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MyEF-3, a Developmentally Controlled Brain-Derived Nuclear Protein Which Specifically Interacts with Myelin Basic Protein Proximal Regulatory Sequences

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Myelin basic protein (MBP) gene contains the upstream regulatory sequence that confers cell type- and stage-specific transcription to MBP expression in oligodendrocytes during brain development. The MB_1 regulatory motif located between −14 to −50 with respect to transcription start site binds to a brain derived nuclear protein and plays an important role in transcriptional activation of the MBP promoter in transfection assay. Here, we report the isolation of a recombinant cDNA clone, termed *my*elin *e*xpression *f*actor-*3*, (MyEF-3) from a mouse brain expression library that encodes a novel protein which interacts with the MBP MB_1 domain. Computer assisted evaluations of the MyEF-3 sequence revealed several interesting features including four sites for phosphorylation by casein kinase II, a transmembrane domain at the N-terminus, a nuclear localization signal and a Zinc finger domain at the carboxyl terminal. Results from Western and band shift assays indicate that MyEF-3 binds efficiently to double-stranded MB_1 as well as the single-stranded non-coding strand of MB_1. The use of short DNA fragments encompassing the nucleotide base substitutions across the MB1 domain in competition band shift assay revealed that the ten nucleotide sequence, 5′-GCCTGTCTTT-3′ is important for binding of MyEF-3 to DNA. Results from Northern blot studies demonstrate that expression of MyEF-3 is restricted to brain and developmentally regulated during brain maturation. The biological importance of MyEF-3 in the cell type- and stage-specific expression of MBP during brain development is discussed.

Myelin basic protein (MBP) is the major component of myelin sheath of central nervous system (CNS) which is produced by oligodendrocytes (for review see 1, 2). In mouse brain, expression of the MBP gene is developmentally regulated during the course of myelination. Briefly, MBP is first detected at 8 to 10 days postnatal, peaks at 18–20 days, and its levels decrease to approximately 20% of its highest level in adult mouse (for review see 3). Evidently, cell type- and developmental-specific expression of MBP is regulated primarily at the level of transcription (2, 4, 5). Analysis of the MBP genome by cell free in vitro transcription and cell culture transfection assays has led to the identification of a regulatory sequence located between −14 to −50 with respect to the transcription start site (+1) which plays an important role in transcriptional activity of the MBP promoter (6–10). This regulatory sequence has the ability to interact with several DNA binding proteins from mouse brain, some with specific patterns of expression during brain development (11). These observations suggest that the timely regulation of MBP gene expression in brain may depend on the presence of specific regulatory proteins during myelinogenesis, which upon interaction with the specific promoter sequence of MBP, stimulate transcription of this gene.

In this report we present results from our recent studies aimed at identification of a brain-derived transcription factor with the ability to stimulate MBP gene transcription. We have screened a λ-phage expression library made from mouse brain at the peak of myelination, and performed in situ filter binding method to isolate a cDNA encoding a protein which binds the MBP regulatory sequence. The use of oligonucleotide spanning −14 to −50 (MB_1 region) as a probe, has led to the isolation of *my*elin gene *e*xpression *f*actor *3* (MyEF-3) cDNA.

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EXPERIMENTAL PROCEDURES

Screening of a mouse cDNA library. A cDNA expression library in λgt11 derived from 15 day postnatal mouse brain (kindly provided by Dr. A. Campagnoni, MRRC, UCLA) was screened according to the method described previously (12). In brief, bacterial strain Y1090 was incubated with recombinant phages and the control phage, plated and maintained at 42°C for 2-3 h. The nitrocellulose filters pre-treated in 10 mM IPTG were overlaid in the plates and incubated at 37°C for 12 h. The filters containing more than 5×10^5 phages were incubated in binding buffer containing 25 mM Hepes (pH 8.0), 25 μM NaCl, 5 mM $MgCl_2$, and 0.5 mM DTT supplemented with 6 M guanidine hydrochloride. After 30 min, filters were washed in binding buffer with no guanidine hydrochloride followed by incubation in binding buffer supplemented with 5% nonfat dry milk. Binding was carried out in the buffer containing 0.25% nonfat dry milk and [^{32}P] end-labeled double-stranded oligonucleotide representing mouse MBP sequence spanning nucleotides −14 to −50. Filters were then washed in binding buffer with 0.25% milk two to four times.

A positive clone was identified, and after plaque purification, the insert was subcloned in Bluescript II for DNA sequencing. The sequencing of the clones was carried out using the dideoxy chain termination method (13) which proceeded from both ends. The sequences were verified by sequencing of the opposite strand.

Prokaryotic expression system. For production of MyEF-3 in a bacterial system, the EcoRI DNA fragment corresponding to MyEF-3 cDNA from the recombinant phage was cloned into the EcoRI site of pMAL-CRI vector (New England Biolabs). The plasmid was introduced into E. coli HB101 and the transformed cells were cultured in L-broth to OD600=0.5 after which IPTG (0.3 mM) was added and cultures were maintained at 37°C for 2 h. Total bacterial proteins were prepared and fusion proteins were purified by amylose affinity chromatography, according to the manufacturer's instructions (New England Biolabs).

Band-shift assay. Protein-DNA complexes were identified by running the sample on low ionic strength polyacrylamide gel. Approximately 5×10^4 cpm end-labeled MB_1 oligonucleotide probe was mixed with 5 μg of bacterially produced MyEF-3 protein for 20 min in a reaction buffer containing 12 mM Hepes (pH 7.9), 4 mM Tris (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, 0.8 mM DTT, and 2 μg poly[dI-dC]. In competition studies, the binding reaction was carried out in the presence of an excess amount (50-fold molar excess) of unlabeled competitor oligonucleotide. The following oligonucleotides were used in the band shift and Southwestern studies.

MB1	3′ TCCCTCCTGTTGTGGAAGTTTCTGTCCGGGAGACT 5′
MB1A	3′ GTGGAAGTTTCTGTCCGGGAGACT
MB1C	3′ TCCCTCCTGTTGTGGAAGTTTCT 5′

RNA preparation and analysis. RNA from various mouse tissues was isolated by the guanidinium isothiocyanate/cesium chloride method. Approximately 20 μg of total RNA were size fractionated on a formaldehyde/agarose gel, stained with ethidium bromide, and then transferred to nylon membrane (Hybond N, Amersham). MyEF-3 and MBP cDNA probes were labeled by nick-translation with [^{32}P]-dCTP and hybridized at a concentration of 2×10^6 cpm/ml at 65°C in a solution containing 5× SSC, 2× Denhardt's solution, 0.1% SDS, and 0.3 mg/ml salmon sperm DNA. The filters were washed under high stringency conditions (65°C-0.1X SSC-0.1% SDS) prior to autoradiography.

RESULTS AND DISCUSSION

We utilized a [^{32}P]-labeled oligonucleotide probe spanning the MB_1 regulatory region and screened an expression library prepared from a 15 day postnatal mouse brain. A distinct positive clone was isolated and named myelin expression factor 3 (MyEF-3). Figure 1A illustrates the cDNA sequence and deduced amino acid composition of MyEF-3. Complete sequencing of the cDNA fragment revealed only one open reading frame able to produce a protein of 232 amino acids. This open reading frame extends the entire length of the fragment and is in-frame with EcoRI site located at the 3′ end of the β-galactosidase gene. Computer assisted sequence analysis of MyEF-3 revealed no apparent similarity to any clones thus far identified, suggesting that MyEF-3 may represent a novel DNA binding protein which is produced in brain. Computer sequence analysis of the MyEF-3 protein, as shown in Figure 1B, revealed several interesting features which may possess functional importance on the biological activity of this protein. Four distinct regions located between amino acid residues 31-34, 142-145, 185-188, and 201-204 contain putative sites for phosphorylation by casein kinase II (CK II), whereas the regions between amino acids 31-33 and 175-177 are targets for phosphorylation by protein kinase C. A transmembrane domain between amino acid residues 40-60 with the outside-to-inside orientation was predicted by Motif Finder software at ISREC. The region between residues 84-189 is proline-rich (12-38%) and has significant homology with the mouse NFkB p65 protein. The position of the nuclear localization signal was identified between amino acid residues 206-210. The C-terminal end of MyEF-3 possesses a Zinc finger domain (amino acid residues 207-229) which is identical to the oncoretroviral and lentiviral nucleocapsid gag protein with the consensus motif CX_2, CX_4, HX_4C (14). The significance of these structural features is not known, but they may contribute to the DNA binding and/or regulatory activity of MyEF-3.

To estimate the molecular mass of the MyEF-3 and determine its binding activity, the cDNA corresponding to MyEF-3 open reading frame was placed into a prokaryotic expression system pMAL-CR1 allowing production of a maltose-binding protein (MalBP)-MyEF-3 fusion protein. Southwestern analysis of protein extracts obtained from IPTG-induced pmal-MyEF-3 containing bacteria revealed a strong binding affinity of the MalBP-MyEF-3 fusion protein to oligonucleotide MB_1 (Figure 2B, lane 2). As our preliminary filter binding assay revealed strong binding activity of MyEF-3 to the non-coding strand of MB_1, we utilized the non-coding single-stranded sequences of MB_1 (MB_1^{nc}) as a probe. Protein extract from bacteria containing pMAL vector showed no signal indicative of the association of MB_1 probe with MalBP suggesting that the observed association of MB_1 with MalBP-MyEF-3 shown in lane 2 (Figure 2B) is due to MyEF-3 protein. Coomassie blue staining of the size-fractionated extracts obtained from

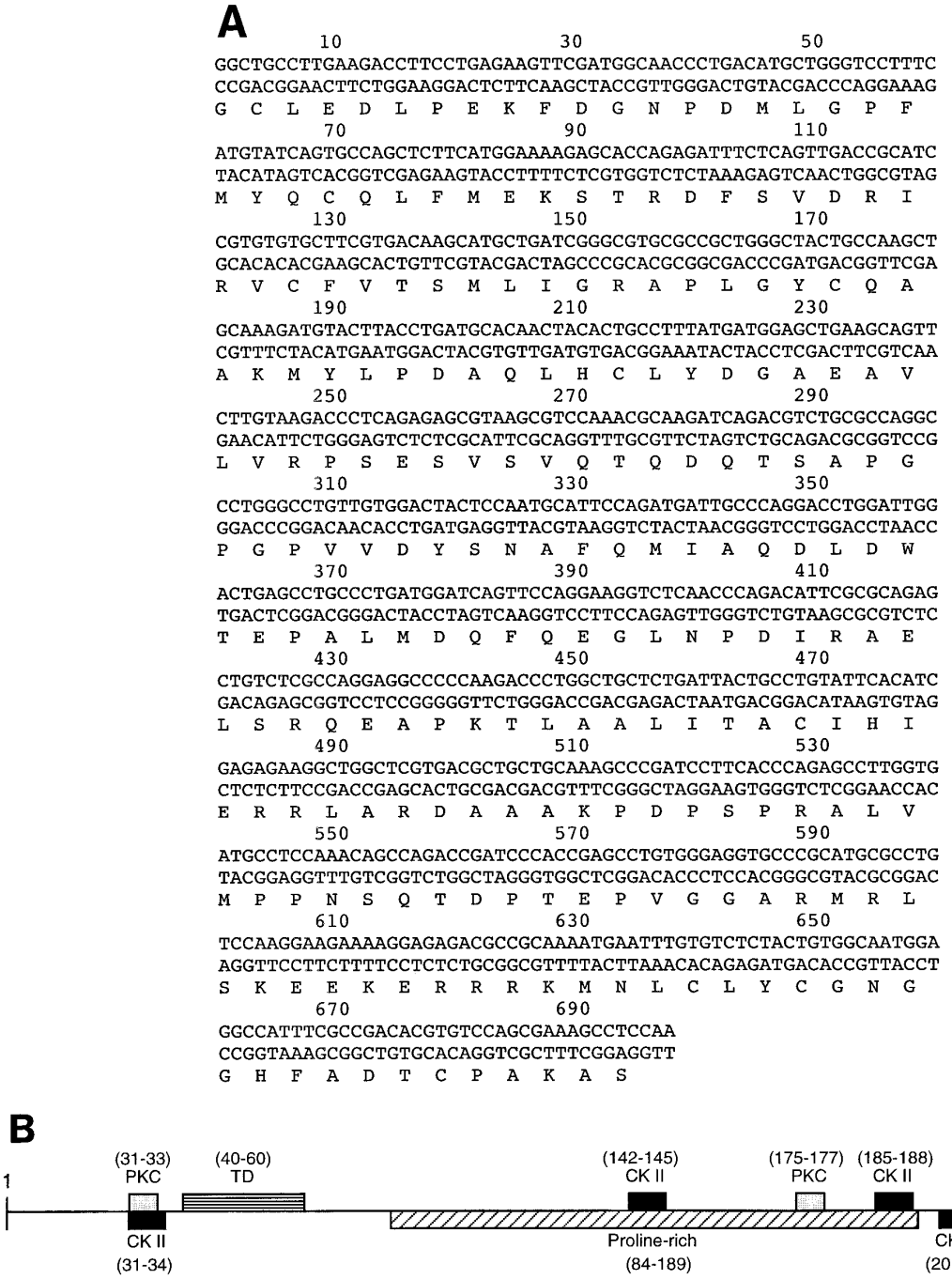


FIG. 1. Nucleotide sequence of MyEF-3 and schematic representation of the putative domains. A. Sequence determination of MyEF-3 cDNA was performed by the method of Sanger et al. (18). Ambiguities were clarified by sequencing of the opposite strand. Translation of the MyEF-3 cDNA predicts a protein of 232 amino acids, which is depicted below the nucleotide sequence. This information is available from GenBank. B. The positions of the four putative sites for phosphorylation by casein kinase II (CK II), protein kinase C (PKC), transdominant domain (TD), proline-rich domain (PRD), nuclear localization signal (NLS), and Zinc finger domain (Zn-finger domain) are depicted. The numbers represent the amino acid residues.

pmal-MyEF-3 and pmal containing bacteria by SDS-PAGE indicated that MalBP-MyEF-3 fusion protein is in the range between 68 to 70 kd in size of which 23-27 kb accounts for the MyEF-3 (Figure 1A). This size is consistent with the number of amino acid residues (Figure 1B). In addition to full-length MB_1, we examined binding of MyEF-3 to two partially overlapping oligonucleotide probes representing the 3′ and 5′ re-

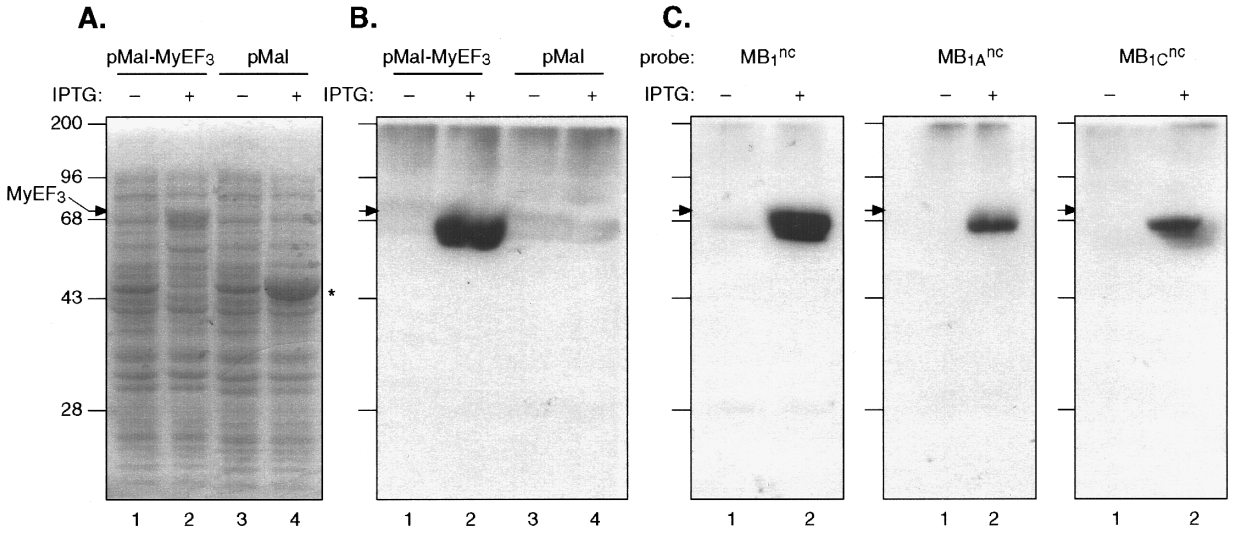


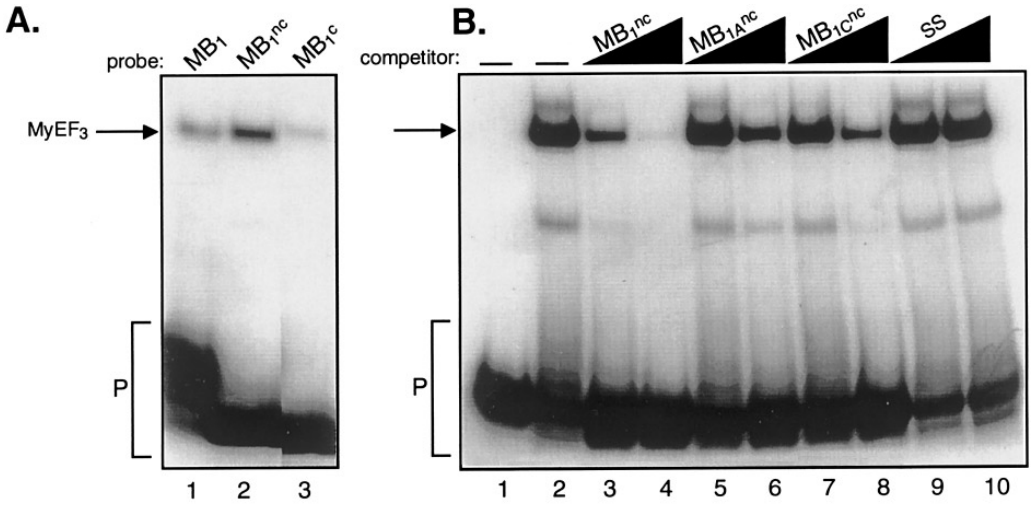
FIG. 2. Overproduction of MyEF-3 in bacteria and Southwestern analysis. A. The proteins produced in bacteria transformed with pmal-MyEF-3 or pMal (induced for 1 h with 10 mM isopropyl-β-D-thiogalactoside, IPTG) were analyzed by 10% SDS-PAGE and stained with Coomassie blue. The arrow points to the position of MalBP-MyEF-3 fusion protein and the asterisk depicts MalBP. B. A parallel PAGE was transferred to nitrocellulose and Southwestern analysis was performed utilizing oligo MB_1^{nc} probe. The arrow shows the position of the MalBP-MyEF-3 bound to the MB1 probe. C. Southwestern blot analysis of protein extracts from uninduced and IPTG induced bacteria containing pMal-MyEF-3 utilizing full length MB_1^{nc} or its deletion variants as described in Experimental Procedures.

gions of MB_1 (shown in Experimental Procedures). As illustrated in Figure 2C, MyEF-3 fusion protein exhibited significant reduced binding affinity to these oligonucleotides in comparison to the full-length MB_1 probe suggesting that stable association of MyEF-3 DNA requires sequences spanning the entire MB_1 domain.

In the next experiment, we performed band shift assay to further investigate binding of MyEF-3 to the MB_1 sequence. In this study, we utilized double-stranded and single-stranded DNA from the non-coding (MB_1^{nc}) and coding (MB_1^{c}) strands of MB_1 as probes. As shown in Figure 3 (left panel), double-stranded and single-stranded DNA corresponding to the non-coding strand showed a modest and strong binding activity to MyEF-3, respectively. In contrast, the DNA probe from the coding strand of MB_1 showed very weak binding activity. To determine the specificity of this interaction, competition experiments were performed utilizing unlabeled oligonucleotides corresponding to the non-coding strand of MB_1 or its variant along with unrelated single-stranded oligonucleotides. As shown in Figure 3 (right panel), inclusion of the unlabeled non-coding strand of MB_1 completely abrogated association of the MB_1 probe to MyEF-3. The intensity of the complex was noticeably decreased in the presence of an excess amount of unlabeled MB_{1A}^{nc} and MB_{1C}^{nc}. The addition of unrelated single-stranded (SS) DNA to the binding reaction showed no effect on the intensity of the nucleoprotein complex. These data suggest that MyEF-3 has the capacity to specifically interact with the double- and single-stranded MB_1 DNA sequence and has more affinity to the non-coding strand of MB_1.

To further identify the nucleotide requirements for interaction of MyEF-3 with DNA, we have generated a series of single-stranded DNA fragments (M1 to M5) with base substitutions across the MB_1 (shown in Figure 4B), and have utilized them as competitors in band shift assay. As shown in Figure 4A, unlabeled MB_1 and its mutant variants, M1 and M2 at the concentration which is equal to 10-fold molar excess in comparison to the probe severely competed for the association of MyEF-3 with the MB_1 probe. Neither M3 nor M4 showed significant effect on binding of MyEF-3 to the MB_1 probe. Mutant oligonucleotide M5, at a higher DNA concentration was able to decrease the level of MyEF-3 association with the probe. These observations led us to believe that a defined region in the middle of MB_{1A}, with the nucleotide composition 5′-GCCTGT-CTTT-3′, is important for binding of MyEF-3 to DNA. Whether association of MyEF-3 with the MB1 promoter induces expression of MBP during the course of myelination, remains to be elucidated. However, our preliminary transfection studies indicated that ectopic expression of MyEF-3 in oligodendrocytic cells leads to modest increase in transcription of the MBP reporter construct containing MB_1 motifs (Steplewski, A., unpublished observations).

In the next study, we designed experiments to determine the level of expression of MyEF-3 RNA in various tissues, as well as its temporal expression in brain.



C.

MB_1: 5'- AGGGAGGACAACACCTTCAAAGACAGGCCCTCTGA -3 (coding)
3'- TCCCTCCTGTTGTGGAAGTTTCTGTCCGGGAGACT -5 (non-coding)

MB_{1A}^{nc}: 3' GTGGAAGTTTCTGTCCGGGAGACT -5' (non-coding)

MB_{1C}^{nc}: 3'- TCCCTCCTGTTGTGGAAGTTTC] -5' (non-coding)

FIG. 3. DNA binding specificity of bacterially produced MyEF-3. Left panel: Bacterially produced MyEF-3 was incubated with [^{32}P]-labeled double-stranded MB1 or single-stranded DNA presenting non-coding (MB_1^{nc}) or coding (MB_1^{c}) strands of MB_1. Right panel: Binding reactions were carried out in the absence (second lane) or presence of 5-fold molar excess of the competitor oligonucleotides from MB1nc, MB1Anc, MB1Cnc and unrelated single-stranded (SS) DNAs. In the first lane, the probe was mixed in binding buffer without protein extracts and competitors.

Results from Northern blot analysis of 20 μg of total RNAs from brain at various stages of development, heart, kidney, lung, and spleen of 30 day old mice revealed the presence of a major RNA of approximately 6 kb in size which was predominant in brain tissue (Figure 5, middle panel). Of particular interest was the notion that the intensity of the band corresponding to MyEF-3 transcript increased during brain development, as first detected in RNA samples from 14 day old brain, maximized in brain at 22 days, and significantly decreased at 60 days postnatal. The pattern of MyEF-3 expression was very similar to that obtained for the MBP gene utilizing the same RNA preparations shown in Figure 5 (right panel). Ethidium bromide staining of the gel demonstrated that similar amounts of RNA were loaded on the gel (data not shown).

In a separate approach, we utilized the more sensitive RT-PCR technique to examine the level of MyEF-3 mRNA in brain, heart, lung, liver, spleen, kidney, and spinal cord. In agreement with the Northern blot data, a significant level of MyEF-3 RNA was detected in brain (data not shown). In addition, results from RT-PCR analysis of the RNA corresponding to the actin transcript showed a consistent level of this message in brain and other tissues ruling out the possibility of RNA degradation. Of interest, we obtained a moderate level of MyEF-3 transcript in the spinal cord.

In general, it is believed that the combined action of transcriptional activation and repression limits expression of a gene to a specific cell type or dictates its stage-specific expression. Previously, we have identified and characterized negative and positive transcriptional factors that modulate expression of the MBP promoter in CNS cells (8, 15, 16). Similar to MyEF-3, the previously characterized positive regulator, MEF-1/Pur α (8), and the negative factor, MRF-2 (16, 17), exhibited affinity to the MBP proximal regulatory sequence, i.e. MB_1. However, results from Northern blot analysis indicate that expression of MyEF-3 is more restricted to brain and that it is developmentally regulated, coinciding with that observed for the myelin gene. Similarities between the developmental profile of MyEF-3 expression in mouse brain and that of MBP, and the ability of MyEF-3 for binding to the MBP genome and enhancing MBP promoter activity suggest that this protein may be involved in the activation of the myelin gene in vivo. Currently we are in the process of isolating a genomic clone for MyEF-3 for use in knock-out studies in order

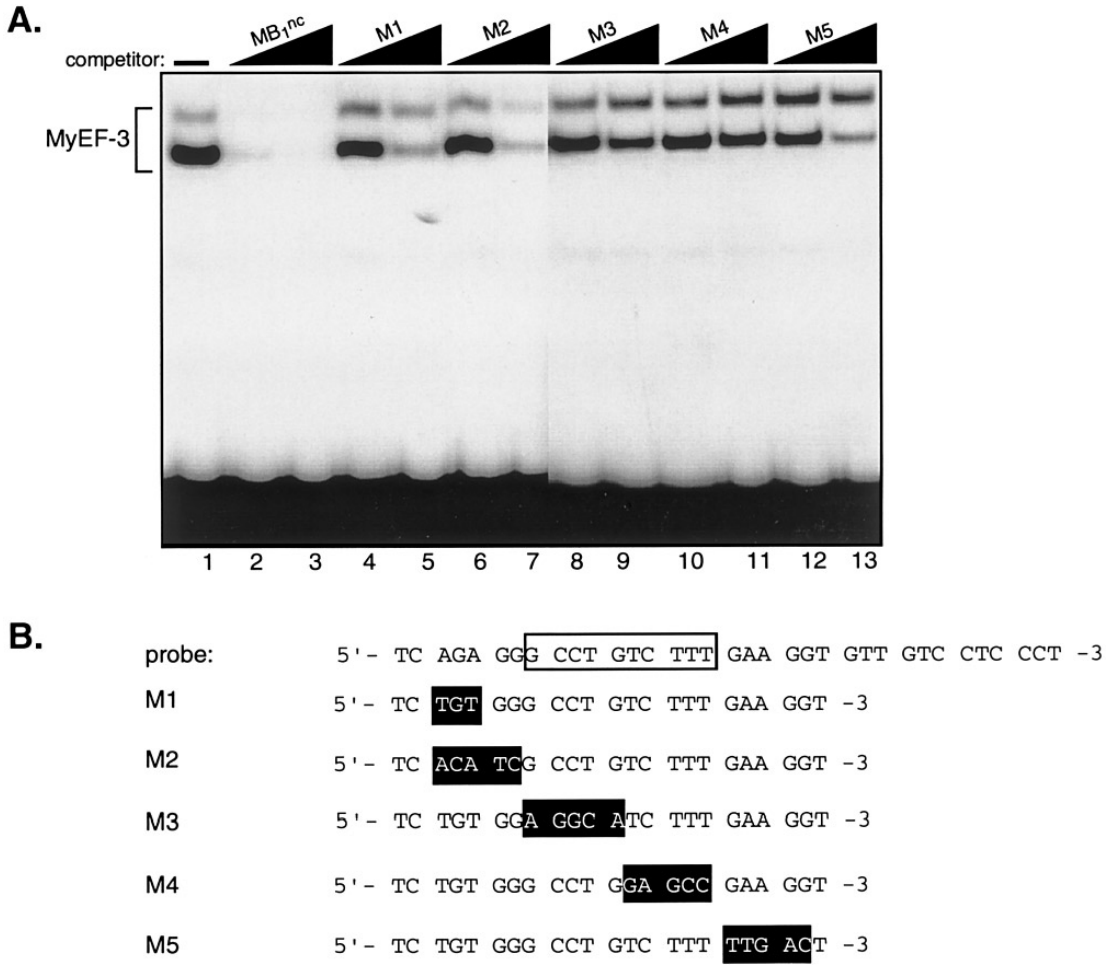


FIG. 4. Identification of the MyEF-3 binding nucleotides. A. Band shift competition assay utilizing bacterially produced MyEF-3, [^{32}P]-labeled single-stranded MB_1 probe and 2.5- and 10-fold molar excess of unlabeled competitors as depicted above the lanes. B. Nucleotide compositions of the probe (MB_1^{nc}) and the various competitor mutants. The nucleotide base substitutions in each mutant DNA (M1 to M5) are shown in the black boxes. The open box in the probe depicts the nucleotide sequences required for MyEF-3 binding.

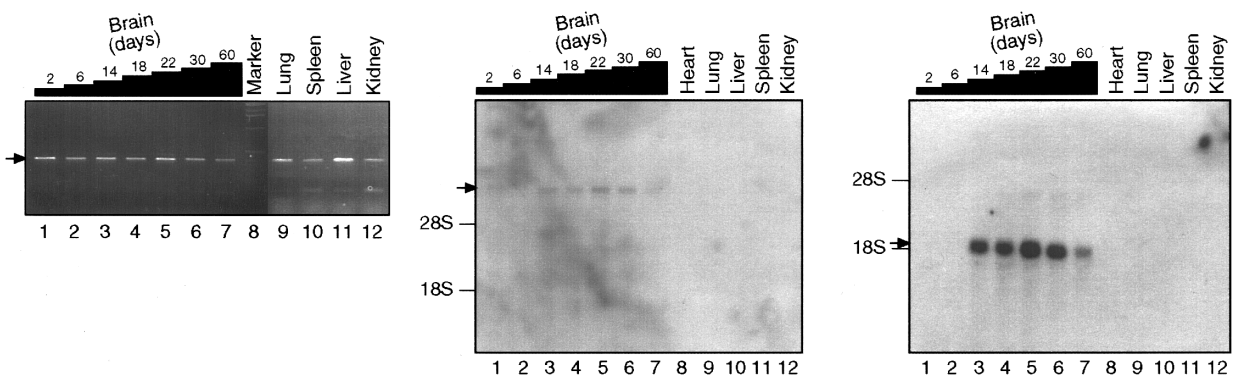


FIG. 5. Developmental accumulation and tissue distribution of MyEF-3 transcript. Northern blot analysis of 20 μg of total RNA derived from brain tissue of mice at various ages and from various tissues of 30 day old mice, as depicted above each lane, utilizing MyEF-3 (middle panel), or MBP (right panel) cDNAs as probes. Positions of the MyEF-3 and MBP transcripts are shown by the arrows. The panel on the left illustrates RT-PCR analysis of RNA samples from brain and other tissues to determine the level of control actin RNA as described previously (19).

to determine the biological significance of MyEF-3 in brain. As binding of MyEF-3 overlaps, although partially, with MEF-1 and MyEF-2, the positive and negative regulators, respectively, studies are designed to investigate the possible interplay of these regulators at the MB_1 motif and its consequence on transcription of the MBP gene.

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REFERENCES

1. Campagnoni, A. T. (1988) *J. Neurochem.* **51,** 1–14.
2. Campagnoni, A. T., and Macklin, W. B. (1988) *Mol. Neurobiol.* **2,** 41–89.
3. Lemke, G. (1988) *Neuron* **1,** 535–543.
4. Dubois-Dalcq, M., Behar, T., Hudson, L., and Lazzarini, R. A. (1986) *J. Cell Biol.* **102,** 384–392.
5. Zeller, N. K., Behar, T. N., Dubois-Dalcq, M. E., and Lazzarini, R. A. (1985) *J. Neurosci.* **5,** 2955–2962.
6. deFerra, F., Engh, H., Hudson, L., Kamholz, J., Puckett, S., Molineaux, S., and Lazzaraini, R. A. (1985) *Cell* **43,** 721–727.
7. Deschamps, J., and Meijlink, F. (1992) *Crit. Rev. Oncogenesis* **3,** 117–173.
8. Haas, S., Thatikunta, P., Steplewski, A., Johnson, E. M., Khalili, K., and Amini, S. (1995) *J. Cell Biol.* **130,** 1171–1179.
9. Luckow, B., and Schultz, G. (1987) *Nucl. Acids. Res.* **15,** 5490.
10. Miura, M., Tamura, T., Aoyama, A., and Mikoshiba, K. (1989) *Gene* **75,** 31–38.
11. Lashgari, M. S., Devine-Beach, K. A., Haas, S., and Khalili, K. (1990) *J. Clin. Inv.* **86,** 1671–1677.
12. Vinson, C. R., LaMarco, K. L., Johnson, P. F., Landschultz, W. H., and McKnight, S. I. (1988) *Genes Dev.* **2,** 801–806.
13. Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989) *in* Current Protocols in Molecular Biology, Wiley, New York, NY.
14. Rein, A., Ott, D. E., Mirro, J., Arthur, L. O., Rice, W., and Henderson, L. E. (1996) *J. Virol.* **70,** 4966–4972.
15. Haas, S., Gordon, J., and Khalili, K. (1993) *Mol. Cell. Biol.* **13,** 3103–3112.
16. Steplewski, A., Haas, S., Amini, S., and Khalili, K. (1995) *Gene* **154,** 215–218.
17. Haas, S., Steplewski, A., Siracusa, L., Amini, S., and Khalili, K. (1995) *J. Biol. Chem.* **279,** 12503–12510.
18. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
19. Franks, R., Rencic, A., Gordon, J., Zoltick, P. W., Curtis, M., Knobler, R. L., and Khalili, K. (1996) *Oncogene* **12,** 2573–2578.