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Myelin-deficient rat: a point mutation in exon III (A → C, Thr75 → Pro) of the myelin proteolipid protein causes dysmyelination and oligodendrocyte death

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The expression of the proteolipid protein (PLP) gene of the myelin deficient (md) and normal rat was studied during the myelination period. The sizes of the PLP transcripts (1.6 and 3.2 kb) in the md and normal rat were identical although the md PLP messenger RNA level was extremely reduced as shown by *in situ* hybridization and Northern blot hybridization analysis. The structure of the md proteolipid protein gene was analyzed on the cDNA and genomic level. The molecular basis of the myelin deficiency phenotype has been elucidated: a point mutation in exon III (A → C transversion) verified by cDNA and genomic DNA sequencing causes a mutation of Thr75 to Pro and creates an additional *Ava*II restriction site in exon III of the md rat. The threonine to proline mutation located within the second transmembranal α-helix might induce a conformational change and thereby prohibit the integration of PLP into the membrane with the clinical manifestation of dysmyelination leading to premature death within 3-6 weeks.

Key words: dysmyelination/myelin-deficient rat/rat PLP gene structure/PLP membrane integration/point mutation

Introduction

Dysmyelinating diseases of the CNS with mutations inherited in an X-linked recessive mode occur in a number of species. Among them the jimpy mouse (Sidman *et al.*, 1964), the myelin-deficient (md) rat (Csiza and De Lahunta, 1979), the shaking pup (Inuzuka *et al.*, 1986) and Pelizaeus-Merzbacher disease in man (Seitelberger, 1970) have been studied most intensively. Profound cytological abnormalities are observed in oligodendrocytes, the myelin-producing neuroglia cell type, in these dysmyelinating genetic diseases.

The structural elucidation of the proteolipid protein (PLP), the main integral membrane protein of myelin (Stoffel *et al.*, 1983, 1984), its cloning (Schaich *et al.*, 1986), the analysis of its gene organization (Diehl *et al.*, 1986) and X-chromosomal assignment (Willard and Riordan, 1985; Diehl *et al.*, 1986), facilitate investigations on the biochemistry and molecular basis of these dysmyelinating diseases.

Recently the jimpy defect of the mouse has been described as a 74 bp deletion in the mRNA of PLP (Morello *et al.*, 1986; Nave *et al.*, 1986). An A → G transition mutation at the splice acceptor site of intron IV induces an aberrant splicing associated with the loss of exon V coding amino acids 208-232 and a frame shift leading to the synthesis of a 243 amino acid PLP polypeptide with a missense C terminus (amino acids 206-243) (Hudson *et al.*, 1987). The md male rat shows similar CNS abnormalities in light microscopy, ultrastructurally and biochemically, with an almost total lack of CNS myelin and abnormal oligodendrocytes drastically reduced in number during myelination (Csiza and De Lahunta, 1979; Dentinger *et al.*, 1982, 1985; Yanagisawa *et al.*, 1986; Duncan *et al.*, 1987). A reduced size of the large 3.2 kb PLP mRNA associated with the lack of the 1.6 kb PLP message in md rat brain has been reported recently (Kumar *et al.*, 1988).

In this study we demonstrate that the md rat reveals a point mutation in exon III of the proteolipid protein gene. A transversion of A → C leads to the substitution of threonine (residue 75 of the primary translation product of PLP) by proline. This transversion was ascertained by three independent methods: sequence analysis on (i) the mRNA (cDNA) level, (ii) the genomic DNA level and (iii) the identification of a new restriction site for *Ava*II created by the point mutation (A → C transversion).

The mRNA of brains of 18 day old male wild-type and md rats contained both the large (3.2 kb) and small (1.6 kb) transcripts identical in size but strongly reduced in the md brain as demonstrated by quantitative Northern blot hybridization analysis.

Comparative Southern blot hybridization analysis of restricted genomic DNA of the normal and md rat probed with two *Pst*I fragments of a full-length rat brain PLP-specific cDNA clone gave identical hybridization patterns. All exon and intron sizes, determined by exon-primed PCR, proved to be identical in md and normal rats.

Analysis of the 5′ non-coding region of the md rat PLP gene revealed an identical size with that of the normal rat. A strong sequence homology was observed with the mouse PLP gene upstream from the translation start to base -970 and again from -1539 of the mouse sequence. A 569 bp sequence is missing in the rat.

The point mutation in the second transmembranal α-helix (Thr75 → Pro) might cause a dramatic conformational change of the α-helical structure of the second transmembrane helix, thereby disturbing the membrane integration into the myelin lipid bilayer and the association of the myelin components.

Results

md rat brain has a strongly reduced number of oligodendrocytes with traces of PLP and myelin basic protein messenger RNA

During the myelination period male littermates of md carrier Wistar female rats with the md mutation develop a severe axial body tremor often combined with seizures. These symptoms become apparent around day 10 and lead to premature death between 3 and 6 weeks after birth.

For *in situ* hybridization, anesthetized 18 day old male md rats were perfused with fixative and the brain, optical

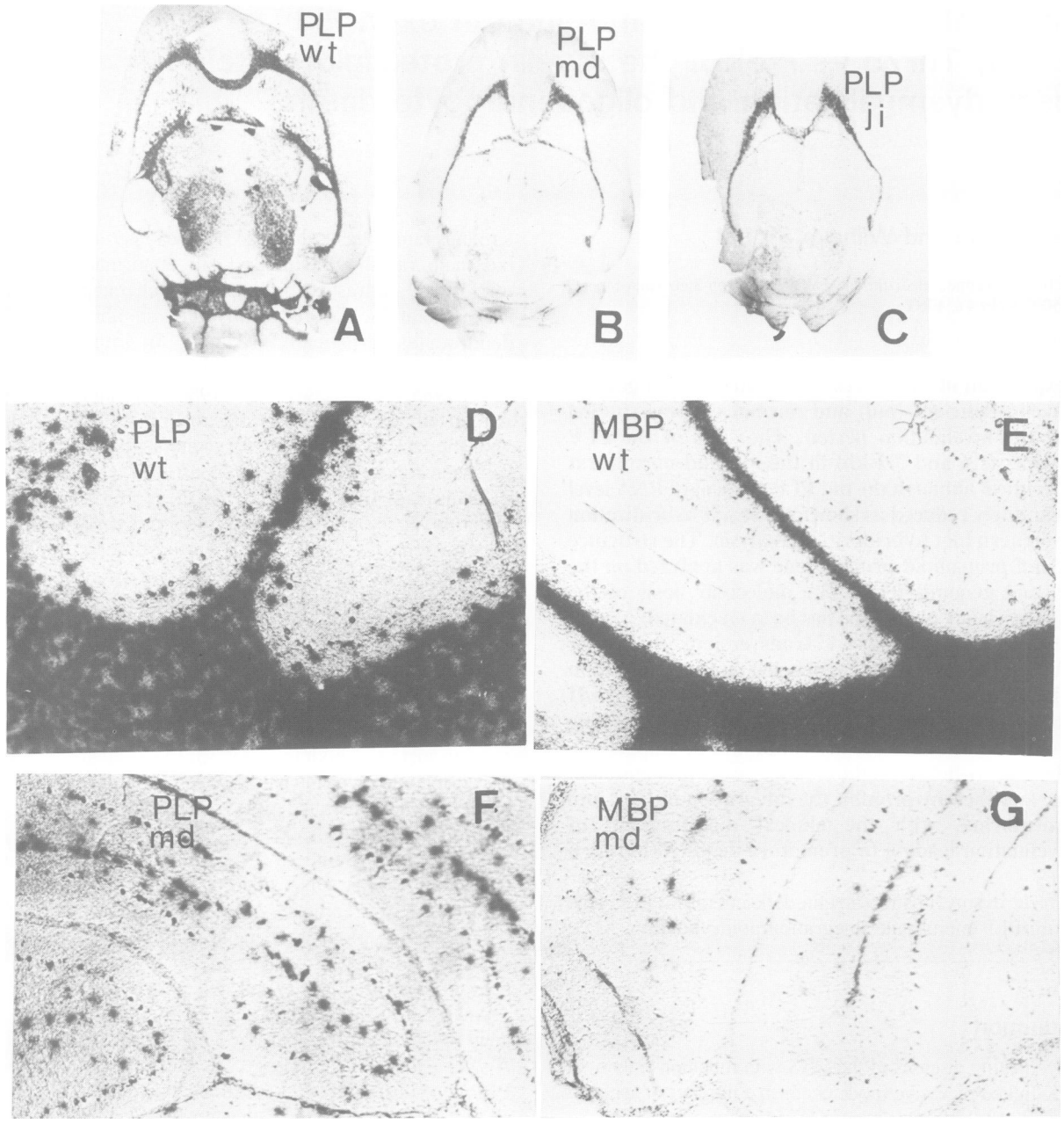

Fig. 1. *In situ* hybridization of horizontal, 6 μm cryosections of brains of 18 day old rats. They were probed with UDPS ^{35}S-labelled antisense rat PLP RNA and rat MBP RNA: (**A**) normal rat brain hybridized with antisense PLP RNA; (**B**) md rat brain hybridized with antisense PLP RNA; (**C**) jimpy mouse brain hybridized with antisense PLP RNA. Cerebellar gyri of normal rat: (**D**) hybridized with antisense PLP and **E** with antisense MBP RNA. Cerebellar gyri of md rat: (**F**) hybridization with antisense PLP and (**G**) antisense MBP RNA. Sp. acts of probes: PLP probe 1.4×10^5 Ci/mmol, MBP probe 0.9×10^5 Ci/mmol. 10^6 c.p.m. per section were used for hybridization.

nerve and spinal cord were isolated for cryo-sectioning. Antisense PLP RNA was synthesized with the 2.9 kb *Hin*dIII/*Eco*RI fragment representing the complete coding region of the PLP-specific cDNA template. This fragment has been cloned into the *Hin*dIII/*Eco*RI-restricted pGEM2 vector, and likewise for antisense myelin basic protein (MBP) RNA synthesis a 612 bp *Pst*I fragment of MBP cDNA, encoding the complete sequence of MBP. *In situ* hybridizations of thin sections of normal and md rat brain were performed with antisense PLP and MBP RNA probes labelled with [^{35}S]UDPS. Their autoradiograms are shown in Figure 1.

These antisense RNA probes led to a heavy staining of the PLP and MBP mRNA in oligodendrocytes in the horizontal slices of normal myelinating rat brain (Figure 1A), whereas the md brain (Figure 1B) and jimpy mouse brain (Figure 1C) were almost devoid of PLP and MBP message. The autoradiogram of the cerebellar gyri, resolved at higher magnification, showed a heavy labelling of the oligodendrocytes of wild-type (wt) brain with PLP (D) and MBP (E) antisense RNA probes whereas these probes detected their respective mRNA only in a few oligodendrocytes of the md cerebellum.

Northern blot hybridization analysis of normal and md rat brain mRNA

Total and poly(A)$^+$ RNA of normal (Figure 2A, lanes 1 and 2) and of md rat brain (lane 3) respectively were

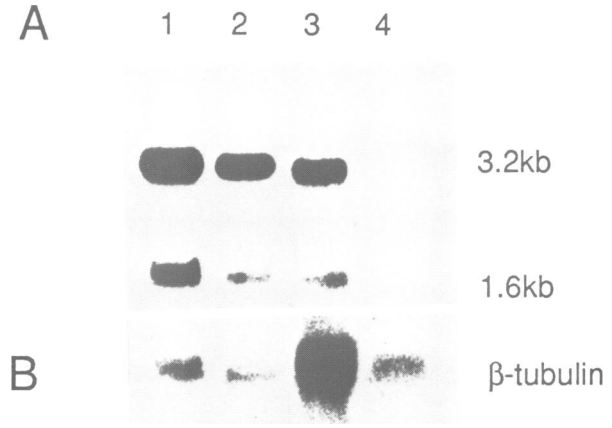

Fig. 2. Northern blot hybridization analysis. (**A**) **Lane 1**, 30 μg of total RNA from normal rat brain; **lane 2**, 15 μg of total RNA from normal rat brain; **lane 3**, 5 μg poly(A)$^+$ RNA from md rat brain of 18 day old males; **lane 4**, control with 1 μg rat liver poly(A)$^+$ RNA was separated electrophoretically on a 1% formaldehyde–agarose gel (Lehrach *et al.*, 1977), transferred to nitrocellulose filters, prehybridized and hybridized to the ^{32}P-labelled *Pst*I fragment (740 bp) encoding a large part of the N-terminal PLP sequence. The position of the large 3.2 kb and the small 1.6 kb PLP transcripts is indicated. (**B**) The blot was also probed with ^{32}P-labelled β-tubulin cDNA to normalize for variations in the amount of mRNA loaded into each well. The autoradiograms were analyzed by quantitative laser densitometry.

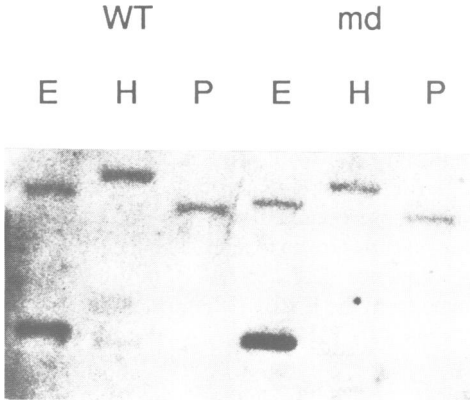

Fig. 3. Southern blot hybridization analysis of normal rat (wt) and md rat (md) genomic DNA. Restriction enzymes used: *Eco*RI = E; *Hin*dIII = H; *Pst*I = P. Aliquots of 10 μg of genomic DNA were digested with restriction enzymes, separated by agarose gel electrophoresis (0.7%), blotted to nitrocellulose filters and hybridized with ^{32}P randomly primed rat brain PLP cDNA.

separated by formaldehyde–agarose gel electrophoresis and hybridized to the 740 bp randomly primed [^{32}P]*Pst*I fragment of our rat PLP cDNA clone. For quantitative densitometry the labelled PLP probe was washed off and the RNA blot hybridized to ^{32}P-labelled β-tubulin cDNA (Feinberg and Vogelstein, 1983) (Figure 2B).

The two main transcripts of the PLP gene of 3.2 and 1.6 kb are present in the normal and the md rat brain mRNA. The sizes of the transcripts of normal and md PLP gene are identical. Densitometric quantitation, however, indicates that the PLP 1.6 and 3.2 kb transcripts in the md RNA are present at extremely reduced levels, the 1.6 kb transcript at 6 ± 1.6% and the 3.2 kb transcript at 2.7 ± 0.7% of normal rat.

Southern blot hybridization analysis of md rat genomic DNA

Genomic DNA of normal and md rat. Genomic DNA isolated from the liver of normal and md rats was digested with the restriction enzymes given in Figure 3, separated by agarose gel electrophoresis and hybridized to the 740 and 620 bp *Pst*I fragments of our rat PLP cDNA insert. These fragments encode exons I–VII. Normal and md rat genomic DNA show identical restriction patterns. Therefore a gene rearrangement can be excluded and the previous finding of only one PLP gene was confirmed.

md rat genomic library. This library was constructed by ligation of partially digested and size-fractionated (sucrose gradient) md rat DNA into the *Bam*HI site of the EMBL3 vector. Two PLP clones with ~15 kb long inserts were isolated by screening ~7.5 × 10^5 plaques, one encoding the 5′ non-coding region up to the *Bam*HI site −73 bp upstream from the translation start codon, and the other encoding exons IV–VII and introns IV–VI. Nucleotide sequences of exons IV–VII and their adjacent intron sequences were determined by the chain termination sequence analysis using the double-strand sequencing strategy (Chen and Seeburg, 1985). The coding sequences were absolutely homologous to the normal rat. The splice donor and acceptor sites showed the characteristic sequences around the exon–intron junctions (Breathnach and Chambon, 1981; Padgett *et al.*, 1986), which are summarized in Table I.

No mutation in either the splice donor or acceptor site or the coding sequences were found. Thus a splicing defect analogous to the jimpy defect could be excluded.

Table I. Splice donor and acceptor sites at the exon–intron junction

Exon	Length of		Acceptor	Splice site	Donor	Conserved bases	
	Exon	Intron	(Intron)	(Exon)	(Intron)	Acceptor	Donor
I		ND					
II	187	680	ND	G . . .GT	GTAAGT	ND	6
III	262	1150	GTATGTCATTGCAG	G . . .AG	ND	11	ND
IV	169	590	TCAATTGTTTTTAG	T . . .TG	GTGAGT	11	7
V	74	730	TTTCTGCATCTTAG	G . . .AG	GTAAGT	13	8
VI	66	880	ACATTTTCCTGCAG	T . . .TG	GTAAGT	12	7
VII	72		TCTGTTCCCTACAG	C . . .		13	
Consensus			YYYYYYYYYYN$^{C}_{T}$AG	G . . .AG	GT$^{A}_{G}$AGT	15	8

The sequences around the splice sites of exons II–VII are compared with the consensus sequences (Breathnach and Chambon, 1981). The number of conserved bases is shown at the right.

Table II. Oligonucleotide primers used for PCR amplifications demonstrated in Figures 4–7

(I) Sense primer, homologous to 5′ end of exons I–VII (*Eco*RI linker)

I sense	AGAGAGAAAAAGTAAAGGACAGAAG
IIE	GAATTCGTTTGTTAGAGTGTTGTGCT
IIIE	GAATTCAGGATTCATGCTTTCCAGTGTG
IVE	GAATTCAGTTTGTGGGCATCACCTATGC
VE	GAATTCAGGTGTTCTCCCATGGAATGCT
VIE	GAATTCAGTTCCAAATGACCTTCCACCTG
VIIE	GAATTCAGCTCACCTTCAAGTATTGCTGC

(II) Antisense primer, homologous to 3′ end of exons II–VII (*Sal*I linker)

IIS	GTCGACACATTAATGAGATACTCATA
IIIS	GTCGACCTTGTCGGGATGTCCTAGCCG
IVS	GTCGACCATACAATTCTGGCATCAAGCA
VS	GTCGACCTCGGCTGTTTTGCAGATGGA
VIS	GTCGACCCAGGGAAACTAGTGTGGCCG
VIIS	GTCGACTCAGAACTTGGTGCCTCGGC
VIIS	homologus to 3′ end of coding region of exon VII

(III) Primers for PCR of 5′ untranslated region

'5′ md S': sense primer, 133 bases 5′ of deletion
5′ GAGGGCAGTCCTAGGAAGGCATGGGTCTT 3′

'3′ md AS': antisense primer, 458 bases 3′ of deletion
5′ TGTGTAATGTCATGTGAAATGGTAGCCCTG 3′

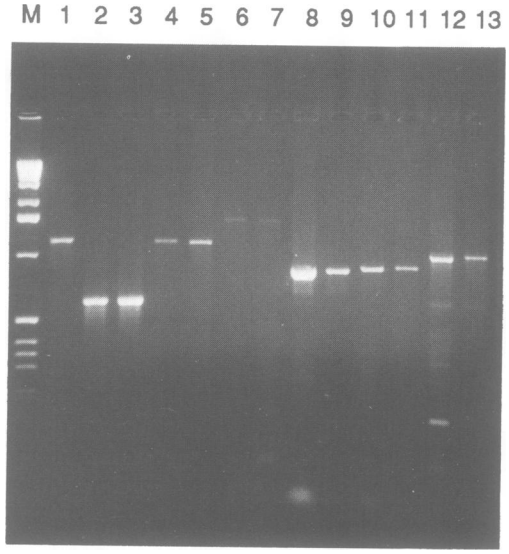

Fig. 4. Comparison of exon and intron sizes of the PLP gene of md and normal rat by PCR amplification using the primers listed in Table II. The 5′ region of mouse PLP gene (**lane 1**), md rat PLP gene (**lane 2**), normal rat PLP (**lane 3**); exon II–intron II–exon III of md rat (**lane 4**), normal rat (**lane 5**); exon III–intron III–exon IV of md rat (**lane 6**), normal rat (**lane 7**); exon IV–intron IV–exon V of md rat (**lane 8**), normal rat (**lane 9**); exon V–intron V–exon VI of md rat (**lane 10**), normal rat (**lane 11**); exon VI–intron VI–exon VII of md rat (**lane 12**), normal rat (**lane 13**). M. marker, kilobase ladder. The PCR reaction with 100 pmol of the respective oligonucleotide primers was mixed with 300 ng DNA. The solution contained 200 μM of each dNTP, Taq polymerase buffer (Stratagene) and Taq polymerase (2.5 units) (Stratagene) in a total volume of 100 μl. Thirty cycles were run. Ten per cent of the incubation mixture was used for agarose gel electrophoresis (1%).

PCR amplification of genomic DNA

The sense and antisense primers listed in Table II were used for PCR amplification of the 5′ untranslated region of the mouse (Figure 4, lane 1), md rat (lane 2) and normal rat

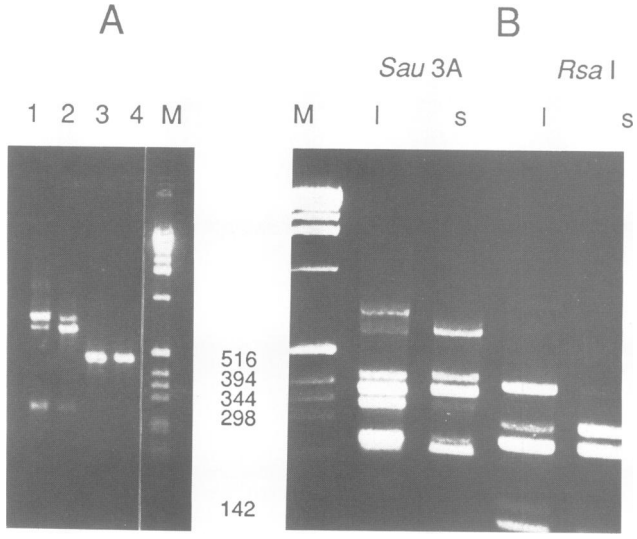

Fig. 5. PCR amplification of PLP-specific cDNA constructed from poly(A)$^+$ RNA of 18 day old md rat brain. (**A**) Oligonucleotide primer I sense (5′) and antisense primer IV S (3′ end of exon IV, Table II) were used. Two fragments of ~750 bp corresponding to exons I–IV were obtained (**lanes 1** and **2**). Using primers IIE and IIIS within these two PCR fragments, embracing exons II and III, identical 450 bp fragments were obtained (**lanes 3** and **4**) to prove the identity of the 750 bp fragments. (**B**) The large (l) and small (s) fragments were separately digested with *Sau*3A and *Rsa*I for subcloning and sequencing. M, marker, kilobase ladder.

(lane 3) PLP gene; of exon II–intron II–exon III of md and wild-type rat (lanes 4 and 5); exon III–intron III–exon IV (lanes 6 and 7); exon IV–intron IV–exon V (lanes 8 and 9); exon V–intron V–exon VI (lanes 10 and 11) and exon VI–intron VI–exon VII (lanes 12 and 13).

The PCR fragments with the md rat genomic DNA as template and those obtained with the normal rat genomic DNA show no differences in size. This is in support of the nucleotide sequence data derived from our genomic PLP clone isolated from the EMBL-3 library of the md rat, which encodes exons IV–VII.

PCR amplification of PLP-specific cDNA

For the PCR amplification of cDNA synthesized from poly(A)$^+$ RNA from 18 day old male md rat brains, the oligonucleotides primer I sense (Table II) homologous to the 5′ end and antisense primer IVS homologous to the 3′ end of exon IV were used. Two fragments of ~750 bp corresponding to exons I–IV (Figure 5a, lanes 1 and 2) were formed. Priming the reamplification of the isolated 750 bp fragment with the primers IIE and IIIS hybridizing within this fragment yielded the expected amplification of the 450 bp of exons II and III (Figure 5a, lanes 3 and 4). The 750 bp PCR fragments comprising exons I–IV have been restricted in separate digestions with *Sau*3A and *Rsa*I (Figure 5b) and cloned into the *Bam*HI and *Sma*I sites of pUC13 respectively for supercoil sequencing. The 370 bp (−72 to 298) *Sau*3A fragment encoding exon I, II and partially III overlapped with the *Rsa*I fragment (125–521) which encodes part of exon II, exon III and part of exon IV. The sequence analyses of these subclones revealed an A → C transversion at position 344 within the codon of Thr^{75} of the primary translation product of the PLP polypeptide mutating Thr to Pro (Figure 6). This mutation creates an *Ava*II restriction site which was confirmed by *Ava*II digestion

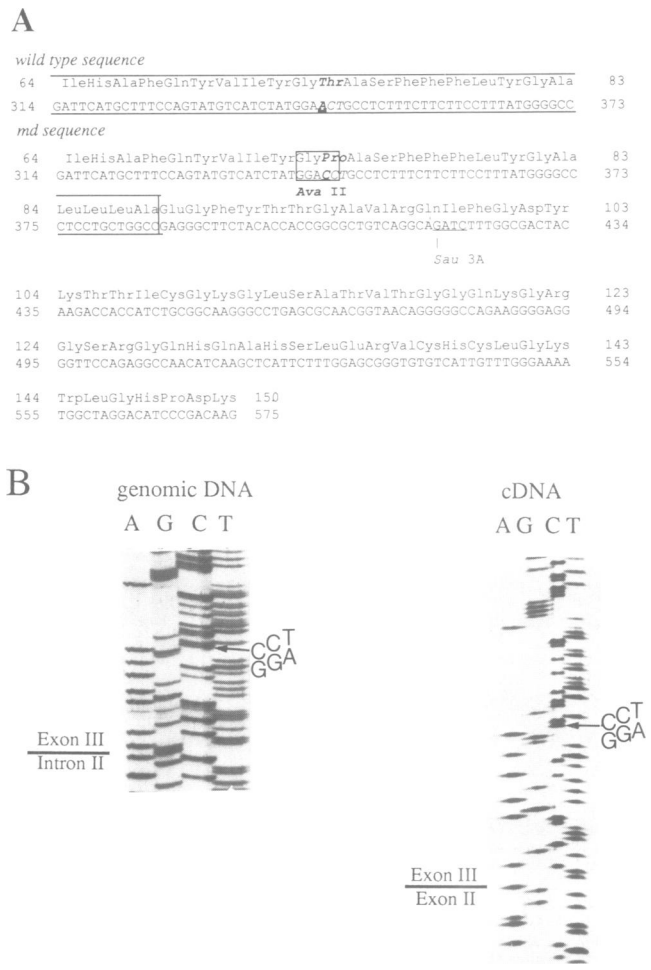

Fig. 6. (**A**) Nucleotide sequence of exon III and derived amino acid sequence of wild-type and md rat PLP gene. The transmembrane sequence is encased. The A → C transversion and the Thr75 to Pro mutation together with the newly generated *Ava*II site are marked. (**B**) Autoradiogram of sequencing gels of cDNA and genomic DNA (exon III) around the site of the point mutation. The arrows indicate the mutation.

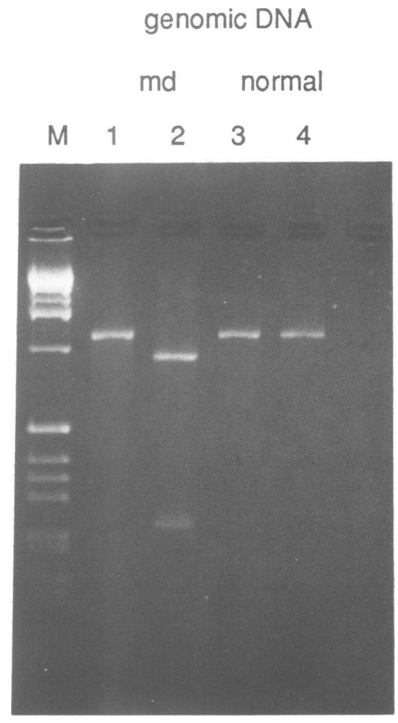

Fig. 7. PCR amplification and restriction with *Ava*II of genomic DNA of md and normal rat. The 1180 bp PCR fragment (primers IIE and IIIS) corresponding to exon II−intron II−exon III exclusively of the md rat exhibits an *Ava*II polymorphism. The 960 and 220 bp subfragments are absent in the restricted PCR fragment of genomic DNA of the normal rat. **Lanes 1** and **3**, undigested fragments; **lanes 2** and **4**, fragments digested with *Ava*II. M, marker, kilobase ladder.

of genomic PCR fragments of md versus normal rat.

Genomic DNA of normal and md rat was amplified by PCR using primers IIE and IIIS. A 1180 bp fragment corresponding to exon II, intron II and exon III was amplified. *Ava*II cleaved this fragment into a 960 and 220 bp subfragment only when md-specific genomic DNA served as template in the PCR, whereas no cleavage occurred in the wild-type control. The *Ava*II polymorphism is documented in Figure 7.

The mutation was further documented by sequencing the *Alu*I fragments of the genomic 1180 bp PCR fragment after cloning into pUC13. The same A → C transversion as observed in the cDNA-derived sequence was established. In addition this analysis also revealed two exon−intron junctions around the splice donor and acceptor sites of intron II (Table I).

Analysis of the regulatory region of the PLP gene of the md rat

A clone encoding the 5′ regulatory region of the PLP gene was isolated from our md rat genomic EMBL3 library with the 1.45 kb long *Bam*HI fragment of the 5′ non-coding region of the mouse PLP gene as probe (Figure 8). The 1.1 kb *Eco*RI/*Bam*HI fragment was excised from the 15 kb insert, cloned into the multicloning site of the pGEM3Z vector, and sequenced using the T7 and SP6 pGEM-primers and in addition the primers indicated in Figure 8. The nucleotide sequence of the 5′ region of the md rat showed strong homology with the mouse sequence between the translation start and bp 950. The 5′ upstream region of the wild-type and md rat PLP gene shows a 589 bp deletion in comparison to the mouse gene, followed again by strongly homologous upstream sequences in both species (Figure 8).

This deletion becomes very obvious by a comparison of the amplification products of this upstream region using the primers del S (around −1650) and del A (around −480) for the PCR (Figures 4 and 8).

Discussion

Myelinogenesis of the rat starts around birth and is characterized by a co-ordinated differentiation and maturation programme of oligodendrocytes. This process is primarily governed by gene expression of myelin-specific proteins and of enzymes for the biosynthesis of lipids required for the assembly of the myelin membrane.

The X-chromosome-linked recessive inheritance of dysmyelination of the CNS of the md rat prompted us to examine the PLP gene that has been previously assigned to the X-chromosome (Willard and Riordan, 1985; Diehl *et al.*, 1986). Our main interest was to study (i) the expression of the PLP gene during the myelination period at the peak of

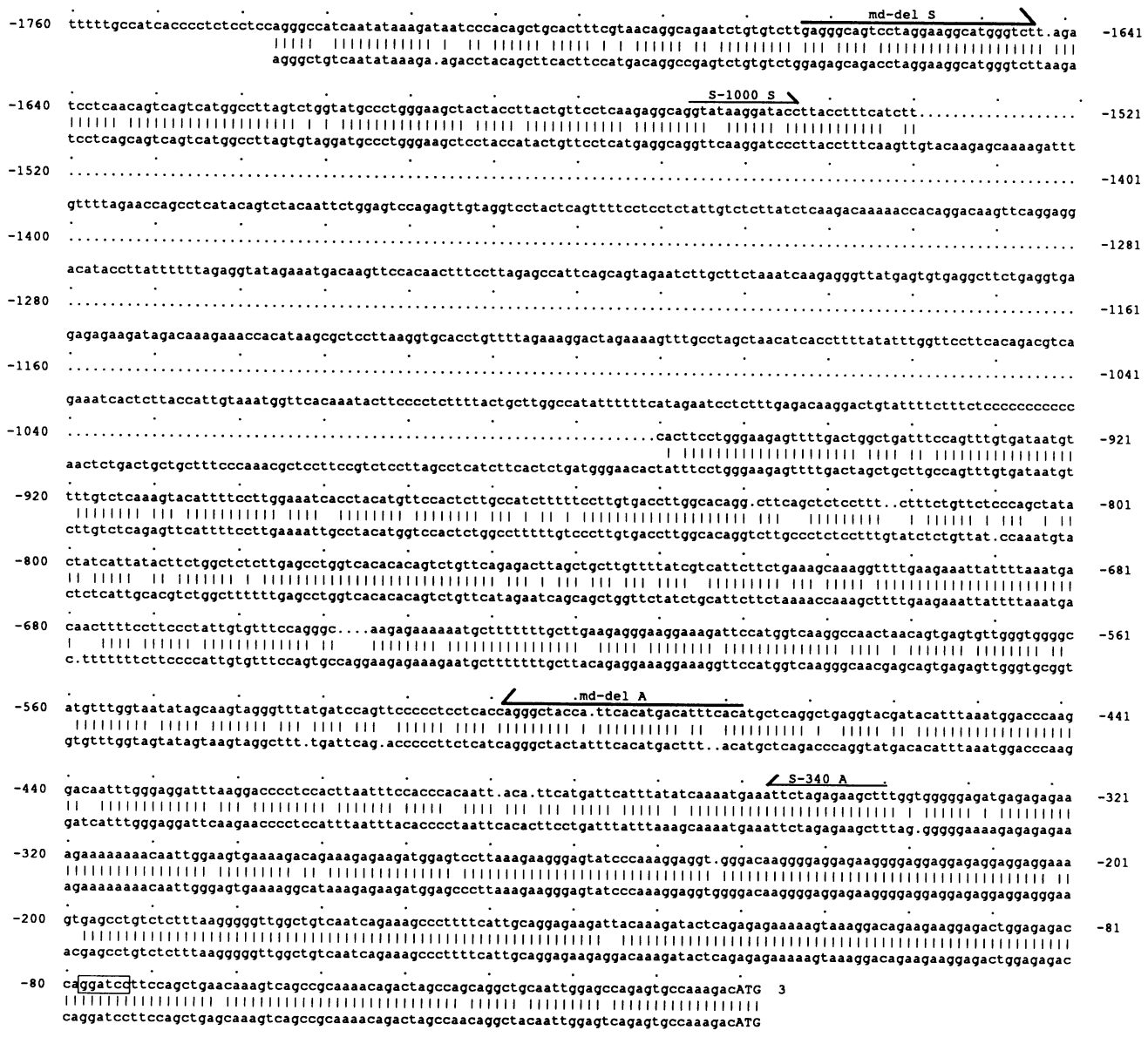

Fig. 8. 5′ non-coding region of the PLP gene of md rat and mouse. Comparison of nucleotide sequences of md rat and mouse 1735 bp upstream from the translation start, md sequence upper line, mouse lower line. Notice the 589 bp sequence missing in the normal and md rat PLP sequence. The location of the PCR primers md-del A and S flanking the deletion and the sequencing primers S-1000S and S-340A are indicated. The *Bam*HI site is boxed.

the development of the clinical symptoms around day 18 (axial body tremor, seizures) and particularly (ii) to focus on the genetic defect leading to this dysmyelination.

PLP has not been detected in brain whereas axons of the spinal cord of the md rat are only scarcely myelinated (Yanagisawa *et al.*, 1986); in addition to the PLP marker, a severely depressed level of MBP-specific RNA in the spinal cord of md rats has been reported (Duncan *et al.*, 1987). The myelin deficit is associated with a strong reduction of glycolipids in the total brain of md rat (Csiza, 1982) and likewise in the glycolipid synthesizing enzymes (Hof and Csiza, 1982).

In situ hybridization of horizontal thin sections of the brain of md rat, normal rat and jimpy mouse using antisense RNA of the two oligodendrocyte markers PLP and MBP as probes proved the severely impaired synthesis of PLP and MBP in the md rat (Figure 1). The similarities in the morphological, biochemical and genetic analysis between md rat and jimpy mouse suggested a similar mutation in the PLP gene. A missing 1.6 kb PLP message and an undefined small size alteration in the 3.2 kb transcript of the md PLP gene has been related to the md defect (Kumar *et al.*, 1988).

Our *in situ* hybridization studies clearly indicate that the extremely low expression of PLP and MBP is due to the low number of surviving oligodendrocytes in the white matter. Also our Northern blot hybridization of the PLP-specific mRNAs isolated from the md and normal rat underline the observations made in the *in situ* hybridization brain sections of 18 day old md and normal rats. Contrary to a previous report (Kumar *et al.*, 1988) the two characteristic 3.2 and 1.6 kb PLP transcripts are detectable with no size alteration in the mutant transcript. Comparative quantitation by laser densitometry of the two PLP transcripts in md rat brain mRNA with β-tubulin as an internal marker

independent of development revealed a level ~2–5% of normal rat brain PLP mRNA. Our quantitative data roughly agree with those reported previously (Kumar *et al.*, 1988).

Rearrangement of the single-copy PLP gene as the basis of the md defect could be excluded by Southern blot hybridization analysis of restricted md and normal rat genomic DNA. The identical pattern of the md and normal rat DNA excluded gross alterations of the PLP gene organization of the md mutant. Therefore the analysis of the coding sequences and the 5′-untranslated region of the PLP gene was indicated. This analysis included the exon–intron transition sequences.

The nucleotide sequences of exons IV–VII of the wt and md rat PLP gene showed complete homology.

Cloning and sequencing of PCR-amplified cDNA of exons I–IV yielded a point mutation in exon III. An A → C transversion leads to the substitution of Thr75 by Pro. This transversion introduces an additional *Ava*II site, a polymorphism documented in Figure 7. The oligonucleotide primers bordering exons II and III of the genomic md PLP were used for PCR amplification. The analysis of this fragment was in full agreement with the md PLP-specific cDNA analysis regarding the point mutation associated with *Ava*II polymorphism. In contrast to the jimpy defect our analysis of the splice donor and acceptor signals and their adjacent nucleotide sequences proved their unaltered structure and agreed with the consensus sequences (Padgett *et al.*, 1986).

We could also exclude mutations in the 5′ untranslated region of the md PLP cDNA and regulatory domains up to 1700 bp upstream from the transcription start signals. Interestingly this upstream region of the md and normal rat PLP gene shows a 569 bp deletion compared to the mouse PLP gene.

In summary the complex pattern of the neurological defects displayed by the md rat results from a one base transversion within the 17 kb PLP gene.

The implication of this point mutation by which Thr75 is exchanged by Pro can be deduced from the model of the PLP integration into the lipid bilayer of the myelin membrane (Stoffel *et al.*, 1983, 1984) and the assignment of the *trans*- and *cis*-membrane α-helices to exons II–VII (Diehl *et al.*, 1986). The position of Thr75 (position 74 of the mature PLP form) is located within the centre of the second transmembranal α-helix. Substitution by proline is well known to break all *trans* α-helical conformation and lead to partial reversal of the chain direction facilitated by glycine preceding it in the sequence. Proline sterically restricts the conformation of this glycine rendering it more difficult to adopt an α-helical conformation. The unusual dihedral angles at the proline ring give rise to a kink in the main chain. Proline occurs at bends and at the beginning of helices (Cantor and Schimmel, 1980; Schulz and Schirmer, 1979).

Therefore the integration of the mutant PLP polypeptide during myelin membrane assembly may be disturbed due to altered conformation of the second transmembranal α-helix irrespective of the mechanism by which integration of the polytopic PLP occurs: be it a translocation by sequential signal and stop transfer sequences or spontaneously by unique insertion sequences or by a combination of the two integration pathways (Blobel, 1983).

The md mutation apparently leads to premature death of the oligodendrocytes. PLP has a half-life of >88 days (Agrawal *et al.*, 1980). Since no translation product can be detected in the 3 week old md rat we propose that the newly synthesized mutant PLP cannot be integrated into the lipid bilayer due to the proposed conformational change within the second transmembranal α-helix. The mutant protein as a strongly hydrophobic polypeptide might interact with membranes (plasma membrane, Golgi, endoplasmic reticulum) and lead to impaired functions and finally to cell death. Other alterations of myelin components would therefore be secondary to the expression of mutant PLP. It seems unlikely to us that the defect involves the control of cell division and early oligodendrocyte differentiation as proposed for the jimpy effect (Knapp *et al.*, 1986).

The molecular events leading from the point mutation in md rat and jimpy mouse to the cell death of oligodendrocyte is studied presently.

Materials and methods

md rats were obtained by breeding pretested female carriers of the md defect with normal Wistar rats. The female carriers were kindly provided by Dr C.K.Csiza, New York State Department of Health, Albany, NY. Male md littermates were identified by tremor and seizures developing around day 10 after birth.

In situ hybridization

Eighteen day old animals were anaesthetized with Nembutal and perfused through the left ventricle first with 20 ml saline followed by 20 ml 4% paraformaldehyde, then 30 ml 3% glutaraldehyde fixative with the right atrium punctured. The perfused brain was soaked in 3% glutaraldehyde in PBS overnight at 4°C and transferred to a solution of 30% sucrose until the dishes sank to the bottom (4°C). Brains were rinsed with PBS and mounted in the cryostat in OCT (Tissue Tek, Miles Scientific, Naperville). Slices of 5–7 μm thick normal, md rat and jimpy mouse brain were hybridized with ^{35}S-labelled antisense RNA, using a full-length PLP cDNA clone (2.9 kb) and MBP cDNA cloned into the pGEM2 vector. The sp. act. of the PLP probe was 1.4×10^5 Ci/mmol and of the MBP probe 0.9×10^5 Ci/mmol; 10^6 c.p.m. per section were used for the hybridization. Slide preparation, prehybridization, hybridization, washings and autoradiography was carried out as described before for paraffin-embedded tissue (Tourtellotte *et al.*, 1987).

Northern blot and Southern blot analysis

RNA was isolated by published methods (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was prepared using oligo(dT)–cellulose chromatography (Aviv and Leder, 1972).

RNA (concentrations as indicated in the legends of figures) was electrophoresed in 1% agarose gels containing 0.2% formaldehyde and blotted onto nitrocellulose. These blots were hybridized with PLP-specific ^{32}P-labelled fragments (Feinberg and Vogelstein, 1983) (sp. act. 10^6 c.p.m./pmol) in 50% formamide at 42°C, washed in 2 × SSC, 0.1% SDS at 60°C and exposed to Kodak XAR film using an intensifying screen at −80°C. Subsequently the blots were washed for 5 min in boiling 1% sodium dodecylsulphate to remove the hybridization probe and again hybridized to β-tubulin, an internal standard for quantitative laser densitometry. The autoradiograms were scanned with the Laser densitometer LKB model 220L.

Construction of md rat brain cDNA and genomic library

Poly(A)$^+$ RNA of md rat brain was reversely transcribed into double-stranded cDNA according to a modified Gubler–Hoffman procedure (Gubler and Hoffman, 1983) with *Eco*RI adaptor for cloning into the *Eco*RI site of the λ-zap vector according to the recommendation of the manufacturer (Pharmacia-LKB).

Isolation of md and normal rat DNA and the construction of a genomic library were performed according to Kaiser and Murray (1985). md rat genomic DNA was partially restricted with *Sau*3A, size-fractionated and cloned into the *Bam*HI site of the EMBL 3 vector.

A 5′ clone was isolated after screening 400 000 and a 3′ clone 750 000 plaques. Restriction fragments were cloned into the multicloning site of pGEM3-Z for double-strand sequencing (Chen and Seeburg, 1985).

Polymerase chain reaction

A Perkin-Elmer DNA Thermal Cycler was used for the amplification of

the DNA sequences. Synthetic 30^{mer} oligonucleotides listed in Table II were used as sense and antisense primers as described in Results. Hybridization temperatures, times and number of steps in the PCR of the original paper (Saiki *et al.*, 1985, 1988) were adapted to the DNA to be amplified and the oligonucleotide used.

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