

# A 39-kD DNA-binding Protein from Mouse Brain Stimulates Transcription of Myelin Basic Protein Gene in Oligodendrocytic Cells

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**Abstract.** The MB1 regulatory sequence of the myelin basic protein (MBP) gene spanning between nucleotides -14 to -50 with respect to the transcription start site is critical for cell type-specific transcription of the MBP gene, which encodes the major protein component of myelin sheath in cells derived from the central nervous system (CNS). This regulatory sequence has the ability to interact with a developmentally controlled DNA-binding protein from mouse brain that stimulates transcription of MBP promoter in an in vitro system (Haas, S., J. Gordon, and K. Khalili. 1993. *Mol. Cell Biol.* 13:3103–3112). Here, we report the purification of a 39-kD protein from mouse brain tissue at the peak of myelination and MBP production that binds to the MB1 regulatory motif. Following partial amino acid sequence analysis, we have identified a complementary DNA encoding a 39-kD DNA-binding protein called

*pur*  $\alpha$ . Expression of *pur*  $\alpha$  cDNA in the prokaryotic and eukaryotic cells resulted in the synthesis of a protein with characteristics similar to the purified brain-derived 39-kD protein in band shift competition assays. Cotransfection of the recombinant *pur*  $\alpha$  expressor plasmid with MBP promoter construct indicated that Pur  $\alpha$  stimulates transcription of the MBP promoter in oligodendrocytic cells, and that the nucleotide sequence required for binding of the 39-kD Pur  $\alpha$  to DNA within the MB1 region is crucial for this activity. Moreover, transient expression of *Pur*  $\alpha$  caused elevation in the level of endogenous MBP RNA in oligodendrocytic cells. Thus, Pur  $\alpha$ , a sequence-specific DNA-binding protein upon binding to MB1 regulatory region may play a significant role in determining the cell type-specific expression of MBP in brain.

**M**YELIN basic protein (MBP)<sup>1</sup> is a major component of the myelin sheath composing greater than 30% of the total myelin protein in the central nervous system (CNS) (6, for review see 5). This protein has several isoforms, all of which are encoded by alternative splicing of a single major transcript from a single gene on mouse chromosome 18 (7, 23, 33, 35, 36, 43). 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

(PNS), Schwann cells are responsible for myelin formation and expression of the MBP gene (31). The cell type and developmental expression of myelin basic protein has been demonstrated to be regulated at the level of transcription (6, 10, 47). Initial transgenic studies using a transgene that contained the entire MBP genomic locus including all introns and exons, as well as 4 kb of the 5' flanking sequence and 3 kb of the 3' flanking sequence revealed restricted expression of the transgene in brain with the proper developmental pattern (34). In subsequent studies, as little as 1.3 kb of the MBP 5' flanking sequence has been shown to direct specific and developmentally regulated expression of the MBP cDNA-coding sequence (24) or a heterologous reporter gene (12, 14, 28). In addition, transient transfection and in vitro transcription studies have been used by several laboratories including ours to identify regulatory sequences that may participate in the restricted expression of the MBP gene (8, 9, 29, 44). These studies have indicated that the MBP regulatory region is composed of multiple *cis*-acting elements, some of which stimulate transcription of the reporter gene in CNS-derived

This work represents equal contributions of the first two authors.

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1. *Abbreviations used in this paper:* CNS, central nervous system; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAG, myelin-associated glycoprotein; MBP, myelin-basic protein; NF-1, nuclear factor 1; PLP, proteolipid protein; PNS, peripheral nervous system; PVDF, polyvinylidene difluoride.

cells. The proximal element, termed MB1, spans the sequence from nucleotides -14 to -50 bp (relative to the RNA start site at +1 bp) and has been shown to direct glial cell type specific expression of a heterologous promoter. Due to its position directly upstream of the transcription start site, the MB1 element was initially thought to be involved in a direct interaction with TATA box binding protein and serve as a core promoter element for the assembly of the preinitiation complex. Sequence analysis of this region, however, indicated that the MB1 element lacks a typical TATA box, and further, *in vitro* transcription studies suggest that this TATA box-like sequence (TTCAA) is not responsible for tissue-specific transcription of the MBP proximal promoter (45). The distal regulatory element of the MBP promoter between nucleotides -93 to -209 contains multiple positive and negative regulatory elements including a binding site for the nuclear factor 1 (NF-1) family of transcription factors (21), and differentially regulates MBP promoter activities in glial and non-glial cells. Previous studies from our laboratory indicated that the proximal MB1 regulatory region has the ability to interact with a 38–41-kD protein from crude nuclear extract derived from mouse brain tissue and stimulates transcription of the MBP promoter in an *in vitro* system (18). Of particular interest was the observation that the 38–41-kD MB1-associated proteins are expressed in a tissue-specific and developmental stage-specific pattern that overlaps with the pattern of MBP transcription in the developing mouse.

In this study, we have purified a 39-kD, MB1-binding protein from mouse brain at the peak of myelination to homogeneity, identified a cDNA with the potential for encoding the 39-kD protein, and evaluated its ability to stimulate transcription of the exogenous and endogenous MBP promoter in oligodendrocytic cells.

## Materials and Methods

### Nuclear Extract Preparation

Mice were obtained from a commercial vendor (Jackson ImmunoResearch Labs., Inc., West Grove, PA) or from the Jefferson Cancer Institute transgenic facility (kindly provided by Dr. L. Siracusa, Jefferson Cancer Institute), and mouse brain tissue was stored at -80°C before use. Nuclear extracts were prepared from mouse brain tissue essentially by the method of Ahmed et al. (1). Omission of the final step, concentration of the protein sample by precipitation with ammonium sulfate (0.33 g/ml), was found to increase the yield of subsequent chromatography.

### Protein Purification

**Phosphocellulose Chromatography.** Phosphocellulose slurry, 200 ml, was freshly packed in a 2.5 × 15-cm column (Bio-Rad Labs., Richmond, CA) to give ~90 ml packed bed volume and equilibrated with five volumes (~500 ml) buffer D<sub>100</sub> containing 10 mM Hepes (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 100 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 10% glycerol, before loading. Nuclear extract was diluted with buffer D<sub>0</sub> (D<sub>100</sub> without KCl) to a final salt concentration of ≤100 mM, as determined by conductivity measurement, and loaded by gravity flow. The column was washed with buffer D<sub>100</sub> until OD<sub>260</sub> of the eluate reached baseline, and was eluted with a linear salt gradient of buffer D (400 ml total volume) containing 100–800 mM KCl. Fractions containing MB1-binding activity were identified by quantitative band shift assay.

**Preparation of DNA Affinity Gels and Chromatography.** DNA affinity supports were prepared essentially according to the method of Kadonaga and Tjian (22). Cyanogen bromide activated sepharose (Pharmacia), 1.5 g, was swelled in 20 ml 1 mM HCl and washed with 300 ml 1 mM HCl on a sin-

tered glass funnel. The gel was immediately resuspended in 7.5 ml coupling buffer (0.1 M Na-phosphate, pH 8.0; 0.5 M NaCl) containing the DNA ligands and incubated overnight at room temperature with gentle rocking. Single-stranded oligodeoxynucleotides were purified by preparative gel electrophoresis before coupling. Following coupling, excess reactive groups in the gel were blocked by incubation of the gel in 1.0 M ethanolamine (pH 8.3) for 6 h at room temperature with gentle rocking. Uncoupled DNA was removed from the gel by washing with three cycles of buffer E1 containing 0.1 M HOAc (pH 4.0) and 0.5 M NaCl, and buffer E2 containing 0.1 M Na-phosphate (pH 8.3) and 0.5 M NaCl. The coupling efficiency was monitored by incorporation of radioactivity into the gel, and was typically 10–30% for double-stranded DNA and 1–3% for single-stranded DNA.

DNA affinity gels were packed in a 1.5 × 5-cm column (Bio Rad) resulting in a packed bed volume of 4–5 ml. DNA affinity columns were washed with two cycles of buffer E1 and E2 before each use, then equilibrated with 10 bed volumes (~50 ml) of buffer X<sub>150</sub> containing 12 mM Hepes (pH 7.9), 5 mM MgCl<sub>2</sub>, 150 mM KCl, 0.5 mM DTT, and 10% glycerol before loading. Protein samples were diluted with buffer X<sub>0</sub> (X<sub>150</sub> without KCl) to a final salt concentration of ≤150 mM, as determined by conductivity measurement, and loaded by gravity flow. The column was washed with excessive (at least 40 ml) buffer X<sub>150</sub> until the OD<sub>260</sub> of the eluate reached baseline, and proteins were eluted by washing the column sequentially with 20 ml buffer X<sub>500</sub> (X<sub>150</sub> except containing 0.5 M KCl) then 20 ml buffer X<sub>1000</sub> (X<sub>150</sub> except containing 1.0 M KCl). Fractions containing MB1-binding activity were identified by quantitative band shift assay. DNA affinity gels were reused as many as 30 times with no apparent loss of their binding capacity.

For concentrating the active fractions, pooled fractions were dialyzed overnight against three changes, 50 volumes each, of a buffer containing 5 mM Hepes (pH 7.9), 10 mM NaCl, 1 mM MgCl<sub>2</sub>, and 0.5 mM DTT. Dialysis served to remove salt and glycerol from column fractions following chromatography. Samples were concentrated by centrifugation under vacuum (Speedvac) to 20% of their original volume. Concentration to lesser volumes resulted in increased sample viscosity and poor electrophoretic mobility of samples, presumably due to incomplete dialysis before concentration.

For amino acid analysis, second pass affinity-purified MB1-binding protein, 25 pmol as estimated by quantitative gel shift assay, was concentrated, resolved on SDS-10% PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore Corp., Bedford, MA) in a buffer containing 25 mM Tris (pH 7.8), 192 mM glycine and 15% methanol according to the manufacturer's instructions. Following transfer, the blot was stained for 15 min in 45% methanol/10% HOAc/0.1% Coomassie brilliant blue, and destained twice, 10 min each, in 45% methanol/7% HOAc. Bands corresponding to the 37- and 39-kD proteins were excised from the blot, washed five times in HPLC-grade water (1 ml per wash) with extensive vortexing, and dried completely under vacuum (Speedvac). Samples were sent to Dr. W. Lane (Harvard Microchem, Cambridge, MA) for quantitation by amino acid analysis. Amino acid sequencing of the 39-kD MB1-binding protein was done according to the procedure described previously (20).

### Quantitative Band Shift Assay

Band shift assays were performed essentially as previously described (18) except that poly dI-dC was omitted to obtain maximal binding. Specific activity of the single-stranded MB1 end-labeled probe was determined by precipitation with trichloroacetic acid (2) and was generally 0.5–2.0 × 10<sup>6</sup> cpm/μg. Complexes were detected by autoradiography of the dried gels, and the bands were excised from the gels and quantitated by liquid scintillation counting. The amount of protein present in each band was calculated based on the assumption of 1:1 stoichiometry of protein/DNA in the complex.

### Prokaryotic Expression of *pur α*

Plasmid pMAL-*pur α* was generated by insertion of 1.14-kb EcoRI restriction fragment of *pur α* cDNA into the EcoRI site of pMAL-cRI (New England Biolabs, Boston, MA). *E. coli* containing pMAL-*pur α* or pMAL-cRI control plasmid were grown usually in a 500-ml culture, total bacterial proteins were collected and fusion proteins were purified by amylose affinity chromatography according to the manufacturer's instructions (New England Biolabs). For band shift assay, ~2 × 10<sup>4</sup> cpm of the <sup>32</sup>P-end-labeled single-stranded MB1 was incubated with 5 μg of maltose-

binding protein-Pur  $\alpha$  fusion protein according to the procedure described previously (18), and the complexes were analyzed on a native 9% PAGE.

### Eukaryotic Expression of pur $\alpha$

Pur  $\alpha$  cDNA (1.14-kb EcoRI fragment) was placed downstream of the CMV promoter in the pcDNA<sub>3</sub> expression vector (Stratagene Inc., La Jolla, CA). For pur  $\alpha$  expression in eukaryotic cells, 30  $\mu$ g of the plasmid DNA was introduced into the N20.1, an oligodendrocytic cell line conditionally immortalized with a retroviral vector bearing a thermolabile SV-40 T-antigen (46). This cell line expresses MBP and PLP messages in addition to the early oligodendrocyte marker 2',3'-cyclic nucleotide 3'-phosphodiesterase mRNA. 48 h after transfection, cells were harvested and nuclear proteins were prepared and used in band shift and competition experiments by the procedure described previously (18).

### Site-directed Mutagenesis

The DNA fragment encompassing nucleotide -86 relative to the transcription start site to +21 bp downstream of the transcription initiation site of mouse MBP that was cloned in the M13 phage DNA was used for site-directed mutagenesis in the MB1 region. The mutation was designed to reduce the binding of the 39-kD protein within the MB1 region (see Fig. 1 C, oligo MB1A\*). The detail strategy for site-directed mutagenesis, and selection of the mutants by growing the transformed cells in uridine containing medium is described previously (2). Once the mutants were identified, double-stranded phage DNA containing the mutations in the 39-kD binding site was isolated and transferred to the eukaryotic expression vector containing the chloramphenicol acetyl transferase (CAT) reporter gene. The integrity of the final construct was checked by direct sequencing before its utilization in transfection studies.

### Transient Transfection and CAT Assay

Transfections were performed by the calcium phosphate precipitation methods (15) using the previously characterized oligodendrocytic cells N20.1 (46). The salient feature of these cells is in their ability to produce MBP. After transfection, cells were maintained at 39°C to suppress expression of SV-40 T-antigen, and after 3 h, cells were harvested and CAT assay was carried out by the method described previously (13).

### Northern Blot Analysis

Total RNA from pCMV-pur  $\alpha$  transfected cells was prepared by hot phenol method (2), and after DNase I digestion to remove the endogenous DNAs in the samples, equal amounts of total RNAs (20  $\mu$ g) from various samples were analyzed by Northern blot procedure (2) using cDNAs from mouse MBP (gift of Dr. Jeffrey Green, Frederick Research & Development Center, Frederick, MD) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as probes.

## Results

### Nuclear Proteins from Mouse Brain Recognize Single-stranded MB1 DNA

Results from our previous studies have led to the identification of a nuclear protein from mouse brain that, upon interaction with the MB1 regulatory sequence of MBP, stimulates transcription of MBP promoter in an in vitro system (18). To better understand the requirements for interaction of the MB1-binding protein to its target DNAs, mouse brain nuclear extract was probed with both double- and single-stranded MB1 oligonucleotides in a band-shift assay. As shown in Fig. 1, a major B-complex was detected with the double-stranded MB1 probe (A and B, lane 1). As described earlier, two other minor bands, A and C, were detected upon prolonged exposure of the autoradiogram. The single-stranded noncoding MB1 probe bound to the nuclear proteins and formed complexes with similar electrophoretic mobilities as A, B, and C (Fig. 1 A, lane 2).

The coding strand of the MB1 showed an intermediate binding ability to the nuclear proteins (Fig. 1 B, lane 2). Competition analysis demonstrated that the participant nuclear proteins bound more tightly to the single-stranded than to the double-stranded MB1 element, since the single-stranded DNA-protein complexes could be effectively competed with homologous single-stranded DNAs, but not as efficiently with double-stranded MB1 DNA (Fig. 1 A, compare lanes 3 and 4 with 7 and 8 and B, compare lanes 3 and 4 with 5 and 6). Addition of single-stranded competitor that was complementary to the probe DNA resulted in reconstitution of double-stranded probe DNA and abolishment of the complexes (Fig. 1 A, lanes 5 and 6 and B, lanes 7 and 8). Binding of the nuclear proteins to the noncoding strand of the MB1 element is in a sequence-specific manner, since the activity could be competed with homologous noncoding MB1 DNA, but not with an unrelated, single-stranded oligonucleotide (Fig. 1 A, compare lanes 7 and 8 with 9 and 10). Conversely, binding to the coding strand appeared nonspecific, since equivalent competition was observed with the homologous coding strand DNA and an unrelated competitor (Fig. 1 B, compare lanes 5 and 6 with 9 and 10).

In spite of their similar electrophoretic mobilities in the band-shift assay (shown in Fig. 1, A and B, lanes 1 and 2), one could argue that the single-stranded and double-stranded MB1-binding proteins may not be identical. However, further competition studies indicated that the formation of the A, B, and C complex requires the same sequence specificity for the single-stranded DNA as was previously described for the developmentally regulated nuclear proteins in an assay using double-stranded MB1 probe DNA (18), supporting the notion that the participant single- and double-stranded proteins are identical. As shown in Fig. 1 D, the three complexes binding to the MB1 noncoding strand (Fig. 1 C, bottom strand) can be efficiently competed with noncoding strands from MB1, MB1A, less efficiently with MB1A\*, but not with MB1E competitors. These observations demonstrate that the binding of the nuclear proteins to MB1 DNA is sequence specific, and has the target nucleotides within the MB1A oligonucleotide and that binding can be affected, at least partially, by three point mutations present in the MB1A\*.

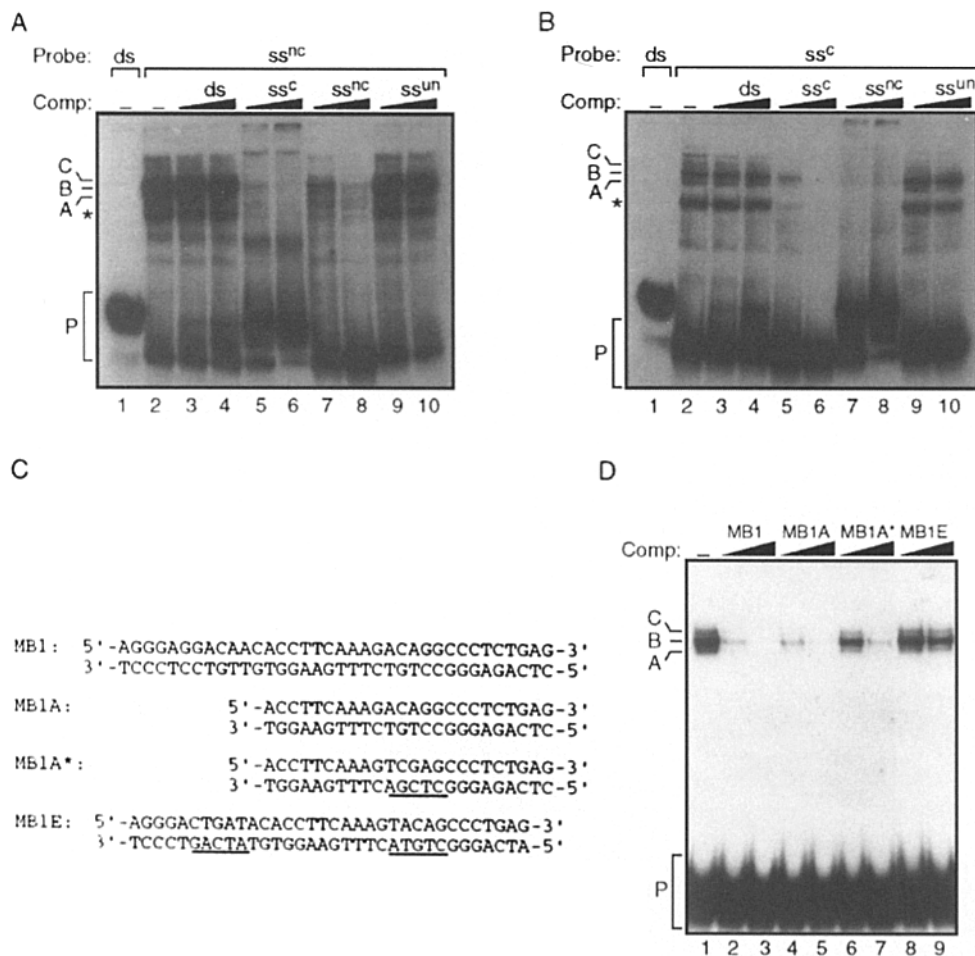
### Purification of the Brain-derived MB1-binding Protein

The purification procedure for the MB1-associated proteins compromised a combination of phosphocellulose and DNA affinity chromatography. For preparation of the starting material, brains were collected from mice aged 18–60 d and protein extracts were prepared by the procedure described previously (1). Nuclear proteins (5 mg/ml bed volume) were applied to phosphocellulose resin in 50 mM KCl, and the column was eluted by a linear gradient of 100–700 mM KCl. Throughout the fractionation procedure, the activity was followed by using quantitative band-shift assay with MB1 single-stranded noncoding MB1 probe. The peak of the specific DNA-binding activity that assembled in the B-complex was eluted at 350–500 mM KCl (Fig. 2 A, lanes 13–18). This chromatographic step resulted in fourfold purification of the MB1-associated complex (shown in Table I), and separation of these proteins

from other DNA-binding activities present in the extract. In addition to the fourfold enhancement of specific activity, phosphocellulose chromatography also removed lipids and fine particles from the extract. Purification of the MB1-associated proteins to homogeneity was performed using DNA affinity chromatography. Toward this end, an oligonucleotide corresponding to the noncoding strand of the MB1 was covalently linked to sepharose beads. The active protein fractions from phosphocellulose were pooled and applied to the affinity column, and eluted with buffer containing 0.15, 0.5, and 1.0 M KCl. All three complexes, A, B, and C, were eluted in the 0.5 M KCl wash, however, a small amount of the slowest migrating complex (C-complex) was retained at 0.5 M KCl and eluted at 1.0 M KCl (Fig. 2 B). Two rounds of affinity chromatography led to 210-fold purification of the phosphocellulose fraction and 842-fold purification relative to nuclear extract (Table I). Through this procedure, 1,094 pmol (~44 µg) of purified MB1-binding protein was obtained that corresponds

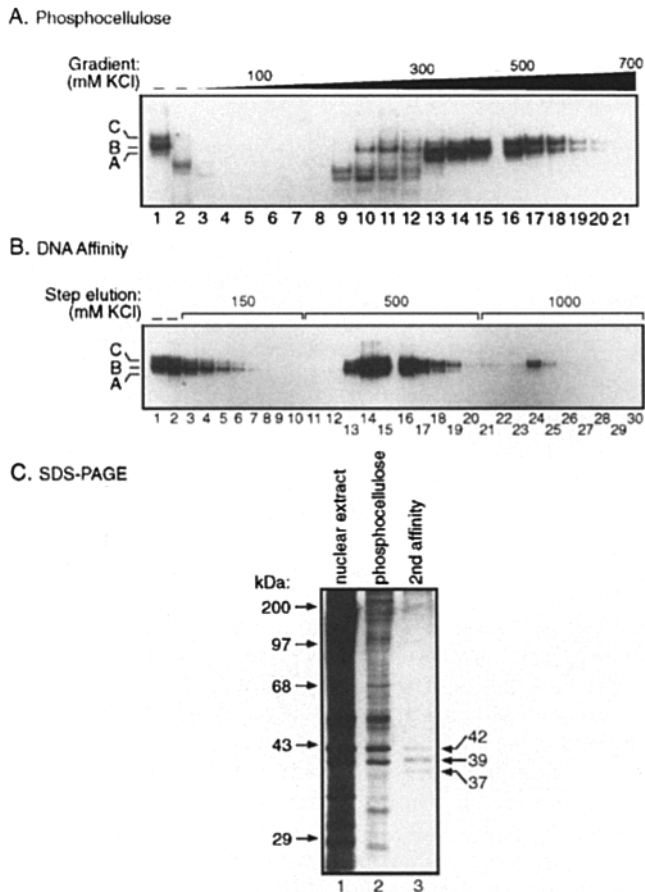
to a 9% total yield (Table I). The highly purified protein samples were visualized with apparent molecular masses of 37, 39, and 42 kD (Fig. 2 C).

For amino acid analysis, the more abundant protein, the 39-kD species, was digested with trypsin and the cleavage products were isolated by HPLC. Two peptides were sequenced and the results indicated a perfect match with predicted amino acid sequence of human *pur α* gene (Fig. 3). The *Pur α* is a sequence-specific single-stranded DNA-binding protein that has a high affinity for the purine-rich motif (GGN)<sub>n</sub> (3, 4). The *pur α* sequence predicts a protein of 322 amino acids in size, which is in good agreement with the 39-kD apparent molecular mass of the MB1-binding protein, indicating that the purified 39-kD MB1-binding protein is likely the mouse homologue of human *Pur α*. More recently, sequence analysis of mouse *Pur α* protein indicated extraordinary high sequence conservation between mouse and human *pur α* genes (27). In fact, only two amino acid alterations between the human protein



**Figure 1.** Single-stranded DNA-binding activity of the MB1-binding protein MEF-1. (A) Binding to the noncoding strand of the MB1 element. Band shift analysis using end-labeled double-stranded (lane 1) or single-stranded noncoding strand (lanes 2–10) MB1 DNA in the presence of 10 µg mouse brain nuclear extract. For competition analysis, binding reactions were incubated with competitor DNA before the addition of probe. Competitors were double-stranded MB1 DNA (lane 3, 10 µg; lane 4, 50 µg), single-stranded coding strand MB1 DNA (lane 5, 10 µg; lane 6, 50 µg), single-stranded noncoding strand MB1 DNA (lane 7, 10 µg; lane 8, 50 µg), and unrelated single-stranded DNA derived from the CAT gene coding region (lane 9, 10 µg; lane 10, 50 µg). A, B, and C indicate the complexes corresponding to the MB1-binding protein MEF-1, and P indicates the position of unbound single- and double-stranded MB1 DNA probe. (B) Binding to the coding strand of the MB1 element. Identical to A with

the exception that the single-stranded probe DNA (lanes 2–10) is derived from the coding strand of the MB1 DNA. (C) Sequence structure of MB1 and its sub-regions that were used in these studies. (D) Sequence specific binding of MEF-1 to single-stranded DNA corresponding to the noncoding strand of the MB1 element. Band shift analysis using end-labeled single-stranded noncoding strand MB1 DNA in the presence of 10 µg mouse brain nuclear extract. For competition analysis, binding reactions were incubated with competitor DNA before the addition of probe. All competitors were single-stranded DNAs derived from the noncoding strand of the MB1 element. Competitors were MB1 (lane 2, 10 µg; lane 3, 100 µg), MB1A (lane 4, 10 µg; lane 5, 100 µg) MB1dIA (lane 6, 10 µg; lane 7, 100 µg), MB1dIE (lane 8, 10 µg; lane 9, 100 µg). The letters, A, B, and C, indicate the complexes corresponding to the developmentally regulated MB1-binding protein MEF-1 (18) and the P indicates the position of unbound probe DNA.



**Figure 2.** Purification of MB1-binding protein. (A) Purification of MB1-binding protein by phosphocellulose chromatography of mouse brain nuclear extract. Phosphocellulose chromatography was performed as described in the text, with the sample loaded in a buffer containing 100 mM KCl and eluted with a linear salt gradient of the same buffer containing 100–800 mM KCl. Fractions (1- $\mu$ l aliquots) were analyzed by band shift assay using end-labeled single-stranded noncoding strand MB1 DNA ( $1-5 \times 10^5$  cpm) as a probe. An outline of the chromatographic procedure and the corresponding fraction numbers are shown above the gel. The letters, A, B, and C, indicate the complexes corresponding to the developmentally regulated MB1-binding protein MEF-1 (18), which appear to coelute as a broad peak between 350–500 mM KCl. Faster migrating complexes are also seen, which elute at 200–300 mM KCl. (B) Purification of MB1-binding protein by DNA affinity chromatography. DNA affinity chromatography was performed as described in the text, with pooled fractions from the phosphocellulose column loaded onto the MB1 (single-stranded, noncoding) affinity column in a buffer containing 150 mM KCl. The column was eluted with a step gradient of 0.5 M and 1.0 M KCl in the same buffer. Fractions (1- $\mu$ l aliquots) were analyzed by band shift assay, using end-labeled single-stranded noncoding strand MB1 DNA ( $1-5 \times 10^5$  cpm) as probe. An outline of the chromatographic procedure is shown above the gel, and the corresponding fraction numbers are shown below. The letters, A, B, and C, indicate the complexes corresponding to the developmentally regulated MB1-binding protein, MEF-1 (18), which appear to coelute with 0.5 M KCl. The complex with the slowest mobility seems to have a higher affinity for the column than the others, with a portion of this protein eluting only at the highest salt concentrations (1.0 M). (C) SDS-PAGE analysis of the purified fractions. Nuclear extract, 20  $\mu$ g (lane 1), phosphocellulose fraction, 7  $\mu$ g (lane 2) and two times affinity purified

and the mouse protein (321 amino acids) were identified. The structural organization of Pur  $\alpha$  revealed three repeats of a 23-amino acid motif (class I) separated by two repeats of a 26-amino acid motif (class II). As shown in Fig. 3, the cleavage products of the MB1-associated 39-kD protein exhibited perfect identity to the regions from class I and class II.

### Binding of Pur $\alpha$ to MB1 Regulatory Motif

To evaluate the ability of Pur  $\alpha$  to bind to the MB1 sequence, the protein was produced in prokaryotic cells using the pMAL-cR1 expression vector that permits synthesis of the protein as a fusion to maltose-binding protein. The binding ability of the Pur  $\alpha$  fusion protein was investigated by band shift assay utilizing single- and double-stranded MB1 oligonucleotide probes. As shown in Fig. 4 A, Pur  $\alpha$  showed strong binding activity to the noncoding strand of MB1 and exhibited a substantially reduced affinity to the coding strand and the double-stranded MB1 region (Fig. 4 A, compare lane 1 with lanes 3 and 4). The sequence specificity of this interaction was determined by competition analysis with unlabeled oligonucleotides derived from various regions of the noncoding strand of MB1. Results from these studies indicated that the unlabeled competitor corresponding to MB1A effectively blocked association of Pur  $\alpha$  with the probe (Fig. 4 B, lanes 1–3). Under similar conditions, unrelated single-stranded competitor exhibited no inhibitory action on the assembly of MB1/Pur  $\alpha$  complex (Fig. 4 B, lanes 4 and 5). These data demonstrate that prokaryotically produced Pur  $\alpha$  binds to the noncoding strand of the MB1 element in a sequence-specific manner.

Next, the DNA-binding activity of the cloned *pur*  $\alpha$  gene product that is expressed by a eukaryotic vector in oligodendrocytic cells was examined by band shift assay. In this study, N20.1 oligodendrocytic cells were transfected with a recombinant *pur*  $\alpha$  expression plasmid, pCMV-*pur*  $\alpha$ , and after 48 h nuclear extracts were prepared and used in the binding assays containing single- or double-stranded MB1 DNA probes. Results shown in Fig. 5 A indicated that the eukaryotically produced Pur  $\alpha$  prefers the noncoding strand of MB1 rather than the coding strand and the double-stranded DNAs. Fig. 5 B illustrates results from band shift analysis of nuclear extract from untransfected N20.1 cells with MB1 DNA probe. From the intensity of the band (shown in lane 2) it appears that N20.1 cells produce significantly low levels of Pur  $\alpha$ . Results from competition studies of nuclear extracts from cells overproducing Pur  $\alpha$  (upon transfection of pCMV-Pur  $\alpha$ ) (Fig. 5 C) indicated that binding of Pur  $\alpha$  produced by the transgene in oligodendrocytes to the MB1 DNA exhibits a similar sequence

MEF-1,  $\sim 0.5$   $\mu$ g as estimated by quantitative band shift assay (lane 3) were analyzed by SDS-PAGE and proteins were visualized by silver staining. Three bands, of 42, 39, and 37 kD apparent molecular mass are observed in the affinity-purified MEF-1 preparation. In other preparations, only the 39- and 37-kD bands were reproducibly observed, while the presence of the 42-kD species was variable.

**Table 1. Purification of MEF-1 by Column Chromatography Using Phosphocellulose and Single-Stranded MB1 (Noncoding Strand) DNA Affinity**

	Total protein	Total activity	Specific activity*	Fold purification	Total yield
	mg	u	u/mg		%
NE	482 <sup>‡</sup>	1.18 × 10 <sup>4</sup>	24	1	100
P11	52 <sup>‡</sup>	5.38 × 10 <sup>3</sup>	104	4	46
1st affinity	1.0	1.28 × 10 <sup>3</sup>	1.28 × 10 <sup>3</sup>	53	11
2nd affinity	55 × 10 <sup>-38</sup>	1.09 × 10 <sup>3</sup>	2.19 × 10 <sup>3</sup>	842	9

\*One unit of bonding activity is defined as the amount of protein that will bind one pmol of radiolabeled oligonucleotide in the quantitative band shift assay.

<sup>‡</sup>Total protein on the basis of Bradford assay.

<sup>§</sup>Total protein is estimated by SDS-PAGE and silver staining, by comparison with known quantities of marker proteins.

specificity to that observed for the 39-kD protein shown in Fig. 1. Of note is that our binding results indicate that nucleotides at juxtaposition of GGC of the MB1 play an important role in binding of Pur  $\alpha$  to DNA since alterations of these nucleotides in the MB1A\* decrease Pur  $\alpha$  binding to MB1 sequence. Thus, based on the molecular mass of the purified protein, amino acid data, and the requirements for binding to the DNA, we concluded that the 39-kD MB1-binding protein is closely related to the cloned pur  $\alpha$  gene product.

### Pur $\alpha$ Stimulates Transcription of the MBP Gene in Oligodendrocytes

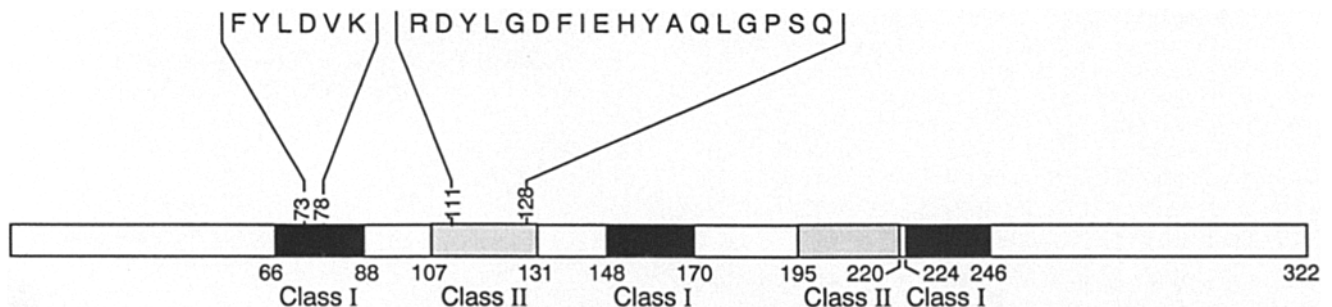
The capacity of the 39-kD Pur  $\alpha$  to modulate transcription of the MBP gene was evaluated by cotransient transfection of N20.1 oligodendrocytes with the reporter p86-CAT and pCMV-pur  $\alpha$ . The p86-CAT contains 86 bp upstream of the MBP transcription start site to +21 bp, downstream cloned at 5' position of the reporter CAT gene. As shown in Fig. 6 A, the basal transcriptional activity of the p86-CAT was increased in cells expressing moderate levels of Pur  $\alpha$  (compare lane 1 to lanes 2 and 3). At higher levels, however, Pur  $\alpha$  showed adverse effect, most likely due to the previously described squelching effect. Inclusion of the expresser plasmid containing pur  $\alpha$  in the antisense orientation showed no significant effect on MBP gene transcription (Fig. 6 A, lanes 6–9). The activation of MBP promoter by Pur  $\alpha$ , requires the intact binding site for the 39-kD protein in the MB1A region, since alteration of 5'-GCCTGTC-3' to 5'-GCTCGAC-3' as shown in MB1A\* in the reporter p86mut-CAT results in the loss of its responsiveness to Pur  $\alpha$  (Fig. 6 B). Again, antisense pur  $\alpha$  showed no

detectable effect on the transcriptional activity of the mutant promoter (Fig. 6 B, lanes 6–9). As shown in Fig. 6 C, optimum concentration of Pur  $\alpha$  increases transcription of the MBP promoter and that this induction requires intact Pur  $\alpha$  binding site within the MB1A promoter element.

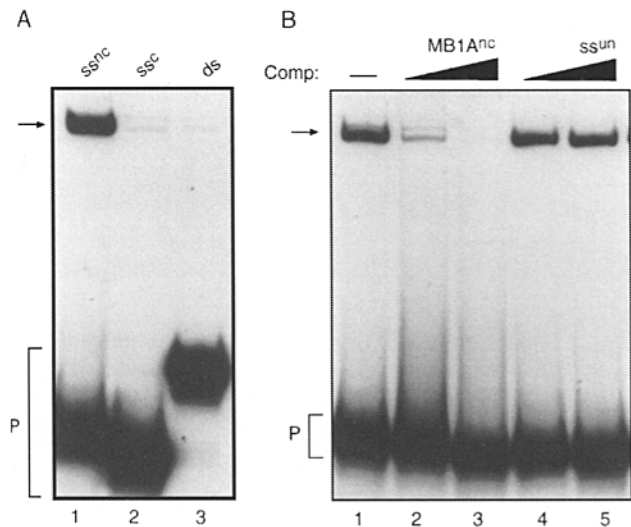
The effect of Pur  $\alpha$  on expression of the endogenous MBP gene was assessed by Northern blot analysis of RNA derived from N20.1 cells and cells transfected with the pur  $\alpha$  expresser plasmid. As shown in Fig. 7, transient expression of pur  $\alpha$  in this oligodendrocytic cell line resulted in elevated levels of MBP RNA in the cells (Fig. 7 A). Under similar conditions, the level of control GAPDH RNA remained constant (Fig. 7 B) and served as the baseline for the quantitative analysis shown in Fig. 7 C. Thus, the effect of Pur  $\alpha$  on MBP promoter activity is not limited to exogenously and transiently expressed messages. Rather by affecting endogenous MBP promoter activity, Pur  $\alpha$  appears to increase transcription of MBP gene in oligodendrocytes.

### Discussion

The myelin sheath is a specialized plasma membrane structure generated by the myelin-forming cells, oligodendrocytes in the central nervous system, and Schwann cells in the peripheral nervous system. Oligodendrocytes are small cells with many processes that in white matter of the brain participate in myelination and in gray matter surround the cell bodies of neurons. A single oligodendrocyte forms myelin sheath around many axons by wrapping its plasma membrane around the axons. The initiation and maintenance of myelin sheath formation follows a complex regulatory program that is tightly controlled during brain development.

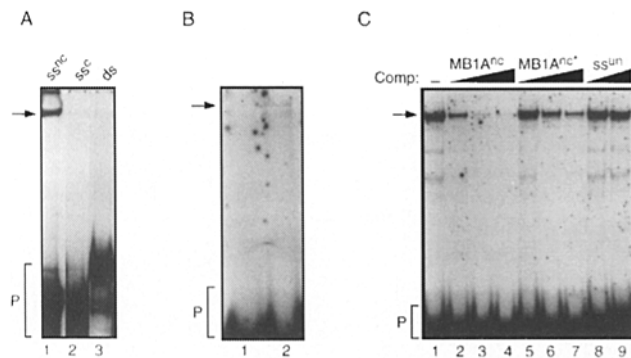


**Figure 3. Structural organization of the 39-kD Pur  $\alpha$  protein.** Peptide sequences from a HPLC fraction that contains 39-kD MB1-binding protein. The peptide sequences were compared with Pur  $\alpha$  (4). Sequence identity of the primary structure of the 39-kD-derived peptides and the regions within class I (residues 73–78) and class II (residues 111–128) of Pur  $\alpha$  are shown.



**Figure 4.** Binding of prokaryotically produced Pur  $\alpha$  to MB1 sequence. (A) 5'-end-labeled single-stranded and duplex oligonucleotides from MB1 region were analyzed for their ability to form complexes with maltose-binding protein (MalBP) fused to Pur  $\alpha$ . Lanes 1 and 2 present binding of the single-stranded probes from noncoding and coding strands of MB1, respectively, to the purified fusion protein, whereas, lane 3 shows binding of the double-stranded MB1 to the MalBP-Pur  $\alpha$ . The arrow shows the position of the complex and the P depicts the position of the unbound probe. (B) Specificity of the Pur  $\alpha$  binding to the noncoding MB1 sequence was tested by preincubation of the MalBP-Pur  $\alpha$  fusion protein with 10 and 100 ng of noncoding strand of MB1A (lanes 2 and 3), and 10 and 100 ng of an unrelated single-stranded DNA (lanes 4 and 5). The arrow and P show the positions of the complex and the free probe, respectively.

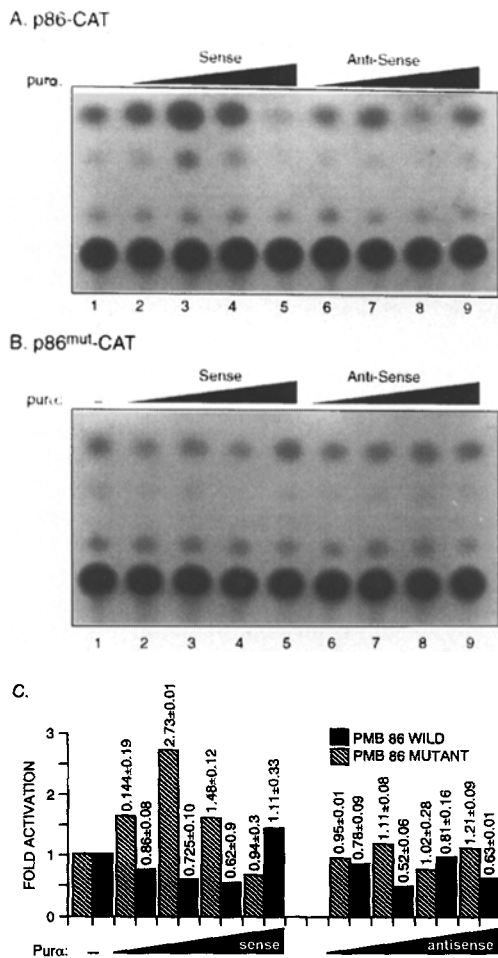
Expression of the proteins that comprise myelin sheath, including MBP perfectly overlaps with the programmed production of myelin sheath. The MBP control region that regulates the rate of MBP gene transcription during brain



**Figure 5.** Binding of eukaryotically produced Pur  $\alpha$  to MB1 sequence. (A) Oligodendrocytic cell line N20.1 (46) maintained at 39°C was transfected with pCMV-pur  $\alpha$  (30  $\mu$ g per 100-mm dish) and after 48 h nuclear extracts were prepared and used in binding assays using single-stranded (lanes 1 and 2) and double-stranded (lane 3) MB1 oligonucleotides. (B) Nuclear extracts from untransfected N20.1 cells were incubated with MB1A probe and the resulting nucleoprotein complexes were resolved by native PAGE. The arrow indicates association of endogenous nuclear protein (Pur  $\alpha$ ) with the probe. (C) Competition experiments using 10, 50, and 100 ng of the competitors from the noncoding strand of MB1A (lanes 2–4), MB1A\* (lanes 5–7), and 50 and 100

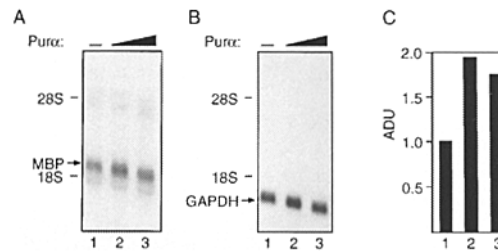
development contains multiple *cis*-acting elements that are responsible for its unique pattern of expression. It is postulated that similar to the other eukaryotic genes, interplay between the *cis*-acting elements of MBP genome and the regulatory proteins that are produced in the oligodendrocytes balances the level of transcription of MBP during myelinogenesis. It is also conceivable to speculate that the tight regulation of the MBP gene transcription is mediated by a combination of positive and negative regulatory mechanisms. In an earlier effort to identify the regulatory proteins that mediate developmental expression of MBP by binding to these regulatory sequences, we employed Southwestern blot and identified a sub-region within the MB1 proximal element of MBP called MB1A (nucleotides -14 to -37) that binds to 39 and 37-kD protein components in young adult brain cells (26). Through partial purification and *in vitro* transcription studies, we demonstrated that the MB1A-associated protein has the ability to increase transcription of the MBP promoter (18). Here we extended those observations and by using conventional and DNA affinity chromatography, we have purified a 39-kD DNA-binding protein that upon interaction with the specific sequences within the MB1 regulatory element up-modulates transcription of the MBP promoter in oligodendrocytes. This is an interesting observation in light of the recent discovery of a repressor protein, MyEF-2, which by binding to the MB1 sequence that partially overlaps with the 39-kD protein binding site, down-regulates MBP promoter activities. Of interest, unlike the 39-kD activator protein, which is more abundant only in brain during and after the period of active myelination (18), the suppressor MyEF-2 is produced in all mouse tissues, and efficiently reduces the MBP promoter activity in nonoligodendrocytic context (17, 41). These observations suggest that the MB1 element may function not only to stimulate brain-specific transcription, perhaps by binding to the activators such as Pur  $\alpha$ , but could also restrict transcription of the MBP gene in other cells by interacting with repressors, including MyEF-2. Thus, programmed expression of MBP via MB1 domain may be accomplished by complex interplay of the activator(s) and suppressor(s). Perhaps it should be noted that several DNA-binding proteins including SCIP (30), CTF-NF1 (21), and thyroid hormone receptor transcription factor (11) have been shown to regulate transcription of the MBP promoter. It is also likely that interaction of Pur  $\alpha$  and other transcription factors become important parameters in the overall transcriptional regulation of MBP genome in oligodendrocytes. The availability of the cDNA clones for the participant activators including, Pur  $\alpha$ , and the suppressor, MyEF-2, (41) and SCIP should enable us to design mechanistic studies that include parallel structural and functional analysis of MBP gene expression by these regulatory proteins in oligodendrocytic cells. Examination of the effect of these regulatory proteins on transcription of other myelin-

ng of unrelated single-stranded DNA (lanes 8–9). The arrow shows the position of the complex and P indicates the position of the unbound probe. The sequence of the oligonucleotide competitors and the single-stranded noncoding strand of MB1 are shown in Fig. 1 C.



**Figure 6.** Activation of MBP promoter by Pur  $\alpha$  in oligodendrocytic cells. Transient cotransfection analysis of Pur  $\alpha$  transcriptional activity on wild type MBP promoter containing -86 to +21 of the MBP sequence at 5' of the reporter CAT gene (A) or on its mutant variant that contained no binding site for Pur  $\alpha$  at the MB1 region (B). 2.5  $\mu$ g of the reporter plasmids were introduced into N20.1 cells alone (lane 1) or together with 0.5, 2.5, 5.0, and 10.0  $\mu$ g of pCMV-*pur*  $\alpha$  expresser plasmid (lanes 2-5), or with equal amounts of the pCMV construct containing *pur*  $\alpha$  in the antisense orientation by the Ca-phosphate precipitation method (15). After 48 h, extracts were prepared and CAT enzymatic activity was determined (13). C illustrates results of these studies that examine the effect of *pur*  $\alpha$ , the antisense *pur*  $\alpha$ , on transcription of the wild type and mutant MBP promoters. Quantitation of the CAT assays represent an average of multiple experiments.

associated genes including proteolipid protein (PLP), myelin-associated glycoprotein (MAG), etc. is also of great significance. Here we have reported identification of a single-stranded DNA-binding protein, Pur  $\alpha$ , which by binding to its target sequence enriched in purine, potentiates transcription of MBP promoter. It appears that Pur  $\alpha$  belongs to a growing family of single-stranded DNA-binding proteins that exhibit a distinct affinity to the structure and nucleotide composition of the DNA. Recent observations in our laboratory identified proteins with the capacity to bind single-stranded DNA that modulate transcription of the JC virus promoter (42). In addition, two well-characterized transcriptional factors, the myogenic determination



**Figure 7.** Transcriptional activation of MBP gene by Pur  $\alpha$  in oligodendrocytic cells.  $5 \times 10^5$  cells were transfected with 10  $\mu$ g (lane 2) and 30  $\mu$ g (lane 3) of pCMV-*pur*  $\alpha$ , and after 48 h total RNAs were isolated and after DNase I treatment, fractionated on formaldehyde gel, transferred to nitrocellulose and hybridized to MBP cDNA (A) or GAPDH (B) probes. Lane 1 illustrates RNAs from cells that were transfected with pCMV vector without the *pur*  $\alpha$  gene. (C) The intensity of the bands corresponding to MBP in the control and Pur  $\alpha$  producing cells was normalized to GAPDH and rated on an arbitrary scale of 0 to 2.0 adsorption unit (ADU).

factor, MyoD (38) and the estrogen receptor have been shown to bind single-stranded DNA (25, 32). At present, the biological significance of Pur  $\alpha$  binding to the noncoding strand of the MB1 motif remains unclear. It is possible that Pur  $\alpha$ , by binding to the single-stranded DNA, induces DNA binding and thus facilitates interaction of the upstream activators and the basal transcriptional complexes assembled at the initiation site of MBP transcription. This hypothesis is supported by the notion that several DNA-binding proteins, which stimulate transcription of eukaryotic promoters, have a unique ability for binding DNA molecules (16, 19, 37, 39, 40). Experiments are currently in progress in our laboratory to investigate the biological significance of Pur  $\alpha$  binding to the DNA at the level of transcription, DNA replication, and recombination.

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