seemed ideal to produce transgenic mice producing DM-20 or

PLP from the integrated transgene and to transfer those transgenes into *jimpy* mice by crossing the transgenic mice with the *jimpy* heterozygotes. I expected to observe partial or full recovery of *jimpy* phenotype by forcing production of wild type PLP and/or DM-20 in *jimpy* mouse. Previously, I generated transgenic mice carrying the PLP minigenes (1.5 kb of mouse PLP-promoter, 1.4 kb of rat PLP-cDNA, 2.1 kb of the 3'-region of the mouse PLP gene). However, the regulatory regions of the transgene were apparently insufficient for its expression and there was no expression from the transgene. In this study, I cloned the whole PLP genome from the cosmid library and used to produce transgenic mice. The PLP-transgenic mice overexpressing the wild type PLP gene at a level 5 times more than the wild type control showed dysmyelination similar to *jimpy*, and less overexpressing mice (3-fold) showed demyelination in the CNS. This observation is interesting since mutations within one gene and overexpression of the particular gene caused a similar phenotype. These results strongly indicate that DM-20 is not only one of the structural proteins of myelin, but plays a crucial in the glial cell differentiation and/or its survival.

RESULTS

Cloning of the PLP gene.

Since the PLP-minigene was not expressed in the transgenic mouse system, I planned to use entire PLP genome to produce transgenic mouse. My PLP genomic clones λ23 and λ38 did not encompass entire PLP gene (Ikenaka *et al.*, 1988). The lambda clone λ23 contained the promoter region, the first exon and a part of the first intron of the PLP gene, while λ38 contained the rest of the exons. Thus, I cloned the PLP gene from the cosmid library (Little, 1987; Okano *et al.*, 1991). The wild type mouse genomic cosmid library constructed from the DNA of C57BL/6 mice liver (6 x 10^5 colonies) were screened with the ^{32}P-labeled rat PLP cDNA probe, and 16 candidates were selected. Next, an oligonucleotide probe specific to the 7th exon of the PLP gene (probe; XH-primer) was used for the 2nd screening, and 9 candidates were obtained. Finally, an oligonucleotide probe specific to the 1st exon of the PLP gene (probe; PLP-promoter) was used for the 3rd screening, and 6 positive clones were isolated. DNAs from these clone were digested with BamHI, EcoRI and HindIII, and the pattern of digested fragments were compared with those from λ23 and λ38 (Ikenaka *et al.*, 1988). I obtained 4 clones which carry the whole seven exons of PLP gene. The cosmid clone PLP-4e was used in the following experiments.

To map the restriction sites of these clones, DNA from

PLP-4e was digested with BamHI, EcoRI, HindIII and PvuII, and were fractionated by electroforesis through 0.7% agarose gel. They were transferred to a nylon membrane filter and hybridized with the DNA fragments obtained by restriction enzyme digestion of the clone, PLP-4e, to determine the overlapping regions of these fragments. The clone PLP-4e contained a 40 kb genomic DNA fragment encompassing all of the seven exons of the mouse PLP gene as well as 20 kb of 5'-flanking and 4 kb of 3'-flanking sequence (Fig. 12), which was expected to include entire transcriptional regulatory elements. Three other independent clones had the same restriction sites in the overlapping region (Fig. 12).

Construction of the PLP transgenic mouse.

The cosmid plp-4e DNA was digested with the λ-terminase, and the resulting protruding ends were filled in with Sequenase™ (United States Biochemical Corporation). The linearized DNA was introduced into the germ line of BDF1 (C57/BL6J x DBA/J F1 hybrid) mice by microinjection into the pronucleus of fertilized eggs (Hogan *et al.*, 1986). Sixty-five mice were born and the presence of the transgene was identified by polymerase chain reaction (PCR) analysis of the region indicated by '4ePCR' in Fig. 12. Only one transgenic mouse line was obtained. The genomic DNA from this mouse was further analyzed by the Southern blotting method using various fragments of cosmid plp-4e as probes indicated in Fig. 12 (data shown in Fig. 13). The transgene was inher-

Fig. 12. A physical map of the cosmid clone plp-4e.

Open boxes indicated seven exons of the PLP gene (I-VII). Closed box indicated cosmid vector Lorist B. The thick underlines indicated fragments used in the Southern blot analyses. Genotypes of all transgenic mice and *jimpy* were determined by the PCR-amplification of the regions indicated by brackets, 4ePCR and jpPCR (5, 21, 24), respectively. Alphabets show the restriction enzyme recognition sites. Three other independent clones had the same restriction sites in the overlapping region. B; BamHI, C; λ-terminase, E; EcoRI, H; HindIII.

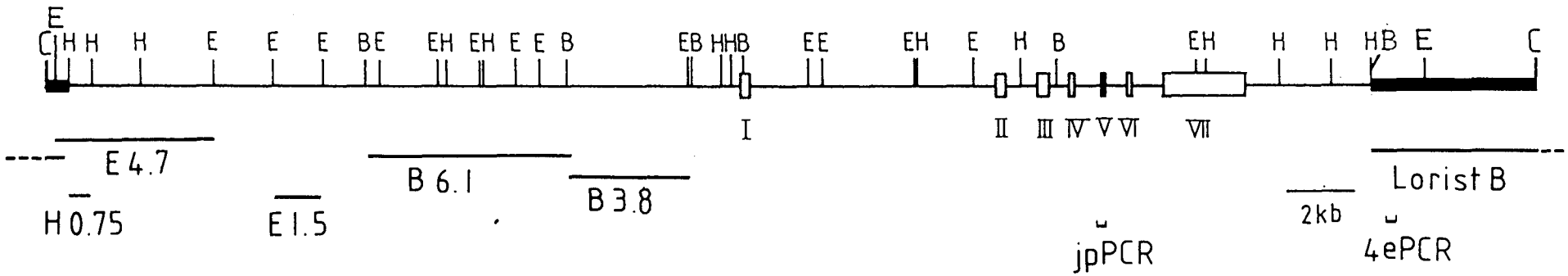
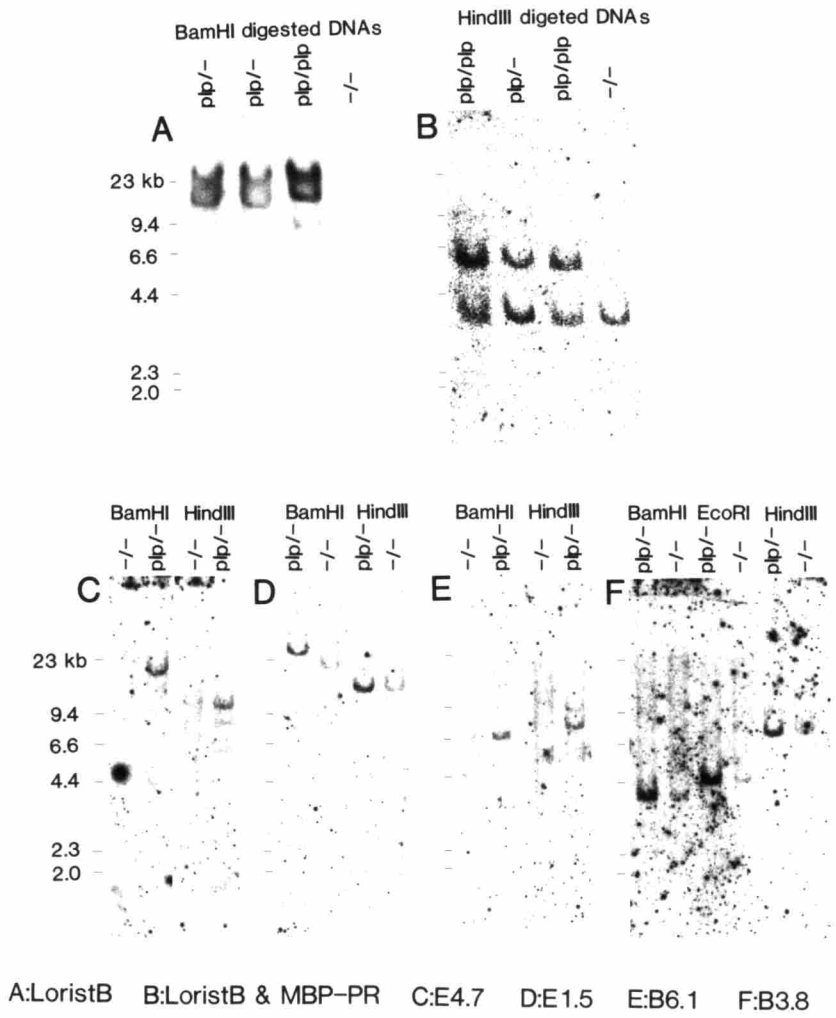

E
C H H H E E E B E E H E H E E B E B H H B E E E H E H B E H H H B E C
I
II III IV V VI VII
E 4.7
H 0.75
E 1.5
B 6.1
B 3.8
jpPCR
2kb
4ePCR
Lorist B

Fig. 13. Southern blot analysis of DNAs from transgenic mice.

The method for Southern blotting is described in "MATERIALS AND METHODS". The DNA fragments of cosmid clone PLP-4e used as probes are indicated in Fig. 10. Restriction enzymes used in individual blottings are described above the figures. Each data suggested that the copy number of the integrated PLP transgenes to be two. A: LoristB as a probe. This data suggested that the integrated transgene was 2 copies and that they were tandemly repeated. B: LoristB and 1.5 kb of a 5' flanking region of the mouse MBP gene as probes. This experiment was used to discriminate between homozygotes and heterozygotes. C: the region indicated E4.7 as a probe. D: the region indicated E1.5 as a probe. E: the region indicated B6.1 as a probe. F: the region indicated B3.8 as a probe.



A:LoristB B:LoristB & MBP–PR C:E4.7 D:E1.5 E:B6.1 F:B3.8

ited in an autosomal fashion (Fig. 14) and its copy number was estimated to be two (Fig. 13A and B). For reliable discrimination between homozygote and heterozygote, Southern blot analysis was performed using two probes simultaneously; vector region of 'LoristB' and the promoter region of MBP gene as an internal control (Fig. 12 and 13B).

Introduction of the PLP transgene into the *jimpy* mouse.

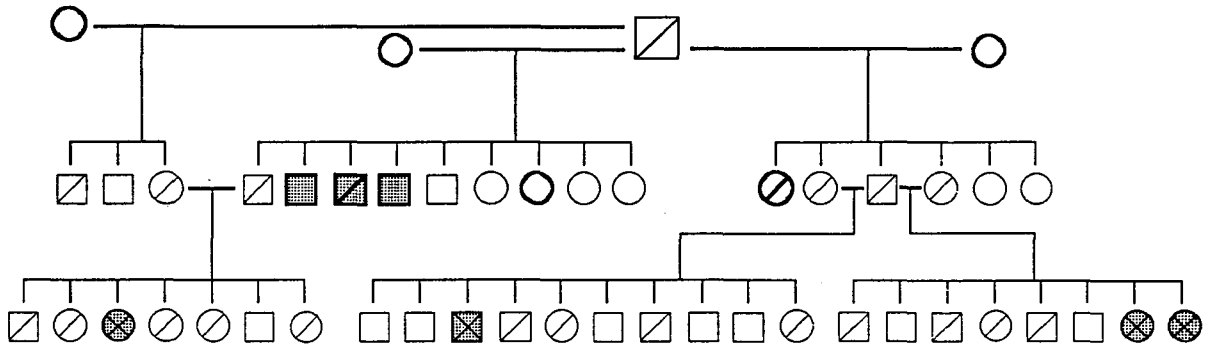
To investigate whether introduction of the wild type PLP gene rescue the defects in *jimpy*, the founder male mouse (X/Y;, *plp*/-) was backcrossed to *jimpy* female heterozygote (*jp*/X;-/-) so that the PLP transgene can be transferred into *jimpy* male hemizygote (*jp*/Y; *plp*/-), which does not contain wild type PLP gene endogenously. Genotypes of all transgenic mice and *jimpy* mice were determined by the PCR analysis (see Materials and Method, Fig. 12. The *jimpy* mice carrying exogenous wild type PLP genes (2 copies) showed intentional tremor and died before P30 by tonic convulsion. Therefore, the defects in *jimpy* did not recover by introducing wild type PLP gene.

Tissue specific expression of the PLP transgene.

Introduction of the PLP transgene was not able to recover the defects of *jimpy* mouse. I thus examined the expression of the PLP transgene in various tissues. Total RNA from tissues of the transgenic *jimpy* mouse heterozygous for the PLP transgene and control mice (X/Y and *jp*/Y) were

Fig. 14. Family tree for the production of the transgenic mice homozygous for the PLP transgene (*plp/plp*).

The transgene was inherited in an autosomal fashion. The transgenic mice homozygous for the PLP transgene (*plp/plp*) generated in wild type background exhibit a quite similar phenotype to that of *jimpy*. Shade; striking phenotype, thick line; jimpy type PLP gene carrier.



	X/Y	X/X	jp/Y	jp/X
-/-	□	○	□	○
plp/-	▨	⊘	▨	⊘
plp/plp	⊠	⊗		

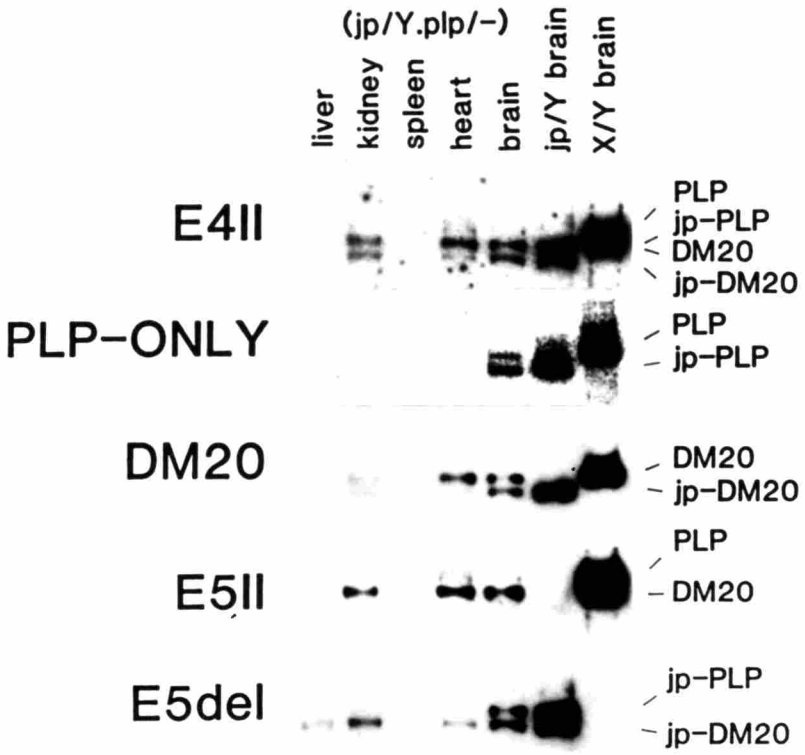
prepared, and they were analyzed by 30 cycles of reverse transcription-PCR (RT-PCR), which amplify the entire PLP coding region. The PCR products were fractionated on a polyacrylamide gel, electrotransferred to a nylon membrane and hybridized with various probes to detect all 4 types of the PLP gene products as described in Part I (Fig. 9). Expression of the wild type PLP transgene can be detected independently from that of the endogenous *jimpy* type PLP gene by using E5II probe. The transgene as well as the endogenous *jimpy* PLP gene was expressed mainly in the brain and in some other tissues (PLP in liver and kidney, and DM-20 in liver, kidney and heart) (Fig. 15). Both the wild type PLP transgene and *jimpy* type PLP gene were expressed in the brain of *jimpy* mouse carrying the PLP transgene (*jp*/Y, *plp*/-), although the level of transcripts from the transgene was far less than that from the endogenous PLP gene of control wild type littermates (compare lane brain of *jp*/Y; *plp*/- and X/Y in Fig. 15, E5II). These results suggest that expression from the PLP transgene was insufficient to rescue the *jimpy* phenotype, if there were no mutations in coding region of the transcripts from the PLP transgene.

Cloning and sequencing of the PLP- and DM-20-cDNA from exogenous PLP gene transcripts.

Total RNA from brain of the transgenic *jimpy* mouse heterozygous for the PLP transgene was prepared, and entire PLP coding region was amplified by 25 cycles of RT-PCR with

Fig. 15. RT-PCR analysis of the PLP transgene expression.

Total RNAs from various tissues of mice brains were amplified by RT-PCR and hybridized with E4ll oligonucleotide probe, which can detect all PLP gene transcripts, or probes specifically detecting DM-20 (DM-20), PLP (PLP-ONLY), the fifth exon (E5ll), and the region for exon 4-6 junction (E5del). Positive controls, which were amplified from *jimpy* and wild type brain RNAs, are shown in the right lanes.


(jp/Y.plp/-)
liver
kidney
spleen
heart
brain
jp/Y brain
X/Y brain
E4II
PLP
jp-PLP
DM20
jp-DM20
PLP-ONLY
PLP
jp-PLP
DM20
DM20
jp-DM20
E5II
PLP
DM20
E5del
jp-PLP
jp-DM20

PLP-PCR1 and PLP-PCR2 as PCR primers. The PCR products were purified by polyacrylamide gel electrophoresis, and the DNA fragments were electroeluted. The fragments digested with BamHI and SacI were purified with Geneclean II system (Bio101), and were inserted into a BamHI-ScaI site of a plasmid vector BluescriptKS$^-$. The extracted plasmid DNAs were checked by digestion with BamHI and SacI, and the PLP- and DM-20-cDNAs were isolated. Five clones of PLP-cDNA and one clone of DM-20-cDNA were sequenced by dideoxy-chain termination method of Sanger *et al.* (1980), from M13 primer, reverse primer and primers hybridizing to appropriate regions of PLP-cDNA. No mutations were detected in coding region of the transcripts from the PLP transgene.

Phenotype of the transgenic mouse homozygous for the transgene (*plp/plp*).

I thus tried to increase the PLP transgene dosage by producing mice homozygous for the integrated PLP transgene in the *jimpy* genetic background (*jp*/Y; *plp/plp*). First, I generated the transgenic mouse homozygous for the PLP transgene (*plp/plp*) in wild type background to raise the efficiency of its production. Surprisingly, the transgenic mice homozygous for the PLP transgene (*plp/plp*) obtained by mating male and female heterozyous for the transgene (*plp*/-), exhibited an unusual phenotype after about 2 weeks of ages; ataxia, intentional tremors, and tonic convulsions, which finally lead them to early death at about 30 days of

age, just like the *jimpy* mouse except that the symptoms were also observed in the female mice. Appearance of this unexpected phenotype was inherited in a typical autosomal Mendelian fashion, while the original PLP mutants were X-linked (Fig. 14).

PLP-production in brains of homozygote (*plp/plp*) and its wild type littermate was studied immunohistochemically at 19 days of age, by which time PLP gene expression achieve its maximum. The wild type mouse (-/-) brain was immunostained for PLP intensely in the white matter of the cerebellum and the medulla oblongata (Fig. 16). Although brain from the transgenic mice (*plp/plp*) harbor one copy of endogenous wild type PLP gene and 4 more copies of wild type PLP transgene (total 5 copies), they were mostly negative for PLP-staining (Fig. 17), just like *jimpy* (*jp*) (Fig. 18). At higher magnification of the white matter of cerebellum from homozygote (*plp/plp*), the PLP staining was strikingly reduced (Fig. 19A), as compared with that from control mouse (-/-), in which the staining was observed along nerve fibers, indicating PLP localization in myelin (Fig. 19B). To ask whether myelin was normally formed in the transgenic homozygote (*plp/plp*), I immunostained adjacent section for myelin basic protein (MBP), another major myelin protein. MBP production in homozygote (*plp/plp*) brain was also much less than that in wild type control (-/-)(compare Fig. 19B with D), however it seemed to be less severely affected than the PLP production (compare Fig. 19B with A). These phenó-

Fig. 16. Immunochemical staining for PLP of sagittal sections from the wild type mouse (-/-) brain at P19.

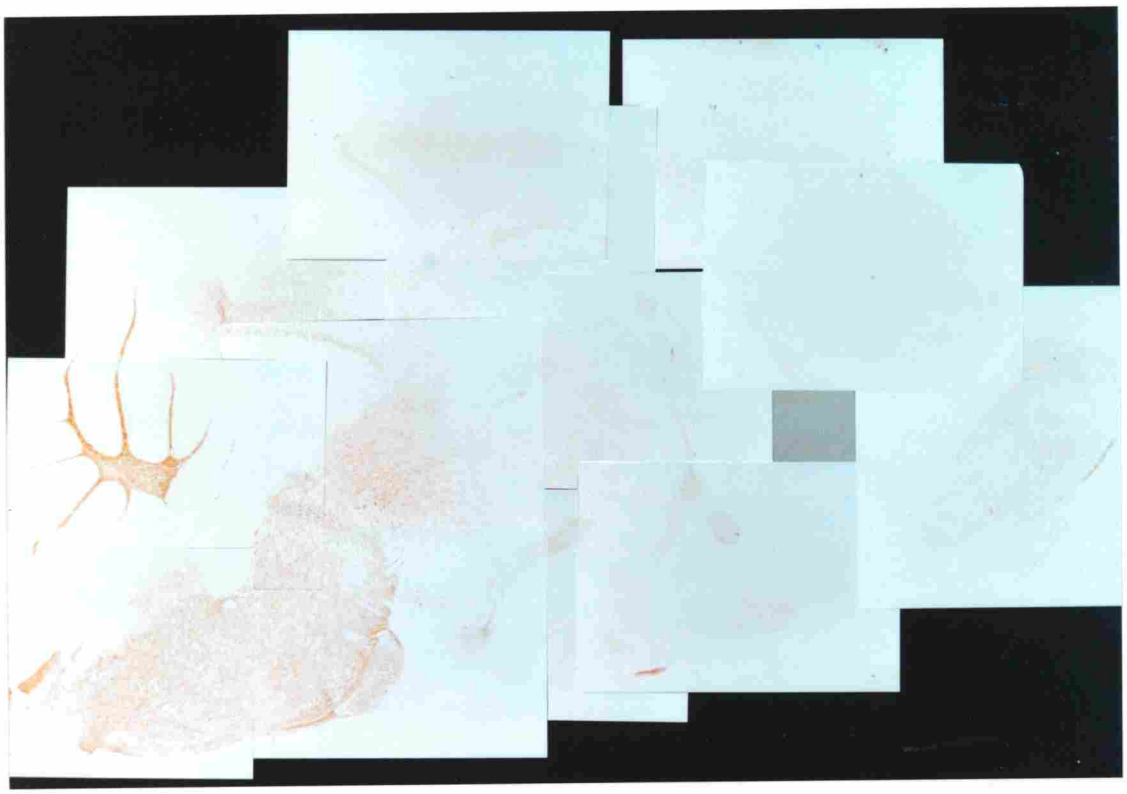


Fig. 17. Immunochemical staining for PLP of sagittal sections from the homozygote (*p/p/p/p*) brain at P19.

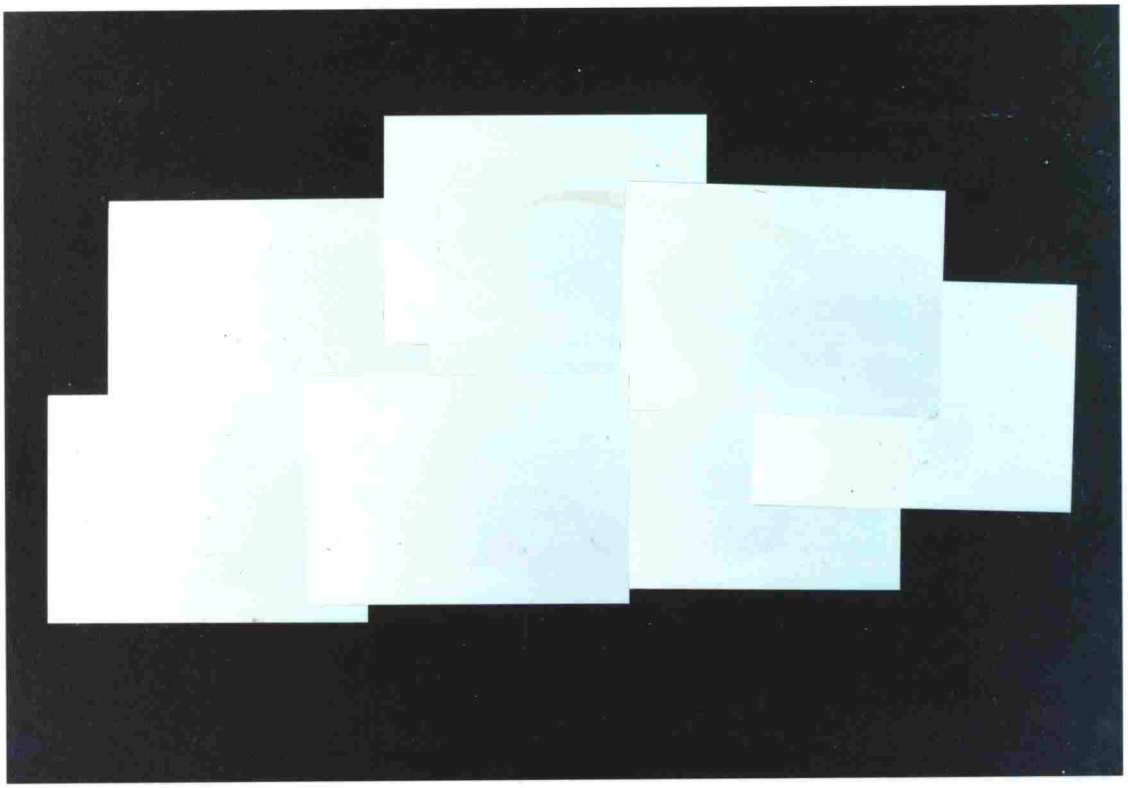


Fig. 18. Immunochemical staining for PLP of sagittal sections from the *jimpy* hemizygote (*jp*/Y) brain at P19.

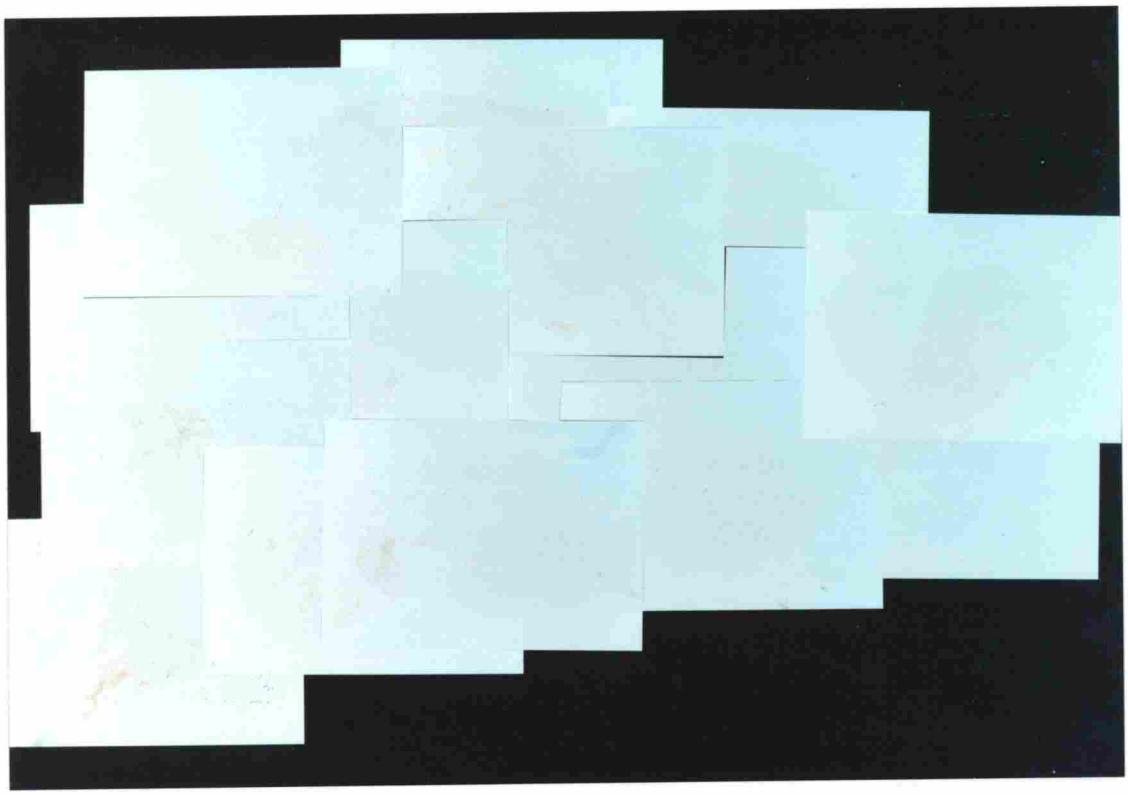
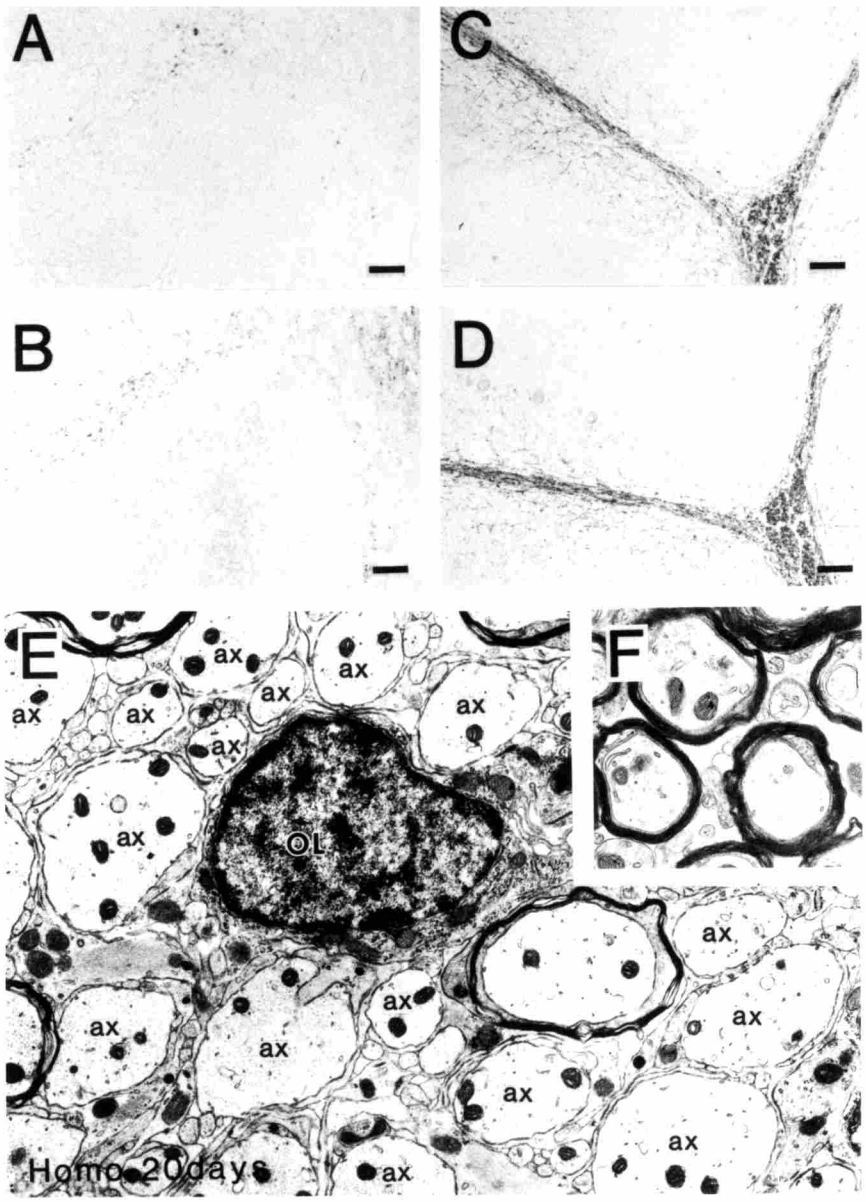


Fig. 19. Immunohistochemical studies and electronmicrographs of sagittal sections of cerebella from 20-day-old homozygote (*plp/plp*) and control mouse (-/-).

Paraffin sections from homozygote (A and B) or control mice (C and D) were immunostained for PLP (A and C) and MBP (B and D), respectively (25). E is an electronmicrograph of white matter from homozygote cerebellum, which revealed the presence of numerous unmyelinated axons. Axons of age-matched control mouse were well myelinated (F). OL, degenerated oligodendrocyte: ax, unmyelinated axons. Bar = 50 μm. Electronmicrographs (E and F) were taken by Prof. Inoue (Hokkaido Univ.).

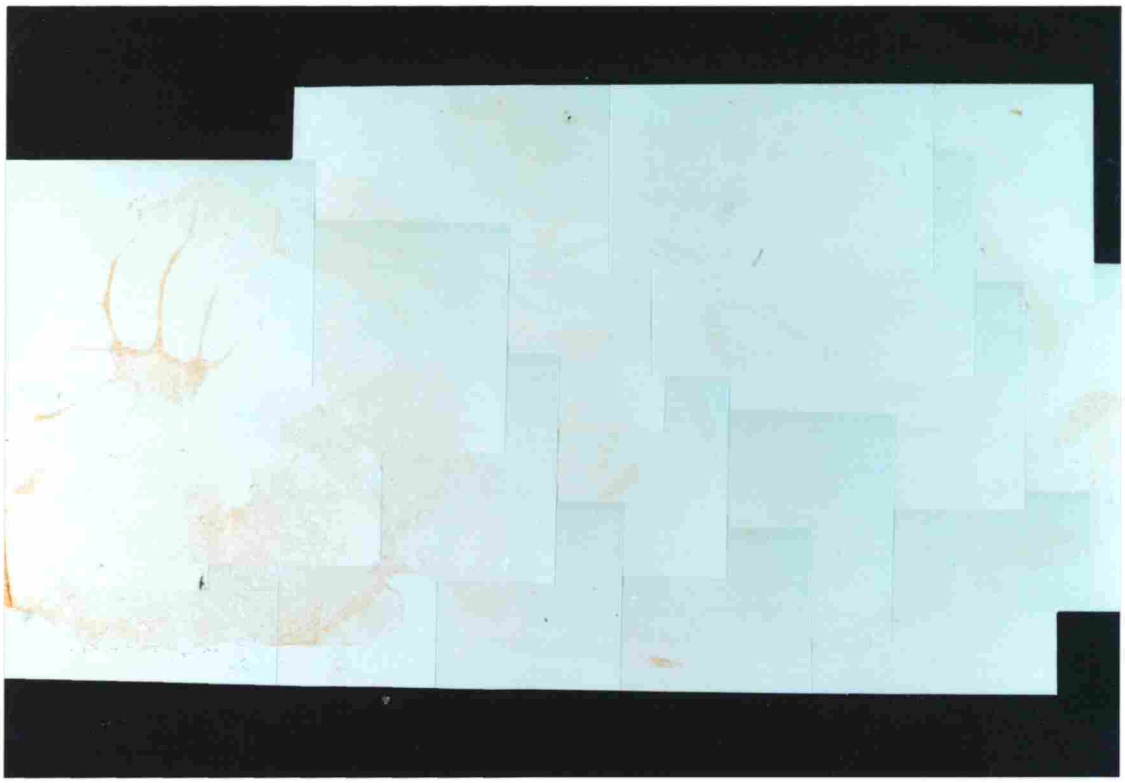

A
C
B
D
E
F
ax
OL
Homo 20days

type was strikingly similar to that of *jimpy* mice (Fig. 18 for PLP-staining). Wild type littermate (-/-) was strongly positive for both PLP and MBP immunostaining. Electronmicroscopic analysis of the homozygote (*plp/plp*) showed that the majority of axons were either naked or surrounded by a disproportionally thin myelin sheath in white matter of the transgenic mouse (*plp/plp*) cerebellum (Fig. 19E). Degenerating oligodendrocytes were also observed, which was rarely observed in wild type mice (-/-). These phenotypes were quite similar to those of *jimpy* (Mikoshiba *et al.*, 1989).

Phenotype of the transgenic mouse heterozygous for the transgene (*plp/-*).

The transgenic mice heterozygous for the transgene (*plp/-*) (with 2 more wild type PLP gene) exhibited normal behavior which was indistinguishable from the wild type littermates. However, they began to show hind limb tremor after several months of age, and soon later convulsion occurred all over the body. Brains of the heterozygotes (*plp/-*) were immunostained for PLP. There were no great difference in the PLP-staining pattern between heterozygotes (*plp/-*)(Fig. 20) and wild type littermates (-/-)(Fig. 16) at P19, although the staining was slightly decreased in the heterozygotes (*plp/-*). Production of the myelin proteins, when heterozygote (*plp/-*) began to exhibit tremors, was next examined. In wild type mouse (-/-) brain, immunostaining for PLP increased at 6 months of age (-/-) compared to the

Fig. 20. Immunochemical staining for PLP of sagittal sections from the heterozygote (*p/p/-*) brain at P19.



staining at P19 (Fig. 21). Heterozygote (*plp*/-) brain showed unusual decrease of PLP-staining (Fig. 22). At higher magnification, wild type mouse (-/-) brain showed intense staining for PLP along nerve fibers (Fig. 23, -/-), whereas heterozygote (*plp*/-) showed non-uniform patchy pattern of staining, suggesting seemed to show destruction of myelin (demyelination, Fig. 23, *plp*/-). To investigate the abnormality in myelin structure in the heterozygote (*plp*/-), the section from each stage was immunostained for MBP. At P20, MBP was produced in both heterozygote (*plp*/-)(Fig. 24A) and wild type mouse (-/-)(Fig. 24B) brains at almost the same level. Electronmicrographic analysis showed that myelin formation was slightly reduced at P20 and most of oligodendrocytes contained degenerated vacuolar structures (Fig. 24C), eventhough the mice exhibited normal behavior. However, several months later when convulsion occurred all over the body, severe paucity of MBP-staining was observed as inthe case of PLP-staining (Fig. 24D), indicating destruction of myelin (demyelination). Electron micrographic analysis of the heterozygotes (*plp*/-) at this stage clearly showed degenerated myelin sheaths and numerous demyelinated axons (Fig. 24F). Thus, demyelination occureed in the transgenic mouse carrying two extra copies of wild type PLP gene.

PLP gene expression in the transgenic mice.

Next I examined the level of PLP gene transcripts at E16 and P18. Total RNAs were extracted from brains at lit-

Fig. 21. Immunochemical staining for PLP of sagittal sections from the wild type (-/-) brain at 5 months after birth.

Fig. 22. Immunochemical staining for PLP of sagittal sections from the heterozygote (*p/p/-*) brain at 5 months after birth, which showed hind limb tremor.

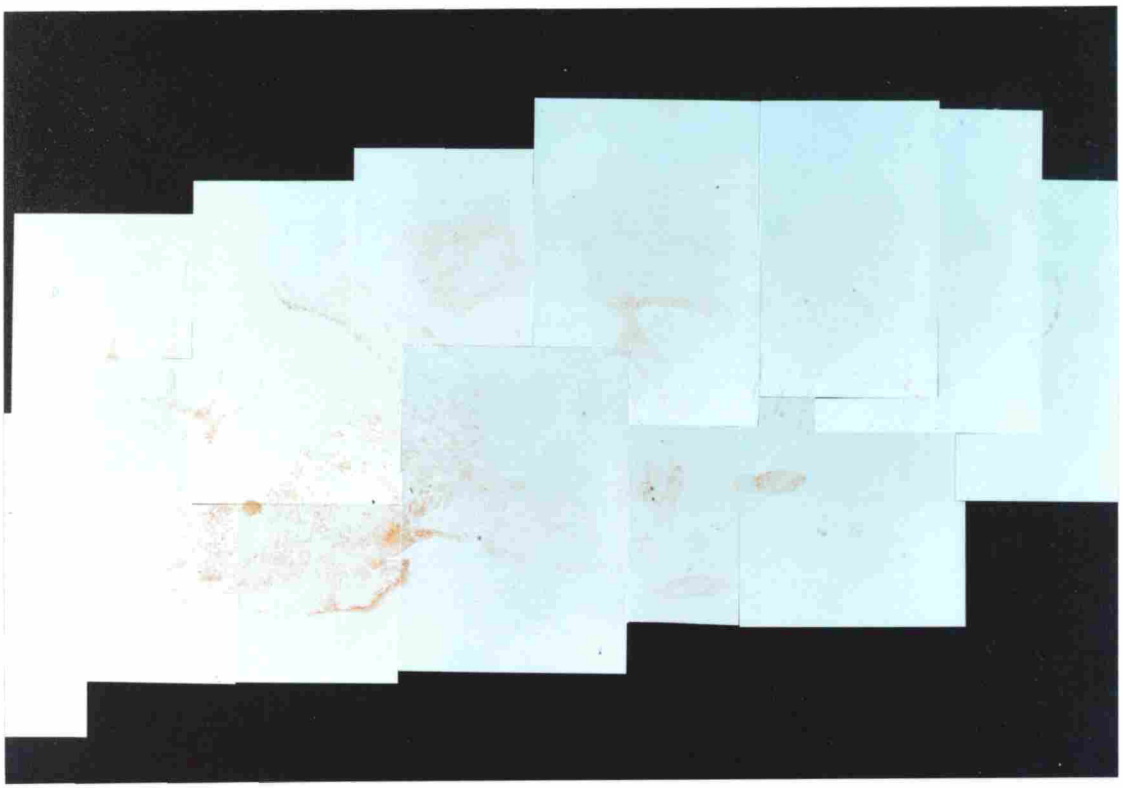


Fig. 23. Immunochemical staining of heterozygote (*p/p*/-) and wild type mouse (-/-) brains at 6 months after birth.

Sagittal sections of cerebella from heterozygote (*p/p*/-, left panels) and wild type mouse (-/-, right panels) were prepared and stained immunohistochemically using antibody against PLP (D2).

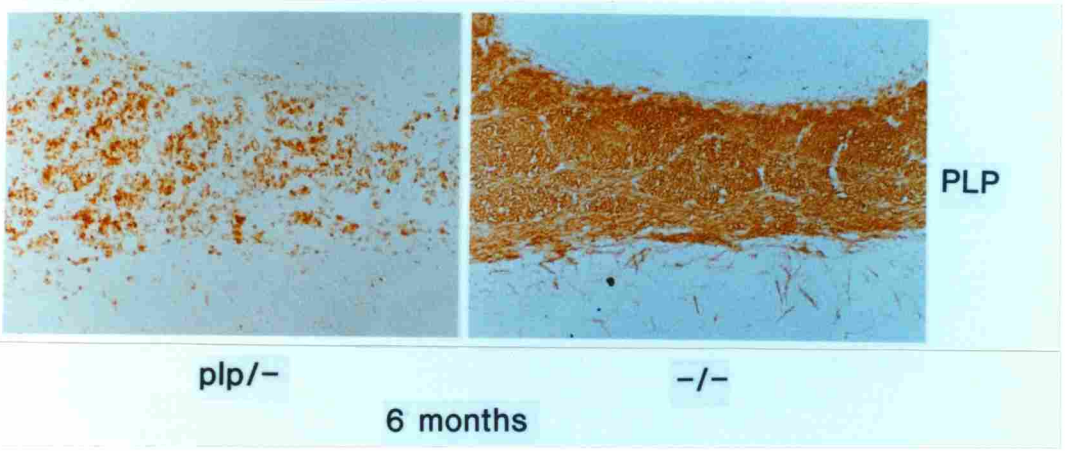
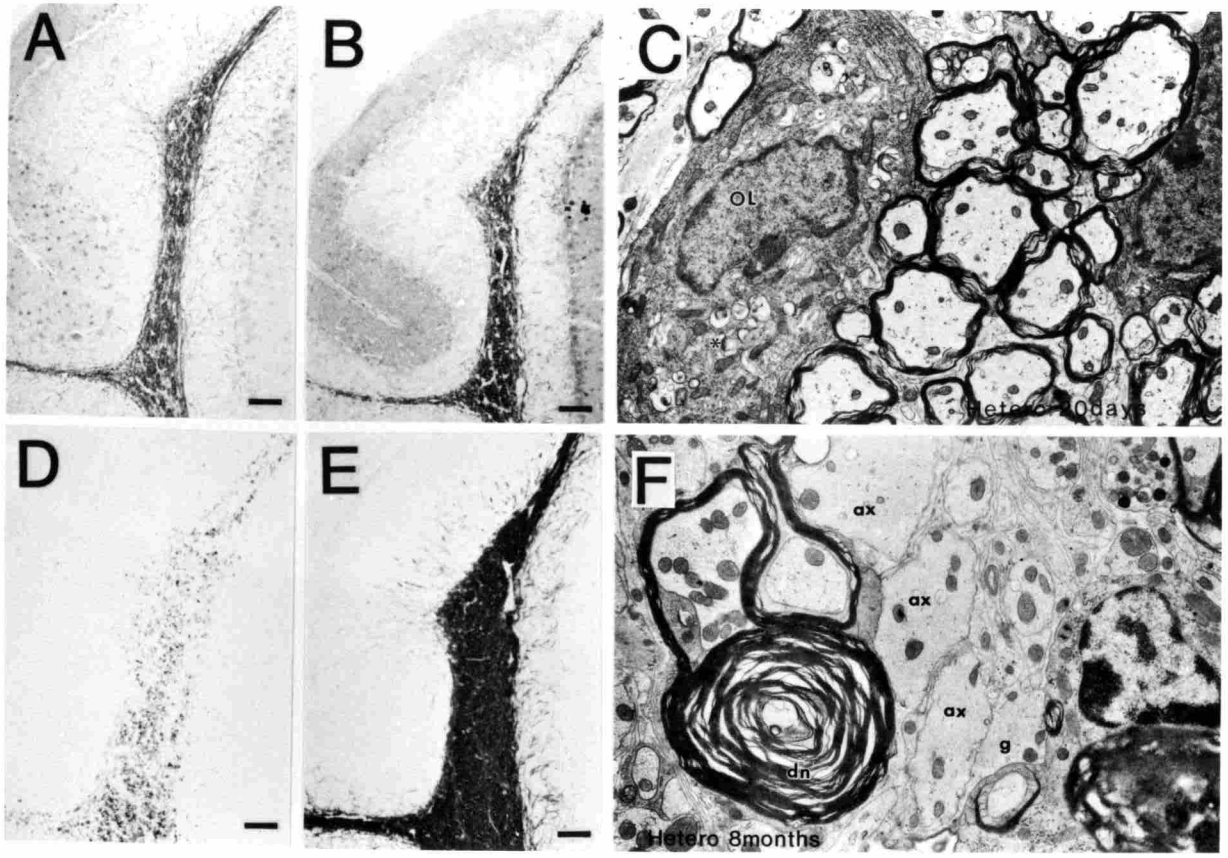

PLP
plp/–
–/–
6 months

Fig. 24. Immunohistochemical staining for MBP and electronmicrographs in sagittal sections of cerebella from heterozygote (*p/p*/-) and control mouse (-/-).

Paraffin sections of 17-day-old (A and B) and 6-months-old (D and E), heterozygote (plp/-)(A and D) and its wild type littermate (-/-)(B and D) were immunostained for MBP, respectively. C and F are electronmicrographs of white matter from 20-day-old and 6-months-old heterozygote (plp/-) cerebella, respectively. OL, degenerated oligodendrocyte: *, vacuoles not seen in a control mouse: ax, unmyelinated axons: dn, degenerated axon and myelin: g, glial fiber. Bar = 50 μm. Electronmicrographs (C and F) were taken by Prof. Inoue (Hokkaido Univ.).


A
B
C
OL
Hetero 10days
D
E
F
ax
ax
ax
g
dn
Hetero 8months

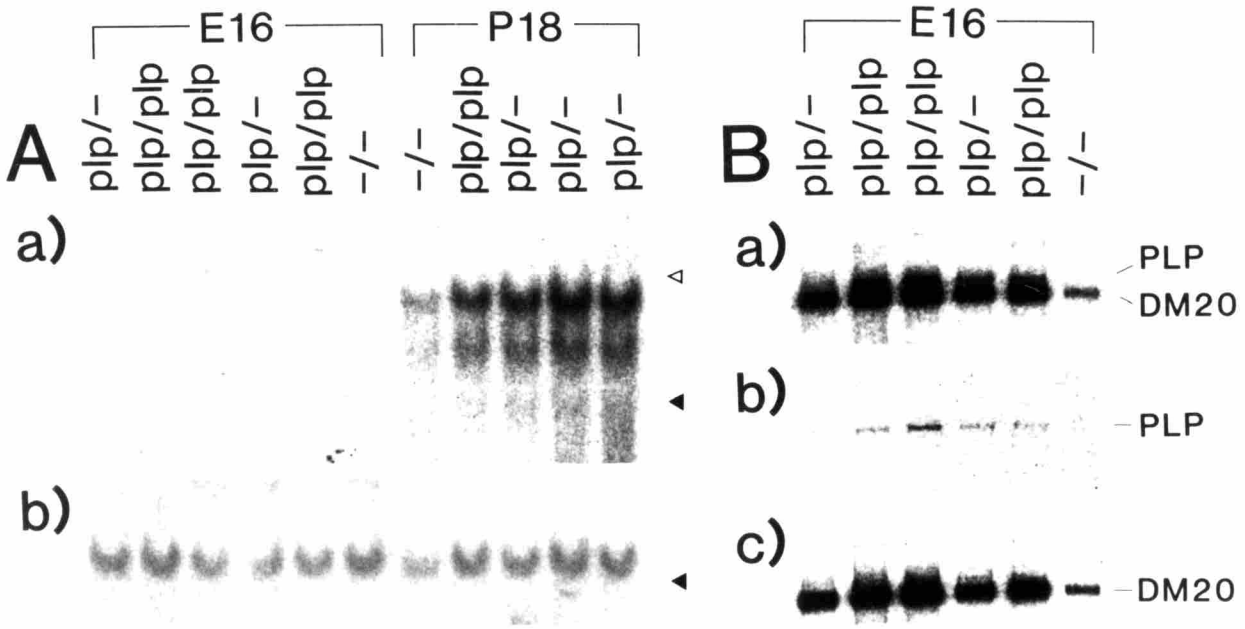
termate mice of each stage, and analyzed by the Northern blotting with a radiolabeled PLP cDNA probe (Ikeneka et al., 1992), which can detect both PLP and DM-20 mRNAs. PLP gene expression at E16 were too low to be detected by the Northern blot analysis (Fig. 25A). Three species of PLP- and DM-20-mRNA were detected in P18 mouse brain; 3.2 kb, 2.4 kb and 1.6 kb in size, known to be produced by alternative usage of polyadenylation sites (Campagnoni et al., 1988; Ikenaka et al., 1988). The ratio of these three mRNAs in transgenic mouse brain was essentially the same as that in normal mouse (Fig. 25A). Total amounts of PLP- and DM-20-mRNA in heterozygous transgenic mouse brain (*plp/-*) increased three to four times more than those in normal mouse brain (-/-) reflecting the over dosage of the PLP gene in these mice (3 copies, compare Fig. 25A, P18 lanes *plp/-* with -/-). The PLP/DM-20-mRNA level in the transgenic mice homozygous for the transgene (*plp/plp*) was approximately twice as much as that in wild type control (Fig. 25A, P18 lanes *plp/plp*) even though this mouse carries 5 copies of the PLP gene. Degeneration of oligodendrocytes responsible for production of most of the PLP gene transcripts in the homozygote might be the reson for this discrepancy.

To detect the PLP gene expression at the embryonic stage, more sensitive RT-PCR method was applied. I have shown that the PLP gene products can be detected semiquantitatively (Fig. 5) by choosing appropriate number of amplification cycle. I used probes that specifically detect PLP

Fig. 25. PLP gene expressions in transgenic and wild type mouse brains.

A: Northern blot analysis using mouse PLP-cDNA (a) or chicken β-actin (b) as a probe. Three species of PLP- and DM-20-mRNA were detected in P18 mouse brain; 3.2 kb, 2.4 kb and 1.6 kb in size, known to be produced by alternative usage of polyadenylation sites. The ratio of these three mRNAs in transgenic mice brains were essentially the same as that in normal mice. Triangles indicate the positions of the ribosomal RNAs.

B: RT-PCR analysis using E4ll (a), PLP-ONLY (b) or DM-20 (c) as a probe. Twenty-five cycles of PCR was performed.


A
E16
P18
plp/–
plp/plp
plp/plp
plp/–
plp/plp
–/–
–/–
plp/plp
plp/–
plp/–
plp/–
a)
b)
B
E16
plp/–
plp/plp
plp/plp
plp/–
plp/plp
–/–
a)
b)
c)
PLP
DM20
PLP
DM20

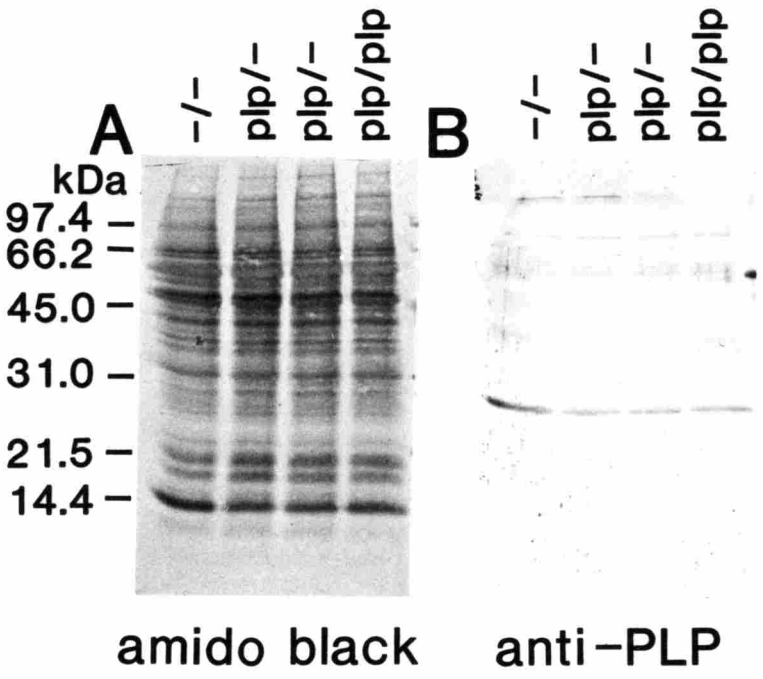
mRNA, DM-20-mRNA or both. In this case, the level of DM-20-mRNA roughly followed the PLP gene dosage. That is 5 times more DM-20-mRNA level in homozygotes (*plp/plp* in Fig. 25B) and 3 times more in heterozygotes (*plp/-* in Fig. 25B) than in wild type mice. The level of DM-20-mRNA at E16 seems to determine the severity of the symptom appearing later in these mice; 5 times higher level of DM-20-mRNA causes dysmyelination and 3 times higher level causes demyelination. Moreover, PLP-mRNA was detected in the transgenic mice brains. In wild type mouse brain, PLP-mRNA becomes detectable after oligodendrocytes maturation and can never be detected at these early stages.

Western blotting analysis.

The PLP/DM-20 mRNAs were overproduced in the brain of the transgenic mouse homozygous for the PLP transgene (*plp/plp*) at P18, whereas very little amount of PLP prtein was detected immunohistochemically. To investigate reason for this discrepancy, Western blot analysis, a more quantitative method than immnohistochemical detection, was applied. Whole brain from littermate mice at P20 were homogenized in solubilizing buffer containing 2% SDS solution, immersed in a boiling water bath, and ran on 17.5% polyacrylamide gel. After electrotransferring to a nitrocellulose membrane, PLP was detected with an anti-PLP antibody against sequence 110-127 of rat PLP (D2)(Fig. 26B). Anti PLP antibody (D2) could not detect DM-20 protein because most of the

Fig. 26. Western blot analysis of myelin proteins in transgenic mouse brains.

Whole homogenates of brains from each mice were analyzed by Western blotting method using antibodies against PLP (B). The amounts of PLP were almost normal in the brains of both homozygote (plp/plp) and heterozygote (plp/-) at P20 (B). A: amido black staining.


A
-/-
plp/-
plp/-
plp/plp
kDa
97.4
66.2
45.0
31.0
21.5
14.4
B
-/-
plp/-
plp/-
plp/plp
amido black
anti-PLP

region recognized by D2 antibody is deleted in DM-20 (amino acid residue 116-150 of PLP). The level of PLP was almost normal in the brains of both homozygote and heterozygote at P20 (Fig. 26B). This result is consistent with the results obtained from the Northern blotting analysis or the immuno-histochemical analysis.

DISCUSSION

Overexpression of the PLP gene before myelinating stage causes abnormal differentiation/maturation of oligodendrocytes.

In "Part I" of my study, I presented several circumstantial evidence that the PLP gene products, especially DM-20, play an important role in glial cell differentiation.

As a next step to investigate the function of the PLP gene products, I overexpressed the PLP gene in mouse brain by producing transgenic mouse with extra mouse PLP genes. Surprisingly, the transgenic mice homozygous for the PLP transgene (bearing 4 more PLP gene) exhibited oligodendrocyte degeneration and dysmyelination in the CNS, while heterozygotes (with 2 more PLP gene) showed normal myelination at active myelinating stage, but later developed demyelination.

It is still unknown why the oligodendrocytes degenerated in the transgenic mouse homozygous for the PLP transgene (*plp/plp*). The level of DM-20-mRNA at E16 correlated well with the gene dosage as well as the severity of the symptom that later appear in these mice, while the level of PLP gene expression at P18 did not. When DM-20-mRNA was produced at a level 5 times more than the wild type control in embryonic mouse brain, degeneration of oligodendrocyte was observed even before the myelinating stage, resulting in dysmyelination. At a 3-fold level, oligodendrocyte differentiated to

form myelin, but later, oligodendrocyte degenerated and caused demyelination. These results suggested that overproduction of DM-20 at the embryonic stage causes abnormal oligodendrocyte differentiation/maturation.

Since I could get only one PLP-transgenic mouse strain, it is possible that all of the results described above was caused by insertion mutagenesis of a gene that is important for oligodendrocyte differentiation. One argument against this is an autosomal dominant inheritance of the abnormal phenotype, although possibility of haploid insufficiency still remains. Another strong argument was obtained by K.-A. Nave et al., who also generated another PLP transgenic mouse exhibiting similar phenotype to our transgenic mouse (personal communication). Therefore, overdosage of the PLP gene, which led to overproduction of DM-20 at embryonic stage, caused abnormal oligodendrocyte differentiation or survival.

The *jimpy* PLP gene shows dominant effect over wild type PLP gene.

I introduced wild type PLP gene into the *jimpy* genetic background (*jp*/Y; *plp*/-) to ask whether the PLP transgene was sufficient to rescue the *jimpy* phenotype, however it could not rescue the *jimpy* phenotype. The expression level from the PLP transgene might be insufficient to rescue the *jimpy* phenotype, or the *jimpy* PLP gene products might cause some abnormalities in glial cells even in the presence of wild type PLP gene products. There are some lines of evi-

dence for this dominance of the *jimpy* PLP gene over wild type PLP gene. While the PLP transgene introduced into wild type mouse was expressed as much as the endogenous PLP gene (Fig. 25), the same transgene introduced into *jimpy* was expressed at much lower level than the endogenous PLP gene in the wild type mouse brain (compare Fig. 15, lane brain from *jp*/Y; *plp*/- with X/Y brain). This indicates that the *jimpy* PLP gene products worked inhibitory for the wild type transgene to be expressed. This speculation was supported by another transgenic study by Nave et al.(personal communication), who introduced *jimpy* type PLP-cDNA into wild type mouse and showed that this transgenic mouse behaved similarly to *jimpy*. Therefore, the phenotype of *jimpy* mouse is caused by the presence of abnormal DM-20/PLP rather than the absence of normal DM-20/PLP. However, all of the PLP mutants are not affected by their abnormal PLP gene products, because complete deletion of the PLP gene has been found in one case of PMD (Raskind *et al.*, 1991).

Possible mechanism for oligodendrocyte degeneration in mice over expressing the PLP gene.

Recently, it was shown that DM-20 expressed in non-oligodendroglial cells was present in the rough endoplasmic reticulum or Golgi apparatus (Timsit *et al.*, 1992). Another group reported that *jimpy* mutation affects the transport of PLP and DM-20 through the Golgi apparatus (Roussel *et al.*, 1987). In my transgenic mice, degenerated vacuolar struc-

ture, which may be generated from Golgi membrane, were found. Since PLP/DM-20 is a very hydrophobic protein, its overproduction may change the membrane structure, which results in abnormal transport of proteins via Golgi apparatus. In the brains of homozygotes at E16, a small amount of PLP-mRNA were also produced, which might be one of the reason for the abnormal Golgi structure.

Some other functions of PLP/DM-20 were also reported previously. PLP/DM-20 could be involved in the regulation of calcium permeability, thus affecting the calcium level in myelin (Diaz *et al.*, 1990). If this is true, PLP/DM-20 overexpression would result in abnormal calcium level, in PLP/DM-20 producing cell, which could exert toxic effects to the cell.

Translational or Post translational regulation of the PLP gene expression.

In this PLP transgenic mouse study, there was a discrepancy between the PLP message level and its protein level at P18-20. Immunohistochemical studies showed that PLP level was strikingly reduced in the homozygote (Fig. 17), while Western blotting analysis showed that the PLP level in the homozygotes (*plp/plp*) was comparable to that in the wild type control (Fig. 26)(though this mouse carries 5 copies of the PLP gene). This discrepancy might be caused by the difference in the process for sample preparation between these two methods. To obtain paraffin sections for immuno-

histochemistry, the transgenic mouse brain went through series of dehydration steps. The organic solvents might have extracted PLP present in abnormal region since there are no myelin membranes, where it is normally transported.

Moreover, the PLP/DM-20-mRNA level in the homozygotes (*plp/plp*) was approximately twice as much as that in wild type control, although this mouse produced only normal level of PLP (Western blotting analysis in Fig. 26). This might have caused by reduced translation rate or by rapid degradation of PLP since there are no myelin where they should assemble. However, the extract mechanism remains to elucidated.

Overexpression of one gene and mutations within that gene can results in similar phenotype.

Point mutations in the PLP gene in several PMD patients has been discovered (Gencic *et al.*, 1989; Hudson *et al.*, 1989; Weimbs *et al.*, 1990). However, in some cases no mutations have been found in PLP coding region of PMD patients (Doll *et al.*, 1992; Patt *et al.*, 1992). My results open a possibility that PLP gene overexpression to be a cause for the PMD without any defects in the PLP coding region. These observations are very similar to what have been reported for the pmp-22 mutants, which exhibit abnormal myelin formation in the peripheral nervous system (PNS). The pmp-22 (PAS-II/SR13/GAS-3) gene was suggested as a candidate gene for *trembler* mouse (Suter *et al.*, 1992) 11) and Charcot-Marie-

Tooth disease type 1A (CMT1A)(Timmerman *et al.*, 1992; Valentijn *et al.*, 1992; Matsunami *et al.*, 1992; Patel *et al.*, 1992) in human. This pmp-22 was initially identified as a growth arrest specific (GAS-3) gene (Schnider *et al.*, 1988), and later shown to encode a myelin protein considered to be a PLP/DM-20 analog in the peripheral nervous system (PNS)(Welcher *et al.*, 1991; Hayasaka *et al.*, 1992). The *Trembler* mouse carries a point mutation in the pmp-22 gene, while CMT1A patients have a gene duplication in about 1.5 Mb region on the chromosome 17p11.2 containing the pmp-22 gene, which is similar to the situation in our present study. However, since the duplicated region is very large, it had to be proven whether duplication of the pmp-22 gene, which presumably results in its overproduction, is the direct cause of the CMT1A disease. Our study clearly proved it to be true.

CONCLUSIONS

DM-20 has been widely believed to be merely a minor form of PLP, and no function other than that of PLP has been considered (Lees and Brostoff, 1984). The results presented in this study strongly suggest that DM-20 is not only one of the structural proteins of myelin, but plays some other roles, possibly in the pathway involved in glial differentiation, for the following reasons. First, it is detected in the embryonic mouse brain long before myelinating stage and even in non-oligodendroglial cells (Part I). Second, overexpression of the PLP gene (as DM-20-mRNA) before the stage for myelination, as well as mutations within the PLP gene, affected oligodendroglial cell differentiation, which resulted in hypomyelination (Part II).

MATERIALS AND METHODS

Antibodies and chemicals.

Antipeptide polyclonal antibody directed against the sequence 110-127 of rat PLP (D2)(Shiota *et al.*, 1991) and anti-chicken MBP polyclonal antibody (BP-2, Mikoshiba *et al.*, 1983) were prepared in our laboratory. Dulbecco's modified Eagle's medium (DMEM/high glucose) was obtained from Heizelton Inc. The reagents for immunochemical studies were obtained from Vector Lab., Inc. ECL system for Western blot was obtained from Amersham Japan, Inc. Most of the restriction enzymes otherwise indicated were obtained from TaKaRa Shuzou Co., Ltd. Oligonucleotides were synthesized using an *Applied Biosystems model* 380B DNA *synthesizer*. *Taq* DNA polymerase (Replitherm™) was obtained from Epicentre Technologies. General regents were obtained from Wako Pure Chemical Ind., Ltd.

Wild-type mice and mutant mice.

The ICR mice strain was obtained from Japan SLC, Inc. and used for analysis of the PLP gene expression. The *jimpy* (*jp*) mutant mice (B6CBA/J-*jp*)(Hogan and Greenfields, 1984) were gift from Dr. S. Tsuji (Wakayama Medical College), originally obtained from Jackson laboratories, and were maintained in our laboratory. The *jp* males (*Tajp*/Y and *jp*/Y), females jp carriers (*Tajp*/X and *jp*/X) and normal mice (X/X and X/Y) were identified by gene diagnosis de-

scribed in another paragraph.

Cell culture.

Rat C6 astrocytoma cells, mouse N18-TG neuroblastoma cells, NG108-15 cells (hybridoma of C6 and N18-TG), mouse G-26 oligodendroglioma cells, rat RN-22 Schwannoma cells, mouse Neuro 2A neuroblastoma cells, mouse B16 melanoma cells, rat B104 neuroblastoma cells, and mouse NIH3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM/high glucose) supplemented with 10% fetal calf serum (FCS; Bioproducts, Inc., lot. OM0032). To induce the PLP gene expression in C6 cells by serum deprivation the cells were cultured in DMEM without FCS. Rat PC12h pheochromocytoma cells were maintained in DMEM supplemented with 10% FCS and 5% horse serum. The PLP-producing NIH3T3 cells were obtained by transfection with a PLP-SPUD plasmid [PLP-minigene, Moloney murine leukemia virus-long terminal repeat (MoMLV-LTR): mouse PLP cDNA: SV40-promoter: E.coli-lacZ: MoLV-LTR)(see Fig. 6. for its structure). Details of this procedure will be described in the following section.

PLP-cDNA transfection into fibroblast cells: Calcium phosphate co-precipitation method (Gorman, 1985).

The packaging cells, ψ2 (a derivative of NIH3T3) were plated on a 25 cm^2 dish at 0.5×10^4 cells/cm^2. On the next day, 10 μg of the plasmid PLP-SPUD and 1 μg of the plasmid pSV2neo were mixed with 0.47 ml of 1x HBS (0.818% NaCl,

0.594% Hepes, and 0.02% Na_2HPO_4 pH7.12) and 31μl of 2M $CaCl_2$, and the resulting precipitates were pipetted onto the cells. After incubating for 4 hours in a CO_2 incubator, the cells were washed with serum-free medium, and incubate for 2 minutes in 0.5 ml of 15% glycerol in HBS. Then the glycerol solution was replaced with DMEM containing 10% FCS. After 2 days, G418 (GIBCO) was added at a final concentration of 1 mg/ml to obtain G418-resistant transformants. Two weeks later, the cells were treated with 0.5 ml of a 1:1 mixture of 2 mM fluorescein-di-β-D-galactopyranoside (FDG) and DMEM for 1 min at 37°C. Ice-cold DMEM with 10% FCS (4.5 ml) was added, and the cells were incubated for 60 min on ice. Generated fluorescein was detected with an inverted microscope (IMT-2, Olympus, Tokyo, Japan) equipped with a confocal laser scanning system (MRC-500 Confocal Imaging System, Nippon Bio-Rad Laboratories, Tokyo, Japan). Positive colonies of fluorescent cells were isolated, expanded and used for further experiment.

Preparation of High Molecular Weight mouse DNA.

Genomic DNAs from mouse tissues were extracted according to the method described in "Molecular Cloning" (Sambrook *et al.*, 1989). Mouse liver was homogenized with 10-20 times volume of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 20 mM EDTA, and 1% SDS or 1% sarcosyl containing 100 μg of proteinase K (Sigma, P-0390) per ml, and incubated at 50°C for 4 hours with gentle agitation. Then, equal volume of phenol-

chloroform [TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA)-saturated phenol mixed with an equal volume of chloroform] was added and gently shaked for 30 min. The mixture was separated by centrifugation at 10,000 r.p.m. for 10 min, and the upper aqueous layer without any interphase was transferred to a fresh tube. This extraction step was repeated 3 times. To remove remaining organic reagents, the aqueous solution was dialyzed overnight against TES (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM NaCl) overnight. After transferring the solution into a fresh tube, RNase A (Sigma, R-5125) was added at final concentration of 100 μg/ml and incubated at 37°C for 2 hours. The phenol-chloroform extraction procedure was repeated again as mention above and the aqueous solution was dialyzed against TE. The DNA concentration was determined by measuring U.V. absorption at 260 nm.

Cosmid Library Construction and Screening.

The cosmid library was constructed according to Ish-Horowicz and Burke (1981). High molecular weight DNA was extracted from C57BL/6 mouse liver, and partially digested with HindIII. Fragments of approximately 30-50 Kb were isolated after ultracentrifugation through 10%-40% sucrose gradient, and dephosphorylated by a calf intestine alkaline phosphatase (CIP, Boehringer Mannheim, Molecular biology grade). A LoristB vector was digested with SstI or BstEII, and dephosphorylated by CIP treatment. Both vector samples were further digested with HindIII and ligated with the

HindIII-digested DNA fragments described above. The ligation mixture was subsequently packaged into phage particles, mixed with a culture of E. Coli. strain ED8767 and plated on L-Broth plates containing 40 mg/l of kanamycin.

Colonies ($6x10^5$) were transferred onto Colony/Plaque Screens (E.I. du Pont de Nemours & Co.), fixed with 0.5 N NaOH for 5 min, and hybridized with 1.5-Kb BamHI-SacI fragment of rat PLP cDNA (p27; Milner *et al.*, 1985) ^{32}P-labeled with the random priming method as described below. Twenty-five ng of the DNA fragment was dissolved in 9 μl of distilled water and boiled for 15 min. One μl each of 0.5 mM dATP, 0.5 mM dGTP and 0.5 mM dTTP, 2 μl of 10 x reaction mixture, which contained 62.5 O.D. units/ml random hexamer DNA, 0.5 M Tris-HCl pH 7.2, 0.1 M $MgCl_2$, 1 mM dithioerythoritol and 2 mg/ml of bovine serum albumin, and 1 μl of 2 units/μl Klenow fragment (Boehringer Mannheim, DNA labeling grade) were added. Second and 3rd screenings were carried out with ^{32}P-end-labeled oligonucleotide probes, a PLP-XH-primer (5'-GTTATCTGTTATATTA-3') derived from the 3'-region of the mouse PLP gene (Ikenaka *et al.*, 1988) and PLP-Promoter (5'-GGAAACGAGCCTGTCTC-3') derived from the 5'-flanking region of the mouse PLP gene.

Production of Transgenic Mice.

Hybrid mouse strain C57/BL6J x DBA/J, obtained from Japan SLC, Inc., was used for production of transgenic mice (Hogan *et al.*, 1986) and kept in a room with light period of

7 am-7pm. Four- to 6-week old BDF1 (C57/BL6J x DBA/J F1 hybrid) mice were injected intraperitoneally with 5 IU of gonadotropin from pregnant mare's serum (PMS, Sigma, SIG. 4877) dissolved in 0.1 ml of 0.9% NaCl at 3 pm, and 46-47 hours later with 5 IU of human chorionic gonadotropin (hCG, Sigma, SIG. CG2) dissolved in 0.1 ml of 0.9% NaCl. Each superovulated female was placed with a stud male, and was checked for a copulation plug the next morning. After cervical dislocation, fertilized eggs were dissected out from the oviduct of the female at about 10-11 am.

Sterile stud male mice for generation of pseudopregnant recipients were produced by vasectomy. The vas deferens of a male mouse was cut off, under anesthesia with 10 x [body weight(g) + 11]μl of 2.5% Avertin (100% Avertin; 10 g of tribromoethyl alcohol with 10 ml of *tert*-amyl alcohol). To confirm the sterility of the operated males, each male was examined by mating with at least 2 females before using in this study. Pseudopregnants were produced by mating with these sterile males.

Seven μg of the cosmid clone PLP-4e was digested with λ-terminase (λ-terminase system; Amersham) and the resulting protruding end was filled-in with 75 μM dNTPs and 13 units of Sequenase™ (United State Biochemical Corporation). After phenol extraction, the DNA solution was dialyzed against 50 mM NaCl, 5 mM Tris-HCl (pH 7.4), and 0.1 mM EDTA, to concentration of 5 μg/ml, and microinjected into the pronuclei of fertilized one-cell-stage eggs from superovulated SLCBDF1

mice oviducts (C57BL/6JCr x DBA2Cr F1, Japan SLC, Inc). About 100 copies of DNA were injected into the pronucleus. The eggs were washed in M2 medium and were kept in M16 medium (Whittingham, 1971). After injection they were incubated for several hours in M16 medium under 5% CO_2 atmosphere at 37°C. The eggs were transferred to the oviducts of pseudopregnant SLCBDF1 female mice.

Preparation and PCR analysis of the Tail DNA.

Mouse tail (1 cm in length) was cut off under ether anesthea and was incubated at 50°C overnight with gentle agitation in a solubilizing solution [50 mM KCl, 10 mM Tris-HCl (pH 8.3) 2.5mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP40 and 0.45% Tween 20 containing 200 μg/ml Proteinase K (Sigma)]. The reaction was stopped by immersing the tubes in a boiling water bath for 15 min. Ten μl of the solution was used for polymerase chain reaction (PCR). The reaction mixture contained in a final volume of 100 μl 1x Replitherm™ buffer [10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.005% Tween20, 0.005% NP-40, 0.001% Gelatin] 10 nmol each of dNTPs, 100 pmol each of 2 pairs of the PCR primers, I4I and jp-PCR (the expected amplified region is indicated as jpPCR in Fig. 11), or 4e-primer-2 and 4e-primer-3 (indicated as 4ePCR in Fig. 11), and 2.5 units of Replitherm™ thermostable DNA polymerase (Epicentre Technologies). The PCR was carried out for 30 cycles in a programmable heat block (Techne, PHC-1) as follows: 95°C for 1 min, 55°C for 2

min, and 72°C for 3 min with the final extension step for 10 min. PCR-products were fractionated by polyacrylamide gel electrophoresis [5% acrylamide, 0.17% N,N'-methylene-bisacrylamide, 1x TBE (89 mM Tris-borate and 2 mM EDTA, pH8.0), 0.083% ammonium persulfate and N,N,N',N'-tetramethylethylenediamine). and stained by ethidium bromide.

For *jp* gene diagnosis, 1 or 2 μl of the PCR products were dot blotted onto 2 sets of Nylon membrane filters (Zeta-Probe, Bio-Rad), and treated with 0.5 N NaOH and neutralized 2x SSC (20xSSC: 17.5% NaCl, 8.8% sodium citrate, pH 7.0). One filter was hybridized with 5'end-labeled oligonucletide probe, jp-E5, at 50°C in a solution containing 20 times moles of non-labeled oligonucleotide competitor, PLP-E5, 5xSSC, 50mM sodium phosphate buffer (pH 6.5) 1% SDS, 10xDenhart's (1xDenhart's; 0.02% Ficoll, 0.02% polivinylpyrolidone, 0.02%BSA), and 100 μg of herring sperm DNA. Another one was hybridized with 5'end-labeled PLP-E5 probe instead of jp-E5 in a same solution containing non-labeled jp-E5 instead of PLP-E5. These were then washed and exposed to an imaging plate of Bio-Image Analyzer BAS2000 (Fuji Film). The sequences of the oligonucleotides used in this experiment were as follows: 141, 5'-TTATGAAGTTTACTCTGGCTGCT-3'; jp-PCR, 5'-CACTCTTCTCATTCACTTACCT-3'; jp-E5, 5'-TGGGAGAACACCCAASGATACATAA-3'; PLP-E5, 5'-TGGGAGAACACCIAAGATACATAA-3'.

Southern Blotting Analysis.

Chromosomal DNA isolated from mouse livers (P18) or

whole bodies (E16) were digested with BamHI or HindIII, separated by electrophoresis on 0.7% agarose gel, and transferred to a nylon membrane (Zeta-Probe, Bio-Rad) with 0.4 N NaOH by the vacuum blotting method. DNA fragments for probes (the region indicated in Fig. 12) were ^{32}-labeled with Random Primer Labeling Kit (α^{32}P-dCTP, Amersham. Random primed cDNA labeling kit, Boehringer Mannheim). Blots were prehybridized at 42°C for 4-6 hours in 50% formamide, 2x SSPE (1x SSPE; 0.15 M NaCl, 10mM NaH2PO4, 1mM EDTA, pH 7.4), 1% SDS, 0.5% skimmilk, 0.5 mg/ml salmon sperm DNA and 10% dextran sulfate, and then hybridized with ^{32}P-labeled probes (1x 10^6 cpm/ml). After hybridization, blots were washed 3 times in 2x SSC containing 0.1% sodium dodecyl sulfate (SDS) for 5 min at room temperature and 3 times in 0.1x SSC containing 0.1% SDS for 15 min at room temperature. Filters were subjected to autoradiography using an imaging plate for Bio-Image Analyzer BAS2000, or exposed against an X-ray film for 4 days (XAR, Kodak). For rehybridization, the filters were washed in 50% formamide, 2x SSPE and 1% SDS without drying.

Preparation of the total RNA from various cells and tissues.

Total RNA was extracted from mouse tissues and cells according to Sambrook *et al.* (1989). Tissues were homogenized and with approximately 10 times volume of 4.0 M guanidiniumthiocyanate (GTC), 0.1 M Tris-Cl (pH 7.5), 1% β-mercaptoethanol and 0.5% sodium lauryl sarcosinate. After

shearing, they were layered onto a cushion of 5.7 M CsCl, 0.01 M EDTA (pH 7.5) and centrifuged at 48000 rpm for 5.5 hr (Beckman L8-H, 50Ti). The RNA pellets were dissolved in TES (10 mM Tris-Cl, 1 mM EDTA and 0.1% SDS, pH 7.6) and precipitated with 70% ethanol. The RNAs were stored in 50% ethanol at -20°C.

PCR detection of the PLP gene transcripts.

Two μg of a total RNA was spin-dried and dissolved in 20 μl of ice-cold reverse transcriptase buffer (50 mM Tris-HCl pH 8.3, 6 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol) containing 10 units of RNase inhibitor (human placental RNase inhibitor, Boehringer Manheim), 100 pmols of random hexamers (TaKaRa) 1 mM each of the dNTPs, and 24 units of avian myeloblastosis virus reverse transcriptase (Life Science).

The reaction mixture was incubated for 1.5 h at 42°C, and then mixed with 80 μl of PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mg/ml gelatin) containing 100 pmol of PCR primers (PLP-PCR1 and PLP-PCR2), 0.125 mM each of the dNTPs and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Promega). One ng each of mouse PLP-cDNA clone, pKIf171 (isolated by K. Ikenaka, unpublished result), and mouse DM-20-cDNA clone, pC11 (Nave et al., 1987a), were also amplified in the same buffer. Fifteen cycles of PCR were carried out in a programmable heat block (Techne, PHC-1) set to heat at 94°C for 1 min, cool at 55°C for 2 min,

and incubate at 72°C for 3 min. After the addition of fresh *taq* polymerase (2.5 units), 30 more cycles of PCR were carried out.

The PCR products were fractionated by electrophoresis through 5% polyacrylamide gel (described above) and transferred to a nylon membrane filter (Zeta-Probe, Bio-Rad). After treatment with 0.5 N NaOH, the filter was hybridized with 5'end-labeled oligonucleotide probes in a solution containing 10% dextran sulfate, 6xNET (1xNET; 150 mM NaCl, 15 mM Tris-HCl, 1 mM EDTA, pH 7.5), 0.5% SDS, and 5x Denhardt's. The following temperatures were used for hybridization: E4II (5'-CAAGACCTCTGCCAGTAG-3'), 50°C; DM20 (5'-TGCCCACAAACGTTGCGCTC-3'), 62°C; PLP-ONLY (5'-TCGGGATGTCCTAGC-3'), 44°C; E5II (5'-AGATGGACAGAAGGTTGG-3'), 44°C; E5del (5'-TCATTTGGAACATACATTCT-3'), 50°C. Rehybridization were carried out after washing the filters with 50% formamide, 2xSSPE, and 1% SDS.

Northern Blotting Analysis.

Twenty μg of RNA were dissolved in 4.5 μl of distilled water, and followed by addition of 2.0 μl of 10x formaldehyde gel-running buffer (1x buffer; 0.02 M MOPS (pH 8.0), 8 mM sodium acetate and 5 mM EDTA (pH 8.0)), 3.5 μl of formaldehyde, 10.0 μl of formamide, 2 μl of loading buffer (10 mM sodium phosphate (pH 7.0), 0.25% bromophenol blue, and 50% glycerol) and 1 μl of 1 mg/ml ethidium bromide. The samples were separated on 1.5% agarose gel with 1x formalde-

hyde gel-running buffer containing 2.2 M formaldehyde and capillary-transferred to a nylon membrane (Zeta-Probe, Bio-Rad) with 10x SSC. The filters were baked at 80°C for 2 hours. Hybridization and Rehybridization protocol was the same as that for Southern blotting analysis described above.

Immunohistochemistry.

Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were dissected out and post-fixed overnight in the same fixative at 4°C. After paraffin embedding, serial sagittal sections (6 μm) were prepared on a microtome. After deparaffinization they were rinsed and incubated with 0.5% solution of skim milk (DIFCO) for blocking of non-specific bindings. Tissue sections were incubated overnight at 4°C in 1/200 dilution of rabbit anti-MBP antiserum (BP-2, Mikoshiba *et al.*, 1983), or in 1/200 dilution of rabbit anti-peptide antibody D2 directed against sequence 110-127 of rat PLP (Shiota *et al.*, 1989). They were incubated in 1/200 dilution of biotinylated anti-rabbit IgG at 37°C for 30 min, then incubated in 1/100 dilution of Avidine-Biotin-Peroxidase complex (ABC, Vectastain). They were immersed in a 0.005% solution of diaminobenzidine containing 0.02% hydrogen peroxide. Finally, sections were rinsed, dehydrated in serial alcohols and xylene, and mounted in Permount.

Western Blot Analysis.

Mouse brains were homogenized in distilled water, followed by addition of equal volume of 2x SDS buffer (50 mM Tris-HCl pH 6.8, 10% β-mercaptoethanol, 4% SDS and 5% glycerol) and sheared. Ten μg of the samples were electrophoresed. They were transferred to Hybond ECL nitrocellulose filter (Amersham) using semi-dry electroblotter (Sartorius). The filter was incubated with 5% of skim milk (DIFCO) in PBS-T (PBS containing 0.2% Tween 20) for blocking of non-specific bindings at 4°C for 2 hours and incubated overnight at 4°C with the anti-PLP-antibody (D2) described above. The immunoreactive products were visualized according to the ABC method as described previously, and developed in DAB (3,3'-diaminobenzidene) or ECL system (Amersham).

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