MOLECULAR AND CELLULAR BIOLOGY, July 1989, p. 3122–3126
0270-7306/89/073122-05$02.00/0

Vol. 9, No. 7

Tissue-Specific In Vitro Transcription from the Mouse Myelin Basic Protein Promoter

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Received 5 December 1988/Accepted 9 March 1989

The mouse myelin basic protein promoter was transcribed in brain nuclear extracts. The distal promoter region from −253 to −54 directed preferential transcription in brain extracts, whereas the same region repressed transcription activity in liver extracts. Stimulation of transcription was observed when the distal region was located only in a native orientation. The proximal region downstream from −53 alone still directed preferential transcription. It is suggested that cooperative function by the two promoter regions may be required for higher specificity.

Myelinogenesis is one of the essential processes for nervous activity of the vertebrates, and many diseases that cause defects in this process in animals and humans have been documented (10, 14, 15, 23, 24). Myelin basic protein (MBP) is a major protein of myelin in the central nervous system, and we are studying molecular mechanisms of transcription control of the MBP gene. Among genes relating to the nervous system, that for MBP is a particularly good example for the study of brain-specific gene expression, since the promoter activity is very strong in the brain (20) and since fundamental promoter functions of the MBP gene have been well characterized (6, 16, 21, 22, 25–27). In addition, transgenic mice studies have already specified a 1.3-kilobase region of upstream sequence necessary and sufficient for nervous tissue-specific expression of the MBP gene (12). In vitro study is a powerful method for analysis of interaction of *cis*-acting elements with their *trans*-acting factors. In this study, we established tissue-specific transcription from the MBP promoter in brain-derived nuclear extracts and investigated promoter regions required for specific transcription.

Nuclear extracts from the brain of mice and rats were prepared as previously described (7). The MBP promoter sequences have also been determined (16). Structures of a series of the 5′-deletion mutants of the mouse MBP promoter-carrying plasmids used in this study are illustrated in Fig. 1A. In all experiments, closed circular DNAs were used as templates. We used pBP1318 DNA, which contained a region similar to that of the MBP promoter used in transgenic mice studies, to investigate transcription competency of the brain nuclear extract. In vitro transcription was carried out for 45 min at 30°C. Preincubation mixture contained 8 μl of extract (10 mg of protein per ml), 4 μl of the dialysis buffer (7), and 1.5 μl of template DNA (300 ng/μl). After preincubation for 10 min on ice, 6.5 μl of a mixture containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.6), 9% glycerol, 80 mM KCl, 18 mM $MgCl_2$, 0.08 mM CTP, 2 mM ATP, 2 mM UTP, 2 mM GTP, and 8 μCi of [α-^{32}P]CTP was added to start transcription. Reaction was terminated by sodium dodecyl sulfate, and RNAs were purified by phenol-chloroform extraction. Transcripts were analyzed by a modified S1-mapping procedure (9) using single-stranded DNA probe. For modified S1 mapping, RNA was hybridized with 2.5 μg of corresponding single-stranded DNA probe at 37°C for 30 min in the presence of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.5), 1 mM EDTA, 0.4 M NaCl, and 50% formamide and then denatured at 85°C for 10 min. Single-stranded nucleic acids were digested with 220 U of S1 nuclease in a buffer containing 30 mM sodium acetate (pH 4.5), 3 mM $ZnSO_4$, and 0.4 M NaCl at 37°C for 30 min. Digestion was terminated by adding 33 mM EDTA and 0.33 M Tris hydrochloride (pH 8.0), and S1-resistant RNAs were analyzed through a 5% sequencing gel. The MBP promoter in pBP1318 yielded a strong signal of 415 bases, demonstrating the mouse brain nuclear extract to be apparently transcription competent (Fig. 1B). This signal is α-amanitin sensitive, and the transcription start point has been confirmed to be identical to that of the MBP mRNA (data not shown). The amounts of transcripts from pBP652 and pBP253 were similar to the amounts from pBP1318. However, the transcript of pBP53 decreased to 25% of the pBP253 transcript. Positive transcription elements functioning in the brain extract were found to be located downstream from −253. The same results were obtained with rat brain extracts (data not shown). We chose pB253 to study brain-specific transcription.

In this study, we did not put any promoter in the reaction mixture as an internal control because this could cause interferences in experimental transcripts. Addition of pML DNA as an internal control generated nonspecific signals and preferentially enhanced transcription of pBP253 (Fig. 2, lane 3). Moreover, the internal MLP signal in lane 4 of Fig. 2 was 1.7 times stronger than that in lane 3. Without pML DNA in the reaction mixture, the ratio of transcripts of pBP253 to those of pB53 (lanes 1 and 2) was 3.7. However, the ratio changed to 6.3 in the presence of pML DNA (lanes 3 and 4). We may conclude that the exclusion of internal control is a reasonable procedure for analyzing authentic amounts of transcripts.

To demonstrate promoter-selective transcription, we conducted in vitro transcriptions with three promoters, including adenovirus type 2 major late protein (MLP) (pML), mouse albumin (pALB), and mouse MBP (pBP253) promoters in rat brain and rat liver extracts (Fig. 3A). Since pML contains a short MLP sequence around the TATA box and

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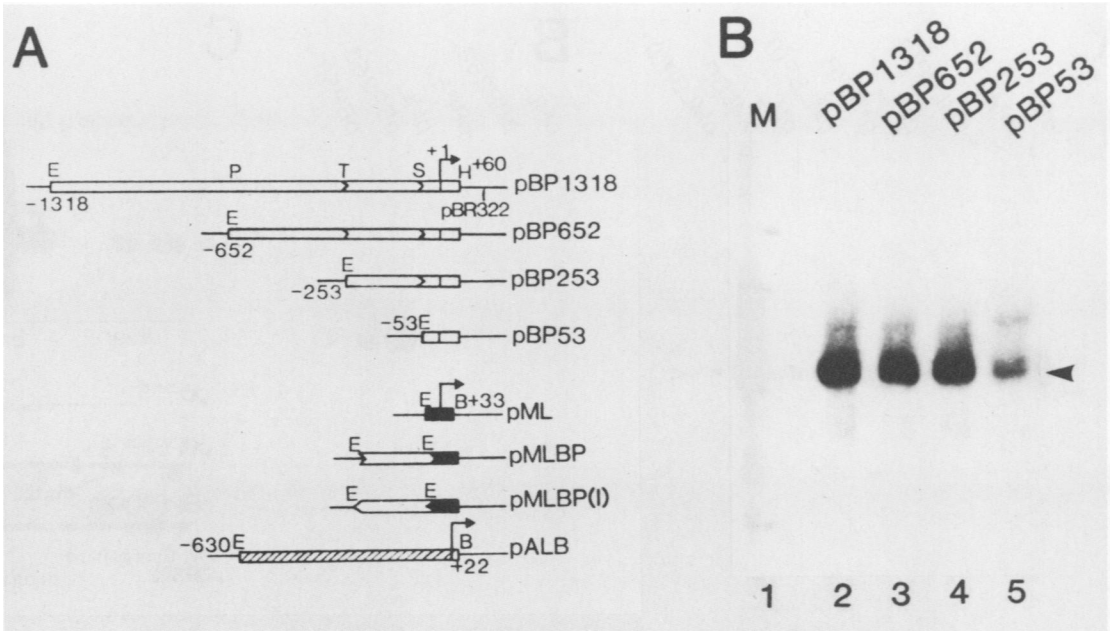

FIG. 1. Structure of recombinant DNAs (A) and in vitro transcription from the MBP promoter in the mouse brain nuclear extract (B). (A) Sequences shown are for pBR322 vector (——), MBP (16) (□), MLP (1) (■), and mouse albumin (▨) (7). Restriction sites are abbreviated as follows: E, *Eco*RI; P, *Pst*I; T, *Stu*I; H, *Hin*dIII; S, *Sal*I; B, *Bam*HI. pML contains MLP sequences from −34 and +33 (27). Single-stranded DNA probes, each of which has appropriate transcribed sequences, were used to detect specific transcription signals 415, 316, and 307 bases long for the MBP, MLP, and albumin genes, respectively. (B) pBP series DNAs were transcribed in the brain nuclear extract of 11-day ICR mice as described in the text. The signal for the MBP transcripts (415 bases long) is indicated by an arrowhead. Lane M, pBR322-*Msp*I marker.

the transcription initiation site (−34 to +33) (Fig. 1A), it is considered a "minimum promoter" with no activating elements. The transcription ability of each extract was normalized by assuming that transcripts of pML were produced at similar levels by both extracts. This standardization was verified with other control promoters (unpublished results). MLP was active in both extracts (Fig. 3A, lanes 2 and 5). However, the albumin promoter was active in the liver extract but not in the brain extract, in agreement with the results of Gorski et al. (7). In contrast, the MBP promoter was preferentially transcribed in the brain extract. Thus, in

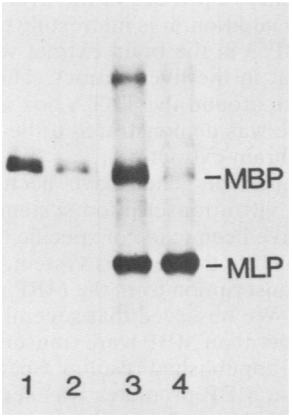

FIG. 2. Effects of internal control in the in vitro transcription system. pBP253 (lanes 1 and 3) and pBP53 (lanes 2 and 4) (450 ng each) were transcribed in the mouse brain nuclear extract in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of pML DNA (450 ng). The reaction conditions were as described in the legend to Fig. 1B.

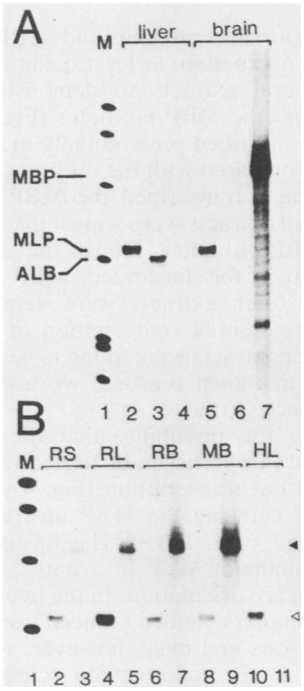

FIG. 3. Tissue-specific transcription of the MBP promoter. (A) MLP (pML) (lanes 2 and 5), mouse albumin (pALB) (lanes 3 and 6), and MBP (pBP253) (lanes 4 and 7) were transcribed in liver and brain nuclear extracts of Wistar rats as described in the legend to Fig. 1B. (B) pML (◁) and pBP253 (◀) were transcribed in nuclear extracts of rat spleen (RS), rat liver (RL), rat brain (RB), mouse brain (MB), and HeLa cells (HL). Lane M, Molecular weight markers.

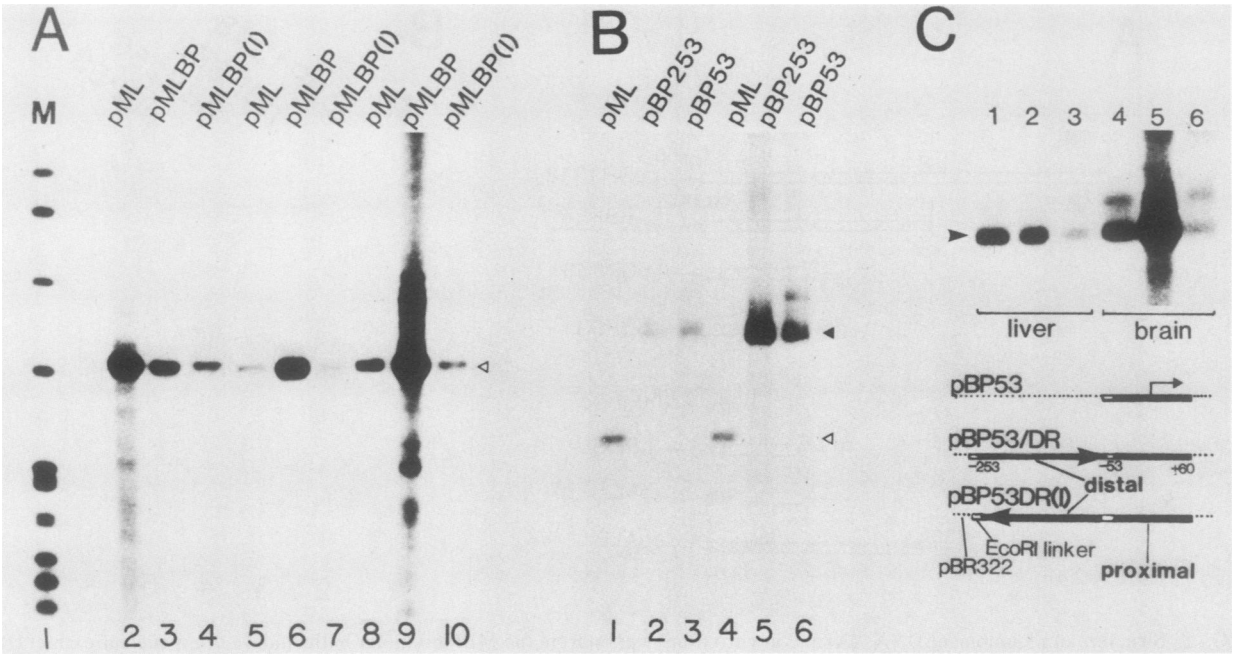

FIG. 4. Effect of the distal and proximal promoter regions of the MBP promoter. The reaction conditions were as described in the legend to Fig. 1B. Signals of MLP (◁) and MBP (◀) are indicated. (A) MLP and MBP-MLP chimeric promoters (Fig. 1A) were transcribed in rat liver (lanes 2 through 4), rat brain (lanes 5 through 7), and mouse brain (lanes 8 through 10) extracts. Lane M, Molecular weight markers. (B) Effect of elimination of the distal region from the proximal region on transcription activity in rat liver (lanes 1 through 3) and mouse brain (lanes 4 through 6) extracts. (C) pBP53 (lanes 1 and 4), pBP53-DR (lanes 2 and 5), and pBP53-DR(I) (lanes 3 and 6) were transcribed in the rat liver or mouse brain extract. Configurations of the distal and proximal MBP promoter regions and *Eco*RI linkers (10-mer) in each template are illustrated. Transcripts are indicated by an arrowhead.

vitro transcription from albumin and MBP promoters can mimic the in vivo situation, at least qualitatively.

We used several extracts to demonstrate preferential transcription from the MBP promoter (Fig. 3B). The MBP promoter was transcribed preferentially in brain extracts of rats and mice. Compared with the rat brain extract, rat liver and spleen extracts transcribed the MBP promoter inefficiently. HeLa cell extracts were almost inactive in transcribing from the MBP promoter, though the same extract was significantly active for linearized MBP templates (27). Mouse and rat brain extracts were demonstrated to be equivalent in preferential transcription of the MBP gene. Since mouse liver extracts were found to be not transcription competent, for unknown reasons, we used rat liver as a source of reference extract.

To investigate the possibility that the MBP upstream region itself could drive tissue-restricted in vitro transcription, we carried out transcription (Fig. 4A) using chimeric MBP promoters carrying the MBP upstream region from −253 to −54 and pML DNA. The upstream region was joined to the minimum MLP in a native (pMLBP) or an inverted [pMLBP(I)] orientation. In the liver extract, both of the chimeric promoters yielded reduced transcripts. In brain extracts of both rats and mice, however, we found considerable enhancement in transcription of pMLBP compared with that of pML. No appreciable transcription stimulation was observed when the upstream region was ligated in the inverse orientation. These results demonstrate that the upstream sequence of the MBP promoter alone functions in the brain extract to elevate transcription from the MLP. In the brain extracts, the MBP upstream region was also able to stimulate transcription from the MBP promoter at −53 to +60 when it was joined in a native orientation by *Eco*RI linkers (Fig. 4C, lane 5). However, when joined in an inverted orientation, the upstream region did not enhance transcription (Fig. 4C, lane 6). In liver extracts, we observed no activating effect by the upstream region in any orientations.

We assayed transcription of pBP253 and pBP53 in both liver and brain extracts. As shown above (Fig. 1B), pBP53 was more weakly transcribed than pBP253 in the brain extract (Fig. 4B, lanes 5 and 6). Quantitative analysis revealed that the transcription efficiency of pBP53 was 25% that of pBP253. However, transcription activity of pBP53 was higher than that of pBP253 in the liver extract (Fig. 4B, lanes 2 and 3). In addition, it is interesting that the transcription signal of pBP53 in the brain extract was still 2.2 times stronger than that in the liver extract. The MBP promoter carrying sequence around the TATA box and the transcription initiation site was demonstrated to be transcribed preferentially in the brain extract.

A limited number of genes have been investigated by tissue-specific in vitro transcription systems (7, 13, 17, 29), but no reports have been made of specific in vitro transcription of genes related to the nervous system. In this paper, we report specific transcription from the MBP promoter in brain nuclear extracts. We observed that several nervous system-specific genes other than MBP were transcribed well in brain nuclear extracts (unpublished results). Specific in vitro transcription from the MBP promoter was seen when we used closed circular DNA templates (data not shown). A major positive *cis*-element of the MBP promoter is found downstream from −253, and similar results have been obtained in transfection experiments (16). Sequences downstream from −253 may be important for MBP expression in vivo, and this idea is not inconsistent with results of the transgenic mice

study (12). We observed no differences between mouse and rat brain extracts in specific transcription of the MBP gene. For the mouse albumin gene, we found that the promoter is preferentially transcribed in rat liver extracts, which agrees with results with a previous report (7).

We divided the MBP promoter into two domains referred to as the distal promoter, from −253 to −54, and the proximal promoter, from −53 to +60. The distal region was able to direct specific transcription by itself, even when linked to the heterologous MLP core promoter, suggesting that the distal region contains some tissue-specific *cis*-acting elements and that the brain is rich in the *trans*-acting cognate factors. Brain-specific transcription activation by the distal region may be conducted during the transcription preinitiation process. Neither postinitiation events nor stability of transcripts was expected to result in preferential in vitro transcription (Fig. 4A). In contrast to upstream activating sequences and enhancers of other genes, the MBP distal region seems to function in a direction-dependent manner. Only a native orientation of the distal region to the MLP core promoter and the MBP proximal promoter can govern enhanced transcription in brain extracts (Fig. 4A and C). Cooperative interaction of transcription factors in the distal and proximal regions may be required for tissue-specific transcription. Alternatively, inhibitory sequences in an upper part of the distal region may contribute to the different profiles of transcription activation. Indeed, we suggest that the distal region functions to repress the transcription in liver extracts. Repressive *cis*-acting elements have been found in other genes (2, 4, 18, 19). Transcription inhibitors associated with the distal region may be dominant in nonexpressing tissues.

We demonstrated that even the proximal promoter region alone directs specific transcription in vitro. Thus, there may be tissue-specific activating sequences in the proximal region as well. However, we could not find any known factor-binding sites between −53 and the TATA box at −34 (16, 27). There are other studies suggesting core promoter-driven tissue-specific transcription (5, 28). It has been suggested that eucaryotic TATA box factors are heterogeneous (3, 8). The TATA box sequence of the MBP promoter does not fit the common consensus sequences (16, 27). Therefore, the MBP promoter may utilize a specific TATA box factor.

We thank S. Takiya, K. Ikenaka, T. Furuichi, A. Miyawaki, and S. Yoshikawa for valuable discussion; Y. Ohya for technical assistance; and U. Schibler for providing plasmid DNAs, advising on preparation of extracts, and critically reading the manuscript.

This work was supported by a grant-in-aid for scientific research on priority areas from the Japanese Ministry of Education, Science and Culture and by a grant from Yamada Foundation.

LITERATURE CITED

1. **Baker, C. C., and E. B. Ziff.** 1981. Promoters and heterogeneous 5′ termini of the messenger RNAs of adenovirus serotype 2. J. Mol. Biol. **149**:189–221.
2. **Bouvagnet, P. F., E. E. Strehler, G. E. White, M.-A. Strehler-Page, B. Nadal-Ginard, and V. Mahdavi.** 1987. Multiple positive and negative 5′ regulatory elements control the cell-type-specific expression of the embryonic skeletal myosin heavy-chain gene. Mol. Cell. Biol. **7**:4377–4389.
3. **Chen, W., and K. Struhl.** 1988. Saturation mutagenesis of a yeast *his3* "TATA element": genetic evidence for a specific TATA-binding protein. Proc. Natl. Acad. Sci. USA **85**:2691–2695.
4. **Colantuoni, V., A. Pirozzi, C. Blance, and R. Cortese.** 1987. Negative control of liver-specific gene expression: cloned human retinol-binding protein gene is repressed in HeLa cells. EMBO J. **6**:631–636.
5. **Dierich, A., M.-P. Gaub, J.-P. LePennec, D. Astinotti, and P. Chambon.** 1987. Cell-specificity of the chicken ovalbumin and conalbumin promoters. EMBO J. **6**:2305–2312.
6. **Ferra, F., H. Engh, L. Hudson, J. Kamholz, C. Puckett, S. Molineaux, and R. A. Razzarini.** 1985. Alternative splicing accounts for the four forms of myelin basic protein. Cell **43**:721–727.
7. **Gorski, K., M. Carneiro, and U. Schibler.** 1986. Tissue-specific *in vitro* transcription from mouse albumin promoter. Cell **47**: 767–776.
8. **Grass, D. S., and J. L. Manley.** 1986. Effects of the adenovirus 2 late promoter on simian virus 40 transcription and replication. J. Virol. **57**:129–137.
9. **Hirose, S., M. Tsuda, and Y. Suzuki.** 1985. Enhanced transcription of fibroin gene *in vitro* on covalently closed circular templates. J. Biol. Chem. **260**:10557–10562.
10. **Hogan, E. L., and S. Greenfield.** 1984. Animal models of genetic disorders of myelin, p. 489–534. *In* P. Morell (ed.), Myelin. Plenum Publishing Corp., New York.
11. **Ikenaka, K., T. Furuichi, Y. Iwasaki, A. Moriguchi, H. Okano, and K. Mikoshiba.** 1988. Myelin proteolipid protein gene structure and its regulation of expression in normal and *jimpy* mutant mice. J. Mol. Biol. **199**:587–596.
12. **Katsuki, M., M. Sato, M. Kimura, M. Yokoyama, K. Kobayashi, and T. Nomura.** 1988. Conversion of normal behavior to *shiverer* by myelin basic protein antisense cDNA in transgenic mice. Science **241**:593–595.
13. **Lichtsteiner, S., J. Wuarin, and U. Schibler.** 1987. The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. Cell **51**:963–973.
14. **Matthieu, J. M., and F. X. Omlin.** 1984. Murine leukodystrophies as tools to study myelinogenesis in normal and pathological conditions. Neuropediatrics **15**(Suppl.):37–52.
15. **Mikoshiba, K., K. Takamatsu, and Y. Tsukada.** 1983. Peripheral nervous system of *shiverer* mutant mice: developmental change of myelin components and immunohistochemical demonstration of the absence of MBP and presence of P2 protein. Dev. Brain Res. **7**:71–79.
16. **Miura, N., T. Tamura, A. Aoyama, and K. Mikoshiba.** 1989. The promoter elements of the mouse myelin basic protein gene function efficiently in NG108-15 neuronal/glial cells. Gene **75**: 31–38.
17. **Monaci, P., A. Nicosia, and R. Cortese.** 1988. Two different liver-specific factors stimulate *in vitro* transcription from the human alpha-1-antitrypsin promoter. EMBO J. **7**:2075–2087.
18. **Nabel, G. J., C. Gorka, and D. Baltimore.** 1988. T-cell-specific expression of interleukin 2: evidence for a negative regulatory site. Proc. Natl. Acad. Sci. USA **85**:2934–2938.
19. **Nelson, J. A., C. Reynolds-Kohler, and B. A. Smith.** 1987. Negative and positive regulation by a short segment in the 5′-flanking region of the human cytomegalovirus major immediate-early gene. Mol. Cell. Biol. **7**:4125–4129.
20. **Norton, W. T.** 1981. Biochemistry of myelin, p. 93–121. *In* S. G. Waxman and J. M. Ritchie (ed.), Advance in neurobiology. Raven Press, New York.
21. **Okano, H., M. Miura, A. Moriguchi, K. Ikenaka, Y. Tsukada, and K. Mikoshiba.** 1987. Inefficient transcription of the myelin basic protein gene possibly causes hypomyelination in *myelin-deficient* mutant mice. J. Neurochem. **48**:470–476.
22. **Okano, H., T. Tamura, M. Miura, A. Aoyama, K. Ikenaka, M. Oshimura, and K. Mikoshiba.** 1988. Gene organization and transcription of duplicated MBP genes of *myelin deficient* (*shi*^{mld}) mutant mouse. EMBO J. **7**:77–83.
23. **Readhead, C., B. Popko, N. Takahashi, H. D. Shine, R. A. Saavedra, R. L. Sidman, and L. Hood.** 1987. Expression of myelin basic protein gene in transgenic *shiverer* mice: correction of the dysmyelinating phenotype. Cell **48**:703–712.
24. **Ritchie, J. M.** 1984. Physiological bases of conduction in myelinated nerve fibers, p. 117–1146. *In* P. Morell (ed.), Myelin. Plenum Publishing Corp., New York.
25. **Roach, A., N. Takahashi, D. Pravtcheva, F. Ruddle, and L.**

Hood. 1985. Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in *shiverer* mutant mice. Cell **42**:149–155.

26. **Takahashi, N., A. Roach, D. B. Teplow, S. B. Prusiner, and L. Hood.** 1985. Cloning and characterization of the myelin basic protein gene from mouse: one gene can encode both 14 kd and 18.5 kd MBPs by alternate use of exons. Cell **42**:139–148.
27. **Tamura, T., M. Miura, K. Ikenaka, and K. Mikoshiba.** 1989. Analysis of transcription control elements of the mouse myelin basic protein gene in HeLa cell extracts: demonstration of a strong NFI-binding motif in the upstream region. Nucleic Acids Res. **16**:11441–11459.
28. **Theill, L. E., O. Wiborg, and J. Vuust.** 1987. Cell-specific expression of the human gastrin gene: evidence for a control element located downstream of the TATA box. Mol. Cell. Biol. **7**:4329–4336.
29. **Tsuda, M., and Y. Suzuki.** 1981. Faithful transcription initiation of fibroin gene in a homologous cell-free system reveals an enhancing effect of 5′ flanking sequence far upstream. Cell **27**:175–182.