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Conversion of Normal Behavior to Shiverer by Myelin Basic Protein Antisense cDNA in Transgenic Mice

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Myelin basic proteins (MBPs) are coded by the single gene necessary for myelin formation in the central nervous system of the mouse. An antisense MBP mini-gene was constructed and used to determine the function of antisense DNA in transgenic mice. Several transgenic offspring of a founder transgenic mouse, AS100, were converted from the normal to mutant shiverer phenotype. Antisense MBP messenger RNA was expressed in these mice, and the endogenous MBP messenger RNA, the MBP, and the myelination in the central nervous system were reduced.

ANTISENSE RNA (MINUS-STRAND RNA complementary to a particular RNA) has been shown to repress the expression of specific genes in cells of some species including mammals (*1*–*7*). The expression of these antisense RNAs and the repression of the targeted gene functions in transgenic animals provide a means for studying the biological functions of cloned genes.

We chose the myelin basic protein (MBP) gene for repression by the antisense DNA for two reasons: (i) the MBP gene promoter affects the expression of MBP cDNA, tissue specifically, in transgenic shiverer mouse brain and can rescue the shiverer phenotype (*8*), and (ii) an MBP-deficient and hypomyelinating mouse that has an autosomal recessive mutation in the MBP gene, the shiverer (*9*–*13*), is available for comparisons with transgenic mice having the antisense MBP DNA.

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To examine the myelination in the transgenic mice having antisense MBP DNA, we constructed a plasmid pMP302AS. This plasmid contained the mouse MBP promoter region (approximately 1.3 kb) followed by a portion of the rabbit β-globin gene carrying the mouse MBP cDNA for the smallest MBP (14 kD) in the antisense orientation and by the polyadenylation sites of rabbit β-globin and simian virus 40 (SV40) early genes (Fig. 1A). The DNA fragment, antisense MBP mini-gene, that was injected into mouse zygotes was prepared from plasmid pMP302AS DNA that had been digested with Hind III and Sal I. Fertilized eggs heterozygous for the shiverer mutation (*shi*/+) were used to produce the transgenic mice. Inhibition of the endogenous sense MBP mRNA expression by the antisense RNA is expected to be more pronounced in heterozygous (*shi*/+) than in wild-type mice (+/+), as the amount of MBP mRNAs in the heterozygous mouse is half that in the wild type (*14*). The method of producing transgenic mice was basically that described by Gordon *et al.* (*15*). Five transgenic mice with the antisense MBP mini-gene were obtained. All five founder mice, each of which had been independently produced, appeared to be normal.

Table 1. Number of transgenic mice with the tremor phenotype in relation to genetic background. A and B represent the two types of transgene in the offspring of AS100 mouse.

Genotype	Phenotype	
	Tremor	Normal
shi/+ (A, B)	7 (3, 4)	5 (3, 2)
+/+ (A, B)	3 (1, 2)	6 (0, 6)
Total (A, B)	10 (4, 6)	11 (3, 8)

The transgenic male mouse, AS100 (*shi*/+), was mated with a wild-type female mouse (+/+, B6c F1 hybrid) and 50 offspring were born (AS100-1 to AS100-50). The transgene of the AS100 mouse was transmitted to 21 of them. There were two types of transgene, A and B. The A type had extra bands as well as the B-type bands as shown by DNA blot analyses (Fig. 1B). These two types were segregated to A and B strains and stably transmitted to their offspring. There also were two different genetic backgrounds for these offspring, *shi*/+ and +/+, shown by the presence of the truncated restriction fragment found only in the heterozygous shiverer mouse (*shi*/+) (*13*) because of the large deletion of the MBP gene in shiverer mutation (*10, 11*).

Surprisingly, 10 of the 21 transgenic mice began to show shivering at about 2 weeks after birth. There was some variation in the severity of the tremors, which became progressively pronounced. Further analysis of the AS100 transgenic offspring showed that

Table 2. Correlation between MBP expression and the tremor phenotype in transgenic mice.

Mouse	Genetic background	Transgene*	Antisense MBP mRNA†	Endogenous MBP mRNA (%)‡	MBP in cerebellum§	Tremor phenotype‖
AS100-10	+/+	−	−	100	+	−
AS100-9	*shi*/+	−	−	50	+	−
AS100-11	+/+	B type	+	50	+	−
AS100-12	*shi*/+	A type	++	30	± (m)	±
AS100-14	*shi*/+	B type	++	20	∓ (m)	+

*A and B type represent the types of transgene transmitted; − indicates a nontransgenic mouse. †Antisense MBP mRNA in the brain was detected by RNA blot analysis with sense MBP RNA as the probe. ‡Relative amount of MBP mRNA in the brain, the wild type (+/+) being taken as 100%. §+ indicates that the MBP was detected uniformly in the cerebellar tissue by anti-MBP; ± indicates relatively sparse distribution of MBP; ∓ indicates intermediate distribution of MBP between − and ±; and m means mosaic expression of MBP. ‖− indicates normal behavior; + indicates tremor phenotype; and ± indicates the faint tremor phenotype.

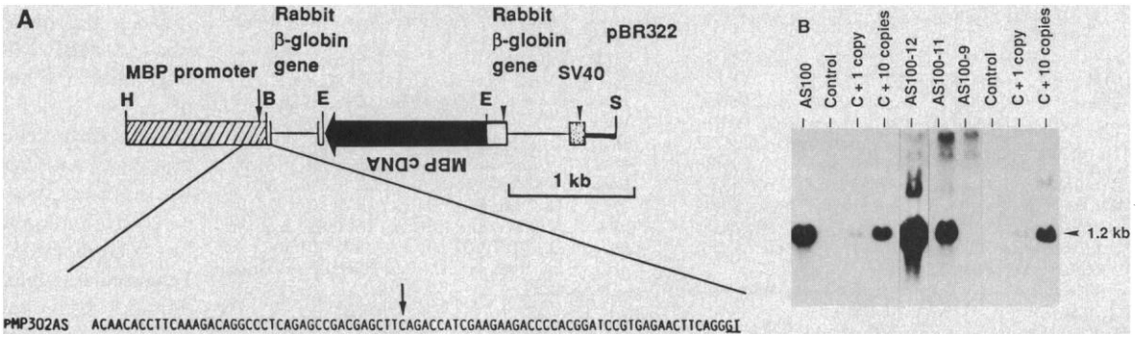

Fig. 1. Antisense MBP mini-gene structure and DNA blot hybridization of transgenic mice DNAs. (**A**) Diagram of the antisense MBP mini-gene structure. A 1.2-kb Eco RI fragment corresponding to the cDNA of the smallest MBP (14 kD) was removed from plasmid pMP302, which consisted of 1.3 kb of MBP gene promoter sequence, rabbit β-globin second intron, and polyadenylation sites of the rabbit β-globin third exon and SV40 early gene (*8*). It was recloned at the same Eco RI site in an antisense orientation (pMP302AS). A 4.2-kb Hind III–Sal I fragment was purified from plasmid pMP302AS DNA and injected into fertilized mouse zygotes heterozygous for the shiverer mutation (*shi*/+) obtained by in vitro fertilization. Offspring (+/+ or *shi*/+) of the founder transgenic mouse (*shi*/+) were obtained by crossing the founder with a B6c F1 hybrid (+/+). (**B**) DNA (5 μg per mouse) that had been extracted from tails of its offspring of the AS100 transgenic mouse was digested with Eco RI, then separated by electrophoresis on 0.7% agarose gels, and the fractions were transferred to a nylon membrane (MSI:N04HY312F5). The transferred DNA was hybridized in formamide buffer with ^{32}P-labeled MBP cDNA (1.2 kb) as the probe (*11*). The control was *shi* F1 mouse DNA. The C + 1 copy represents control DNA with one copy of antisense MBP mini-gene DNA per diploid mouse DNA, and C + 10 is control with ten copies. The DNA from AS100 and from its offspring (AS100-9, AS100-11, and AS100-12) was analyzed at different times. DNA blot hybridization (*19*) patterns of the transgenic AS100 offspring were of two types: AS100-12 (A type) had the B type with additional bands and the expected 1.2-kb band; AS100-11 had only the B type. Bands indicative of large molecular size, which appeared also on the lanes of nontransgenic mouse AS100-9, were derived from the endogenous MBP gene.

this abnormal phenotype was obtained from mice having either the A or B type transgene (Table 1). The AS100-14 mouse exhibited weak tremors at 35 days of age, but severe ones at 60 days of age. The AS100-13 mouse, which had severe tremors at about 30 days of age, began to have tonic convulsions at about 35 days of age and died soon afterward. The time of the onset of tonic convulsions in the AS100-13 mouse was much earlier than in homozygous shiverer mice (*shi*/*shi*) whose tonic convulsions began about 2 months after birth. The AS100-12 mouse showed faint tremors that did not progress further. Neither tremors nor tonic convulsions were observed in nontransgenic littermates.

We then examined the difference in the manifestation of abnormal behavior among the AS100 transgenic offspring in order to determine whether it was attributable to the genetic background's being the shiverer mutation, wild type (+/+), or heterozygous shiverer (*shi*/+). Of 9 transgenic mice with the genetic background +/+, 3 appeared to be tremor phenotypes, whereas 5 of 12 with *shi*/+ appeared to be normal (Table 1). Therefore, a different genetic background in the case of the shiverer mutation could not have been the only reason for the differences in the manifestation of tremors. To determine why abnormal behavior appeared in transgenic offspring of the AS100 mouse, we measured the presence of both antisense transcripts (Fig. 2A) and endogenous MBP mRNA (Fig. 2B).

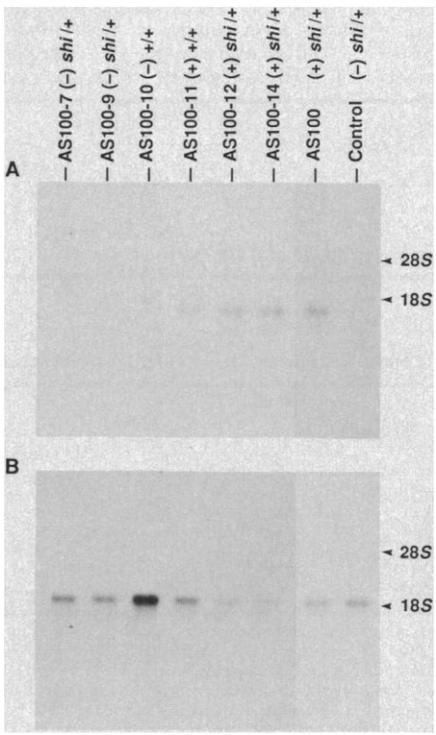

Fig. 2. Antisense and sense MBP mRNA expression in transgenic mice. Total RNA was isolated (*20*) from the transgenic mice at 65 days after birth (offspring) and 100 days of age (AS100). RNA samples (2.5 μg) were denaturated in glyoxal (*21*) and subjected to electrophoresis on 0.9% agarose gels, and the products were transferred to nylon membranes (*22*). (**A**) RNA blot analysis of the AS100 mouse and that of its offspring with sense MBP RNA as the probe. (**B**) RNA blot analysis of the same mice with double-stranded MBP cDNA as a probe. RNAs extracted from the brains were hybridized with ^{32}P-labeled 1.2-kb MBP cDNA. After dehybridization (*22*), the filter was probed with ^{32}P-labeled single-strand sense MBP RNAs that had been synthesized by the SP6 polymerase system (Promega) (*23*). These RNAs hybridized only with the antisense MBP RNAs. The filter was washed twice in 2× saline sodium citrate (SSC) and 0.1% SDS at room temperature for 5 min; then it was treated with ribonuclease A at room temperature for 30 min and washed once in 0.1× SSC and 0.1% SDS at 65°C for 90 min.

We measured the amounts of endogenous MBP mRNAs in the brains of transgenic mice and their nontransgenic littermates of the same age by RNA blot analyses with 1.2-kb MBP cDNA as the probe (Fig. 1A). The transgenic AS100 (founder, *shi*/+), AS100-11 (B type, +/+), AS100-12 (A type, *shi*/+), and AS100-14 (B type, *shi*/+) mice had decreased amounts of endogenous MBP mRNAs (2.3 kb) when compared with their nontransgenic littermates; these transgenic mice had about 40, 50, 30, and 20%, respectively, of the value for the nontransgenic normal (+/+) mouse (AS100-10) (Fig. 2B). The amounts found in nontransgenic heterozygous (*shi*/+) mice (AS100-7 and AS100-9) also showed a reduction of accumulated MBP mRNAs of approximately 50% as compared to the amount found for a normal littermate (AS100-10) (+/+).

After the dehybridization of the MBP cDNA probe, we used the same sample filter to analyze antisense transcripts of the antisense MBP mini-gene in the brains of transgenic mice. The single-stranded sense MBP RNA, which was prepared by the SP6 polymerase system, was used as the probe because it hybridized specifically to the antisense transcripts, but not to the endogenous sense mRNAs (Fig. 2A). Although the amount of antisense RNA in the various samples of brain differed, the transcript band of approximately 1.9 kb was detected in all the transgenic mice. This would be expected if the transcription started in the MBP promoter region of the transgene at the same site as that in the endogenous MBP

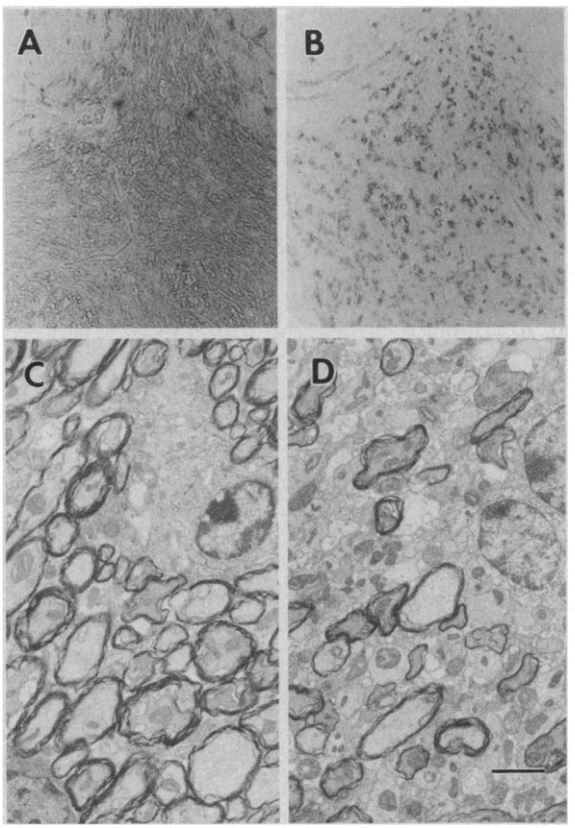

Fig. 3. Repression of MBP expression in the brain of a transgenic mouse with the antisense MBP mini-gene. The immunohistochemical (**A** and **B**) and the morphological analysis (**C** and **D**) of cerebellar tissue of normal and transgenic mice. Immunoperoxidase staining was done with the rabbit antibody to mouse MBP as the first antibody and horseradish peroxidase–labeled antibody to rabbit immunoglobulin G as the second. (A and B) Part of the cerebellum of a 65-day-old nontransgenic mouse (*shi*/+) (A) and affected transgenic mouse, AS100-14 (*shi*/+) (B). MBPs were stained uniformly in the nontransgenic (A) and reduced in the transgenic mouse brain (B). Mosaic expression of MBP, which indicates that some cerebellar tissues were stained and others not, was present only in the transgenic mouse brain. Electron micrographs of the cerebellar tissue indicate that the multilamellar structure of myelin appeared to be normal in the nontransgenic littermate (C), but to be mosaic in the affected transgenic mouse (D). Bar, 5 μm.

gene and if the primary transcripts were spliced by the intron of the rabbit β-globin gene followed by the poly(A) addition site of the SV40 early gene (Fig. 1A). No transcript of this size was detected in any of the nontransgenic littermates even after long exposure.

The reduced amount of MBP mRNAs seems to be correlated with the appearance of the abnormal phenotype (Table 2). Thus, immunohistochemical analyses with antibodies to mouse MBP (anti-MBP) were made to identify MBPs in the brains of the transgenic and nontransgenic mice (Fig. 3, A and B). In the nontransgenic mouse cerebellum the white matter was uniformly stained by the anti-MBP, whereas in the AS100-14 transgenic mouse, a large part of the white matter was not stained, but appeared to be a mosaic of MBP in the cerebellar tissue. Moreover, the myelination in the cerebellar tissue also appeared to be mosaic in affected transgenic mouse, but not in nontransgenic normal mouse (Fig. 3, C and D). Therefore, the affected transgenic mouse brain might be demyelinated at the particular site of the central nervous system where it is responsible for the shivering behavior even if more than 30% of MBP mRNA was expressed in the transgenic mice as compared to that in the nontransgenic mice (Table 2) (*16*). There was a qualitative correlation between the decreases of MBP mRNAs, of MBPs, and of myelination and the appearance of the tremor phenotype (Table 2 and Figs. 2 and 3). Therefore, the phenotypic alterations in these mice were the result of expression of antisense MBP RNA, and this expression may have been responsible for the reduction in the amount of endogenous MBP mRNA.

The reason for the various degrees of alteration in phenotype among the transgenic mice is not clear. Possibly the genetic background may regulate the function of the antisense gene (*17, 18*) because AS100 and its offspring had different genetic and epigenetic backgrounds (F2 and F3 hybrid of B6c). The data in Fig. 3 indicate that the degree of repression of MBP synthesis and of myelination by antisense RNA differed in each oligodendrocyte.

Although the exact mechanism of the reduction of the amount of endogenous MBP mRNA in transgenic mice has yet to be determined, our findings may provide the opportunity to control gene expression by the introduction of antisense mini-gene as a dominant gene into mammalian systems.

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