MEBA Derepresses the Proximal Myelin Basic Protein Promoter in Oligodendrocytes*

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The central nervous system expression of myelin basic protein (MBP) is restricted to oligodendrocytes and is developmentally regulated; these regulatory features are transcriptionally mediated. We have previously shown that the proximal 149 nucleotides of the MBP promoter were both necessary and sufficient to activate the transcription of MBP in cultured oligodendrocytes, but not in other cell types. Sequences within the distal portion of this promoter, which contains a nuclear factor 1 (NF1) binding site, repressed activation of the MBP promoter in Cos-7 cells, but not in oligodendrocytes. We now describe a sequence upstream of and partially overlapping the NF1 site that activates the MBP promoter in oligodendrocytes, but not in Cos-7 cells. A protein complex binds to this site, designated MEBA (myelinating glia-enriched DNA binding activity), and is enriched in nuclear extracts prepared from the brain, oligodendrocytes, and Schwann cells. The amount of MEBA parallels MBP expression and myelination in the developing brain and parallels new MBP expression as purified oligodendrocytes differentiate. Mutational analyses of binding and function distinguish MEBA, an activator, from NF1, a repressor of MBP transcription, and suggest that MEBA consists of at least two proteins. Because the binding sites of MEBA and NF1 overlap, we suggest that MEBA may either compete with or modify NF1 binding, thereby activating the MBP promoter in oligodendrocytes.

Myelin basic protein (MBP)1 is a structural protein of myelin expressed only by oligodendrocytes or Schwann cells. In the central nervous system, MBP expression by oligodendrocytes is required for normal myelogenesis (1). MBP mRNA expression in the rodent brain rises approximately 100-fold between developmentally regulated expression of reporter genes in transgenic mice (5–8). Several studies in vitro have sought to identify specific DNA sequences within the proximal MBP promoter that might be necessary for cell-specific expression, but most of these studies were performed using cells that do not transcribe MBP or nuclear extracts prepared from the brain, which contains a complicated mixture of cell types (9–11).

We have previously shown that 750 nt of human MBP promoter were sufficient to activate oligodendrocyte-specific, developmentally regulated expression of lacZ during active myelogenesis in transgenic mice (3). In addition, we showed by transient transfection analysis in primary cultures of oligodendrocytes that 750, 420, or 149 nt of human MBP promoter was sufficient to activate a 5–10-fold increase in the expression of CAT reporter gene in oligodendrocytes, but not in other cell types (12). We also showed that the 149-nucleotide region contained two subregions with opposing functional activities: (a) the proximal 102-nt region activated the expression of CAT in most cell types; and (b) the more distal region, from −149 to −102 nt, silenced the expression of CAT in them. Interestingly, the distal region did not silence the expression of CAT in oligodendrocytes. This distal region contained a consensus nuclear factor 1 (NF1) site; a deletion that removed half of the NF1 site activated the expression of CAT in oligodendrocytes. Based on these results, we hypothesized that a specific alteration of NF1 may alleviate repression and contribute to the oligodendrocyte-specific activation of MBP transcription (12).

We now describe a DNA sequence that flanks and partially overlaps the NF1 site, which activates the MBP promoter in oligodendrocytes, but not in Cos-7 cells. This sequence is bound by a unique set of nuclear proteins enriched in the brain, oligodendrocytes, and Schwann cells, designated MEBA (myelinating glia-enriched binding activity). The appearance of MEBA parallels both myelination in the developing brain and MBP mRNA expression in differentiating oligodendrocytes. Mutations within the region of MEBA and NF1 binding distinguish MEBA from NF1 and suggest that MEBA consists of at
least two proteins. Because these binding sites with opposing functions overlap, we suggest that MEBA may either compete with or alter the NF1 binding of the proximal MBP promoter and derepress the MBP promoter in oligodendrocytes.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—All deletions of the proximal 149–nt MBP promoter were prepared by a modification of the exonuclease III/mung bean nuclease technique (Stratagene), as described previously (12). Transversion mutations spanning from 149 to 110 nt of the MBP promoter were prepared by polymerase chain reaction-mediated mutagenesis, as described previously (12). All mutations were confirmed by sequence analysis. pRSVZ (12) was a gift from Dr. G. MacGregor (Baylor College of Medicine, Houston, TX).

**Cell Culture and Transfection Analysis**—CG4 cells, a bipotential glial cell population derived from primary cultures of rat brain, were cultured as described previously (14). In medium conditioned by B104 neuroblastoma cells (B+, 30% in Dulbecco’s modified Eagle’s medium; plus N1 and biont), these cells resemble oligodendrocyte precursors, in the absence of B104-conditioned medium (B−−), these cells are induced to differentiate into oligodendrocytes. A1.20 (SV40 T-antigen-transformed oligodendrocytes), COS-7, HeLa, C6, HJC, A7, or L cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum or as described previously (12). Rat Schwann cells from the sciatic nerve were purified and cultured in the presence or absence of 4 μM forskolin as described previously (15). Primary oligodendrocyte cultures were prepared as described by Grinspan et al. (16) and maintained in 2 ng/ml platelet-derived growth factor for 7 days before transfection.

Cultured cells were transfected with 20 μg of supercoiled DNA (either 10 μg of pBluescript carrier DNA and 10 μg of supercoiled DNA or, for cotransfections with pRSVZ, 2.5 μg of pRSVZ, 7.5 μg of carrier DNA, and 10 μg of DNA) using the CaPO4 procedure in Dulbecco’s modified Eagle’s medium or the CaPO4 procedure in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum for 4 h as described previously (12). The cultures were washed twice, refed with their original medium, and harvested for CAT assays 60 h later. CAT and β-galactosidase enzymatic assays and Southern blot analysis for the transfected plasmid were performed as described previously (12, 17). Autoradiographic signals were quantitated by densitometry. Relative CAT activity was determined as the ratio of the percentage of 14C converted into monoclonal and di-acetylchloramphenicol products to either the amount of the transfected plasmid DNA or to the β-galactosidase activity. For example, the experiments shown in Fig. 1 were performed using either Southern blot analysis for the transfected plasmid or transfected plasmid DNA, and with pRSVZ as controls for transfection efficiency. The changes in CAT activity for the progressive deletions were reproduced in most cases, were repeated several times with separate plasmid preparations. The relative CAT activities were expressed as the mean ± S.E. for n replications.

**Electrophoretic Mobility Shift Assay (EMSA)**—10 μg of liver, brain, or brainstem (includes cerebellum, from the cervical-medullary junction to the midbrain) were harvested from Sprague-Dawley rats of various ages. After homogenization in buffer containing 2.0 M sucrose, 10 mM HEPES (pH 7.6), 15 mM KCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 80 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1% w/v low-fat milk, nuclei were pelleted through a sucrose cushion containing the same buffer without low-fat milk by centrifugation in a SW-28 rotor at 24,000 rpm (75,000 × g) for 60 min at 4 °C. Nuclear extracts were prepared from tissue nuclei or from 50–100 million cultured cells as described previously (12), except that extraction Buffer C contained 420 mM NaCl.

EMSA was performed as described in Ref. 12, with the following modifications: (a) probes were prepared from synthetic double-stranded oligonucleotides containing portions of the proximal human MBP promoter (Fig. 2) by end-labeling with both α-32P-dCTP and α-32P-dGTP using Klenow enzyme; (b) competitors included NF1 5′-ATTTGGCTT-GAAACCATAAT-G′, NF1 MBP 5′-AGTCCGAGATGGCACAAG′-3′, NF1 5′-AAGTCTGATTAAGGTCGTCGGC′-3′, and NF1 5′-ACGAGATCCTGACGTCCTG′-3′, which were used at a 100-fold molar excess; (c) the binding reactions contained 100 mM KCI, 2 μg of poly(dexoyinosinic-deoxycytidylic acid), 1–4 μg of nuclear protein in 1 μl, and 60,000 cpm of probe (approximately 0.4 ng of DNA); and (d) protein-DNA complexes were resolved on low ionic strength 8% polyacrylamide gels in 4× Tris-acetate EDTA buffer.

**DNase Footprint Analysis**—End-labeled sense or antisense fragments from −187 to −25 nt of the proximal MBP promoter (20,000 cpm of probe corresponding to 1–2 ng of DNA) were used in binding reactions with increasing amounts (10, 20, and 40 μg) of rat adult brain and liver nuclear extracts as described previously (18). Briefly, binding reactions were carried out for 45 min at 4 °C in a 50-μl reaction volume containing 90 mM KCl, 3 mM MgCl2, 20% (v/v) glycerol, 20 mM Tris-Cl (pH 7.9), 1 mM dithiothreitol, and 1 μg of poly(dexoyinosinic-deoxycytidylic acid) and then treated with 300 ng of DNase I for 90 s at room temperature and stopped by the addition of EDTA/SDS (20 mM and 0.7%, final concentration). In the NF1 competition assays, 40 μg of nuclear extract were incubated with either a 100-fold or a 200-fold molar excess of NF1 competitor oligonucleotides (see above) for 20 min at 4 °C before the binding reaction. The samples were phenol-extracted, precipitated, and resolved on 6% polyacrylamide sequencing gels. Maxam-Gilbert G+A sequence ladders were generated from the same probes by the standard protocols (19).

**RESULTS**

**Northern Blot Analysis**—Total RNA was prepared from rat forebrain or brainstem by the method of Chirgwin et al. (20) and from cells by the method of Chomczynski and Sacchi (21). Northern blot analysis of 10 μg of total RNA was performed as described in Ref. 12 using either rat MBP (12), rat glyceraldehyde-3-phosphate dehydrogenase (22), or rat proteolipid protein cDNA (23) as a template to generate probes. All quantitation of autoradiographic signals was performed by densitometry (Molecular Dynamics 300A densitometer).

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**Fig. 1. Progressive deletional analysis of the proximal MBP promoter.** A, transient transfection analysis of plasmids containing progressive deletions of the 149-nt MBP promoter in oligodendrocytes (OL) and Cos-7 cells. CAT activities for each plasmid (e.g. pSN149) are expressed relative to that of p600CAT = 1. B, a diagrammatic representation of these results shows that in both cell types, the proximal segment of the MBP promoter (flanked by an arrow) activates CAT transcription, whereas the NF1 site in the distal segment is associated with the silencing of CAT transcription. Note that the segment from 149 to 128 nt activates CAT transcription in oligodendrocytes, but not in Cos-7 cells. (In all figures, number represents the position (in nt) upstream of the initiation of MBP transcription.)
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Competitions with probe B. Note that the amount of nuclear extract is only 2 μg in the NF1 competitions with probe B.

MBP transcription. Deletion from −149 to −128 nt reduced CAT levels approximately 50% in oligodendrocytes, but not in Cos-7 cells. Similar results were obtained for the five other cell lines tested. These data suggest that there is an oligodendrocyte-specific activating sequence upstream of the NF1 site.

To identify a corresponding DNA binding upstream of the NF1 site, we performed the EMSA using double-stranded oligonucleotides containing the sequence from −149 to −105 (probe A) and from −149 to −118 (probe B). The results of this experiment are shown in Fig. 2. EMSA of the nuclear extract prepared from CG4 oligodendrocytes, which transcribe MBP (see below), with probe A generates a complex pattern of at least six bands (lanes 10 and 11). This binding was sequence specific, because the six bands were abolished by competition with excess unlabeled A oligonucleotide (lane 12). Four of the six bands were abolished by competition with an oligonucleotide containing either the consensus NF1 sequence or the MBP NF1 sequence (lanes 13 and 14). Conversely, the two remaining bands were abolished by competition with oligonucleotide C, which contained a truncated NF1 site and the upstream sequence, whereas the first four NF1-related bands were not competed (lane 15). These results suggest that there is nuclear protein binding upstream of and distinct from NF1.

EMSA of CG4 extract using oligonucleotide B as a probe directly confirmed this. Fig. 2 (lanes 2–4) shows that a doublet was generated with a mobility similar to that of the two bands remaining after NF1 competition of probe A. This binding was specific, as shown by competition with unlabeled oligonucleotides B or A (lanes 5 and 8), but was only partially competed by

The Upstream DNA Binding Site Overlaps the NF1 Site—The partial competition of the doublet by cold NF1 oligonucleotide suggested that its binding site could contain a portion of the NF1 sequence. In keeping with this, the EMSA of CG4 nuclear extracts using probe C, which contained the sequence from −149 to −124 nt, revealed no binding, showing that the 5′ portion of the NF1 site is necessary to generate the doublet (Fig. 3). The specific sequence of the 5′ portion of the NF1 site was required for binding, because EMSA with probe D, which contained a transversion mutation of the nucleotides from −124 to −118 nt, produced a nonspecific band of a different mobility that was not competed by cold D oligonucleotide (Fig. 3, lanes 15–17).

MEBA Is Enriched in Oligodendrocytes and Schwann Cells—To analyze the tissue distribution of this binding activity, we performed an EMSA of nuclear extracts prepared from various tissues and cell types using probe B. As shown in Fig. 4A, the doublet was present in the brain, in CG4 B(−) cells, and in Schwann cells, all of which transcribe MBP. On the contrary, the doublet was not present in the liver, A1,20, C6, or Cos-7 cells, which do not transcribe MBP. Therefore, we designated this binding activity MEBA.

MEBA Correlates with Oligodendrocyte Differentiation and Myelination—To see whether MEBA is associated with the induction of MBP expression in the developing brain, we correlated the EMSA of brain nuclear extracts with the Northern blot analysis of total RNA, each of which was prepared from the forebrain of rats of various ages. As shown in Fig. 4B, MEBA was present 2 days after birth but then disappeared during the next week. MEBA reappeared at 10 days after birth and increased in amount over the subsequent 8 weeks. Similarly, MBP mRNA was first detected at 10 days after birth and rose to much higher levels in the 18-day-old and 10-week-old forebrain (Fig. 4D). We then correlated the presence of MEBA and MBP mRNA in the brainstem and forebrain at 5 and 18 days after birth, because MBP mRNA appears first in the brainstem and later in the forebrain, as shown in Fig. 4D. EMSA analysis for MEBA showed the same trend; MEBA levels were significantly higher in the brainstem than in the forebrain at 5 days after birth (see Fig. 4C, lanes 1 and 4, in which 2 μg of nuclear extract produced an unsaturated signal), whereas the levels were more similar in the brainstem and the forebrain at 18 days after birth. Thus, MEBA appears as MBP expression is induced during myelination in the developing forebrain and brainstem, although the early postnatal appearance of MEBA does not correlate with myelination.

The early postnatal appearance of MEBA in the brain could be explained by a contribution of nuclear proteins from cells other than oligodendrocytes. To directly correlate the appearance of MEBA with MBP expression in pure differentiating oligodendrocytes, we analyzed CG4 oligodendrocytes. CG4 cells model oligodendrocyte differentiation well, because they up-regulate myelin-specific gene expression when cultured in the absence of growth factors, and they form myelin when transplanted into rodent brain (24). The MEBA doublet appeared at much higher levels (Fig. 5A) only in the differentiated CG4
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oligodendrocytes, as measured by the up-regulation of MBP and proteolipid protein mRNA levels (Fig. 5B). Thus, in pure oligodendrocytes, the appearance of MEBA and MBP mRNA are strictly correlated.

Interestingly, at a low dosage of nuclear extract from differentiated (B104−) CG4 cells, the upper band of the MEBA doublet is present along with a lower-intensity lower band (Fig. 5A). In contrast, at a high dosage of nuclear extract from undifferentiated (B104+) CG4 cells, a more intense lower band is seen, but the upper band is not present. This suggests that the upper band does not simply appear as a consequence of high dosage of the lower band.

DNase I Footprint Analysis of MEBA Binding—To locate the MEBA binding site, we performed DNase I footprint analysis of the MBP promoter using brain and liver nuclear extracts. Fig. 6 shows that both brain and liver nuclear extracts protected the sequence from −132 to −106 nt, an extension of the NF1 binding site from −124 to −111 nt. Extended footprints have been reported for purified NF1 in several other promoters (see Ref. 25 for example). Instead, hypersensitive sites upstream of the NF1 protection at nucleotides −135 to −136, −143, and −147 to −148 appeared more prominently with brain than with liver nuclear extract, although no protection upstream of nt −132 could be detected. These results were confirmed in analysis of the opposite strand (data not shown). To better reveal MEBA binding, we performed footprint analysis in the presence of NF1 oligonucleotide competitor. As shown in Fig. 6B, a protection from −133 to −127 nt appeared with the brain extract, but not with the liver extract, as the NF1 footprint was diminished by competition. Thus, MEBA binds upstream of the NF1 site as predicted by the EMSA analysis of oligodendrocyte nuclear extracts.

Mutational Analysis Correlates MEBA Binding and Function—In a further attempt to localize the binding of MEBA, we synthesized a series of oligonucleotides containing transversion mutations from −149 to −124 nt and investigated the resulting effects on MEBA in an EMSA of oligodendrocyte nuclear extracts. As shown in Fig. 7, mutations between −146 and −141 nt or between −132 and −125 nt abolished all sequence-specific binding, whereas a mutation between −140 and −133 nt did not. However, the mutation between −140 and −133 nt produced a sequence-specific binding that shifted with a slower mobility (Fig. 7). Thus, MEBA binds two noncontiguous sequences between −149 and −124 nt.

To correlate the effects of these mutations on binding and function, we reproduced these same mutations in the wild-type pSN149 as well as a transversion of the NF1 site between −124 and −110 nt and performed a transient transfection analysis in primary cultures of oligodendrocytes. As shown in Fig. 8, the wild-type pSN149 produced a relative CAT activity 10-fold above the promoterless construct p0SNCAT. pSN149A with a...

FIG. 4. Cell-specific and developmental appearance of MEBA. A, EMSA analysis with probe B shows that MEBA is present in nuclear extracts (NE) from brain (Br), differentiated oligodendrocytes (CG4 B(−)), and Schwann cells (SC) cultured in the presence (F(+)) or absence (F(−)) of forskolin, but not in the liver (Liv), undifferentiated oligodendrocytes (CG4 B(+)), A1.20, C6, or Cos-7 cells. B, EMSA analysis of forebrain (FB) nuclear extracts at various postnatal ages. C, EMSA analysis of nuclear extracts prepared from various brain regions in development shows that MEBA is enriched in 5-day-old brainstem (5d BS) as compared with 5-day-old forebrain (5d FB; compare lanes 1 and 2 with lanes 4 and 5), whereas it is present more uniformly in 18-day-old brainstem (18d BS) and 18-day-old forebrain (18d FB; compare lanes 7 and 8 with lanes 10 and 11). This parallels the topographical appearance of MBP mRNA in developing brain as shown by Northern blot analysis in D. D, parallel Northern blot analysis for MBP mRNA. Note that the MEBA doublet reappears as MBP mRNA expression is induced at 10 days after birth. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) signals demonstrate equal loading of RNA.

FIG. 5. MEBA appears as oligodendrocytes differentiate. A, EMSA analysis with probe B of increasing amounts of nuclear extracts prepared from undifferentiated (B104+ ) or differentiated (B104− ) CG4 oligodendrocytes shows that the MEBA doublet appears at high levels only in differentiated oligodendrocytes, in parallel with the appearance of MBP and proteolipid protein mRNA by Northern blot analysis in B. 28 s, 28S ribosomal RNA. NS, nonspecific oligonucleotide competitor.
mutated NF1 site produced CAT levels 400% higher than that of wild-type pSN149, confirming that NF1 acts as a repressor of MBP transcription. Instead, the mutations between 2146 and 2141 nt or between 2132 and 2125 nt reduced the relative CAT levels by 70 or 60%, respectively, as compared with that of the wild-type pSN149, whereas the mutation between 2140 and 2133 nt did not reduce CAT levels significantly. Thus, the same two noncontiguous sequences upstream of the NF1 site that bind MEBA are also necessary for the activation of the MBP promoter in oligodendrocytes.

DISCUSSION

Silencers play an important role in determining neuron-specific (26–28) and astrocyte-specific (29) gene expression. In this study, we show that silencing may also be an important mechanism for restricting gene expression to oligodendrocytes. We and others have previously shown that short fragments of proximal MBP promoter are sufficient to mediate oligodendrocyte-specific transcription in transgenic mice (3, 7), and that both positively and negatively acting DNA sequences regulate activation of the proximal MBP promoter in primary cultures of oligodendrocytes (12). Here, we showed that an NF1 site in that region silences the MBP promoter in many cell types that do not transcribe MBP. Instead, in oligodendrocytes, we identified an activating sequence upstream of and overlapping the NF1 site in the MBP promoter. A protein complex, MEBA, binds this site and is enriched in nuclear extracts from the brain, oligodendrocytes, and Schwann cells in which MBP is transcribed. Mutational analyses of MEBA correlate its binding and function and suggest that MEBA binds two noncontiguous sequences to activate the MBP promoter. Our results suggest that unlike many neuron-specific genes that are regulated via the reciprocal expression of activators in neurons and repressors in other cells, oligodendrocyte-specific genes such as MBP are regulated by the interaction of activators and repressors expressed simultaneously in oligodendrocytes, whereas the repressors are expressed ubiquitously in other cells. Finally, si-
lencers are also used to restrict gene expression to glia in *Drosophila* (30). Thus, an emerging body of work suggests that the restriction of gene expression by lencers is a general, evolutionarily conserved mechanism used to differentiate neurons and various glia in the central nervous system.

Association of MEBA with MBP Transcription in Vivo and in Vitro—We found MEBA in oligodendrocytes and Schwann cells, both of which transcribe MBP and synthesize myelin. In the brain, however, the appearance of MEBA is not perfectly correlated with myelination in development, as we found MEBA in forebrain nuclear extracts at 2 days after birth, when MBP transcription and myelination are minimal. One potential explanation is that cells other than oligodendrocytes contribute nuclear proteins whose binding is similar to that of MEBA. In fact, MEBA appeared robustly in tight correspondence with MBP mRNA as cultured CG4 oligodendrocytes differentiated. Another possibility is that MEBA acts in combination with other transcription factors to activate the MBP promoter, because DNA elements outside of the proximal MBP promoter are required for full activation of MBP transcription during peak myelogenesis (3). Thus, this combination of factors may correlate with MBP transcription and myelination, even if the presence of its component factors, such as MEBA, does not.

Finally, an EMSA of nuclear extracts prepared from cultured Schwann cells also produces bands with the mobility of MEBA. Although we showed previously that DNA sequences in the proximal MBP promoter activate transcription differently in Schwann cells as compared with oligodendrocytes (17), MEBA could play an important role in both. Further identification and purification or cloning of MEBA constituents will create the reagents necessary to clarify these important issues.

**MEBA Binds a Novel cis-acting Sequence**—Extensive functional and biochemical studies of the proximal MBP promoter have not previously revealed MEBA or its binding site. However, many of these studies were performed using cells that do not transcribe MBP (reviewed in Ref. 12), and here we show that MEBA is not present in those cells. In the few other functional studies performed in oligodendrocytes, deletional and mutational analysis targeted areas that would not have revealed MEBA (91, 92). Of note, *in vitro* transcription analysis of the proximal MBP promoter in HeLa nuclear extracts has revealed a potential repressor, Myef-2. However, its target silencer is in a more proximal region of the MBP promoter, and Myef-2 function has not been characterized in oligodendrocytes as compared with cells that do not transcribe MBP (33, 34); therefore, what role, if any, it may play in restricting MBP expression to oligodendrocytes is not clear.

Biochemical analyses of the region from −150 to −100 nt of the MBP promoter include footprint analysis of brain nuclear extracts that revealed an extended protection upstream of the −124 to −110 nt NF1 site that could represent MEBA (10). However, NF1 footprints typically extend beyond the consensus TGGN,C binding site. For example, the same extended footprint from −130 to −105 nt was produced using purified NF1 (35). In keeping with this, NF1 binding in other promoters has limited the recognition of nearby binding by other transcription factors (36). In fact, our footprint analysis in the presence of excess competitor NF1 oligonucleotide revealed a protection from −133 to −127 nt with brain nuclear extract, but not with liver nuclear extract. This provides direct evidence of MEBA binding upstream of the NF1 site and coincides well with one of the two binding sites (−132 to −125 nt) predicted by the functional and EMSA binding analysis of transversion mutants (see Figs. 7 and 8). Downstream of the NF1 site, EMSA analysis of the sequence from −128 to −94 nt has revealed binding (37). A cDNA encoding for a protein, MRF-1, with similar nucleotide binding specificity was subsequently identified (38). However, it is highly unlikely to be a component of MEBA, because MRF-1 is a single-stranded RNA/DNA-binding protein, and methylation interference analysis identified the majority of nucleotides required for its binding to be downstream of the NF1 site; they are not contained in probe B that we used to identify MEBA. Finally, MEBA is not simply a variant of NF1, because it is clearly distinct from NF1, based on the cross-competition data of Fig. 2. That NF1 partially competes MEBA binding of probe B suggests only that some of the same sequences are necessary for both NF1 and MEBA binding.

Precise identification of the MEBA binding site is difficult with crude nuclear extracts, because NF1 binding obscures MEBA binding in footprint analysis (see above), and MEBA shifts relatively few counts in EMSA analysis (see Fig. 4, lane 4). For example, EMSA analysis of two-base transversions from −149 to −116 nt produced variable alterations in MEBA (data not shown). However, the binding site identified by the EMSA analysis of eight nucleotide transversion mutations is a discontinuous, nonpalindromic sequence that suggests binding by distinct proteins. Also, dosage analysis of the bands in the MEBA doublet show that the upper band is not stoichiometrically related to the lower band, suggesting that MEBA consists of at least two proteins, not multimers of one protein (see Fig. 5). Finally, Signal Scan analysis for previously reported cis-acting sequences (Ref. 39; http://bimas.dcri.nih.gov/molbio/signal/) did not reveal an exact match to the MEBA sequence other than the 5′ half of a NF1 binding site. Taken together, these findings suggest that the MEBA binding site is novel, and that MEBA may comprise a unique combination of factors.

**Model of MEBA and NF1 Interactions**—Our analysis of the
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MBP promoter performed in oligodendrocytes is in keeping with and extends the model proposed by Mikoshiba and colleagues (11) based on in vitro transcription analysis performed with brain nuclear extracts. Mouse MBP promoter from −256 to −53 nt (contains the composite MEBA/NF1 binding site) activated transcription in brain nuclear extracts and silenced transcription in liver nuclear extracts when placed upstream of the −53 to +60 nt basal MBP promoter (11). Moreover, mutation analysis showed that nucleotides CAG from −122 to −119 were required for transcription in brain extracts. Because these nucleotides lie in the 5′ half of the NF1 site, these authors concluded that NF1 is required for brain-specific activation of the MBP promoter (10). They further identified a novel NF1 isoform in the brain and speculated that brain-specific NF1 was responsible for brain-specific activation of the MBP promoter (40). However, they also found by transfection analysis that the sequence containing the NF1 site silenced the MBP promoter in cells not transcribing MBP (see Fig. 6 in Ref. 40). Also, we note that these nucleotides are contained in the MEBA/NF1 overlap sequence that we showed was required for MEBA binding and function. Therefore, we would modify their model to suggest that MEBA and NF1 interact at this sequence to activate MBP transcription.

How might NF1 play a role in the activation of MBP transcription? NF1 sites are bound by a group of protein isoforms encoded by four different genes that interact to form either homodimers or heterodimers. Recently, cDNAs encoding NF1 isoforms have been cloned from mouse, and the expression of these isoforms was measured in various tissues and during development (41). In the brain, the differential expression of NF1 genes is developmentally and topographically regulated. For example, only three of the four NF1 genes are expressed in white matter, although the pattern of NF1 expression in oligodendrocytes has not yet been examined. Our previous EMSA analysis of this region with NF1 competitors suggests that the NF1 binding in oligodendrocytes differs from that of other cells (12). In addition to differential expression, the NF1 isoforms also exhibited differential function on a target mouse mammary tumor virus promoter; some isoforms activated the mouse mammary tumor virus promoter; some isoforms repressed the mouse mammary tumor virus promoter much more effectively than others (41). Thus, even though NF1 proteins are present ubiquitously in tissues, the differential expression, interactions, and function of NF1 isoforms make cell-specific promoter activation by NF1 possible.

As reported for other transcription activators (42, 43), NF1 can also mediate repression at some promoters. For example, the glutathione transferase P promoter contains a consensus NF1 site that acts as a silencer and is bound by the NF1-A and NF1-B isoforms (44). NF1A and NF1B isoforms are enriched in white matter in the brain (41), consistent with the idea that NF1 could silence the MBP promoter. Thus, the remarkable activation of the MBP promoter associated with transversion mutation of the NF1 site (Fig. 8) is not surprising.

These data, taken together with our data, raise three possible models (Fig. 9). In all three models NF1 represses MBP transcription in most cells (Fig. 9, bottom), whereas MEBA is enriched and activates MBP transcription in oligodendrocytes. First, MEBA could activate MBP transcription without interacting with NF1. However, because the binding sites of MEBA and NF1 overlap, MEBA could instead compete with NF1 binding and hence derepress the MBP promoter (Fig. 9, top). Finally, in the third model, MEBA could interact with NF1; either MEBA alters the NF1 complex that binds, alleviating repression, or the mix of NF1 isoforms in oligodendrocytes may differ from that of other cells and permit MEBA to bind and activate (Fig. 9, middle). A precedent exists for such interactions in the human aldolase A promoter, where overlapping MEF2 and NF1 sites bind a muscle-specific protein complex containing NF1 to stimulate myotube-specific transcription of aldolase A (36). However, our data favor the second competition model, because either a deletion to −116 or a transversion mutation of the NF1/MEBA overlap sequence activates the MBP promoter in oligodendrocytes. Because either mutation removes both MEBA and NF1 binding, MEBA may not be required as a direct activator; rather, MEBA’s role may be to compete NF1 binding and thereby activate the MBP promoter. Cloning of the genes encoding MEBA proteins and identification of the NF1 isoforms expressed by oligodendrocytes will be required to test these hypotheses.

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\[2\] L. Wraetz and J. Kamholz, unpublished results.

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**Fig. 9. Models of potential MEBA and NF1 interactions.** In oligodendrocytes (OL, top diagram), MEBA may bind to the MBP promoter overlapping the NF1 site and compete for binding with NF1. Alternatively (middle diagram), MEBA may bind cooperatively with NF1, changing its composition or function, or NF1 in oligodendrocytes may differ and permit MEBA binding, leading in either case to activation. Finally, in Cos-7 cells (bottom diagram), either the protein components of MEBA are not present, or the composition of NF1 prevents MEBA binding, leading to repression. \(\tau\) signifies that the interactions between protein components of MEBA are unclear. IC, transcriptional initiation complex; TTCAAA, the TATA-like box.
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