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Evidence for Inhibition of MyEF-2 Binding to MBP Promoter by MEF-1/Pur α

Vandhana Muralidharan,[1] Anna Tretiakova,[1] Andrew Steplewski,[1] Susan Haas,[1] Shohreh Amini,[1] Edward Johnson,[2] and Kamel Khalili[1]*

[1]Center for Neurovirology, Allegheny University of the Health Sciences, Philadelphia, Pennsylvania 19102
[2]Department of Pathology and Brookdale Center for Molecular Biology, Mount Sinai Medical Center, Mount Sinai School of Medicine, New York, New York 10029

Abstract Myelin basic protein (MBP) is a major component of the myelin sheath whose production is developmentally controlled during myelinogenesis. Earlier studies have indicated that programmed expression of the MBP gene is regulated at the level of transcription. Evidently, the MB1 regulatory motif located between nucleotides −14 to −50 plays an important role in transcription of the MBP promoter in both in vitro and in vivo systems. The MB1 element contains binding sites for the activator protein MEF-1/Pur α and the repressor protein MyEF-2. In this study we use bandshift assays with purified MEF-1/Pur α and MyEF-2 and demonstrate that binding of MyEF-2 to its target sequence is inhibited by MEF-1/Pur α. Under similar conditions, MyEF-2 enhances the association of MEF-1/Pur α with MB1 DNA. MEF-1/Pur α binds to MB1 in mono- and dimeric forms. Inclusion of MyEF-2 in the binding reaction increases the dimeric association of MEF-1/Pur α with the MB1 sequence. The use of MEF-1/Pur α variants in the bandshift assay suggests that two distinct regions of this protein may be involved in its binding to the MB1 sequences, and its ability to block MyEF-2 interaction with the MB1 sequence. Based on previous studies on the programmed expression of MEF-1/Pur α and MyEF-2 during myelination and the current findings on their interplay for binding to the MB1 motif, a model is proposed for their involvement in transcriptional regulation of the MBP gene during the course of brain development. J. Cell. Biochem. 66:524–531, 1997.

Key words: MBP; brain development; transcription

INTRODUCTION

Expression of the myelin basic protein (MBP) gene occurs postnatally in the mouse brain such that it is first detected at the end of the first postnatal week, increases dramatically to peak at 18–21 days, and decreases to about 20% of peak levels in the mature animal [Carson et al., 1983]. Steady-state levels of MBP RNA correspond well with the observed pattern of MBP accumulation [Sorg et al., 1987], and a direct measurement of MBP RNA synthesis by nuclear run-on transcription assay has demonstrated that MBP gene expression is regulated primarily at the level of transcription [Shiota et al., 1991; Wiktorowicz and Roach, 1991; Zeller et al., 1984]. Functional dissection of the MBP gene regulatory region has indicated that similar to a prototypical transcription unit in eukaryotic cells, the MBP promoter/enhancer contains a critical cis-acting element positioned in close proximity to the transcription start site and a variety of upstream regulating motifs with the potential to positively or negatively regulate transcription of MBP [Devine-Beach et al., 1990, 1992; Miura et al., 1989; Tamura et al., 1989, 1991; Tamura and Mikoshiba, 1991]. A detailed analysis of the MBP proximal regulatory element named MB1 spanning the sequence from nucleotides −14 to −50 identified a 38- to 41-kDa developmentally controlled DNA binding protein from mouse brain named MEF-1/Pur α that forms a nucleoprotein complex with a DNA fragment containing the MB1 sequence [Haas et al., 1993]. MEF-1/Pur α is a single-stranded DNA binding protein that exhibits specific affinity for a purine-rich sequence positioned in the non-coding (transcribed) strand of the MB1 motif. MEF-1/Pur α possesses the capacity to augment the tran-

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*Correspondence to: Kamel Khalili, Center for NeuroVirology and NeuroOncology, Allegheny University of the Health Sciences, Broad and Vine MS #406, Philadelphia, PA 19102.

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scriptional activity of the MBP promoter in an in vitro transcription system [Haas et al., 1993] and in transient transfection of glial cells [Haas et al., 1995b]. Evidently, the observed transactivation of the MBP promoter by MEF-1/Pur α requires an intact MEF-1/Pur α binding site within the MB1 motif. In addition to MEF-1/Pur α, the MB1 regulatory motif exhibits strong affinity for another sequence-specific single-stranded DNA binding protein, MyEF-2 [Haas et al., 1995a]. A cDNA clone for MyEF-2 was first isolated from a mouse brain cDNA expression library by in situ DNA-protein binding technique utilizing the MB1 DNA as a probe. Expression of MyEF-2 is developmentally regulated in mouse brain with its maximum levels detected at postnatal day 7, prior to the onset of MBP expression. In contrast to MEF-1/Pur α, overexpression of MyEF-2 decreases transcription of the MBP gene when transfected in cell lines [Haas et al., 1995a]. Thus, it appears that the proximal regulatory element of the MBP promoter spanning the MB1 motif binds to at least two distinct classes of regulatory proteins with opposing regulatory effects on MBP gene transcription. Here we present our results from in vitro DNA binding studies aimed at determining the possible intercommunication of MEF-1/Pur α and MyEF-2 pertaining to their interaction with the MB1 regulatory sequence. We demonstrate that the positive regulatory protein MEF1/Pur α is capable of abrogating binding of the negative regulatory factor, MyEF-2, to the MB1 sequence. Results from deletion analysis indicate that the DNA binding activity of MEF-1/Pur α is required for its capability to displace MyEF-2 for the MB1 sequence. We propose a model in which the interplay between MEF-1/Pur α and MyEF-2 and their binding to the MB1 regulatory motif may play a role in the programmed expression of the MBP gene during brain development.

MATERIALS AND METHODS

Protein Purification

Maltose binding protein (MalBP) fused to MEF-1/Pur α and MyEF-2 were expressed in the bacterial strain HB101. Briefly, 5-ml overnight bacterial cultures transformed with pMAL-MEF-1/Pur α or pMAL-MyEF-2 were used to inoculate one liter of L-broth culture medium supplemented with 2 g of glucose. The cells were grown at 37°C in a shaking incubator to A_{600} of approximately 0.4–0.6. The expression of the fusion protein in bacterial cultures was induced by the addition of IPTG to a final concentration of 3×10^{-4} M. After approximately 90 min, cells were harvested and resuspended in lysis buffer containing lysozyme, and incubated for 30 min at 0°C. After sonication, cell lysates were separated from the membrane and cell debris by centrifugation and fractionated on an amylose affinity column for preparation of the fusion protein (New England Biolabs, Beverly, MA) as per manufacturer's instructions. The fusion proteins were cleaved from the MalBP moiety upon treatment with factor Xa and purified on an amylose affinity column [Ausubel et al., 1989]. To remove factor Xa from the recombinant proteins, gel-filtration chromatography on G50 column was performed. The MEF-1/Pur α or MyEF-2 fractions eluted from the affinity column after cleavage with factor Xa were pooled together and loaded on the Sephadex G50 column. The purified MEF-1/Pur α and MyEF-2 proteins were stored at −70°C for later use.

Bandshift Assay

The recombinant MEF-1/Pur α and MyEF-2 proteins were incubated with single-stranded MB1 DNA labeled with ^{32}P (Amersham, Arlington Heights, IL) at the 5′ end. The 30-µl binding reaction mixtures contained 1 µg of poly (dI-dC), 25–1,000 ng of MEF-1/Pur α and/or MyEF-2 proteins, $3–5 \times 10^4$ cpm of MB1 probe. The amount of total protein in the reaction mixture was kept constant by the addition of MalBP or bovine serum albumin (BSA). The binding reaction was carried out at 4°C for 30 min. The protein-DNA complexes were separated from the free DNA probe on 6% or 9% native polyacrylamide gels in 0.5× TBE buffer. Electrophoresis was carried out at 175 V for 2.5–3 h at 4°C.

RESULTS AND DISCUSSION

In order to investigate the DNA binding activity of MEF-1/Pur α and MyEF-2, we used the bacterial expression vector, pMAL-cRI. In this expression system, the MEF-1/Pur α and MyEF-2 are produced as fusion proteins with maltose binding protein (MalBP). As shown in Figure 1A, the fusion of MEF-1/Pur α and MyEF-2 to the control MalBP with a molecular weight of approximately 40 kDa results in the production of 82-kDa and 86-kDa polypeptides, respectively (compare lane 1 with lanes 2 and 3). The minor bands with lower molecular

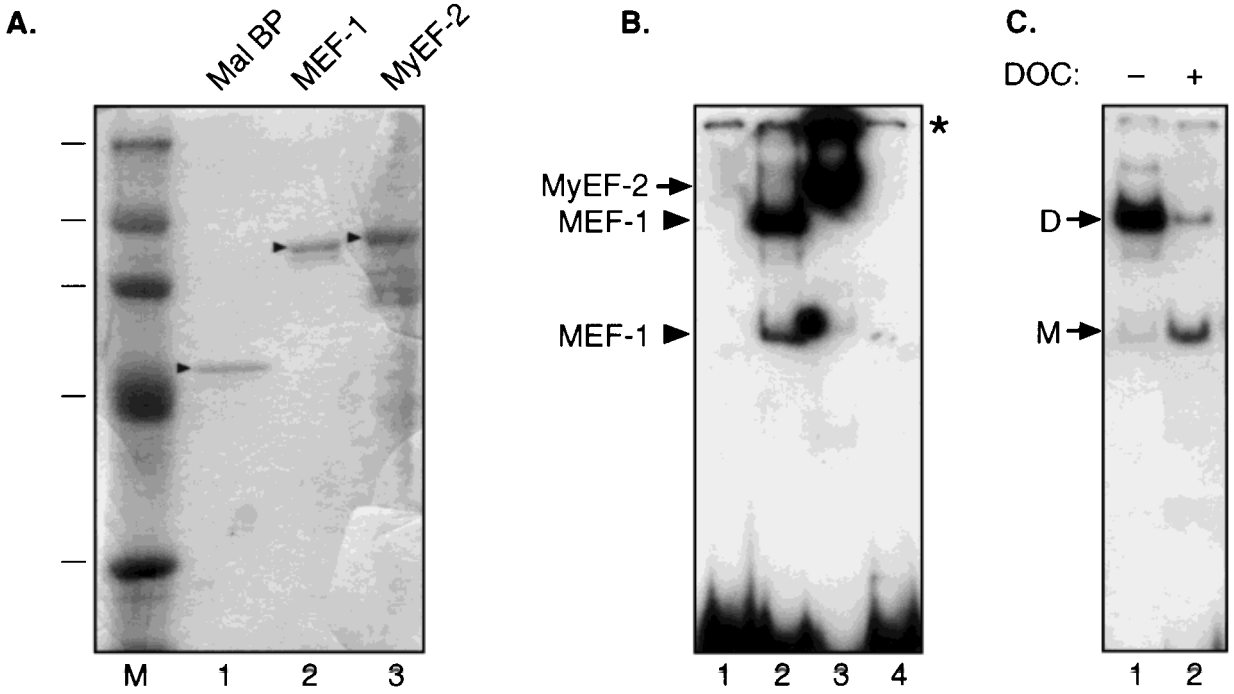

Fig. 1. DNA binding activity of MEF-1 and MyEF-2. **A:** Coomassie Blue staining of purified maltose binding protein, MalBP (**lane 1**) and fusion MalBP-MEF-1/Pur α (**lane 2**), and MalBP-MyEF-2 (**lane 3**). The arrowheads indicate positions of the purified proteins. Molecular weight markers from top to bottom are 200, 97.4, 68, 43, and 29 kDa, respectively. **B:** Bandshift analysis using end-labeled single-stranded MB1 DNA in the presence of 0.1 µg of MalBP (**lane 1**), 0.1 µg of fusion MEF-1 (**lane 2**), and 0.5 µg of MyEF-2 (**lane 3**). **Lane 4** contains no protein. *Arrowheads,* positions of the complexes that appear with MEF-1; *arrow,* MyEF-2 complex. *Asterisk,* nonreproducible aggregates that were detected in this experiment. **C:** Dissociation of the MEF-1:MB1 complex with sodium-deoxycholate (DOC). Binding reactions were performed in the absence (**lane 1**) and presence of 0.5% Na-deoxycholate (**lane 2**). Positions of dimeric (D) and monomeric (M) complexes are shown.

weight than 82 kDa and 86 kDa may represent proteolytic degraded forms of the fusion proteins. The ability of the highly purified control (MalBP) and the fusion proteins to bind DNA was investigated by band-shift assay utilizing ^{32}P labeled single-stranded MB1 probe. As illustrated in Figure 1B, the association of MEF-1/Pur α with MB1 results in the appearance of two complexes with distinct electrophoretic mobility (lane 2). The binding of MyEF-2 to the MB1 probe formed a major nucleoprotein complex and an aggregate which was inconsistently detected at the top of the gel (lane 3). No DNA protein complex was detected upon incubation of the control MalBP to the MB1 probe (lane 1), suggesting that MalBP may not be responsible for the association of the fusion proteins MEF-1/Pur α or MyEF-2 to the MB1 sequence. The formation of two MEF-1/Pur α nucleoprotein complexes with distinct electrophoretic mobility implies that MEF-1 may bind to the MB1 sequence in both the monomeric and dimeric forms. To address this point, binding reactions were performed in the presence of 0.5% sodium deoxycholate (DOC), which prevents protein–protein association. As shown in Figure 1C, the addition of DOC significantly decreased the intensity of the top band and enhanced the appearance of the bottom band, suggesting that the top and the bottom bands are the dimeric (D) and the monomeric (M) forms of the MEF-1/Pur α complex, respectively. A similar treatment with MyEF-2 showed no evidence for dimeric association of this protein with the MB1 sequence (data not shown).

In previous studies, we used several oligonucleotides representing the various regions of the MB1 as the probes in Southwestern analysis and as competitors in the bandshift assay to demonstrate that MyEF-2 binds to the sequences located at the 3′ end of MB1 element [Haas et al., 1995a]. Those studies more precisely localized the MyEF-2 binding site within the MB1c domain [Haas et al., 1995a]. It is of note that a similar approach indicated that MEF-1 interacts with G/A-rich sequences positioned at the 5′ end of this regulatory motif [Haas et al., 1995b]. Based on this information,

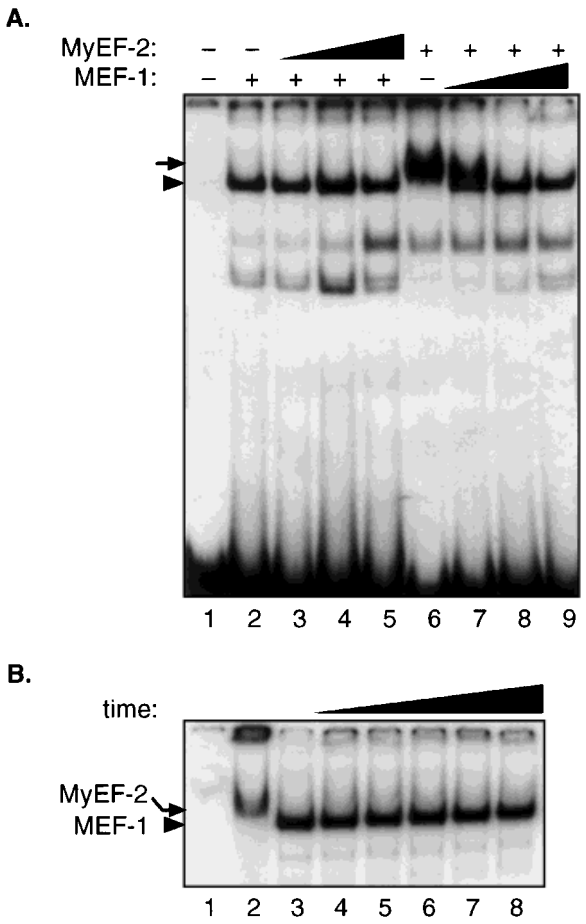

Fig. 2. Effect of MyEF-2 and MEF-1 interplay on their DNA binding activity. **A:** Bandshift analysis in which binding reactions were performed in the absence of protein (**lane 1**), 1 μg of MEF-1 (**lane 2**); 1 μg of MEF-1 plus 0.01, 0.1 and 1 μg of MyEF-2 (**lanes 3–5**); 1 μg of MyEF-2 to MB1 in the absence (**lane 6**); or presence of 0.01, 0.1 and 1 μg of MEF-1 (**lanes 7–9**). Positions of the major bands corresponding to MEF-1 (*arrowhead*) and MyEF-2 (*arrow*) complexes are indicated. **B:** Bandshift analysis in which binding reactions were carried out for 30 min with MyEF-2 (**lane 2**) and MEF-1 (**lane 3**) alone or MyEF-2 plus MEF-1 in which MEF-1 was added to the reaction mixture over a 15-min period. At 1 min (**lane 4**), 2.5 min (**lane 5**), 5 min (**lane 6**), 10 min (**lane 7**), and 15 min (**lane 8**), after the addition of MEF-1, gel loading buffer was added to the reaction mixtures. The positions of the MEF-1 and MyEF-2 are shown on the left.

in the next series of experiments, we investigated the cooperative action of MEF-1/Pur α and MyEF-2 on each other's binding to the MB1 sequence. Binding reactions were performed with a constant amount of MEF-1/Pur α and increasing amounts of MyEF-2, or a constant amount of MyEF-2 and increasing amounts of MEF-1/Pur α. As shown in Figure 2A, the addition of MyEF-2 to the MEF-1/Pur α binding reaction mixture showed no drastic changes in the intensity of the band corresponding to the MEF-1/Pur α:MB1 complex. Of particular interest was the notion that no evidence for association of MyEF-2 with the MB1 probe was detected. The addtion of MEF-1/Pur α to the MyEF-2 binding reaction mixture substantially decreased the formation of the MyEF-2 MB1 complex. Of note, no evidence for formation of a ternary complex MEF-1/Pur α:MB1:MyEF-2 was detected in this study. These observations indicate that cross-communication between MyEF-2 and MEF-1/Pur α may determine their association with the MB1 regulatory motif of the MBP gene. Results from the time course studies shown in Figure 2B indicate that displacement of MyEF-2 from the MB1 probe by MEF-1/Pur α could take place within one minute after the addition of MEF-1/Pur α to the binding reaction.

The above-mentioned studies were carried out with highly purified chimeric proteins in which MEF-1/Pur α and MyEF-2 were fused to maltose binding protein. In order to rule out the involvement of the maltose binding protein moiety of the fusion protein in the above observations, binding assays were performed following dissociation of the maltose binding protein moiety. As shown in Figure 3A, MEF-1/Pur α formed two complexes with the MB1 probe that represent the monomeric (M) and dimeric (D) MEF-1/Pur α:MB1complex. Of note, whereas at low concentrations of MEF-1/Pur α, complexes were preferentially at monomeric form, at higher concentrations of protein more dimeric complex was detected (Fig. 3A, cf. lanes 1 and 2–4). In the presence of MyEF-2 the intensity of the band corresponding to the dimeric MEF-1/Pur α complexes was significantly increased (Fig. 3A, cf. lanes 1 and 6). A similar result was obtained when increasing amounts of MyEF-2 was included in the MEF-1/Pur α binding reaction. As shown in Figure 3B, in the presence of MyEF-2, the intensity of the band corresponding to the dimeric MEF-1/Pur α complex was increased (cf. lanes 5 and 6–9). It should be pointed out that as an influence of cooperativity between these two proteins in binding DNA, the presence of MyEF-2 results in enhanced intensity of both monomer and dimer forms of MEF-1.

To gain some information regarding the requirements for interaction of MEF-1/Pur α and MyEF-2 with MB1 DNA, binding reactions were performed in the absence and presence of diva-

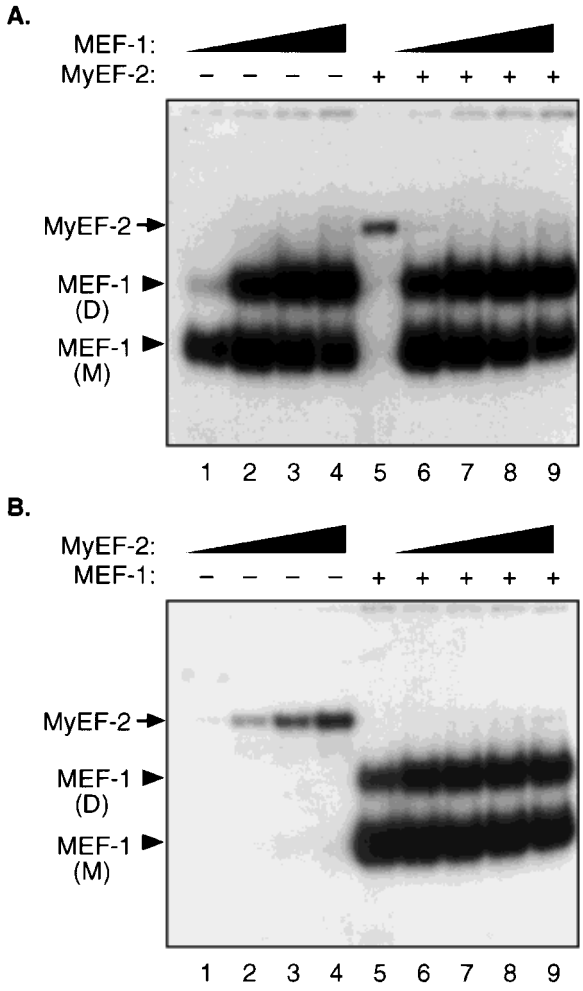

Fig. 3. Influence of purified MEF-1 and MyEF-2 on each other's MB1 binding activity. **A:** 25, 100, 200, and 400 ng of highly purified MEF-1 were incubated with MB1 probe alone (**lanes 1–4**) or in the presence of 500 ng of MyEF-2 (**lanes 6–9**). **Lane 5,** binding of 500 ng of MyEF-2 to MB1. Positions of the MyEF-2 and the dimeric (D) and monomeric (M) forms of MEF-1 are shown on the left. **B:** Binding was performed with increasing amounts of MyEF-2 (200, 400, 600, and 800 ng) in the absence (**lanes 1–4**) or the presence of 100 ng of MEF-1 (**lanes 6–9**). **Lane 5,** binding of 100 ng of MEF-1 with MB1 in the absence of MyEF-2.

lent cations. As shown in Figure 4A, the addition of Mg^{2+} and Ca^{2+} to the reaction mixtures augmented the binding ability of MEF-1/Pur α and MyEF-2 to the MB1 probe. Furthermore, some increase in the co-operation between MEF-1/Pur α and MyEF-2 on each others' binding to the MB1 sequence was observed. Also, we examined the importance of ATP and GTP for association of these proteins with MB1 sequence. The results in Figure 4B suggest that the binding of MEF-1/Pur α to the MB1 sequence remains virtually unchanged in the absence or presence of ATP or GTP. However, it was noticed that inclusion of GTP, but not ATP, in the binding reaction decreased the binding of MyEF-2 to the MB1 probe (Fig. 4B, cf. lanes 4 to 6).

In the last series of experiments, we used a series of MEF-1/Pur α mutant proteins encompassing the various regions of this protein to identify the critical domain that determines its binding to MB1 and perturbs the association of MyEF-2 with the MB1 sequence. As described previously, MEF-1 has several interesting structural features, including three 23-amino acid repeats separated by two 26-amino acid repeats positioned between residues 66–266. This region is believed to be important for its DNA binding activity [Chen et al., 1995]. It is also suspected that the N-terminus of MEF-1/Pur α, a glycine-rich domain (residues 7–53), may play a role in the regulatory functions of this protein. The C-terminus of this protein contains a region of amphipathic helix, two glutamine rich domains, and a psycho motif which binds to several proteins including SV40 T-antigen [Bergemann et al., 1992] (Fig. 5A). The results from binding experiments to evaluate the ability of MEF-1/Pur α variants for binding to the MB1 probe and displacing MyEF-2 from the MyEF-2:MB1 complex are summarized in Figure 5B. It is evident that the important region for the efficient binding of MEF-1 to DNA rests between the residues 216–274. Removal of the region beyond 274 completely abrogates its ability to interact with the MB1 sequence. However, the use of these truncated proteins in MyEF-2 binding studies indicated that a region between residues 167–216 is critical for its negative actions on MyEF-2:MB1 binding. These observations differentiate between the two domains of MEF-1 which play important roles for binding to DNA, and controlling the binding of the other protein, i.e., MyEF-2 to its target sequence on the MB1 motif.

The data presented here indicate that two cellular proteins, MEF-1 and MyEF-2, each with the ability to modulate transcription of myelin-basic protein promoter upon binding to the MBP promoter sequence may influence each other's interaction with their target DNA sequences. Evidently, MEF-1 decreases the binding of MyEF-2 to its target sequence within the MB1 sequence. The inhibition in binding of MyEF-2 to DNA imposed by MEF-1 is unlikely to involve any topological modifications in the

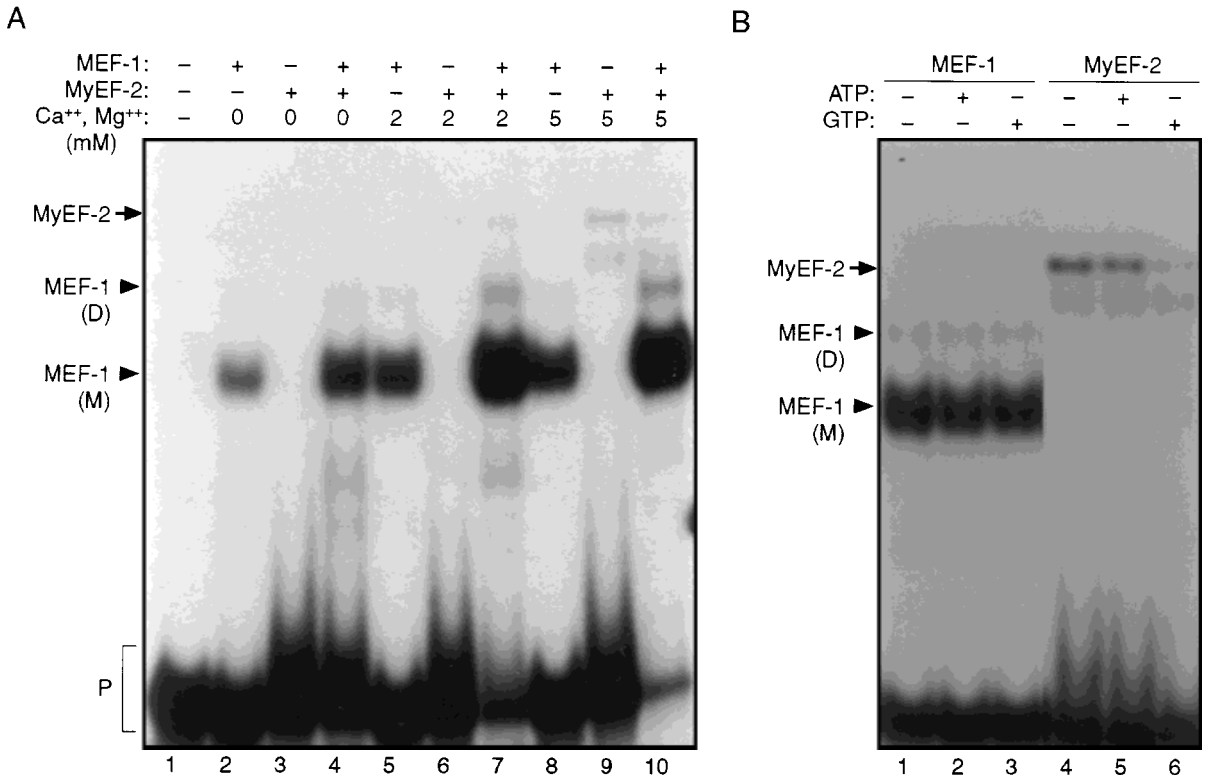

Fig. 4. Effects of divalent cations and ATP, GTP on DNA binding activities of MEF-1 and MyEF-2. **A:** DNA binding reactions were performed in the absence and presence of a mixture containing equal amounts of calcium and magnesium to a final concentration of either 2 or 5 mM using 100 ng of MEF-1 and 200 ng of MyEF-2. **B:** Approximately 1 mM of ATP or GTP was added into reaction mixture containing MEF-1 (**lanes 1–3**) or MyEF-2 (**lanes 4–6**). Positions of the bands corresponding to MEF-1 and MyEF-2 complexes are shown on the left.

target DNA, given the small size of the MB1 DNA probe (36 nucleotides). Results from in vitro protein–protein interaction studies showed no evidence for the association of MyEF-2 and MEF-1 suggesting that transient communication of these two cellular proteins with each other, or together with their target DNA sequence may determine their interaction with the myelin basic protein promoter element. Results from binding studies with MEF-1 mutants indicated that a region of MEF-1 that consists of basic aromatic repeats separated by two acidic leucine-rich repeats (residues 66–248) may play an important role in conferring binding activity to the MB1 sequence. However, to exert its regulatory action on MyEF-2 binding, MEF-1 requires the residues between amino acids 167–216 that contains a basic leucine-rich domain.

These observations may have a functional significance in the programmed expression of the MBP gene during brain development. Earlier studies indicated that MEF-1 binding activity to the MB1 sequence is expressed in a developmental stage-specific pattern that coincides with the pattern of myelin basic protein transcription [Haas et al., 1993]. It was demonstrated that in brain nuclear extract the level of MEF-1 association with MB1 is low at early stages of brain development (3–7 days postnatal), peaks during the phase of myelination (18–20 days), and persists in adults (30 days). Analysis of MyEF-2 mRNA levels during brain development has indicated that expression of the gene is also developmentally regulated in mouse brain, however the pattern of its expression is distinct from those observed for MEF-1 binding. The peak of MyEF-2 expression is detected at the early stages of brain development (7 days postnatal), decreases during the phase of myelination, and remains at low levels throughout the animal's life.

Altogether, these findings are consistent with a model in which the association of MyEF-2 with MB1 at the early phase of brain development suppresses myelin basic protein gene tran-

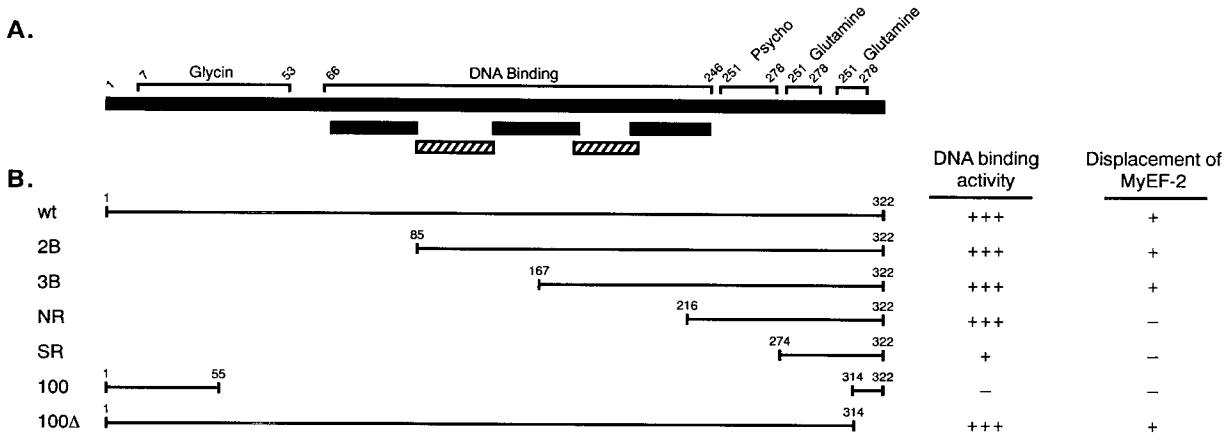

Fig. 5. Determination of the domain of MEF-1 responsible for binding to MB1 and dissociating the MyEF-2 complex. **A:** Structural organization of MEF-1. Positions of the glycine-rich and glutamine-rich domains are shown—psycho motif points to the region which binds to Rb [Johnson et al., 1995] and is located next to the proposed DNA binding domain. The DNA binding domain comprises three 23-amino acid repeats (*solid bars*) separated by two 26-amino acid repeats (*hatched bars*) [Chen et al., 1995]. **B:** Bacterially produced full-length and truncated MEF-1 peptides were mixed with 5′-end labeled MB1 probe and examined for DNA binding activity by bandshift assay. In parallel, the effect of MEF-1 mutants on displacement of MyEF-2 from the MB1 probe was examined by the addition of highly purified MEF-1 peptides to the binding reaction mixture containing MB1 probe and MyEF-2. The position of each mutant with regard to wild-type MEF-1 is indicated by the amino acid residues and their DNA binding activity and the ability for displacing MyEF-2 are indicated on the right.

scription. It is possible that during myelination, the binding of MEF-1 to the MB1 sequence results in the dissociation of MyEF-2 from the MBP promoter and upon its interaction with MB1, stimulates transcription of these genes (Fig. 6). Such a model for the concerted action of positive (MEF-1) and negative (MyEF-2) factors is similar to those mechanisms proposed for accurate cell type specific expression of a variety of genes in muscle, liver, T lymphocyte, and glial cells [Bouvagnet et al., 1987; Colantoni et al., 1987; Nabel et al., 1988; Tada et al., 1989]. In this respect, the unique feature is the ability of a positive regulator protein to influence the binding of a negative factor to its responsive DNA target without stably associating with this complex. Currently, studies are in progress to delineate the mechanism by which MyEF-2 alters the binding ability of MEF-1 to the MB1 domain, and further investigate the global effect of this intercommunication on the expression of other myelin genes during the course of myelination.

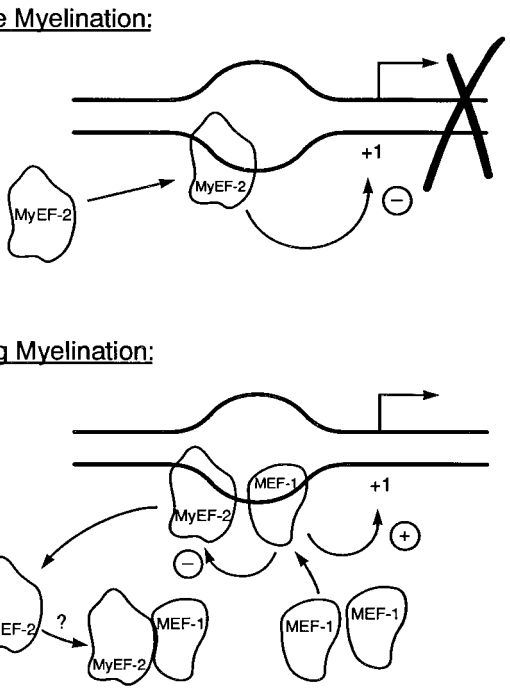

Fig. 6. Proposed model for involvement of MEF-1 and MyEF-2 in programmed transcription of the MBP promoter. Before myelination, the binding of MyEF-2 to its target DNA sequence within the MBP promoter exerts negative effects on transcription of the MBP gene. During the phase of myelination, which is concomitant with high level of expression of MEF-1, binding MEF-1 to its motif within the MB1 dissociates MyEF-2 from the promoter sequence and augments transcription of the MBP gene.

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