

# Nuclear Proteins in Mouse Brain Cells Bind Specifically to the Myelin Basic Protein Regulatory Region

Monir S. Lashgari, Kathleen Devine-Beach, Susan Haas, and Kamel Khalili

Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107-6799

## Abstract

Expression of myelin basic protein (MBP) in mice is regulated in a cell- and stage-specific manner during brain development. The MBP control region contains multiple *cis*-acting elements, shown by *in vivo* and *in vitro* assays, which are responsible for its unique pattern of transcription. Using synthetic DNA fragments spanning the MBP control region, we have analyzed nuclear proteins obtained from newborn (2–3 d), young adult (18–30 d), and adult (60 d) animals; these nuclear proteins form DNA–protein complexes with the MBP regulatory region. Brain extracts from young adult and adult mice showed enhanced binding activities with the sequences supporting transcriptional activation in glial cells. Deletion analysis of the proximal activating sequence located at position -14 to -50 with respect to the RNA initiation site resulted in identification of a small region, located between nucleotides -14 to -37, which is required for formation of the complexes. Southwestern assay revealed a major 39-kD protein from young adult brain extract that recognizes the sequences between nucleotides -14 to -37. An additional minor 37-kD protein, derived from young adult brain extract, was also found to be associated with this proximal activating region. Of particular interest is the observation that the minor 37-kD protein became more abundant in the extract derived from adult brain, whereas the major 39-kD protein became less abundant. The possible role of these proteins in cell/stage-specific transcription of MBP is discussed. (*J. Clin. Invest.* 1990. 86:1671–1677.) Key words: myelin basic protein gene • mouse brain nuclear extract • DNA–protein interaction • developmental regulation

## Introduction

The initiation, maintenance, and termination of myelination in the central nervous system (CNS) follows a complex regulatory program which is tightly controlled in a cell type- and developmental stage-specific manner (1). In the CNS, this process is carried out by oligodendroglial cells which are unique in their ability to form large amounts of myelin around the axons (2). A plateau is reached after sufficient formation of myelin

indicating the cessation of active myelin production (3). The protein composition of myelin is simple compared to other biological membranes, containing relatively few types of proteins. Among these proteins, the myelin basic proteins (MBPs),<sup>1</sup> hydrophobic proteolipid proteins (PLPs), myelin-associated glycoproteins (MAGs), and 2',3'-cyclic nucleotide 3'-phosphorylase (CNP) have been identified (3–5) and their cDNAs isolated (6–9). MBPs account for 30–40% of total protein in myelin sheaths and range in size from 14 to 21.5 kD (10, 11). These forms are produced from a single primary transcript from a single MBP gene through alternative patterns of RNA splicing (6–9, 12–15).

Earlier studies have demonstrated that the level of MBP expression correlates perfectly with the developmental profile of myelination (2, 16–21). Furthermore, it appears that each isoform has a unique developmental pattern of expression and accumulation (17). Northern blot and *in situ* hybridization techniques have been used to show that MBP mRNA is first detected at about 5 d after birth, peaks at 18 d, and progressively decreases to 20% of the peak level in adult mice (18). How the MBP gene is transcriptionally regulated during brain development in oligodendrocytes remains to be determined.

Several studies have indicated that at least two classes of regulatory sequences, promoter and enhancer, mediate the cell- and stage-specific activation of eukaryotic promoters (for review, see reference 22). Activation via promoter and enhancer elements is achieved by an interplay between *cis*-acting DNA sequences and *trans*-acting proteins which recognize these DNA elements (23). Thus, the level of transcription in different cell types and stages of development is governed, at least in part, by the relative abundance of an active tissue- and/or stage-specific transcription factor(s).

An essential step in understanding how MBP transcription is regulated is the identification of *cis*-acting DNA sequences and *trans*-acting factors that bind to these sequences. Unlike most known cellular promoters, MBP has no common *cis*-regulatory elements, such as TATA or CAAT box sequences, within the 300 basepairs of the transcription start site. It is likely that MBP control elements contain unique features that account for programming and tissue specificity of gene expression in brain cells.

Recent observations in our laboratory have indicated that tissue-specific transcription of MBP is regulated by DNA sequences located within the 320 basepairs 5'-upstream of the MBP transcriptional start site (24). Accordingly, Minura et al. (25) have recently demonstrated that the sequences positioned between nucleotides -54 to -253 are important for transcription of MBP promoter in brain cell extracts. In the present study, we have identified protein factors present in mouse brain nuclear extracts that specifically interact with the MBP 5'-upstream sequences. Extracts from mouse brain at different stages of myelination (i.e., 3-, 18/30-, and 60 d) were used in

Address reprint requests to Dr. Khalili, 490 Jefferson Alum Hall, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107-6799.

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1. Abbreviation used in this paper: MBP, myelin basic protein.

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these analyses. We demonstrate that, like many eukaryotic regulatory sequences, the MBP 5'-upstream region is composed of multiple protein binding domains which are able to interact with a sequence-specific nuclear protein(s) that may be a component of a cell- and/or stage-specific MBP regulatory apparatus. Of particular interest is the finding that the brain extracts from 3-, 18/30-, and 60-d-old mice show differential binding activities with the proximal *cis*-acting regulatory sequence.

## Methods

**Mouse brain nuclear extract.** Brain nuclear extracts were prepared from 2-3-, 18-30-, and 60-d-old mice. Briefly, isolated brains were minced and homogenized in PBS and processed according to the method of Wildeman et al. (26). Brain preparations were dialyzed against 20 mM Hepes (pH 7.9), 20 mM KCl, 17% glycerol, and 1 mM DTT. This preparation was used directly as extracts for band-shift and competition assays.

**Preparation of double-stranded radiolabeled oligodeoxynucleotide DNA probes.** Single-stranded complementary oligodeoxynucleotides were hybridized and end-labeled by T4-polynucleotide kinase in the presence of [<sup>32</sup>P]ATP according to the method of Maniatis et al. (27). Labeled DNAs were purified on a 9% polyacrylamide gel.

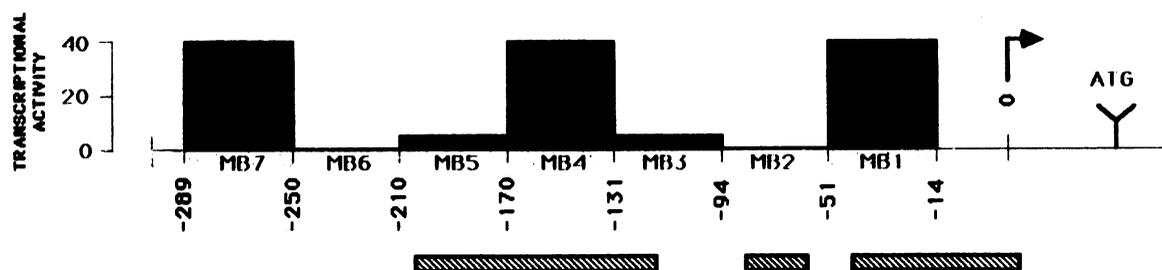
**Band-shift assay.** Protein-DNA complexes were identified by running the samples on a low ionic strength polyacrylamide gel (28). Protein samples (20 μg per reaction) were incubated with 2 × 10<sup>6</sup> cpm/ng of <sup>32</sup>P end-labeled double-stranded DNA in a mixture containing 5 μg of poly(dI-dC) (Pharmacia, Inc., Piscataway, NJ), 17%

glycerol, 12 mM Hepes (pH 7.9), 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 4 mM Tris (pH 8.0), and 0.8 mM DTT. Incubation was carried out for 30 min on ice and samples were purified on a 9% polyacrylamide native gel for 2-3 h at 200 V at 4°C. Gels were dried on 3MM paper (Whatman, Inc., Clifton, NJ) and exposed overnight at -70°C.

Competition analysis was performed by incubating unlabeled competitor DNA with the extract for 30 min on ice before adding the probe to the reaction mixture. The heterologous competitor oligonucleotide was derived from the first intron of MBP gene (40 nucleotides in length). Samples were incubated a second time under identical conditions before transfer to the gel.

**Southwestern blot analysis.** Brain nuclear extract (80 μg in sample buffer) from 18-d-old mice was loaded on a 10% SDS-polyacrylamide gel and electrophoresed overnight at 100 V. Proteins were transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, NH) by a Transblot apparatus (Bio-Rad Laboratories, Richmond, CA) filled with transfer buffer (192 mM glycine, 25 mM Tris-base, and 20% methanol) for 3 h at 400 mA. Filters were processed according to the method of Singh et al. (29). Briefly, the transferred proteins were denatured in buffer containing 50 mM Tris (pH 8.0), 7 M guanidine-HCl, 50 mM DTT, 2 mM EDTA, and 0.25% Blotto (5% nonfat dry milk, 50 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) for 1 h at room temperature. The denaturing buffer was discarded and proteins were renatured by immersing the nitrocellulose filters in renaturing buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% NP40, and 0.25% Blotto) for 24 h at 4°C. Proteins were then incubated for 2 h at room temperature in TNE 50 buffer (20 mM Tris, pH 8.0, 1 mM EDTA, and 100 mM NaCl) before adding 10<sup>6</sup> cpm/ml of <sup>32</sup>P end-labeled double-stranded deoxyoligonucleotides. After incubating for another hour, filters were washed three times in TNE 50 solution, dried, and exposed overnight at -70°C.

**A.**



**B.**

**MB 1** 5'-CAGGGAGGACACACCCCTTCAGGACAGGCCCTCTGA-3'  
3'-GTCCCTCCTGTTGTGGAGGTTTCTGTCG66GAGACT-5'

**MB 2** 5'-CTCAGAGGGCCTGTCCTTTGAGGGTGTGTCCTCCCT-3'  
3'-GAGTCTCCCGGACAGAACTTCACACAGGAGGGG-5'

**MB 3** 5'-TGCCCTGCAAGGATGCCACCCAGCTGACCCAGGGTG-3'  
3'-ACGGACGCTCTACGGGTGGGTCGACTG66TCCAC-5'

**MB 4** 5'-CAGCGGTGACTGACTCCAGGCGCACAGCGGACCCGAG-3'  
3'-GTCGCCACTGACTGAGGTTCCGCTGTCGCTG66CTTC-5'

**MB 5** 5'-GCAGATGCGAACCCACACCAATGGAATCTCCTCTCAGGAC-3'  
3'-CGTCTACGCTTGGTGTGTTACCTTAGAGGGAGGCTCTG-5'

**MB 6** 5'-ACAGGCCCGTATTCATATCTCATTGTTGTTGACAGGAG-3'  
3'-TGCCCGGACATAGATAGAGTAAACAAACGCTCCTC-5'

**MB 7** 5'-CCCCTCAGAGAAAGCTGCTTTGTCG66TCTCAGCTTT-3'  
3'-GGGAGTCTCTTTCCAGCAACAGGCCACGAGTCAAA-5'

**Figure 1.** Schematic illustration of MBP regulatory region. (A) Position of transcriptionally active domains spanning MBP 5'-untranslated region. The arrow indicates the transcription start site at position 0. The vertical solid bars illustrate transcriptional activity of various upstream regions as rated arbitrarily on a scale of 0 to 40 (Devine-Beach et al. [24]). The horizontal striped bars demonstrate transcriptional activity of MBP 5'-upstream sequence as reported by Minura et al. (25). (B) Fragments MB<sub>1</sub> through MB<sub>7</sub> represent synthetic complementary oligonucleotides, 35- to 40-bp in length, derived from myelin basic protein gene sequences between nucleotides -14 to -357 with respect to the transcription start site. MB<sub>1</sub> (-14 to -50), MB<sub>2</sub> (-51 to -93), MB<sub>3</sub> (-94 to -130), MB<sub>4</sub> (-131 to -169), MB<sub>5</sub> (-170 to -209), MB<sub>6</sub> (-210 to -249), and MB<sub>7</sub> (-250 to -288). These sequence data are available from EMBL/GenBank/DBJ under accession number J04746.

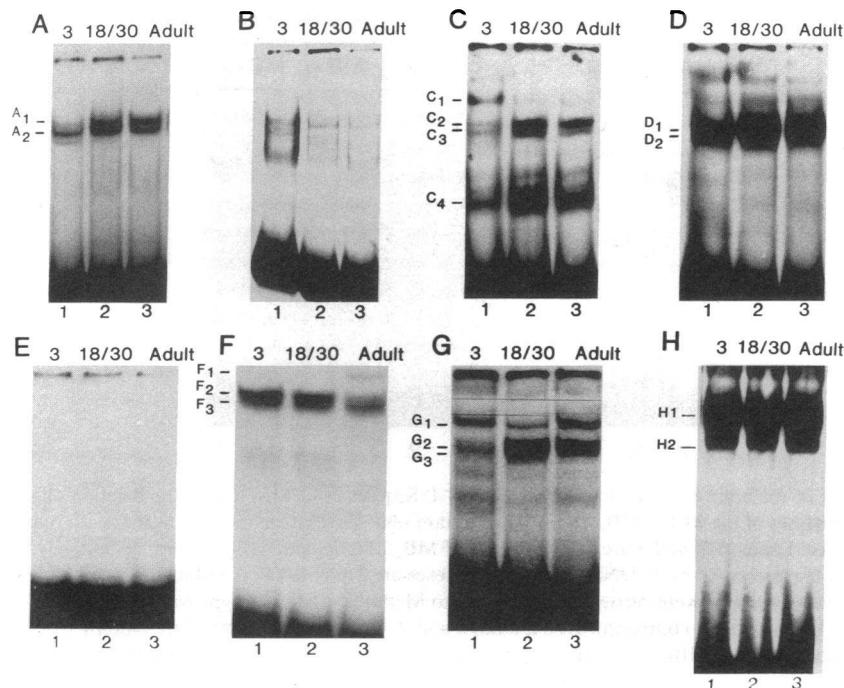
## Results

**Binding of mouse brain nuclear proteins to the MBP regulatory region.** Fig. 1 illustrates the 5'-upstream region of MBP spanning nucleotides -14 to -288, with respect to its transcription start site, and the *cis*-acting regulatory domains that control transcription of the MBP gene in glial cells. To determine whether the MBP 5'-upstream sequence contains any target sites for the binding of mouse brain nuclear proteins, we prepared seven nonoverlapping synthetic oligodeoxynucleotide fragments spanning 275 nucleotides upstream of the MBP regulatory sequence. These fragments were used in the band-shift assay to probe brain nuclear extracts from mice of 2-3, 18-30, and 60 d of age, hereafter referred to as newborn, young adult, and adult extracts, respectively. We chose these periods for our studies because the MBP transcription starts at ~ 5 d after birth, maximizes at 18-25 d after birth, and progressively decreases to 20% of the peak levels in the adult (18). Thus, it is likely that the level of transcription of MBP promoter at different stages of brain development is achieved by the relative abundance of activators that bind to the regulatory domains. Fragment MB<sub>1</sub> (-14 to -50), in the presence of newborn brain extract, formed a complex that resolved as a major band designated A<sub>2</sub> (Fig. 2 A, lane 1). This DNA fragment produced an additional complex (A<sub>1</sub>) with young adult and adult mouse brain extracts (Fig. 2 A, cf. lane 1 with 2 and 3). Fragment MB<sub>2</sub> (-51 to -93) demonstrated similar mobility patterns in all three extracts (Fig. 2 B). Four major complexes with distinct mobilities, C<sub>1</sub>-C<sub>4</sub>, were observed with fragment MB<sub>3</sub> (-94 to -130) (Fig. 2 C, lanes 1-3). Complex C<sub>1</sub> was more prominent in the newborn than in the young adult or adult extracts, whereas complexes C<sub>2</sub>-C<sub>4</sub> were more abundant in the extracts from older mice (Fig. 2 C, cf. lane 1 with 2 and 3). Two closely migrating complexes, D<sub>1</sub> and D<sub>2</sub>, with similar migrating patterns were observed with the MB<sub>4</sub> fragment (-131 to -169) in all three extracts (Fig. 2 D, lanes 1-3). Comparison of band

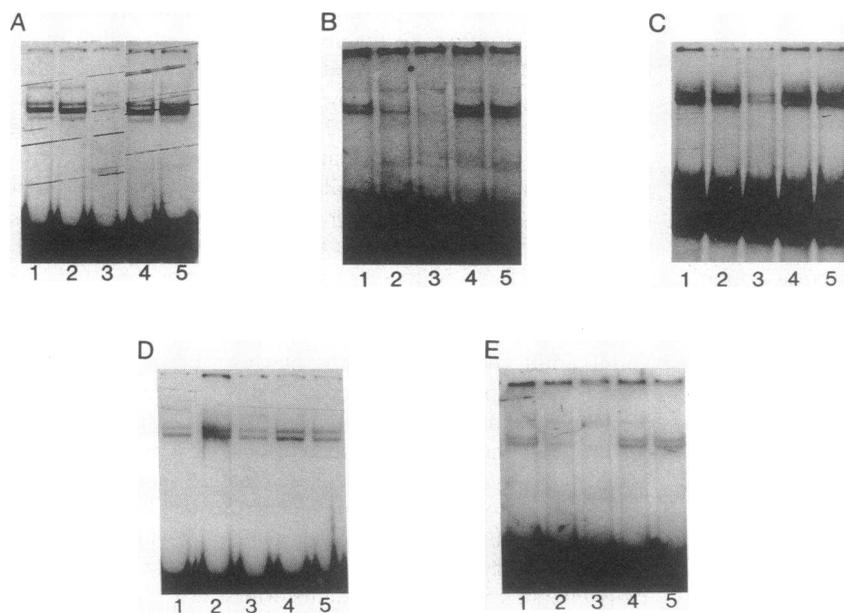
intensity suggested the presence of the relevant protein(s) at higher concentrations in the young adult and the adult brain extracts than in the newborn extract (Fig. 2 D, cf. lanes 2 and 3 with 1). MB<sub>5</sub> (-170 to -209) formed no detectable complex in any of the three extracts (Fig. 2 E). Fragment MB<sub>6</sub>, spanning -210 to -249, produced two major complexes, F<sub>2</sub> and F<sub>3</sub>, with similar mobility patterns in all three extracts. An additional complex (F<sub>1</sub>), although minor, was detected when the MB<sub>6</sub> probe was incubated with the adult brain extract (Fig. 2 F). Fragment MB<sub>7</sub> (-250 to -288) produced minor (G<sub>1</sub>) and two major (G<sub>2</sub> and G<sub>3</sub>) complexes with all extracts (Fig. 2 G). Intensity of the G<sub>2</sub> and G<sub>3</sub> bands was significantly higher in the young adult and adult than the newborn brain extract. The minor G<sub>1</sub> complex was not reproducibly detected in the subsequent experiments (see below, Fig. 3 C). To ensure comparable protein concentration of the various extracts, the binding activity of a synthetic oligodeoxynucleotide representing the central region of the JCV enhancer sequence (30) was examined in parallel. JC is a human polyoma virus which replicates exclusively in human fetal glial cells. The control region of the virus contains enhancer elements which are responsible for tissue-specific transcription of the viral gene (30). As shown in Fig. 2 H, this fragment produces two major complexes (H<sub>1</sub> and H<sub>2</sub>) with identical intensities and mobility patterns in all three brain extracts.

The results of these experiments suggest (a) the presence of multiple distinct protein-binding domains at the 5'-upstream regulatory region of MBP, and (b) the differential interaction of these protein-binding domains with nuclear proteins derived from mouse brain at different stages of myelination.

**Specificity of DNA protein complex.** Previous studies in our (24) and other (25) laboratories have identified three major regulatory elements spanning the proximal region, -14 to -50 (MB<sub>1</sub>), and distal regions, -131 to -169 (MB<sub>4</sub>), and -250 to -288 (MB<sub>7</sub>), with respect to the transcription start site. To confirm the specificity of proteins binding to these major regu-



**Figure 2.** Band-shift analyses of the MBP regulatory sequence with brain nuclear extract from mice at different stages of development. DNA-protein binding activities of the oligodeoxynucleotide probes MB<sub>1</sub> to MB<sub>7</sub> are shown in A-G. The resulting DNA-protein complexes were separated from free fragments by electrophoresis on a 9% native polyacrylamide gel as described in Methods. In lanes 1, 2, and 3, brain nuclear extract was prepared from newborn, young adult and adult mice, respectively. H represents the JCV enhancer DNA sequence used as a control for protein concentration in the three extracts.

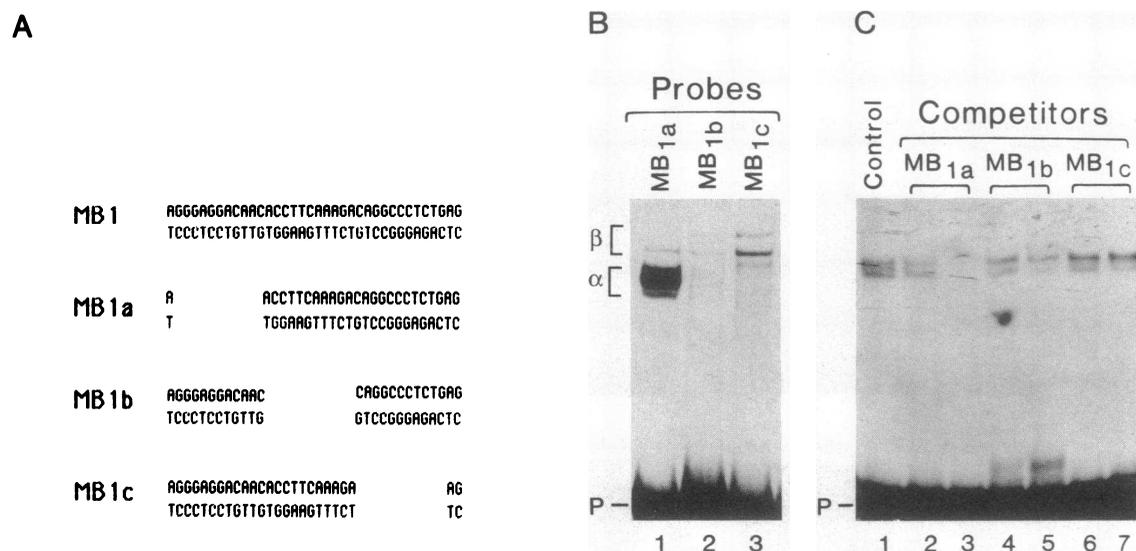


**Figure 3.** Competition analyses of oligodeoxynucleotides MB<sub>1</sub>, MB<sub>4</sub> and MB<sub>7</sub> for protein binding. Competition reactions were carried out as described in Methods. (A, B, and C) Competition reactions of MB<sub>1</sub>, MB<sub>4</sub>, and MB<sub>7</sub>, respectively, with homologous and heterologous competitor DNAs. Lanes 1, no competitor DNA; lanes 2 and 3, binding reactions in the presence of a 50- and 500-fold excess of unlabeled homologous MB domains, respectively; lanes 4 and 5, binding reactions in the presence of 50- and 500-fold excess of unrelated DNA, respectively. (D and E) Cross-competition reactions of MB<sub>1</sub> and MB<sub>4</sub> as probe, respectively. Lanes 1, no competitor DNA; lanes 2 and 3, binding reactions in the presence of 50- and 500-fold excess of unlabeled MB<sub>4</sub>; lanes 4 and 5, binding reactions in the presence of a 50- and 500-fold excess of unlabeled MB<sub>7</sub>.

latory elements, competition experiments were performed using unlabeled homologous or unrelated DNA fragments. Preincubation of the young adult brain extract with a 50- or 500-fold excess of unlabeled MB<sub>1</sub> significantly reduced the intensity of the corresponding complexes (Fig. 3 A, compare lane 1 with 2 and 3), whereas under identical conditions mixing of a 50- or 500-fold excess concentration of the unrelated DNA had no significant effect on complex formation (Fig. 3 A, compare lane 1 with 4 and 5). Similar results were obtained using the MB<sub>4</sub> (-131 to -169) and MB<sub>7</sub> (-250 to -288) fragments (Fig. 3, B and C, respectively). Cross-competition experiments

using MB<sub>1</sub> as a probe and MB<sub>4</sub> and MB<sub>7</sub> as competitor DNAs (Fig. 3 D) and MB<sub>4</sub> as a probe and MB<sub>7</sub> as a competitor (Fig. 3 E) suggested that each regulatory sequence may interact with distinct protein. These results suggest that the protein factor(s) recognized by the MBP *cis*-acting elements has a sequence-specific DNA binding property.

*Targeting of the binding site within the MBP proximal cis-acting element.* A greater cell type-specificity for MBP transcription *in vivo* is strongly associated with the most proximal *cis*-acting element MB<sub>1</sub>, thus, further investigations were focused on this domain. Three DNA fragments were derived



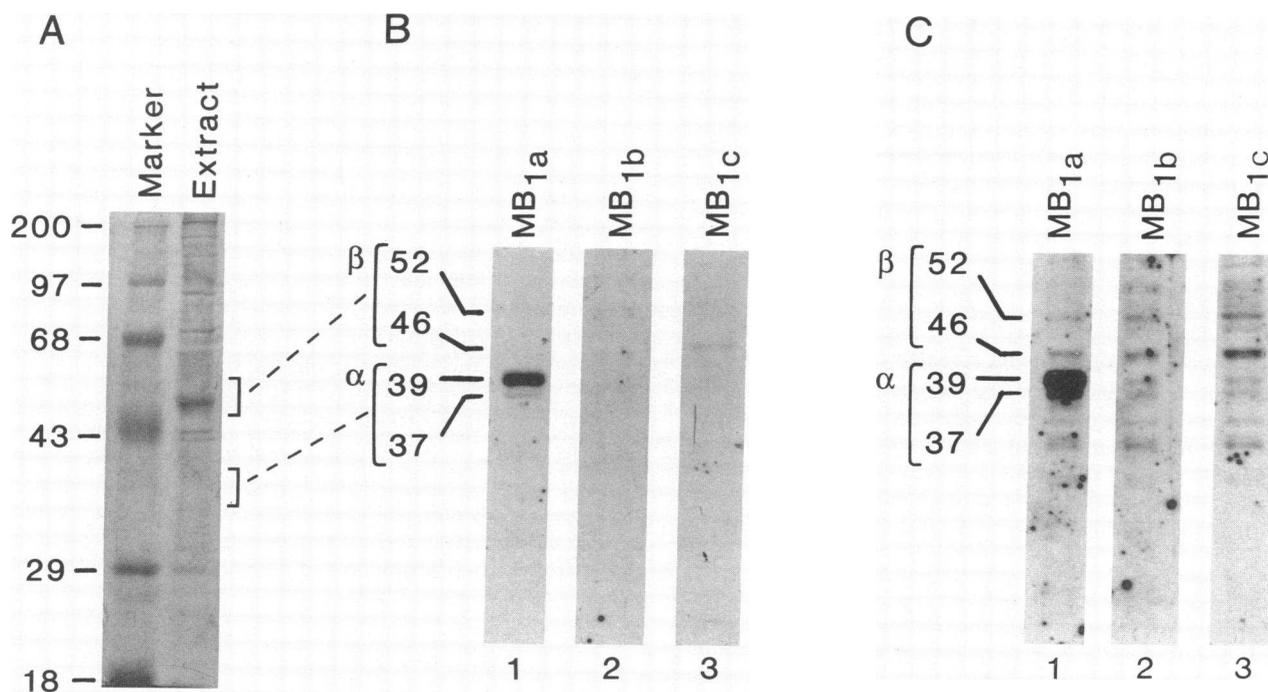
**Figure 4.** Identification of the target region for binding of the brain-protein factor(s) to MB<sub>1a</sub>. (A) Sequences of MB<sub>1</sub> and 11-bp deletion oligodeoxynucleotides MB<sub>1a</sub>, MB<sub>1b</sub>, and MB<sub>1c</sub>. (B) Band-shift analyses of the MB<sub>1a</sub>, MB<sub>1b</sub>, and MB<sub>1c</sub> mutant oligodeoxynucleotides as probes with brain nuclear protein extract prepared from young adult mice. Lanes 1, 2, and 3 are representative of MB<sub>1a</sub>, MB<sub>1b</sub>, and MB<sub>1c</sub> probes, respectively. Free labeled DNA at the bottom left side of the panel is represented by P. DNA-protein complexes are presented as  $\alpha$  and  $\beta$ . (C) Competition analyses of oligodeoxynucleotides MB<sub>1a</sub>, MB<sub>1b</sub>, and MB<sub>1c</sub> mutants were carried out according to Methods. MB<sub>1</sub> wild type was end-labeled as probe in binding to nuclear extract at 18/30 d of age. Lane 1 has no competitor DNA, lanes 2 and 3, 4, and 5, and 6 and 7 are binding reactions in the presence of 50- or 500-fold excess of unlabeled MB<sub>1a</sub>, MB<sub>1b</sub>, and MB<sub>1c</sub>, respectively. These sequence data are available from EMBL/Gen Bank/DDBJ under accession number J04746.

from the MB<sub>1</sub> sequence by deleting 11 nucleotides at the 5' end (MB<sub>1a</sub>), middle (MB<sub>1b</sub>), and 3' end (MB<sub>1c</sub>), as shown in Fig. 4 A. These sequences were synthesized and used as probes in a band-shift assay utilizing extracts prepared from young adult mice. Only MB<sub>1a</sub>, with the deletion at the 5' end, produced a major complex ( $\alpha$ ) (Fig. 4 B, lane 1). In addition, faint slower migrating bands ( $\beta$ ) were also detected with all three probes. The  $\beta$ -complex was consistently more intense with the MB<sub>1c</sub> probe (Fig. 4 B, lane 3).

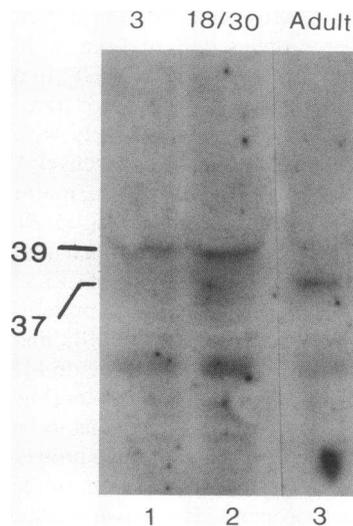
To examine the specificity of the binding, we performed competition experiments using oligo-MB<sub>1</sub> as probe and unlabeled oligo-MB<sub>1a</sub>, oligo-MB<sub>1b</sub>, and oligo-MB<sub>1c</sub> as competitors. The two major complexes shown in Fig. 1 A, lane 2, A<sub>1</sub> (top band) and A<sub>2</sub> (bottom band), were again detected with the MB<sub>1</sub> probe (Fig. 4 C, lane 1). Addition of a 500-fold excess of unlabeled MB<sub>1a</sub> fragment in the incubation mixture abolished the formation of both MB<sub>1</sub> complexes (Fig. 4 C, cf. lane 1 with 3). Addition of 50- to 500-fold excess of MB<sub>1b</sub> or MB<sub>1c</sub> showed a minimum, if any, effect on the intensity of the complexes (Fig. 4 C, lanes 4-7). These observations indicate that at least two protein components from brain of young adult mice interact with the MB<sub>1</sub> region: the major  $\alpha$  complexes, specifically detected with the MB<sub>1a</sub> DNA spanning -14 to -37, and the minor  $\beta$  complexes, formed with the sequences present in MB<sub>1a</sub>, MB<sub>1b</sub>, and MB<sub>1c</sub>.

**Identification of the binding protein.** To identify the nuclear protein(s) involved in binding to the proximal cis-regulatory elements of the MBP promoter, we performed Southwestern

blot analysis (29) using MB<sub>1a</sub>, MB<sub>1b</sub>, and MB<sub>1c</sub> as probes. Fig. 5 A illustrates the nuclear proteins derived from brain extract of 18/30-d-old mice and resolved by SDS-PAGE. In a parallel experiment, the gel-fractionated proteins were transferred to nitrocellulose paper and probed separately with MB<sub>1a</sub>, MB<sub>1b</sub>, or MB<sub>1c</sub> (Fig. 5 B, lanes 1, 2, and 3, respectively). MB<sub>1a</sub>, spanning nucleotides -14 to -37, bound to a major 39-kD and a minor 37-kD protein ( $\alpha$ -complex) (Fig. 5 B). Longer exposure of the *trans*-blot revealed two additional faint bands that migrated along with the marker proteins of size 52 and 46 kD ( $\beta$ -complex) (Fig. 5 C, lane 1). These two proteins also appeared when the *trans*-blots were probed with MB<sub>1b</sub> and MB<sub>1c</sub> (Fig. 5 C, lanes 2 and 3). The intensity of the 46-kD species, in particular, was higher in the MB<sub>1c</sub>-probed blot (Fig. 5 C, lane 3). The specificity of the  $\beta$ -complex remains to be clarified since this complex was detected with all three probes, MB<sub>1a</sub>, MB<sub>1b</sub>, and MB<sub>1c</sub>, and comigrated with a region of the gel containing highly abundant proteins. The above results, together with those of the band-shift analysis shown in Fig. 4 B, suggest that the  $\alpha$ -complex is formed by association of the 39- and 37-kD proteins with a subregion of MB<sub>1a</sub>. To investigate the abundance of the 39- and 37-kD proteins in brain nuclear extracts from mice of 2/3- (newborn), 18/30- (young adult), and 60-d-old (adult), Southwestern blot analysis using MB<sub>1a</sub> as probe was performed. The 39-kD protein was found to be more abundant in the newborn and young adult extracts, whereas the adult brain extract showed a significant increase in the levels of the 37-kD protein (Fig. 6, compare lanes 1 and 2



**Figure 5.** Identification of possible *trans*-acting factor(s) from mouse nuclear protein extract which specifically bind to MB<sub>1</sub> mutants. (A) Coomassie blue staining of prestained high molecular weight standard protein marker (Bethesda Research Laboratories) (left lane) and of nuclear extract from young adult mouse (right lane) on 10% SDS-polyacrylamide gel. Numbers on the left indicate molecular size in kilodaltons. Brackets on the right side indicate the presumed positions of the  $\alpha$ - and  $\beta$ -complexes appearing on transfer blot in B. (B) Southwestern analyses of brain nuclear extract from 18/30-d-old mouse with probes prepared from MB<sub>1</sub> deletions. Lane 1, 2, and 3 represent MB<sub>1a</sub>, MB<sub>1b</sub>, and MB<sub>1c</sub> probes, respectively. Brackets on the left indicate the  $\alpha$  and  $\beta$  DNA-protein complexes. Molecular size in kilodaltons of the bands composing each complex is given on the left. (C) Longer exposure of Southwestern blot analyses. The bands of the complex appear in all three lanes as 46- and 52-kD proteins. Note the intensity of the 46-kD band in lane 3, MB<sub>1c</sub>.



**Figure 6.** Differential bindings of the proximal MBP regulatory sequence with mice brain nuclear extract of various ages. Southwestern analysis of brain nuclear extracts from 2/3-, 18/30-, and 60-d (adult)-old mice (lanes 1, 2, and 3, respectively) with probe prepared from MB<sub>1a</sub>. Molecular size in kilodaltons of the  $\alpha$ -complex bands, i.e., 37- and 39-kD, is given on the left.

with 3). Whether or not this shift in the abundance of the 39- and 37-kD DNA binding proteins account for the programming and/or tissue specificity of MBP gene expression in brain cells remains to be clarified.

## Discussion

Previous transient expression studies with MBP gene 5'-flanking sequences in CAT reporter gene constructs demonstrated that the information necessary for the selective pattern of expression of this gene in cultured cells is contained within its 5'-upstream sequence (24, 25). In those studies, three main regulatory domains were identified: (a) the proximal element (between positions -14 and -50) which is expressed only in glial cells; (b) the first distal element (between positions -131 and -169) which contains three subdomains that independently confer cell specificity to a heterologous promoter as well; and (c) the most distal element (between positions -250 and -288) which, when present, allows expression of a heterologous promoter in glial and nonglial cells. In vitro analysis of MBP transcriptional control elements revealed that the sequences surrounding -34, -93 and located between -106 to -130 are important for activation of the MBP promoter in Hela cell extract (25). In a separate study, it was shown that sequences proximal (-14 to -51) and distal (-51 to -253) to the RNA start site increase MBP transcription in brain extract, whereas, the distal element negatively regulates MBP activity in liver extract (31).

Recently, we have carried out in vitro transcription experiments using homologous (glial) and heterologous (Hela) cell-free extracts to identify precisely the regulatory elements that restrict the transcription of MBP to glial cells. Consistent with our in vivo observations, we find that multiple regulatory elements scattered upstream of the RNA start site differentially contribute to MBP transcription in homologous and heterologous extracts (manuscript in preparation).

Together these results obtained from in vivo and in vitro experiments indicate that MBP has a particular combination of positive and negative *cis*-acting elements located within several hundred nucleotides of the RNA initiation site. Although

our previous results revealed that each element may function independently, it is likely that combinations of these regulatory elements, which are arranged in particular configurations, play a critical role on the differential activation of the MBP promoter during brain development.

In an effort to identify the *trans*-acting regulatory factors that developmentally regulate MBP transcription, we examined the binding of nuclear proteins derived from mouse brain at different stages of myelination, i.e., newborn (2/3 d), young adult (18/30 d) and adult (60 d), to the MBP regulatory sequence. Our results indicate that the MBP 5'-upstream sequence is composed of multiple distinct protein binding regions with differential binding activities to brain nuclear proteins derived from mice at different ages. The three major regulatory elements, regions MB<sub>1</sub>, MB<sub>4</sub>, and MB<sub>7</sub>, revealed higher binding activity with nuclear proteins in the young adult and adult brain extracts than with those present in the extract from newborn brain. Analysis of the MB<sub>1</sub> domain using 11-nucleotide deletions at the 5', 3', and central regions, identified a sub-region positioned between -14 to -37 that binds to 39-kD and 37-kD protein components in young adult brain cells. The differential distributions of the 39- and 37-kD proteins in brain extracts of mice at different stages of duration and MBP synthesis, suggests that these proteins may directly and/or indirectly participate in a developmental pattern of MBP gene expression. Further studies involving purification of proteins to homogeneity and studying their activities in an in vitro transcription system should clarify the role of these proteins in the tissue-specific and stage-specific transcription of the MBP gene. Two minor proteins, 46 and 52 kD in size, bind to the sequences present in all three MB<sub>1</sub> derivatives. Whether the pentanucleotide sequence, AGG(A/C)C, which is shared among the DNA fragments with binding activity for the 46- and 52-kD proteins, is the responsive target sequence for the DNA-protein interactions remains to be determined.

More than 20,000 different genes are expressed in the mammalian nervous system, most of which are neural specific. MBP represents one of the most abundant proteins in CNS, in which its expression is differentially regulated at the level of transcription during brain development. However, the mechanisms that govern tissue-specific transcriptions of this gene during myelination remain unknown. The experiments described here have characterized the binding of specific nuclear protein factors from mouse brain tissue to the MBP regulatory regions. Although the regulatory sequences of MBP are required for its transcription in glial cells, proof that these proteins play a role in the cell/stage-specific activation of MBP promoter awaits more direct functional analyses. The present results should facilitate future efforts to purify these factors and clone their corresponding gene(s) in order to study their differential expression.

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