A Developmentally Regulated DNA-Binding Protein from Mouse Brain Stimulates Myelin Basic Protein Gene Expression

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Transcription of the myelin basic protein (MBP) gene is regulated in a cell-type-specific and developmental stage-specific manner during myelin formation in the murine central nervous system. The 5'-flanking region of the MBP gene contains several regulatory elements that differentially contribute to the cell-type-specific transcription of MBP in cells derived from the central nervous system. The proximal element, termed MB1, which is located between nucleotides -14 and -50 with respect to the RNA start site, has previously been shown to have characteristics of a cell-type-specific enhancer element. In this study, we used band shift and UV cross-linking assays to identify DNA-binding proteins in mouse brain nuclear extract which interact with the MB1 element. Fractionation of these extracts has allowed the identification of a 38- to 41-kDa nuclear protein, derived from mouse brain tissue at the peak of myelination, which specifically binds the MB1 DNA sequence. Fractions enriched in the MB1-binding protein have been shown to stimulate transcription of the MBP promoter in extract derived from HeLa cells. MB1 binding protein activity is expressed in a tissue-specific and developmental stage-specific pattern which coincides with the pattern of MBP transcription, suggesting that this protein may be a biologically relevant transcription factor for the MBP gene *in vivo*.

A typical eukaryotic transcription unit contains an array of *cis*-acting elements positioned in close proximity to the transcription start site (nucleotide +1), including the TATA box and initiator sequences, a variety of upstream regulatory signals positioned within 50 to 400 bp of the start site, and enhancer elements that may be located at great distances upstream or downstream of the start site (28). A number of studies have suggested that DNA enhancer sequences, to a great extent, are responsible for temporal and tissue-specific gene expression (25). Evidently, enhancer elements exert their activities by interacting with *trans*-acting protein factors. In this respect, it is well documented that the immunoglobulin heavy-chain gene and the polyomavirus and simian virus 40 enhancers possess several active motifs, each interacting with distinct cell-specific factors (7, 12, 35, 45). Therefore, a gene can be selectively transcribed according to the relative abundance of these specific transcription factors and the particular arrangement of factor-binding sites present in its regulatory region.

Recent studies have indicated that transcription factors may participate in many developmental processes. Most of these proteins are capable of binding to DNA in a sequence-specific manner and are produced either in a variety of cell types during development, such as the Hox and Pax genes (9, 13), and/or in particular cells and tissues, such as the pit-1, GATA-1, and myoD genes (23, 34, 47). It has been estimated that more than 20,000 different genes are expressed in the mammalian nervous system, most of which are nerve specific and coordinately regulated during brain development (27). However, the regulatory cascades and the participant factors in central nervous system (CNS) development have not been elucidated in detail. To enhance our understanding of cell- and stage-specific gene regulation

during brain development, we have chosen the myelin basic protein (MBP) gene as a model system.

MBP is a major constituent of the myelin sheath, composing 30 to 40% of total myelin protein in the CNS (5). This protein has several isoforms, ranging in size from 14 to 21.5 kDa (8, 30), encoded by alternative splicing of a single major transcript from a single gene on mouse chromosome 18 (31). MBP is expressed in a cell-type-specific manner. In the CNS, myelin formation and MBP gene expression occur in oligodendrocytes, whereas in the peripheral nervous system, Schwann cells are responsible for myelin formation and expression of the MBP gene. The developmental expression of MBP has been demonstrated by Northern (RNA) blot (36) and *in situ* hybridization (21, 44) techniques. Evidently, MBP transcripts first appear in brain tissue at 8 days postbirth in the mouse, peak at 18 days, and progressively decrease to about 20% of the peak level in the mature animal.

Transient transfection and *in vitro* transcription studies have been used by us (11) and others (29, 39) to identify *cis*-acting regulatory elements which participate in cell-type-specific expression of the MBP gene. These results have indicated that the MBP regulatory region is composed of multiple *cis*-acting elements, some of which stimulate transcription preferentially in CNS-derived cell lines. The proximal element, termed MB1, spans the sequences from -14 to -50 bp upstream of the transcription start site and has been shown to direct cell-type-specific transcription of a heterologous promoter (10, 11). The sequences between -93 and -209 bp have been shown to contain multiple cell-type-specific elements (11) and a target sequence for the transcription factor NF1/CTF (17). The most distal element, termed MB7, spans the sequences from -249 to -287 bp and activates transcription in many cell types (11). Recent studies have also identified an element downstream from the transcription start site (+25 to +45 bp) which increases the

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transcriptional activity of the MBP gene in cell extracts derived from brain tissue (40). The finding of multiple elements in the MBP promoter/enhancer region suggests that a complex pattern of regulatory sequences may be required for MBP gene expression. However, the biological relevance of these elements *in vivo* has not yet been established.

Recently, we have shown that the sequence spanning the MB1 region functions as a cell-type-specific enhancer element by transient transfection of CNS-derived cell lines (10). *In vitro* transcription studies of the MBP promoter by others (42, 43) support the importance of this region in tissue-specific transcription. Because of its location with respect to the transcription start site, the MB1 element was initially thought to be involved in a direct interaction with basal transcription factors, e.g., TFIID. Sequence analysis of this region, however, indicates that the MB1 element does not contain a canonical TFIID binding site (TATAAA), and *in vitro* transcription studies suggest that its TATA-like motif (TTCAAA) may not participate in cell-type-specific transcription of the MBP promoter (43). To understand the mechanism by which the MB1 element exerts its activity on brain-specific transcription of the MBP gene, protein purification, DNA binding, and *in vitro* transcription assays were used. Here, we report the identification and partial purification of a 38- to 41-kDa MB1-binding protein, derived from mouse brain tissue at the peak of myelination, which binds specifically to the MB1 element and activates transcription of the MBP gene in cell extract derived from HeLa cells. Of particular interest, we note that this protein is expressed in a tissue-specific and developmental stage-specific pattern which coincides with the pattern of MBP transcription in the developing mouse.

MATERIALS AND METHODS

Plasmids and oligonucleotides. Plasmids pMB₄₀₂, pMB₂₄₆, pMB₁₆₄, and pMB₈₆ contain fragments of the mouse MBP upstream regulatory region spanning the sequences from -402, -246, -164, and -86 bp upstream of the transcription start site, respectively, to +21 bp downstream. These fragments were amplified from the mouse genomic clone Pex-1 (gift from Arthur Roach) by polymerase chain reaction using the following upstream primers: 5'-AAAGCTTCCATGTA TACCAAGCAAGCT-3' (pMB₄₀₂), 5'-AAAGCTTCAGGCC CACATTCATATCTC-3' (pMB₂₄₆), 5'-AAAGCTTGGTGA GAGACTCCAAGCAC-3' (pMB₁₆₄), and 5'-AAAGCTTCA ACTTGATCCGCCTCTTT-3' (pMB₈₆). The downstream primer for all constructs contains the following structural sequence: 5'-AGGTCCTGGGGTCTTCTTGATGGTC-3'. Amplimers were inserted into the *Hind*III-*Bam*HI site of the promoterless reporter plasmid pBLCAT₃ (24), immediately upstream of the chloramphenicol acetyltransferase coding region. The integrity of the polymerase chain reaction-generated inserts was confirmed by direct sequencing of the constructs. Plasmid pMB₁₃₂₀ was constructed by inserting a *Hind*III-*Acc*I fragment of the MBP promoter, derived from Pex-1 (spanning the region from -1320 to -396 relative to the transcription start site), into the *Hind*III-*Acc*I site of plasmid pMB₄₀₂.

The following oligonucleotides were used as probes in DNA binding assays: 5'-AGGGAGGACAACACCTTCAA AGACAGGCCCTCTGAG-3' (MB1) and 5'-GATCGATC GGGGCGGGGCGATC-3' (Sp1). These sense-strand oligonucleotides were hybridized to complementary antisense strands, and double-stranded DNA was isolated by preparative polyacrylamide gel electrophoresis (PAGE).

Nuclear extract preparation. Nuclear extracts were prepared by a modification of the method of Wildeman et al. (46). All procedures were carried out at 4°C. Briefly, fresh tissue was Dounce homogenized with 2 strokes of pestle B, washed with phosphate-buffered saline, and centrifuged for 5 min at 3,000 rpm. The tissue pellet was resuspended in 4 volumes of buffer A containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and incubated for 15 min on ice. The suspension was again Dounce homogenized with 10 to 20 strokes of pestle B and centrifuged for 30 min at 10,000 rpm. The pellet was resuspended in 4 volumes of buffer B containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 25% (vol/vol) glycerol and incubated for 15 min on ice. Following centrifugation for 30 min at 10,000 rpm, the supernatant was precipitated with (NH₄)₂SO₄ (0.33 g/ml). The pellet was resuspended in a minimal volume of buffer D containing 10 mM HEPES (pH 7.9), 100 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF, and 17% (vol/vol) glycerol and dialyzed against two changes, 50 volumes each, of the same buffer. The total protein concentration in the extract was typically 5 to 10 mg/ml, as measured by the method of Bradford (3). The integrity of the extracts was examined by sodium dodecyl sulfate (SDS)-PAGE.

Band shift assay and UV cross-linking. Oligonucleotide probes were prepared by end labeling of commercially synthesized oligonucleotides with [γ -³²P]ATP and T4 polynucleotide kinase (2). DNA-protein complexes were allowed to form during incubation of the protein sample with 20,000 to 100,000 cpm of probe DNA in a 30- μ l reaction mixture containing 1.5 μ g of poly(dI-dC), 12 mM HEPES (pH 7.9), 4 mM Tris (pH 7.5), 60 mM KCl, 5 mM KCl₂, and 0.8 mM DTT. The reaction mixture was incubated for 20 min on ice. For competition, the binding mixture was preincubated with unlabeled competitor oligonucleotides for 10 min on ice, and then the reaction was carried out as described above.

In band shift studies, the complexes were resolved by electrophoresis on a low-ionic-strength (0.5 $\times$ Tris-borate-EDTA) 9% native polyacrylamide gel. Gels were fixed in 10% methanol-10% acetic acid and dried on 3MM paper (Whatman), and complexes were detected by autoradiography. Band intensities were quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) scanning (19) of the dried gels.

In UV cross-linking analysis, the reaction mixture was spotted onto Parafilm and exposed to UV light (254 nm) for 30 min at 4°C. The complexes were resolved on a low-ionic-strength (0.5 $\times$ Tris-borate-EDTA) 9% native polyacrylamide gel and directly detected by autoradiography at 4°C. To analyze the molecular weight of each complex obtained in the band shift, gel slices corresponding to each complex were excised from the gel, weighed, and incubated in 2 $\times$ SDS sample buffer (1 μ l/mg) for 2 h at 37°C. Each sample was heated briefly to 95°C, and then the entire sample (gel slice and buffer) was loaded into the well of an SDS-10% polyacrylamide gel. Following electrophoresis (overnight at 60 V), gels were fixed in 40% methanol-10% acetic acid and dried on 3MM paper (Whatman), and complexes were detected by autoradiography.

Elution and renaturation from SDS-PAGE. Elution and renaturation of proteins from SDS-PAGE were performed by using a modification of the method of Hager and Burgess (14). Gel slices were crushed with a Teflon pestle, and

proteins were eluted with gentle shaking overnight at room temperature in 2.0 ml of elution buffer containing 50 mM Tris (pH 7.9), 0.1% SDS, 150 mM NaCl, 0.1 mM EDTA, 5 mM DTT, and 0.1 mg of bovine serum albumin (BSA) fraction V per ml. Gel pieces were pelleted by centrifugation at 4,000 rpm for 15 min. The supernatant was collected, and eluted proteins were precipitated with 8.0 ml of cold acetone (-20°C) on dry ice for 30 min. Precipitated proteins were collected by centrifugation at 10,000 rpm for 30 min; allowed to air dry for 10 min at room temperature; resuspended in 20 μl of dilution buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg of BSA fraction V per ml, and 20% (vol/vol) glycerol supplemented with 6 M guanidine HCl; and incubated for 30 min at room temperature; 1.0 ml of dilution buffer was then added, and the proteins were permitted to renature overnight at room temperature. Renatured proteins were stored at -80°C .

Chromatography. All chromatographic procedures were carried out at 4°C . Nuclear extract, prepared as described above from the brain tissue of 200 mice, 18 days postbirth, was applied to a 60-ml phosphocellulose (Whatman P11) column equilibrated with buffer D containing 10 mM HEPES (pH 7.9), 100 mM KCl, 1.5 mM MgCl_2 , 0.5 mM DTT, 0.5 mM PMSF, and 10% (vol/vol) glycerol. The column was washed with this buffer until the A_{280} of the eluate reached baseline. The column was eluted with a linear gradient of 0.1 to 0.8 M KCl in the same buffer. Fractions containing peak DNA-binding activity were pooled; dialyzed against buffer E containing 10 mM Tris (pH 7.5), 50 mM KCl, 5 mM MgCl_2 , 0.5 mM DTT, 0.5 mM PMSF, and 10% (vol/vol) glycerol; and applied to a 5-ml DEAE-Sephacel (Pharmacia) column equilibrated with the same buffer. The column was washed with this buffer until the A_{280} of the eluate reached baseline and then eluted with a linear gradient of 0.05 to 0.75 M KCl in buffer E. Peak fractions were pooled and assayed for DNA-binding activity by band shift assay and for protein concentration by the Bradford method (3).

In vitro transcription assay. Whole cell extract was prepared from HeLa cells grown in suspension culture in minimal essential medium (Quality Biochemical) supplemented with 5% horse serum as described by Manley et al. (26). Template DNA was prepared by *EcoRI* (pMB₁₃₂₀, pMB₄₀₂, pMB₂₄₆, pMB₁₆₄, and pMB₈₆) or *SphI* (pB_{JC}; described in reference 1) digestion to generate runoff transcripts of 298 (pMB₁₃₂₀, pMB₄₀₂, pMB₂₄₆, pMB₁₆₄, and pMB₈₆) or 450 (pB_{JC}) nucleotides. Transcription reactions in mixtures of 15 μl containing 0.1 μg (pMB₁₃₂₀, pMB₄₀₂, pMB₂₄₆, pMB₁₆₄, and pMB₈₆) or 0.2 μg (pB_{JC}) of template DNA, 6 μl of HeLa whole cell extract, 20 μCi of [α - ^{32}P]UTP (400 Ci/mmol; Amersham), 1.3 mM ATP, 1.3 mM CTP, 1.3 mM GTP, and 33 μg of poly(dI-dC) per ml were carried out in a buffer containing 20 mM HEPES (pH 7.9), 100 mM KCl, 12.5 mM MgCl_2 , 0.1 mM EDTA, 2 mM DTT, and 17% (vol/vol) glycerol. Runoff transcripts were analyzed on a denaturing 6% polyacrylamide gel and detected by autoradiography. Transcriptional activities were quantitated by PhosphorImager (Molecular Dynamics) scanning (19) of the dried gels and/or laser densitometry of autoradiograms.

Northern blot analysis. Total RNA was isolated from mouse tissue by the guanidine isothiocyanate method (6). RNA samples (20 μg) were separated on a 1% agarose-6.6% formaldehyde slab gel, transferred to nitrocellulose as described by Southern (37), and heated to 80°C for 2 h in vacuo. A rat MBP cDNA probe (gift from Jeffrey Green, Frederick Cancer Research and Development Center, Frederick, Md.), labeled by nick translation with [α - ^{32}P]dATP,

FIG. 1. (A to C) Developmental accumulation of MB1-binding activity. (A) Band shift analysis using MB1 probe DNA in the presence of 10 μg of mouse brain nuclear extract prepared from mice at the indicated stages of development. (B) Band shift analysis using Sp1 probe DNA in the presence of 10 μg of mouse brain nuclear extract prepared from mice at the indicated stages of development. (C) Northern blot analysis of mouse brain RNA probed with MBP cDNA. Total RNA (20 μg per lane) from mouse brain at the indicated stages of development was analyzed. (D and E) Tissue distribution of MB1-binding activities. (D) Band shift analysis using MB1 probe DNA in the presence of 10 μg of nuclear extract prepared from various tissues of mice at 18 days postbirth. (E) Northern blot analysis of total RNA (20 μg per lane) from the same tissues probed with MBP cDNA.

was hybridized at 65°C in $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)- $2\times$ Denhardt's solution-0.1% SDS-0.1 mg of salmon sperm DNA per ml and washed under high-stringency conditions ($0.1\times$ SSC-0.1% SDS).

RESULTS

Developmental and tissue-specific distribution of MB1-binding protein. Previously, we have demonstrated that mouse brain nuclear extract contains proteins which specifically bind to the sequences corresponding to the MB1 region of the MBP promoter (22). To examine the developmental distribution of MB1-binding proteins in mouse brain, band shift assays using double-stranded MB1 oligonucleotide DNA were used to probe nuclear extracts prepared from mouse brain tissue at different stages during development. In this study, we used 10 μg of nuclear extracts derived from mouse brain at 3, 7, 18, 30, and >60 (adult) days postbirth. These ages were chosen because MBP transcription starts at 8 to 10 days postbirth, peaks at 18 days, and decreases to 20% of peak levels in the adult (21, 36, 44). Three DNA protein complexes, designated A, B, and C, were identified in mouse brain nuclear extract (Fig. 1A). Apparently, the protein(s) associated with complex A is constitutively expressed during brain development, with approximately equal abundance at all ages. From the intensity of the band corresponding to complex B, it appears that the level of participant proteins in the B complex is low in the brain prior to the onset of myelination, i.e., 3 and 7 days postbirth, greatly increases in 18 days, and persists at a relatively high

FIG. 2. Specificity of the MB1-protein complexes and localization of the binding site. (A) Map of the 5'-flanking region of the MBP gene, showing the location and sequence of the MB1 element. Sequences of the MB1 deletion mutants are also shown. (B) Band shift analysis using MB1 probe DNA in the presence of 18-day mouse brain nuclear extract (4 µg per sample). Extract was preincubated with 10, 100, and 500 ng of competitor oligonucleotide DNAs prior to addition of the probe. Lanes: 1, probe DNA with no added proteins; 2, no competitor; 3 to 5, MB1 (homologous) competitor; 6 to 8, MB1A competitor; 9 to 11, MB1C competitor; 12 to 14, MB1K competitor.

level in older (30 days) and adult mice. Complex C was observed only in extract derived from brain tissue at or after the peak of myelination, i.e., from mice at 18 days postbirth or from older mice. These results indicate that proteins involved in the formation of complexes B and C are expressed during development in a pattern which correlates well with the developmental pattern of MBP transcription. These observations confirm our earlier results (22) suggesting that the MB1-binding proteins in the brain are developmentally expressed. Extracts were also assayed for Sp1, a sequence-specific DNA-binding protein and transcription factor (4) whose mRNA is expressed at relatively constant levels in the murine cerebrum throughout development (33). As expected, Sp1-binding activity remains constant throughout brain development (Fig. 1B). From these results, we conclude that enhanced MB1-binding activity during development does not result from a general developmental increase in transcription factor expression but rather is specific to MB1-binding activity. Figure 1C illustrates expression of MBP RNAs in mouse brain at various stages of development.

Since MBP transcription is restricted to nervous system tissue, we examined whether the MB1-binding proteins were likewise expressed only in this tissue. Therefore, a panel of nuclear extracts derived from the brain, heart, kidney, liver, and spleen of mice at 18 days postbirth was prepared and screened for the presence of MB1-binding proteins in a band shift assay. As shown in Fig. 1D, a complex comigrating with complex A was detected in all tissues. In heart and kidney extracts, a complex similar in electrophoretic mobility to the B complex of brain, but with a reduced intensity, was detected. Complex C was observed only in brain extract. Again, the B and C complexes were detected at greatest abundance in the 18-day brain extract. Bands with mobilities faster than that of complex A were seen in all tissues. These bands were determined to be nonspecific on the basis of competition with unlabeled oligonucleotides (data not shown). Figure 1E demonstrates tissue distribution of MBP transcripts that correlate with the abundance of the B and C complexes in various tissues.

Localization of the binding site within the MB1 element and

specificity of the DNA-protein complex. To critically identify the DNA sequences required for the formation of the three complexes, competition experiments were performed with various competitor DNAs derived from the MB1 region (Fig. 2A). The synthetic competitor DNAs were constructed such that they divide the MB1 element into three overlapping and nonoverlapping regions. As shown in Fig. 2B, MB1 and MB1A oligonucleotides effectively compete for the formation of all three complexes, whereas MB1C competes much less efficiently than do MB1 and MB1A. The MB1K oligonucleotide appears to compete only for the formation of the A complex. In summary, the affinity of complexes B and C for these DNAs can be described as MB1 = MB1A > MB1C >> MB1K. Complex A, however, binds efficiently to each of these DNAs. These observations suggest that the MB1A oligonucleotide contains the full binding site for complexes B and C, so that at least part of the binding site must be found between -14 and -28 bp. This region of the MB1 element has previously been shown to participate in brain-specific transcription of the MBP promoter in vitro (43), such that the binding site specificity of the B and C complexes is consistent with a role for these complexes in transcription of the MBP gene. Furthermore, complexes B and C bind specifically to the MB1 DNA sequence, since they can be competed for with homologous MB1 DNA but not with MB1K DNA. Complex A was competed for efficiently with all four competitor DNAs, suggesting that either the participant protein(s) binds the central region of the MB1 DNA, which is conserved in all four competitors, or the formation of this complex is not sequence specific.

UV cross-linking analysis of MB1 complexes. To determine the relative molecular weights of the A, B, and C complexes, UV cross-linking of the proteins with the specific target sequences was performed. In this study, the labeled MB1 fragment was incubated with 18-day-postbirth brain extract, cross-linked by UV irradiation, and resolved by SDS-PAGE. A major nucleoprotein band of 56 to 60 kDa and a minor band of 32 kDa were detected by autoradiography (Fig. 3, lane 1).

To more specifically identify the molecular weight of each of the complexes observed in the band shift assay, the

FIG. 3. UV cross-linking analysis of the MB1-binding proteins. Lanes: 1, 18-day mouse brain nuclear extract (10 μ g) probed with MB1 DNA in UV cross-linking analysis (the complexes are resolved by SDS-10% PAGE); 2, band shift analysis of 18-day mouse brain nuclear extract using MB1 DNA as the probe; 3 to 5, covalently cross-linked complexes (A, B, and C, respectively) isolated by native PAGE and separately analyzed by SDS-PAGE.

DNA-protein complexes A, B, and C were cross-linked and then resolved on a native polyacrylamide gel (Fig. 3, lane 2). Bands corresponding to each complex were excised, and their protein contents were separately analyzed by SDS-PAGE. Complex A migrated between 56 and 58 kDa (Fig. 3, lane 3). Complexes B and C both migrated as single broad bands of 56 to 60 kDa (Fig. 3, lanes 4 and 5). The results of this experiment demonstrate that all three complexes contain a DNA-binding protein which forms a DNA-protein complex that migrates with an apparent molecular mass of 56 to 60 kDa. Since the bands observed here correspond to

covalently linked DNA-protein complexes, it is important to note that the protein component of these complexes would be expected to have a molecular weight lower than that detected by this method.

Partial purification of the MB1 binding protein from mouse brain nuclear extract. To examine the biological activity of the MB1-binding protein and its role in transcription of the MBP promoter, the MB1-binding protein was partially purified by conventional column chromatography. To this end, nuclear extract was prepared from the brain of 18-day-postbirth mice and fractionated on P11 phosphocellulose. Proteins were collected from the column by a linear gradient of KCl from 100 to 800 mM (Fig. 4A). Band shift assay suggested that the peak of MB1-binding activity, consisting of the proteins that form complexes B and C, was eluted from 350 to 650 mM KCl (Fig. 4B). In this fractionation, proteins that constitute complex A were eluted at a lower salt concentration. Fractions containing the highest level of MB1 activity were pooled, and quantitative measurement of their binding activity revealed that the MB1-binding protein was purified approximately 10-fold.

The pooled fractions from the P11 column were enriched in the B and C complexes and depleted of the A complex. The pooled P11 fractions were subsequently fractionated by DEAE-Sephacel chromatography. The DEAE-Sephacel column was eluted with a linear gradient of 50 to 750 mM KCl, and peak MB1-binding activity was obtained in fractions between 250 and 500 mM KCl (Fig. 4C). This step allowed a further 2-fold purification of the MB1-binding activity, such that the activity was enriched about 20-fold relative to crude nuclear extract.

Transcriptional activity of the partially purified MB1-binding protein. To evaluate transcriptional activity of the partially purified MB1-binding protein, *in vitro* runoff transcription experiments using whole cell extract derived from HeLa cells were performed. Initially, basal activity of recombinant constructs containing different regions of the MBP promoter were examined. The template DNAs used in this study,

FIG. 4. Chromatographic fractionation of 18-day mouse brain nuclear extract. (A) Summary of the fractionation scheme. The nuclear extract was subjected to phosphocellulose (Whatman P11) chromatography, and the fractions were screened by band shift assay using MB1 DNA as the probe (B). Active fractions from the phosphocellulose column were pooled, fractionated on DEAE-Sephacel, and screened with the MB1 probe in a band shift assay (C).

FIG. 5. Transcriptional activity of the MBP promoter/enhancer in HeLa cell extract and stimulation of MBP transcription by partially purified MB1-binding protein. (A) Map of the MBP promoter/enhancer templates used in transcription reactions. Plasmids pMB₁₃₂₀, pMB₄₀₂, pMB₂₄₆, pMB₁₆₄, and pMB₈₆ contain fragments of the MBP regulatory region spanning from +21 nucleotides to -1320, -402, -246, -164, and -86 nucleotides, respectively. The *EcoRI* fragments shown generate runoff transcripts of 298 nucleotides. The arrowhead marks an endogenous end-labeled species in HeLa cell extract and serves as a control for sample recovery. (B) Template DNA (100 ng) was transcribed in vitro in HeLa cell extract. Lanes: 1, pMB₁₃₂₀; 2, pMB₄₀₂; 3, pMB₂₄₆; 4, pMB₁₆₄; 5, pMB₈₆. (C) pMB₁₃₂₀ (100 ng; top), pMB₁₆₄ (100 ng; middle), and pBJC (200 ng; bottom) template DNAs were transcribed in HeLa cell extract (lane 1) or were preincubated with 0.5 and 2.5 μl of pooled fractions following DEAE-Sephacel chromatography (lanes 2 and 3, respectively) before addition of HeLa cell extract. (D) Quantitation of the transcriptional activities shown in panel C. Transcript intensities were quantitated by PhosphorImager scanning and/or laser densitometry of autoradiograms and expressed as fold transactivation. Results are normalized for basal activity (lane 1) and expressed as means $\pm$ standard errors of the mean for four (pMB₁₃₂₀ and pMB₁₆₄) or three (pBJC) independent experiments.

pMB₁₃₂₀, pMB₄₀₂, pMB₂₄₆, pMB₁₆₄, and pMB₈₆ (Fig. 5A), were linearized with the endonuclease *EcoRI*, thus cleaving these DNAs 298 bp downstream from the major MBP RNA start site (20, 38). A major transcript 298 nucleotides in length is observed (Fig. 5B and C, 6C, and 7B), consistent with faithful initiation of transcription from the MBP promoter/enhancer in HeLa cell extract. As shown in Fig. 5B, comparison of transcription activities from pMB₁₃₂₀ and pMB₄₀₂ templates suggests that a *cis*-acting silencer element located upstream of nucleotide 402 down-regulates transcription of MBP in HeLa extract. Since HeLa cells do not express their endogenous MBP gene, this silencer element may contribute to the suppression of MBP gene expression in HeLa cells and most other cell types. On the other hand, the sequences spanning nucleotides 402 to 164 contain activator elements that increase transcription of MBP in HeLa extracts, and removal of these sequences substantially reduced the level of MBP RNA synthesis in vitro. These observations corroborate our previous findings suggesting that sequences in the upstream region of the MBP promoter contain enhancer elements with the capacity to augment activities of a heterologous promoter in transiently transfected nonbrain cells (11).

Two templates, pMB₁₃₂₀ and pMB₁₆₄, were used to examine transcriptional activity of the MB1-binding protein in HeLa extract. These MBP promoter/enhancer constructs were chosen for study because their low basal transcriptional activity would allow for sensitive detection of tran-

scriptional stimulation. Of particular interest is the pMB₁₃₂₀ construct, encompassing the entire 1.3-kb promoter/enhancer region, which would most likely interact with all other necessary factors, providing the proper molecular context for assessing the transcriptional activity of the MB1-binding protein. In this experiment, template DNAs were preincubated with the partially purified MB1-binding protein, obtained following DEAE-Sephacel chromatography, prior to addition of HeLa extract and the other transcription reagents. After preincubation for 10 min, standard transcription reactions were performed. The specific activity of the MB1-binding protein and HeLa extract in driving the MBP promoters was analyzed by autoradiography, and the intensity of the bands corresponding to the runoff transcripts was quantitated by PhosphorImager scanning (19) and/or laser densitometry. This study indicates that addition of the partially purified MB1-binding protein to the HeLa extract leads to 7.2-fold induction of the pMB₁₃₂₀ template and increases transcriptional activity of the pMB₁₆₄ template approximately 7.3-fold in a dose-dependent manner. In parallel, as a control for promoter specificity, transcriptional activity of the human neurotropic virus promoter JCV_E was examined under similar conditions. Supplementation of the HeLa extract with partially purified MB1-binding protein showed no stimulatory effect on the JCV_E promoter. In fact, we observed a minimal decrease (twofold) in the activity of the JCV_E promoter upon addition of the DEAE fraction. This control demonstrates that the ability of this fraction to

FIG. 6. Evidence that the MB1-binding protein stimulates transcription of the MBP promoter in HeLa cell extract. (A) Silver-stained SDS-polyacrylamide gel of the pooled fractions following each chromatographic step. Lanes: 1, molecular weight standard; 2, 18-day-postbirth mouse brain nuclear extract (5 µg); 3, phosphocellulose fraction (2 µg); 4, DEAE-Sephacel fraction (2 µg). (B) Gel slice fractionation of the partially purified MB1-binding protein following DEAE-Sephacel chromatography. Following elution and renaturation, protein derived from each gel slice was probed with MB1 DNA in a band shift assay. (C) pMB₁₃₂₀ (100 ng) was transcribed in HeLa cell extract (lanes 1 and 8) or was preincubated with 2.5 and 5 µl of protein derived from slice 8 (lanes 2 and 3), slice 9 (lanes 4 and 5) or slice 10 (lanes 6 and 7). (D) Transcript intensities quantitated by PhosphorImager scanning and expressed in arbitrary units. This result represents two independent experiments.

stimulate transcription may not be a general effect on all neurotropic promoters. These observations suggest that the partially purified protein following DEAE-Sephacel chromatography, enriched 20-fold in MB1-binding activity, has the capacity to selectively stimulate transcription of the MBP promoter/enhancer in vitro in HeLa cell extract.

The MB1-binding protein stimulates transcription of the MBP promoter in HeLa extract. To more precisely identify the MB1-binding protein and investigate its transcriptional activity, protein fractions obtained from DEAE-Sephacel were resolved by SDS-PAGE, and eluted bands were analyzed by band shift and in vitro supplementation experiments. Figure 6A demonstrates protein contents of crude nuclear extract and active fractions from phosphocellulose and DEAE-Sephacel chromatography. In parallel, to define the relative molecular weight of the MB1-binding protein, an SDS-polyacrylamide gel of the DEAE-Sephacel fraction was prepared. After resolution of the DEAE-Sephacel fraction, the gel was sliced into 14 sections, and the protein content of each slice was eluted. Following renaturation, the MB1-binding activity of each fraction was examined by band shift assay. Results shown in Fig. 6B demonstrate that proteins derived from slice 9, with electrophoretic mobilities between

38 and 41 kDa, contain binding activity to the MB1 oligonucleotide. Although the DEAE fraction contained both the B and C complexes, activity of only one complex, with an electrophoretic mobility similar to that of complex C, was restored during the renaturation process.

To examine the transcriptional activity of the gel-purified MB1-binding proteins, supplementation experiments using proteins derived from slices 8, 9, and 10 were performed. The ability of HeLa extract to transcribe the MBP promoter was substantially increased when proteins from slice 9, but not from slice 10, were included in the reaction mixture. A low but detectable level of enhancement was obtained upon addition of proteins from slice 8 to the HeLa extract as well, suggesting that this fraction may contain a low level of the activator protein, enough to exert its transcriptional activity but not sufficient to be detected by binding assay. The maximal stimulation of transcription with lower concentrations of renatured proteins suggests squelching of transcriptional activation when excess amounts of these factors are introduced into the system. These data suggest that a 38- to 41-kDa brain-specific DNA-binding protein, in concert with other basal transcription factors present in HeLa cells, increases transcription of the oligodendrocyte-specific MBP promoter in the nonbrain extract.

FIG. 7. Competition analysis confirming the role of the MB1-binding protein in stimulation of MBP transcription. (A) Band shift analysis using MB1 probe DNA in the presence of 30-day mouse brain nuclear extract (3 μ g per sample). Extract was preincubated with 10 and 50 μ g of competitor oligonucleotide DNAs prior to addition of probe. Lanes: 1, no competitor; 2 and 3, MB1A competitor; 4 and 5, MB1d1A competitor. Sequences of the MB1A and MB1d1A DNAs are shown at the bottom. Mutations in the MB1d1A sequences are in lowercase letters. (B) pMB₁₆₄ (100 ng) was transcribed in HeLa cell extract following preincubation with 2.5 μ l of partially purified MB1-binding protein (following phosphocellulose chromatography). Competitor oligonucleotides (10 or 100 ng) were added during preincubation. The arrowhead marks an endogenous end-labeled species in HeLa cell extract and serves as a control for sample recovery. Lanes: 1, no competitor; 2 and 3, MB1A competitor; 4 and 5, MB1d1A competitor. (C) Transcript intensities quantified by laser densitometry and expressed in arbitrary units.

Competition analysis confirms the role of the MB1-binding protein in transcriptional activation. To identify the critical nucleotides within the MB1 sequence which participate in binding of the MB1-binding protein, competition analysis using wild-type and mutant oligonucleotides was performed (Fig. 7A). The wild-type MB1A oligonucleotide completely abrogated formation of complexes with the MB1 DNA probe (see also Fig. 2), whereas the mutant MB1d1A oligonucleotide had no effect on the assembly of the MB1 complexes. Comparison of these sequences reveals that three base pairs are mutated in the MB1d1A sequence, thus identifying these as the base pairs required for binding of the MB1-binding protein to its target sequence.

The role of this protein in transcriptional activation of the MBP promoter/enhancer was examined in an *in vitro* supplementation/transcription assay, using these oligonucleotides to specifically compete for the MB1-binding protein. As shown in Fig. 7B and C, the MBP promoter is efficiently transcribed in HeLa cell extract supplemented with partially purified MB1-binding protein. This transcriptional activity is reduced with the addition of 100 ng of the wild-type MB1A oligonucleotide (approximately 200-fold molar excess) but not with the mutant MB1d1A oligonucleotide (Fig. 7B; compare lanes 3 and 5). These results suggest that the MB1-binding protein binds specifically to the nucleotide sequences within the MB1 region and activates transcription of the MBP promoter.

DISCUSSION

We have previously used Southwestern (DNA-protein) blot analysis to identify 37- and 39-kDa proteins derived from mouse brain tissue which bind the MB1A oligonucleotide (22). The abundance of these proteins varies developmentally, with the 39-kDa protein abundant in newborn (3-day) and young (18- to 30-day) mouse brain and the 37-kDa protein abundant in adult mouse brain. The binding site specificities and molecular weights of these proteins suggest that they may be related, if not identical, to the protein described here. We have also previously identified two distinct MB1-binding proteins present in extract of CNS-derived cell lines (10). However, these apparently differ from the brain-derived MB1-binding protein, since they have a different binding site specificity within the MB1 region. In contrast to the cell line-derived proteins, the brain-derived MB1-binding protein is a potential candidate for a functionally relevant MBP transcription factor as a result of its tissue-specific and developmental stage-specific accumulation.

UV cross-linking analysis has demonstrated that the developmentally regulated (complexes B and C) and constitutively expressed (complex A) MB1-binding factors contain proteins with similar molecular weights. These data raise the possibility that these complexes may result from tissue-specific and developmentally regulated modifications of a single protein. The ability of phosphorylation to modulate transcription factors has been well characterized (reviewed

in reference 16), and glycosylation of transcription factors has also been reported (18). However, the possibility remains that these proteins are unrelated and that their similarity in molecular weight is coincidental. The nature and extent of posttranslational modifications to these proteins are interesting questions which remain to be answered.

Transient transfection experiments have shown that the MB1 element has characteristics of a cell-type-specific enhancer, yet the location of this element relative to the transcriptional start site (−14 to −50 bp) and the presence of the TATA-like sequence motif TTCAAA within the enhancer suggest that the MB1 element may function to establish a basal level of transcription by interacting with general transcriptional machinery. Indeed, the fractionation scheme used here is similar, though not identical, to reported procedures for TFIID purification. Though the MB1-binding protein migrates in SDS-PAGE with an apparent molecular mass of 38 to 41 kDa, similar to that reported for mammalian TFIID (35 to 38 kDa) (15, 41), several lines of evidence argue against the possibility that this factor is identical to TFIID. Band shift analysis has shown that the MB1-binding protein binds the MBP promoter downstream of the TTCAAA motif. Likewise, Tamura et al. (42, 43) have shown by extensive mutational analysis that brain-specific transcription of the MBP proximal promoter is governed by a brain-specific TFIID-like factor which functions through sequences downstream from the dispensable TTCAAA motif. The binding site specificity of this factor suggests that it differs from previously characterized mouse TFIID. Furthermore, our results indicate that the MB1-binding protein stimulates transcription of the MBP promoter, but not a neurotropic viral promoter (JCV_E), suggesting a more specific role for this protein in enhancing brain-specific promoter activity. Further analysis of this regulatory protein, including comparison with the well-characterized general transcription machinery (reviewed in reference 32), awaits purification of this factor to homogeneity and subsequent cDNA cloning and DNA sequence analysis. These proposed experiments, currently ongoing in our laboratory, should elucidate the role of the MB1-binding protein in transcription of the MBP gene.

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REFERENCES

- Ahmed, S., J. Rappaport, H. Tada, D. Kerr, and K. Khalili. 1990. A nuclear protein derived from brain cells stimulates transcription of the human neurotropic virus promoter, JCV_E in vitro. *J. Biol. Chem.* **265**:13899–13905.
- Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. Current protocols in molecular biology. John Wiley & Sons, New York.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Briggs, M. R., J. T. Kadonaga, P. Bells, and R. Tjian. 1986. Purification and biochemical characterization of the promoter-specific transcription factor, Sp1. *Science* **234**:47–52.
- Campagnoni, A. T., and W. B. Macklin. 1988. Cellular and molecular aspects of myelin protein gene expression. *Mol. Neurobiol.* **2**:41–89.
- Chirgwin, J. M., A. D. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299.
- Davison, B. L., J.-M. Egly, E. R. Mulvihill, and P. Chambon. 1983. Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences. *Nature (London)* **301**:680–686.
- deFerra, F., H. Engh, L. Hudson, J. Kamholz, C. Puckett, S. Molineaux, and R. A. Lazzarrini. 1985. Alternative splicing accounts for the four forms of myelin basic protein. *Cell* **43**:721–727.
- Deschamps, J., and F. Meijlink. 1992. Mammalian homeobox genes in normal development and neoplasia. *Crit. Rev. Oncogenesis* **3**:117–173.
- Devine-Beach, K. A., S. Haas, and K. Khalili. 1992. Analysis of the proximal transcriptional element of the myelin basic protein gene. *Nucleic Acids Res.* **20**:545–550.
- Devine-Beach, K. A., M. S. Lashgari, and K. Khalili. 1990. Myelin basic protein gene transcription: identification of proximal and distal cis-acting regulatory elements. *J. Biol. Chem.* **265**:13830–13835.
- Falkner, F. G., and H. G. Zachau. 1984. Correct transcription of an immunoglobulin kappa gene requires an upstream fragment containing conserved sequence elements. *Nature (London)* **310**:71–74.
- Gruss, P., and C. Walther. 1992. Pax in development. *Cell* **69**:719–722.
- Hager, D. A., and R. R. Burgess. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of Escherichia coli RNA polymerase, wheat germ DNA, topoisomerase, and other enzymes. *Anal. Biochem.* **109**:76–86.
- Hoffman, A., E. Sinn, T. Yamamoto, J. Wang, A. Roy, M. Horikoshi, and R. Roeder. 1990. Highly conserved core domain and unique N-terminus with presumptive regulatory motifs in a human TATA factor (TFIID). *Nature (London)* **346**:387–390.
- Hunter, T., and M. Karin. 1992. The regulation of transcription by phosphorylation. *Cell* **70**:375–387.
- Inoue, T., T. Tamura, T. Furuichi, and K. Mikoshiba. 1990. Isolation of complementary DNAs encoding a cerebellum-enriched nuclear factor I family that activates transcription from the mouse myelin basic protein promoter. *J. Biol. Chem.* **265**:19065–19070.
- Jackson, S. P., and R. Tjian. 1989. Purification and analysis of RNA polymerase II transcription factors by using wheat germ agglutinin affinity chromatography. *Proc. Natl. Acad. Sci. USA* **86**:1781–1785.
- Johnson, R. F., S. C. Pickett, and D. L. Barber. 1990. Autoradiography using storage phosphor technology. *Electrophoresis* **11**:355–360.
- Kamholz, J., J. Toffenetti, and R. A. Lazzarrini. 1988. Organization and expression of the human myelin basic protein gene. *J. Neurosci. Res.* **21**:62–70.
- Kristensson, K., N. K. Zeller, M. E. Dubois-Dalcq, and R. A. Lazzarrini. 1986. Expression of myelin basic protein gene in the developing rat brain as revealed by in situ hybridization. *J. Histochem. Cytochem.* **34**:467–473.
- Lashgari, M. S., K. A. Devine-Beach, S. Haas, and K. Khalili. 1990. Nuclear proteins in mouse brain cells bind specifically to the myelin basic protein regulatory region. *J. Clin. Invest.* **86**:1671–1677.
- Li, L., and E. N. Olson. 1992. Regulation of muscle cell growth and differentiation by the myoD family of helix-loop-helix proteins. *Adv. Cancer Res.* **58**:95–119.
- Luckow, B., and G. Schultz. 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids*

- Res. 15:5490.
25. Maniatis, T., S. Goodbourn, and J. A. Fischer. 1987. Regulation of inducible and tissue-specific gene expression. *Science* 236: 1237-1244.
 26. Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gelfand. 1980. DNA-dependent transcription of adenovirus genes in a soluble whole cell extract. *Proc. Natl. Acad. Sci. USA* 77:3855-3859.
 27. Milner, R. J., and J. G. Sutcliffe. 1983. Gene expression in rat brain. *Nucleic Acids Res.* 11:5497-5520.
 28. Mitchell, P. J., and R. Tjian. 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding. *Science* 245:371-378.
 29. Miura, M., T. Tamura, A. Aoyama, and K. Mikoshiba. 1989. The promoter elements of the mouse myelin basic protein gene function efficiently in NG108-15 neuronal/glia cells. *Gene* 75: 31-38.
 30. Newman, S. L., K. Kitamura, and A. T. Campagnoni. 1987. Identification of a cDNA coding for a fifth form of myelin basic protein. *Proc. Natl. Acad. Sci. USA* 84:886-890.
 31. Roach, A., N. Takahashi, D. Pravtcheva, F. Ruddle, and L. Hood. 1985. Chromosome mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. *Cell* 42:149-155.
 32. Roeder, R. G. 1991. The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly. *Trends Biochem. Sci.* 16:402-408.
 33. Saffer, J. D., S. P. Jackson, and M. B. Annarella. 1991. Developmental expression of Sp1 in the mouse. *Mol. Cell. Biol.* 11:2189-2199.
 34. Sharp, Z. D., and Z. Cao. 1990. Regulation of cell type specific transcription and differentiation of the pituitary. *Bioessays* 12:80-85.
 35. Singh, H., R. Sen, D. Baltimore, and P. A. Sharp. 1986. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. *Nature (London)* 319:154-158.
 36. Sorg, B. A., M. M. Smith, and A. T. Campagnoni. 1987. Developmental expression of the myelin proteolipid protein and basic protein mRNAs in normal and dysmyelinating mutant mice. *J. Neurochem.* 49:1146-1154.
 37. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
 38. Takahashi, N., A. Roach, D. B. Teplow, S. B. Prusiner, and L. Hood. 1985. Cloning and characterization of the myelin basic protein gene from mouse: one gene can encode both 14 kD and 18.5 kD MBPs by alternate use of exons. *Cell* 42:139-148.
 39. Tamura, T., A. Aoyama, T. Inoue, M. Miura, H. Okano, and K. Mikoshiba. 1989. Tissue-specific in vitro transcription from the mouse myelin basic protein promoter. *Mol. Cell. Biol.* 9:3122-3126.
 40. Tamura, T., and K. Mikoshiba. 1991. Demonstration of a transcription element in vitro between the capping site and translation initiation site of the mouse myelin basic protein gene. *FEBS Lett.* 286:75-78.
 41. Tamura, T., K. Sumita, I. Fujino, A. Aoyama, M. Horikoshi, A. Hoffman, R. G. Roeder, M. Muramatsu, and K. Mikoshiba. 1991. Striking homology of the 'variable' N terminal as well as the 'conserved core' domains of mouse and human TATA-factors (TFIID). *Nucleic Acids Res.* 19:3861-3865.
 42. Tamura, T., K. Sumita, S. Hirose, and K. Mikoshiba. 1990. Core promoter of the mouse myelin basic protein gene governs brain-specific transcription in vitro. *EMBO J.* 9:3101-3108.
 43. Tamura, T., K. Sumita, and K. Mikoshiba. 1991. Sequences involved in brain-specific in vitro transcription from the core promoter of the mouse myelin basic protein gene. *Biochim. Biophys. Acta* 1129:83-86.
 44. Verity, A. N., and A. T. Campagnoni. 1988. Regional expression of myelin protein genes in the developing mouse brain: in situ hybridization studies. *J. Neurosci. Res.* 21:238-248.
 45. Wildeman, A., M. Zenke, C. Schatz, M. Wintzerith, R. Grundstrom, R. Mathes, K. Takahashi, and P. Chambon. 1986. Specific protein binding to the simian virus 40 enhancer in vitro. *Mol. Cell. Biol.* 6:2089-2105.
 46. Wildeman, A. G., P. Sassone-Corsi, T. Grundström, M. Zenke, and P. Chambon. 1984. Stimulation of in vitro transcription from the SV40 early promoter by the enhancer involves a specific trans-acting factor. *EMBO J.* 3:3129-3133.
 47. Zon, L. I., H. Youssoufian, C. Mather, H. F. Lodish, and S. H. Orkin. 1991. Activation of the erythropoietin receptor promoter by transcription factor GATA-1. *Proc. Natl. Acad. Sci. USA* 88:10638-10641.