Post-transcriptional events are responsible for low expression of myelin basic protein in myelin deficient mice: role of natural antisense RNA

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Communicated by B. Hirt

Myelin deficient mice (mld) are characterized by tandem duplication of the gene coding for myelin basic protein (MBP). The upstream gene contains a large inversion of the 3' region which includes exons 3–7, and therefore it cannot give rise to mature mRNA and functional protein. MBP and MBP mRNA concentrations in mld brains constitute only 2% of the concentrations present in normal mice. The overall transcription rate of the MbP gene is normal. In order to explain the discrepancy between mRNA concentration and transcription rate, we studied transcription of each individual gene. The two genes were transcribed independently, although some uninterrupted transcription could not be excluded. The rate of transcription of the upstream gene was higher than that of the downstream gene. This difference was reflected in the concentration of sense and antisense RNA found in nuclei. Our results indicate that the low concentration of the mature mRNA cannot be caused by transcriptional interference. High concentration of nuclear antisense RNA strongly suggests that post-transcriptional regulation occurs in mld mice through formation of double stranded RNA.

Key words: antisense RNA/myelin basic protein/myelin deficient mice/nuclear RNA

Introduction

Myelin basic protein (MBP) is a functionally important constituent of myelin, the specialized membrane that surrounds and insulates axons. Mutations affecting myelination in mice have been found to be invaluable for understanding the composition, formation and maintenance of this complex structure. Among them, shiverer (Mbp<sup>sh</sup>) and myelin-deficient (Mbp<sup>md</sup>) are allelic (Bourre et al., 1980; Neumann et al., 1989) and present a similar phenotype characterized by a severe myelin deficit in the central nervous system. In shiverer, the Mbp gene is partially deleted, resulting in the complete absence of MBP in homozygous mutants (Roach et al., 1983, 1985; Kimura et al., 1985; Molineaux et al., 1986). In mld mice, MBP synthesis is drastically reduced (Ginalska-Winkelmann et al., 1983; Campagnoni et al., 1984; Matthieu et al., 1984) due to low concentration of normal MBP mRNA (Roch et al., 1986, 1987; Akowitz et al., 1987; Okano et al., 1987; Popko et al., 1987). In contrast to results reported by others (Okano et al., 1987; Popko et al., 1987), we did not find an increase of MBP mRNA concentration in mld mice during development (Roch et al., 1987). In mld heterozygotes (Mbp/Mbp<sup>md</sup>), the levels of MBP mRNA were intermediate between those found in wild-type (Mbp/Mbp) and mld (Mbp<sup>md</sup>/Mbp<sup>md</sup>) homozygous mice (Roch et al., 1986, 1987) throughout development (Roch et al., 1987). These studies indicate that the intact Mbp allele in mld heterozygotes is normally regulated during development (Roch et al., 1987).

Homozygous mld mice carry two Mbp genes which are indistinguishable from the wild-type gene by Southern blot analysis, as well as two additional rearranged genes (Akowitz et al., 1987; Okano et al., 1987, 1988a; Popko et al., 1987; Roch et al., 1988). In the tandem duplication, the upstream gene contains an inversion of exons 3–7 (Okano et al., 1988b; Popko et al., 1988). In spite of normal overall transcription rates of the Mbp genes in mld mutants (Popko et al., 1988; Roch et al., 1988), only 2–5% of the normal concentration of MBP and its corresponding RNA can be detected in the cytoplasm. Recent results demonstrated in vitro that both Mbp genes in mld mice have functional promoters within 1.3 kb upstream from exon I (Okano et al., 1988a). The upstream gene is transcribed into antisense RNA complementary to exons 3 and 7 (Okano et al., 1988b; Popko et al., 1988). In order to determine the cause of low concentration of MBP mRNA in mld mice, we investigated the transcription rates of each Mbp gene and searched for the evidence of a very long primary transcript corresponding to uninterrupted transcription of both genes. In addition, we measured the concentration of the transcripts of each gene in the mld nuclei. Part of this work has been published in abstract form (Tosić et al., 1988, 1989).

Results

Nuclear and cytoplasmic MBP specific RNA concentrations

Total RNA was isolated from the cytoplasm and from the nuclei of 18 day old myelin deficient (Mbp<sup>md</sup>/Mbp<sup>md</sup>) and wild-type (Mbp/Mbp) brains. The concentrations of the MBP specific RNAs were measured by dot blot hybridization with pMBP-1, radiolabeled cDNA probe, corresponding to 14 kd myelin basic protein in rat (Roach et al., 1983). Quantification by dot blot of the cytoplasmic MBP specific RNA showed only ~2% of the concentration present in normal mice (Figure 1), and confirms previous observations (Roch et al., 1986). In contrast, nuclei from mld brains exhibited ~40% decrease in MBP specific RNA compared with normal controls. In order to assure the quantification of MBP specific RNA, the same filters were rehybridized with actin probe, and no difference was observed between mld and wild-type mice. Therefore, specific RNA which is
synthesized at the normal rate (Roch et al., 1989) is not immediately degraded in mld nuclei, but probably inefficiently processed and transported to the cytoplasm.

**Transcription of the 5' region of the Mbp gene**

In order to test whether the Mbp<sup>mld</sup> allele gives rise to one very long primary transcript or to two separate RNAs of normal (or reduced) sizes, we performed run-on experiments in nuclei isolated from 18 day old mld and normal brains. Radiolabeled primary transcripts were hybridized with a clone of DNA from the 5' end of the normal Mbp gene. Clone pEX-1 consists of a 3.4 kb *Hind*III fragment from the 5' end of the wild-type mouse Mbp gene (Takahashi et al., 1985) subcloned into pUC13. The inserted fragment contains 1.4 kb of the 5' flanking region, exon I and part of intron I (Figure 2A). When digested with *Sma*I two fragments result. The 2.1 kb fragment contains all exon I and part of intron I sequences. The larger 3.9 kb fragment contains 1.3 kb of DNA from the region immediately adjacent to exon I in the Mbp gene and the remaining 2.6 kb of the pUC13 vector.

When these two fragments were separated by gel electrophoresis (Figure 2B), transferred to nitrocellulose and hybridized with pulse-labeled nuclear RNA from wild-type brains, the 2.1 kb fragment containing exon I and part of intron I was strongly labeled (Figure 2C). The larger fragment containing only 5' flanking and vector sequences did not hybridize, as expected. Therefore, in normal brains, the 5' flanking region is not transcribed at a detectable rate.

The same hybridization experiment was performed with mld brains (Figure 2D). As for the wild-type transcripts, the pulse-labeled nuclear transcripts hybridized only with the fragment containing exon I and did not hybridize with the 5' flanking region. Previous work has demonstrated that both the upstream (rearranged) and downstream (intact) Mbp genes are transcribed in mld mice (Popko et al., 1988; Roch et al., 1989). However, it was not determined whether transcription of the downstream gene initiates correctly at its own promoter, or is run-through from the upstream gene.
hybridized with the radiolabeled primary transcripts obtained in the nuclei isolated from the wild-type or the mld brains. A strong signal was obtained with SP-42 containing exon I, similar to the one obtained in the previous experiment. However, the higher sensitivity of our test revealed a very low signal with the 5' flanking region both in wild-type and in mld brains. The signal from mld brain RNA was somewhat higher than the one obtained from wild-type brain RNA.

Figure 5. Dot blot analysis of nuclear sense and antisense MBP specific RNAs. 8 µg of the total RNA isolated from the wild-type (Co) and mld brain nuclei or cytoplasm, as well as liver RNA, were spotted onto the nylon membrane. The membranes were hybridized with radiolabeled RNA complementary to the antisense and sense fragments from exon VII.

**Transcription of two Mbp genes in mld mice**

The transcription rate of each individual Mbp gene was measured using single stranded RNA probes which hybridize with sense or antisense MBP specific RNA. Inverted cDNA pMBP-1 fragment subcloned into pSP64 vector (pSP-MBP2) and linearized by StuI restriction enzyme, was transcribed into a 1.1 kb RNA molecule which hybridizes with sense RNA corresponding to exon VII and part of exon V. The XhoI – EcoRI fragment from exon VII was subcloned into pSP64 (pSP-EX VII) and transcribed into a 1060 bp RNA which hybridizes with antisense RNA. These two SP6 transcripts as well as actin specific RNA and 16S and 23S bacterial RNA, used as a negative control, were immobilized on a nitrocellulose filter. This filter was hybridized with radiolabeled primary transcripts obtained from nuclei isolated from 18 day old mld brains. Results from two independent experiments showed hybridization of the primary transcripts with both MBP specific RNAs as well as with actin specific RNA (Figure 4). The upstream Mbp gene, giving rise to antisense RNA, was transcribed at a higher level than the downstream gene.

**Sense and antisense RNA concentrations in the mld nuclei**

In order to distinguish what proportion of the RNA nuclear signal belongs to the transcript from the upstream gene, and
what proportion belongs to the transcript of the downstream gene, we have used strand specific RNA probes in our experiments. Using SP6 transcripts of the clones pSP-MBP2 and pSP-EX VII (described in the previous section), we could discriminate the transcripts from the two genes of the Mbp<sup>mid</sup> allele.

Total nuclear or cytoplasmic RNA from wild-type or mld brains, as well as liver RNA as a negative control, were immobilized on nylon membrane. The RNA was hybridized with pSP-EX VII transcript which recognizes antisense RNA (the transcript from the upstream gene) or with pSP-MBP2 transcript which recognizes only sense RNA (the transcript from the downstream gene) (Figure 5). Antisense RNA was detected only in mld nuclei and not in the cytoplasm. It was not detected in either liver or wild-type brains. Sense RNA gave a very strong signal in the control brains. While cytoplasmic sense RNA concentration in mld brains represents only ~2% of the concentration detected in the normal mice, mld nuclear sense RNA presents ~10% of the concentration measured in normal mice.

**Discussion**

The primary defect in hypomyelinated mld mice lies in the expression of the gene coding for myelin basic protein (MBP). Genomic analysis of different strains of mld mice revealed a very complex structure of the Mbp gene (Akowitz et al., 1987; Okano et al., 1987; Popko et al., 1988; Tosic et al., 1988; Roch et al., 1989). The Mbp<sup>mid</sup> allele is composed of two complete Mbp genes arranged in tandem. The upstream gene contains a large inversion (Okano, 1988; Popko et al., 1988). The inverted fragment corresponds roughly to the fragment which is deleted in another allele of the Mbp gene in mice called shiverer (Mbp<sup>sh</sup>).

In mld mutants MBP and its specific mRNAs are of the same length as those in wild-type mice (Ginalska-Winkelmann et al., 1983; Campagnoni et al., 1984; Roch et al., 1986; Okano et al., 1987). Since the upstream gene contains a large inversion which includes exons 3–7, its transcription cannot give rise to either normal mRNA or to functional protein. Therefore, the downstream, apparently intact Mbp gene is transcribed, and at least some of its primary transcripts are normally processed and efficiently translated. Recent experiments from our laboratory (Roch et al., 1989) and from Popko et al. (1988) showed that the Mbp specific transcription rate in mld mice is comparable to that observed in wild-type mice. Using strand specific probes, Popko et al. (1988) showed that both Mbp genes in the Mbp<sup>mid</sup> allele are transcribed.

Previous studies showed that the extremely low concentration of MBP found in mld mutants was not a consequence of the low translation rate, but caused by a corresponding low concentration of MBP specific mRNA (Roch et al., 1986; Akowitz et al., 1987; Okano et al., 1987; Popko et al., 1987). However, the concentration of the MBP specific RNA in the mld nuclei represents ~60% of the concentration detected in the wild-type nuclei. This finding indicates that the primary transcripts, which are synthesized at a normal rate in mld nuclei, do not get immediately degraded in the nuclei. Thus other post-transcriptional mechanisms are involved which prevent normal processing and transport of the RNA to the cytoplasm. One possible explanation for the arrest of the MBP specific RNA in the nuclei can lie in structure and size of the primary transcripts. We tested the hypothesis that the Mbp<sup>mid</sup> allele is transcribed starting from the promoter of the upstream gene without interruption between the genes. Our hypothesis was based on the fact that the 3' end of the upstream gene is inverted, causing the loss of normal polyadenylation and termination signals. This proposed mode of transcription would result in a very long primary transcript lacking normal processing and transport from the nucleus. Consequently, this molecule would be more easily degraded than the polyadenylated RNA found in wild-type nuclei. We tested for the presence of a transcript corresponding to the 5' flanking region of the gene, absent from wild-type brains.

Our results show discontinuous transcription of the two Mbp genes in the Mbp<sup>mid</sup> allele. The extremely low transcription rate of the 5' flanking region, coupled with data indicating a high rate of transcription of the downstream gene (see Figure 4), indicates that the transcription of the downstream gene starts from its own promoter. Consequently, the Mbp<sup>mid</sup> allele appears to initiate transcription at both Mbp promoters. One of them probably results in spliced mRNA identical to the RNA from the normal Mbp allele, while transcripts from the upstream promoter contain a large antisense region. It is not known at which sites these latter primary transcripts terminate. A very weak hybridization signal was detected with the 5' flanking sequences both in wild-type and mld brains. A low level of transcription initiation upstream of the major initiation site has been reported by others and is probably responsible for this signal (Okano et al., 1988; Newman et al., 1989). The 5' flanking transcription rate was somewhat higher in mld brains. This result may also indicate very low level of uninterrupted transcription starting from the upstream Mbp gene. Possible transcription of this region was reported by Okano et al. (1988b).

The transcription rate of each individual Mbp gene in the Mbp<sup>mid</sup> allele was measured directly using single stranded RNA probes which correspond to the inverted fragment. Both genes are transcribed at a rather high rate. Moreover, the transcription of the upstream gene is higher than the transcription of the downstream gene. The reduced transcription rate of the downstream Mbp gene cannot explain almost complete absence of MBP in mld brains. However, this low rate of transcription is an important prerequisite for the low MBP concentration.

The reason for lower transcription efficiency of the downstream gene copy may lie in transcriptional interference. Although transcription of the 5' flanking region is at an extremely low level, we cannot exclude its interference with the initiation of the downstream promoter. Transcriptional interference between two closely associated promoters has been found in several genes. Cullen et al. (1984) detected overlap interference whenever the polyadenylation site of the upstream gene overlaps with the promoter of the downstream gene. Although we found that most of the upstream Mbp transcripts do not reach the promoter region of the downstream copy defined by Okano et al. (1988a), it is not excluded that upstream Mbp transcription overlaps an enhancer or other regulatory sequences (Miura et al., 1989). Emerman and Temin (1984) described the case of epigenic suppression of one promoter in the proximity of another stronger promoter independently.

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of their relative positions. The two promoters in the Mbp<sup>mld</sup> allele have the same sequences (Okano et al., 1988a) and potentially the same activity, but this does not exclude the possibility of reciprocal partial suppression. Our results are similar to those reported by Garner et al. (1986) for partial tandem duplication of the actin gene in mice. These mutants which carry two active promoters have a much lower rate of actin transcription than the wild type. The mechanism of this partial suppression is not known. One possible explanation for the reduced transcription of each of the two Mbp genes would be a limited amount of trans-acting transcriptional factors as suggested by Miura et al. (1989).

Although transcriptional interference, either reciprocal, due to promoters proximity, or unilateral, due to transcription running through from the upstream gene, may explain a somewhat reduced transcription rate of the downstream gene. Nevertheless, it does not completely explain the drastic reduction of the mRNA concentration. This extremely low concentration of mRNA is the result of some post-transcriptional events.

Independent transcription of the two Mbp genes indicates that the RNA detected in the mld nuclei is most probably a mixture of two distinct transcripts, one of which contains the partial antisense sequence. In order to discriminate between these two types of RNAs, we measured concentrations of the nuclear RNAs using strand specific probes. The antisense RNA was detected only in mld nuclei. This RNA is transcribed from the gene containing an inversion of the 3’ region including the polyadenylation signal. Therefore, the antisense RNA is most probably not polyadenylated and this can be the reason for its defective transport to the cytoplasm.

The MBP sense RNA was found both in nuclear and cytoplasmic mld brain RNA. The proportion of sense and antisense RNA in nuclei reflects the observed transcription rates of upstream and downstream genes. Moreover, a drastic difference between nuclear and cytoplasmic sense RNA concentrations, compared with normal brains, was observed. This difference indicates that the transcript from the downstream gene is abnormally processed or inefficiently transported to the cytoplasm. Since mld brain nuclei contain high concentration of MBP antisense RNA, MBP sense—antisense duplex RNA transcripts may be formed. These duplexes may explain the arrest of sense RNA in mld brain nuclei.

Izant and Weintraub (1985) reported that the formation of stable RNA—RNA hybrids in vivo results in inhibition of RNA transport or of increased rate of RNA turnover. Since this discovery, antisense RNA has been greatly used as a tool for inhibition of gene expression in tissue cultures. Various experiments show that antisense RNA can interfere at different levels of gene expression (Van der Kroel et al., 1988). In nuclei, duplex RNA interferes directly with processing and transport of mRNA. Although natural antisense RNA has been detected in different species (Farnham et al., 1985; Heywood, 1986; Spencer et al., 1986), their regulatory role in gene expression was not identified. The regulatory role of a natural antisense RNA in <i>Antirrhinum majus</i> was reported by Coen and Carpenter (1985). A duplication with an inversion was detected in the gene coding for enzyme chalcone synthase (CHS). The antisense transcript blocks in trans the transcript from the normal gene giving rise to a mutant phenotype. A similar mechanism probably exists in the expression of the Notch gene in <i>Drosophila</i> described by Kidd and Young (1986).

The inhibition of the Mbp gene expression by antisense RNA has been demonstrated in transgenic mice by Katsuki et al. (1988). Introduction of the antisense Mbp cDNA into normal murine embryos inhibits the expression of the Mbp gene and correlates phenotypically with hypomyelination and tremor in adult transgenic mice. Our results show that the downstream gene is transcribed independently from the upstream gene and that the concentration of the sense RNA is relatively high in nuclei. Therefore, the extremely low mRNA concentration in cytoplasm is mostly the consequence of post-transcriptional events. Transcription and rather high nuclear concentration of antisense RNA, together with the experimental evidence from transgenic mice for its possible inhibitory role, strongly suggest that the post-transcriptional regulation occurs through the formation of double stranded RNA. Duplex RNA may eventually interfere with processing or transport of mature RNA to cytoplasm, leading to a low concentration of MBP and hypomyelination.

### Materials and methods

#### Animals

The myelin deficient mice used in this study were derived from the original MDB/Dt strain and had been maintained in our laboratory since 1979 without outcrossing. The mice used in the experiments were 18 day old homozygotes for wild-type (Mbp) or myelin deficient (Mbp<sup>mld</sup>) allele.

#### Nuclear preparation

Brain nuclei from 18 day old wild-type or mld mice were prepared according to the method of McKechnie and Palmiter (1979). The brains were homogenized in 0.32 M sucrose, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1% Triton X-100 and 5 mM HEPES pH 7.4. The homogenate was mixed with two volume<sup>f</sup> of dilution buffer (2.1 M sucrose, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 5 mM HEPES pH 7.4) and centrifuged for 45 min at 4°C onto dilution buffer cushion at 30,000 g. The nuclei were either frozen in 40% glycerol, 40 mM Tris pH 8.0, 20 mM MgCl<sub>2</sub> and 2 mM MnCl<sub>2</sub> and used for probe-labeling experiments, or reprocessed by centrifugation through the cushion of 30% sucrose in 5% citric acid, and used for nuclear RNA isolation.

#### Nuclear and cytoplasmic RNA purification

Repurified nuclei were lysed using the guanidinium method (Chirgwin et al., 1979). The RNA was separated from the other nuclear macromolecules by centrifugation through a CsCl gradient (Gilsin et al., 1973).

Cytoplasmic RNA was prepared by phenol extraction of the cytoplasmic phase which was separated from the intact nuclei by brief centrifugation (Maniatis et al., 1982).

#### Northern hybridization

Nuclear and cytoplasmic RNA were denatured in 50% formaldehyde, 6% formic acid and 1 x MOPS buffer solution at 60°C and spotted onto Gene Screen nylon membrane (NEN-Du Pont) in 10 x SSC solution (20 x SSC = 3 M NaCl; 0.3 M Na<sub>3</sub> citrate).

DNA probes were radiolabeled with [α-<sup>32</sup>P]dCTP (3000 Ci/mmol NEN) using the random priming method (Feinberg and Vogelstein, 1983).

Filters were hybridized and washed in the solution and under the conditions given by the supplier (NEN-Du Pont).

RNA probes were obtained by transcription of the desired fragment with SP6 polymerase in the presence of [α-<sup>32</sup>P]UTP (3000 Ci/mmol, NEN) using the method described by Melton et al., (1984).

RNA—RNA hybridizations were performed at 65°C in the following solution: 50% formamide, 5 x SSC, 50 mM Tris pH 7.5, 0.1% Na-pyrophosphate (NaPP), 1% SDS, 0.2% polyvinylpyrolidone (PVP), 0.2% Ficoll, 5 mM EDTA, 150 ng/ml salmon sperm DNA and 1 x 10<sup>6</sup> c.p.m./ml <sup>32</sup>P-labeled probe. The filters were washed in 2 x SSC, 0.1% SDS and in 0.1 x SSC, 0.1% SDS at the same temperature.
Transcription in isolated nuclei

Nuclear run-on experiments were performed as described by Greenberg et al. (1985). Pelleted nuclei were resuspended in the reaction solution containing: 20% glycerol, 10 mM MgCl₂, 1 mM MnCl₂, 20 mM Tris pH 8, 10 mM phosphatase, 0.1 mg/ml creatine kinase, 1 mM ATP, CTP, GTP, 0.03 mM UTP and 0.25 mM (α-32)PjUTP (3000 Ci/mmol, NEN). After 25 min at 30°C, the nuclei were lysed with 0.5 M NaCl and treated with DNase I and proteinase K. Labeled RNA molecules were separated from unincorporated nucleotides on a Sephadex G50 medium column.

Analysis of primary transcripts

Specific radiolabeled transcripts were detected by hybridization with the probes immobilized onto nitrocellulose filters. DNA fragments were spotted directly onto nitrocellulose using Hybri-Slot™ Manifold (Gibco/BRL) and fixed by heating at 80°C for 2 h.

Prehybridization of the filters was performed in 0.3 M NaCl, 5 mM EDTA, 10 mM Tris pH 7.4, 0.4% PVP, 0.4% Ficoll, 0.5 mM/ml yeast tRNA, 0.1% NaPP and 0.2% SDS. Hybridization with 2 × 10⁶ c.p.m./ml RNA probes were denatured in 50% formamide, 6% formaldehyde, 1 x MOPS at 62°C, 10 mM EDTA, 10 mM Tris pH 7.4, 0.4% PVP, 0.4% Ficoll, 0.1 mg/ml yeast tRNA, 0.1% NaPP and 0.2% SDS. The filters were washed at the same temperature in 0.3 M NaCl, 4 mM EDTA, 0.08 M Tris pH 7.4, 0.1% NaPP and 0.1% SDS.

All the filters were exposed to Agfa-Gevaert Curix RP-1 film for several days. The relative intensity of hybridization signals was quantified using a BioRad videodensitometer model 620.

Acknowledgements

We thank Dr M. Gardiner for critically reading the manuscript. We are very grateful to Dr V. Jongeneel for the gift of actin clone, and Ms S. Cherbuin for technical help. This work was supported by grants from the Swiss National Science Foundation (3.129.88) and from the Multiple Sclerosis Society of Switzerland.

References


Received on October 13, 1989; revised on November 15, 1989.