The c-*myc* Promoter Binding Protein (MBP-1) and TBP Bind Simultaneously in the Minor Groove of the c-*myc* P2 Promoter†

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ABSTRACT: The c-*myc* promoter binding protein (MBP-1) is a DNA binding protein which negatively regulates the expression of the human c-*myc* gene. MBP-1 binds to a sequence which overlaps the binding site for the general transcription factor TBP, within the c-*myc* P2 promoter region. Since TBP binds in the minor groove, MBP-1 might inhibit c-*myc* transcription by preventing the formation of a functional preinitiation complex. In support of this hypothesis, we have demonstrated that MPB-1 is a minor groove binding protein. In order to characterize MBP-1 binding, we substituted A-T base pairs in the MBP-1 binding site with I-C base pairs, which changes the major groove surface without altering the minor groove surface. This substitution did not inhibit the sequence-specific binding of MBP-1 and TBP. On the other hand, G-C to I-C substitution within the MBP-1 binding site alters the minor groove and prevents MBP-1 binding. Competitive electrophoretic mobility shift assays were used to show that berenil, distamycin, and mithramycin, all of which bind in the minor groove, compete with MBP-1 for binding to the MPB-1 binding site. These minor groove binding ligands also effectively inhibit the simultaneous DNA binding activity of both MBP-1 and TBP. We conclude that both MBP-1 and TBP can bind simultaneously in the minor groove of the TATA motif on the c-*myc* P2 promoter. This suggests that MBP-1 may negatively regulate c-*myc* gene expression by preventing efficient transcription initiation.

The c-*myc* protooncogene plays an important role in the regulation of cellular proliferation and differentiation (Marcu *et al.*, 1992; Spencer & Groudine, 1991). The level of c-*myc* expression correlate with the rate of cell proliferation in most cell types, and induction of c-*myc* expression has been associated with the entry of cells into the cell cycle. Overexpression of the c-*myc* gene is common in the phenotypic abnormalities of many maglignant cell types (Cole, 1986); therefore, the mechanisms regulating c-*myc* expression are central to understanding the role of c-*myc* in cell transformation.

In mouse plasmacytomas and in human Burkitt's lymphoma cell lines, the translocated form of the c-*myc* gene is often the only allele which is transcribed, usually in a constitutive and elevated manner (Cory, 1986). This may be caused by the loss of a negative regulatory region during translocation. The explanation correlates with the observations of Grignani *et al.* (1990), who showed that c-*myc* is involved in an autoregulatory loop which is operative in primary and nontumorigenic cell lines but lost in transformed cell lines. They reported that this autoregulation occurs at the level of transcriptional initiation and involves a stable intermediate. This evidence further suggests that the negative regulation of c-*myc* expression may play a very important role in oncogenesis.

The human c-*myc* promoter binding protein, MBP-1, binds the c-*myc* P2 promoter and negatively regulates c-*myc* promoter function in cotransfection assays with *myc*−CAT constructs (Ray & Miller, 1991). Most studies have shown that the P1 and P2 promoters of the c-*myc* gene, which are separated by 160 bp, are subject to independent regulation (Broome *et al.*, 1987; Lipp *et al.*, 1989). MBP-1 is a 37 kDa protein and has been shown to bind in a region +123 to +153 bp relative to P1 on the c-*myc* P2 promoter. The binding site for MBP-1 on the c-*myc* P2 promoter region includes the transcription factor TBP binding site. These features suggest a possible mechanism by which MBP-1 might function and prompted us to compare the groove binding properties of MBP-1 to those of TBP.

The DNA binding antibiotic mithramycin binds as a dimer and spans 6 bp in the minor groove of G-C-rich sequences (Cons & Fox, 1989; Sarker & Chen, 1989; Sastry & Patel, 1993). Mithramycin has been shown to bind the c-*myc* promoter and block transcription initiation *in vitro* (Snyder *et al.*, 1991). One of the strongly protected regions within the c-*myc* promoter is from +110 bp to +128 bp relative to P1 promoter. The large region of the P2 promoter which binds mithramycin indicates that several drug molecules bind adjacent to one another. This region overlaps the first 5 bp of the MBP-1 binding site on the c-*myc* P2 promoter.

Distamycin, berenil, netropsin, and the Hoechst dye 33258 are nonintercalating minor groove binding ligands characterized by their B-DNA and A-T-specific interactions. Distamycin binding spans 5 bp in the minor groove (Coll *et al.*,

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1987; Ford & Rickwood, 1984) and causes changes in the width of the helical minor groove (Fox & Waring, 1984). X-ray analysis of the netropsin−DNA complex has shown that netropsin binds within the minor groove by displacing the water molecules of the spine of hydration, by similar interactions as in the distamycin−DNA complex (Kopka *et al.*, 1985). NMR studies of the DNA binding characteristics of berenil show large changes in the chemical shift for protons in the DNA minor groove (Jenkins *et al.*, 1993). X-ray crystallographic studies on a berenil−DNA complex by Brown *et al.* (1992) have shown that there is no significant alteration in the B-type DNA after berenil binding.

Several investigators have reported the use of minor groove binding ligands as competitors in DNA binding assays with DNA binding proteins for ascertaining their groove binding characteristics (Mattes, 1990; Grokhovskii *et al.*, 1989; Sidorva *et al.*, 1991). Dickinson *et al.* (1992) used distamycin as a competitor for DNA binding of a tissue-specific nuclear matrix/scaffold associating protein, SATB1, using drug−protein competition to study its minor groove recognition characteristics. Distamycin and netropsin as well as Hoechst 33258 effectively compete with HMG-I proteins for binding in the minor groove of DNA (Reeves & Nissen, 1990). Other examples that have been studied by this method include human DNA ligase (Ciarrocchi *et al.*, 1991), binding of polyamines in the minor groove of B-DNA (Schmid & Behr, 1991), sequence-specific synthetic DNA binding peptides which bind in the minor groove (Grokhovsky *et al.*, 1991), topoisomerase I−DNA interaction (Mortenson *et al.*, 1990), and minor groove recognition by histone H1 (Kas *et al.*, 1989).

Starr and Hawley (1991) used a novel approach to show that the general transcription factor TBP binds in the minor groove of the TATA box motif. An artificial TATA box sequence was used in which all of the adenine and thymine residues were replaced by inosines and cytosines. These substitutions alter the surface of the major groove but do not change the surface of the minor groove. The interaction of TBP with the sequence substituted from TATAAA to CICIII was the same as that of the wild-type sequence, confirming minor groove binding. This approach has also been used in studies to elucidate the minor groove binding of the HMG box-containing regulatory proteins: lymphoid enhancer factor 1 by Giese *et al.* (1992) and the high mobility group protein HMGI(Y) by Thanos and Maniatis (1992).

We have used this approch, as well as competition for DNA binding with minor groove binding ligands, to show that MBP-1, like TBP, binds in the minor groove of the c-*myc* P2 promoter. This result lends strong support to the hypothesis that negative regulation of the c-*myc* promoter by MBP-1 is mediated through disrupting or preventing the formation of the transcription initiation complex.

EXPERIMENTAL PROCEDURES

Probes. A 50 bp oligonucleotide (−52 to +2 relative to the P2 start site on the c-*myc* promoter) and its complement oligonucleotide were synthesized and purified by reverse-phase oligonucleotide purification/elution cartridges (Clontech). The oligonucleotide was radiolabeled using [γ-^{32}P]-dATP with T4 polynucleotide kinase (Gibco BRL). It was then annealed to its complement by heating at 90 °C for 10 min in 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and slow-cooling at room temperature to form a radiolabeled double-stranded probe.

The A:T to I:C substitution of the TATA motif was made by substituting TATAA for CICIII; 50 bp oligonucleotides were synthesized and purified in the same manner as the wild-type oligonucleotides. They were then used to make radiolabeled probe as described above, for electrophoretic mobility shift analysis. The mutant DNA fragment used as a competitor in the protein binding assays has been described previously (Ray & Miller, 1991). The G:C to I:C substitution was done at the GC bp −33 relative to the P2 start site, and the complementary 34 bp oligonucleotides with this sequence change were synthesized. The 34 bp oligonucleotides were purified and annealed as before to be used as a competitor in protein binding assays.

The NFkB binding site double-stranded oligonucleotide was purchased from Promega. End-labeling was performed as before, and the DNA fragment was purified by ethanol precipitation.

Minor Groove Binding Ligands. Distamycin A, berenil (diminiazine aceturate), and mithramycin were purchased from Sigma Chemical Co. These compounds were prepared in distilled water as 10^{-2} M stock solutions and stored at −20 °C.

Protein Extracts. The MBP-1 cDNA was cloned in both orientations in the prokaryotic expression vector pGemex-1 (Promega). The pGemex-MBP-1 clones and the pGemex plasmid alone were used to transform the JM109(DE3) *Escherichia coli* strain. Protein extracts were prepared from these transformed strains as follows. The induced bacterial extracts were partially purified by ammonium sulfate fractionation and precipitation. Cells were grown to an OD_{600} of 0.6 and induced with 0.4 mM IPTG for 3 h followed by lysis and fractionation as described by Pognonec *et al.* (1991). The extracts from the MBP-1 cDNA-containing strains had a total protein concentration of 1 μg/μL, and the extracts from the strains containing the plasmid alone had a total protein concentration of 0.5 μg/μL, as estimated by the colorometric Bradford assay (Bradford, 1976).

The bacterial extract containing recombinant TBP was prepared from the BL21(DE3) *E. coli* strain containing the TBP cDNA cloned in a pET expression vector (provided by Dr. R. G. Roeder). Purified TBP and NFkB proteins were purchased from Promega.

Binding Assays for MBP-1. The DNA−protein binding reactions were carried out in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 4% glycerol, and 5 μg of poly[d(I-C)] for 30 min at 37 °C. In some cases, the binding reactions were carried out in the same buffer conditions as for the TBP−DNA binding reactions (described below). Each reaction contained approximately 0.02 pmol of radiolabeled DNA and 1 μL of bacterial extract from the MBP-1 cDNA-transformed strains or 2 μL of bacterial extract from the cells transformed with plasmid alone, in a final volume of 10 or 20 μL. Unlabeled DNA competitor for protein binding was added (wherever mentioned) in 100-fold excess of labeled probe DNA. The protein−DNA binding reactions were analyzed by electrophoresis on a 6% nondenaturing polyacrylamide gel containing 89 mM Tris−borate and 2 mM EDTA.

In the MBP-1−DNA binding assays containing mithramycin, the reactions were performed as above, but with 6.5 mM $MgCl_2$ and at 37 °C for 20 min. The control protein

binding reactions for this set of assays were also done under identical conditions. The MBP-1−DNA binding reactions in the presence or absence of distamycin and berenil were carried out in 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 25 mM KCl, 1 mM DTT, 4% glycerol, and 5 μg of poly-[d(I-C)] at room temperature for 20 min with the same DNA and protein concentrations as above. All binding reactions were resolved on 6% nondenaturing polyacrylamide gels as described earlier.

Binding Assays for TBP and NFkB. The DNA binding assays for NFkB were carried out in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 4% glycerol, and 0.5 μg of poly[d(I-C)] at room temperature for 30 min; 0.02 pmol of radiolabeled DNA probe and 1 gsu of NFkB (Promega) were used for each binding reaction. The NFkB−DNA binding assays which were performed in the presence or absence of mithramycin were carried out in the same buffer with 6.5 mM $MgCl_2$ at 37 °C for 20 min. All reactions were analyzed by nondenaturing polyacrylamide gel electrophoresis as noted above.

The TBP−DNA binding assays, which were performed in the presence or absence of minor groove binding ligands, were carried out in 20 mM Tris-HCl (pH 7.9), 80 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, and 4% glycerol for 15 min at 37 °C (Promega); 0.02 pmol of radiolabeled DNA and 4 ng of TBP (Promega) were used per reaction. The TBP−DNA binding reactions were analyzed on a 6% nondenaturing polyacrylamide gel containing 2.5 mM $MgCl_2$, 0.05% NP-40, 89 mM Tris−borate, and 2 mM EDTA.

Simultaneous Protein Binding Assays. The simultaneous MBP-1 and TBP DNA binding assays, which were performed in the absence or presence of minor groove binding ligands, were carried out in the TBP−DNA binding reaction buffer. All reactions contained 0.02 pmol of radiolabeled DNA probe, 1 μL of bacterial extract containing recombinant MBP-1, and/or 4 ng of TBP (Promega) as before. These reactions were carried out at 37 °C for 20 min and analyzed by nondenaturing polyacrylamide gel electrophoresis as described for the TBP−DNA binding reactions.

RESULTS

Electrophoretic Mobility Shift Analysis. The MPB-1 binding site of the c-*myc* P2 promoter is a 34 bp region which overlaps the TBP binding site. This region was used as a probe in electrophoretic mobility shift assays to study the binding characteristics of MBP-1. Recombinant MBP-1-containing bacterial extracts from cells containing the pGemex-MBP-1 clone were able to specifically retard the electrophoretic mobility of the P2 promoter fragment as shown by the experiment in Figure 1. Induced bacterial extracts from cells which contained the MBP-1 cDNA clone in the negative orientation as well as induced extracts from cells which contained the parent pGemex plasmid did not cause a MBP-1-specific electrophoretic mobility shift (Figure 1, lanes 2, 5, and 7). The induced extract generates a specific and a nonspecific electrophoretic mobility shift when lower nonspecific DNA concentrations are used in the binding reaction. Increasing the concentration of nonspecific DNA as target DNA, in the binding reaction, competes with the nonspecific DNA binding activity in the protein extract but does not affect the specific DNA binding activity by recombinant MBP-1 (data not shown and Figure 6, lanes 2 and 3). Specificity of binding was also determined by competing the MBP-1-specific binding with excess unlabeled DNA fragment, whereas an excess of a mutated unlabeled DNA fragment did not compete for binding (data not shown and Figure 3). The P2 promoter fragment used above was also used in electrophoretic mobility shift assays which demonstrated binding to the TATA binding protein, TBP (Figure 1, lane 3).

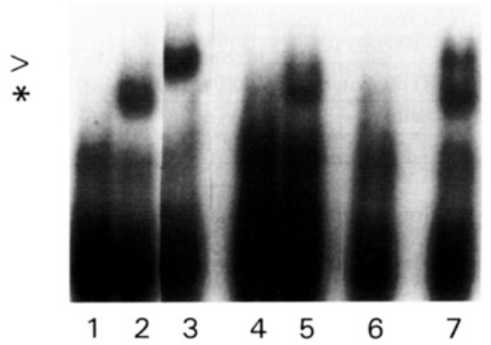

FIGURE 1: Gel mobility shift assay of a 50 bp duplex oligonucleotide containing thec-*myc* P2 promoter region. Lanes 1, 4, and 6, DNA control; lane 2, binding reaction with partially purified extract from cells containing the MBP-1 cDNA cloned in the negative orientation; lane 3, binding reaction with TBP; lane 5, binding reaction with partially purified extract from cells containing the vector plasmid alone; lane 7, binding reaction with partially purified bacterial extract containing recombinant MBP-1 protein. The arrow shows the specific TBP-bound DNA shift in lane 3 and specific MBP-1-bound DNA shift in lane 7, and the asterisk shows the nonspecific protein-bound DNA shifts in lanes 2, 5, and 7.

Recombinant MBP-1 from the pGemex-MBP-1 clone was used to characterize DNA binding at different temperatures and for different binding reaction times. MBP-1−DNA binding was found to be similar at room temperature, 4 or 37 °C, and the binding activity was observed after incubation periods as short as 5 min (data not shown).

Protein Binding to IC-Substituted Oligonucleotides. To determine whether MBP-1 binds to the major groove on the DNA, we substituted the TATAAA motif of the P2 promoter fragment with CICIII, thereby altering the major groove surface and leaving the minor groove surface as before in terms of hydrogen donors and acceptors. TBP was used as a control, since TBP has been shown to bind to the IC-substituted TATA box region on the adenovirus major late promoter (Starr & Hawley, 1991). For the experiment shown in Figure 2A, an induced extract containing recombinant MBP-1 was used in an electrophoretic mobility shift analysis of protein binding to the radiolabeled CICIII-substituted P2 promoter fragment. The electrophoretic mobility shift (Figure 2A, lane 1) indicates that alteration of the major groove had no effect on the DNA binding activity of MBP-1. Figure 2B,C shows the control experiments in which the same DNA fragment can generate an electrophoretic mobility shift by extract containing recombinant TBP and commercially available purified TBP, respectively. These experiments suggest that MBP-1, like TBP, binds on the minor groove surface of the DNA.

As a further test of minor groove binding, the converse experiment was also performed. A single G-C base pair on the MBP-1 binding site in the P2 promoter fragment was substituted by an I-C base pair. This substitution specifically alters the minor groove surface at that base pair but leaves the major groove identical to the normal binding site. The

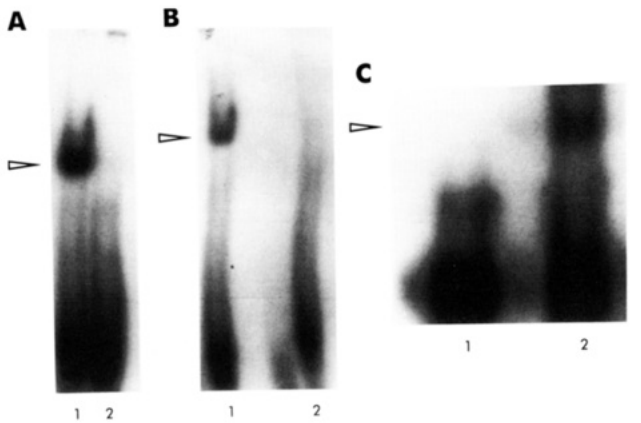

FIGURE 2: Gel mobility shift assay of a 50 bp duplex oligonucleotide containing the c-*myc* P2 promoter fragment with the core TATAAA sequence motif substituted to CICIII. (A) Lane 1, binding reaction with extract containing recombinant MBP-1 protein; lane 2, DNA control. (B) Lane 1, binding reaction with extract containing recombinant TBP protein; lane 2, DNA control. (C) Lane 1, DNA control; lane 2, binding reaction with TBP (Promega).

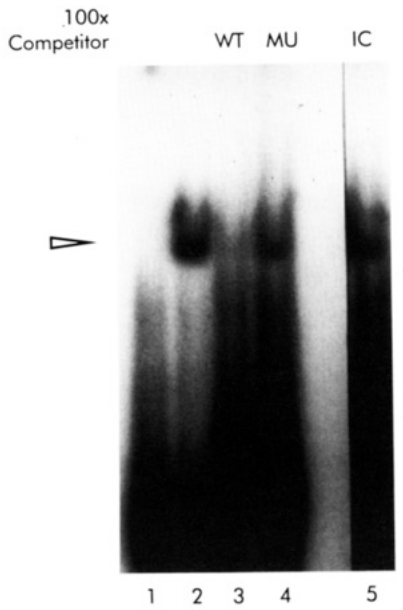

FIGURE 3: Competitive gel mobility shift assays with a 50 bp duplex oligonucleotide c-*myc* P2 promoter fragment (as in Figure 2). Lane 1, DNA control. Lane 2, binding reaction with extract containing recombinant MBP-1 protein. Lanes 3−5, competition for MBP-1 binding by adding excess cold 34 bp duplex oligonucleotides. WT, containing the MBP-1 binding site (lane 3); MU, containing a mutant MBP-1 binding site (lane 4); IC, containing the MBP-1 binding site with a single GC to IC substitution (lane 5).

electrophoretic mobility shift assay with the CICIII-substituted P2 promoter fragment and extract containing recombinant MBP-1 was performed in a competition experiment (Figure 3) using excess unlabeled wild-type binding site (lane 3), mutant binding site (lane 4), and G-C- to I-C-substituted binding site (lane 5). MBP-1 binding is specifically competed only when excess unlabeled wild-type binding site is used, but not when an excess unlabeled mutant binding site or minor groove altered binding site is used. The binding properties exhibited by MBP-1 in these experiments clearly suggest minor groove recognition of the c-*myc* P2 promoter.

Effect of Minor Groove Binding Ligands on MBP-1−DNA Binding. The compounds mithramycin, distamycin, and

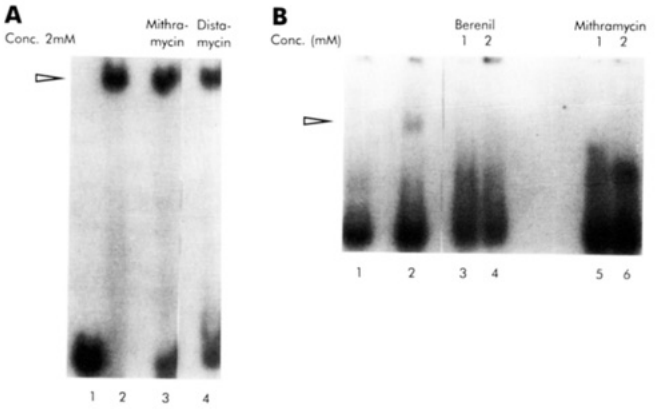

FIGURE 4: Gel mobility shift assay in the presence or absence of minor groove binding ligands. (A) Lane 1, DNA control (NFkB binding site duplex); lane 2, NFkB alone; lane 3, NFkB binding in the presence of 2×10^{-3} M mithramycin; lane 4, NFkB binding in the presence of 2×10^{-3} M distamycin. (B) Lane 1, DNA control; lane 2, TBP alone; lanes 3 and 4, TBP binding in the presence of 1×10^{-3} and 2×10^{-3} M berenil; lanes 5 and 6, TBP binding in the presence of 1×10^{-3} and 2×10^{-3} M mithramycin.

berenil possess and property of minor groove recognition for DNA binding. This property has been used to ascertain the groove binding characteristics of MBP-1. In order to conclusively prove this, a major groove binding protein, NFkB, and a minor groove binding protein, TBP, have been used in a parallel study. Similar DNA concentrations have been used in electrophoretic mobility shift experiments, and the abilities of minor groove binding ligands to compete for DNA binding at similar ligand concentrations have been compared for all three proteins. Partially purified recombinant MBP-1 was used for all ligand binding competition assays.

Figure 4A shows that neither distamycin nor mithramycin is able to compete with NFkB−DNA binding at a concentration of 2 mM. On the other hand, lower ligand concentrations (1 and 2 mM) effectively compete with TBP−DNA binding (Figure 4B). Figure 5B shows that an even lower concentration (0.2 mM) of distamycin and berenil can significantly compete with TBP−DNA binding. Similarly, distamycin and berenil have an inhibitory effect on DNA binding by recombinant MBP-1 in an electrophoretic mobility shift assay at concentrations as low as 0.2 mM (Figure 5A). This further suggests that MPB-1, like TBP, recognizes the minor groove surface on the DNA. We have also successfully tested the ability of two other minor groove binding ligands (netropsin and Hoecsht dye 33258) to compete with the DNA binding activity of recombinant MBP-1 (data not shown).

Effect of Minor Groove Binding Ligands on MBP-1−TBP−DNA Binding. The minor groove binding ligands have been effective in competing with both MBP-1 and TBP for DNA binding, suggesting that both proteins bind on the same surface of the c-*myc* P2 promoter region. Although the MPB-1 binding site is larger and includes the TBP binding site, both proteins bind together on the c-*myc* P2 promoter (Figure 6), raising the question of whether the simultaneous binding of two minor groove binding proteins on the same DNA region can be competed by minor groove binding ligands. This was tested in a competition electrophoretic mobility shift assay, which allows both MBP-1 and TBP to bind simultaneously to the target DNA (Figure 6, lanes 5−12).

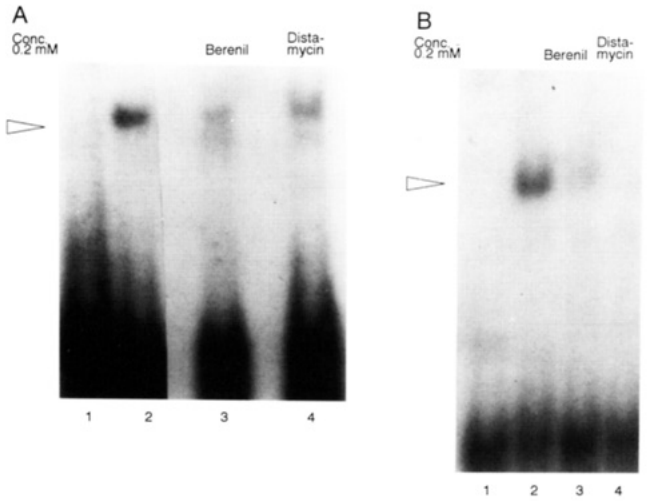

FIGURE 5: Distamycin and berenil compete with MBP-1 and TBP for DNA binding. (A) Lane 1, DNA control (as in Figure 1); lane 2, binding reaction with recombinant MBP-1 alone; lanes 3 and 4, MBP-1 binding reaction with 0.2×10^{-3} M berenil and distamycin, respectively. (B) Lane 1, DNA control; lane 2, binding reaction with TBP alone; lanes 3 and 4, TBP binding reaction with 0.2×10^{-3} M berenil and distamycin, respectively.

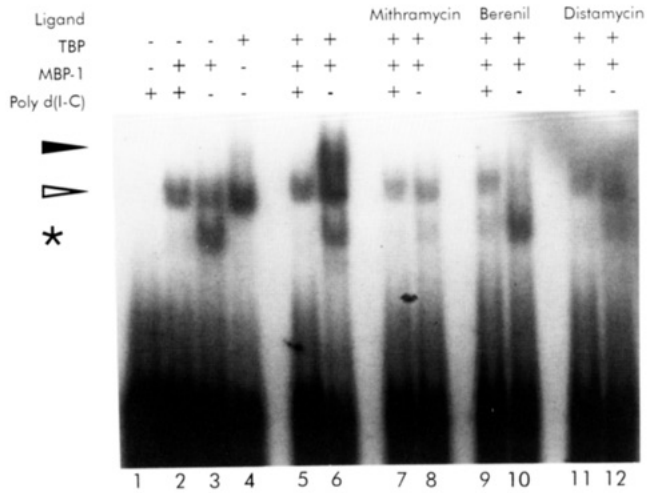

FIGURE 6: Minor groove binding ligands inhibit the simultaneous binding of MPB-1 and TBP on the c-*myc* P2 promoter. Lane 1, DNA control (as in Figure 1); lanes 2 and 3, binding reaction with recombinant MBP-1 in the presence and absence of poly[d(I-C)], respectively; lane 4, binding reaction with TBP; lanes 5−12, binding reaction with both TBP and MBP-1 in the presence of poly-[d(I-C)] (lanes 5, 7, 9, and 11) or in the absence of poly[d(I-C)] (lanes 6, 8, 10, and 12), and with 1×10^{-3} M mithramycin (lanes 7 and 8), berenil (lanes 9 and 10), or distamycin (lanes 11 and 12). The solid arrow shows the dual protein-bound DNA shift, the open arrow shows the single protein-bound DNA shift, and the asterisk shows the nonspecific protein-bound DNA shift.

Partially purified bacterial extracts containing recombinant MBP-1 give a nonspecific band in an electrophoretic mobility shift assay when the binding reaction is carried out in the absence of poly[d(I-C)] (Figure 6, compare lanes 2 and 3; Figure 1). Partially purified bacterial extracts from cells containing the parent pGemex plasmid result in the same nonspecific band upon electromobility shift analysis (Figure 1, lane 5). Figure 7 shows the results of simultaneous protein binding assays using purified TBP and partially purified extracts from cells containing the pGemex vector. The two electromobility shifts seen are the TBP-bound DNA and the

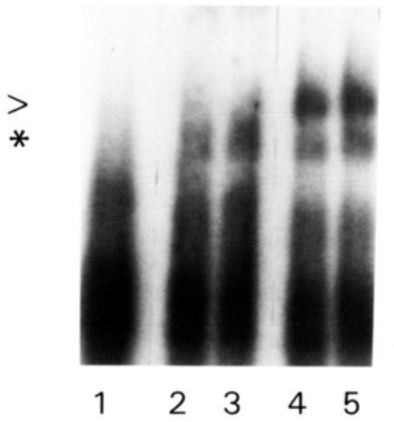

FIGURE 7: Partially purified protein extracts that do not contain recombinant MBP-1 do not form dual protein-bound DNA complex with TBP upon DNA binding. Lane 1, DNA control; lanes 2 and 3, binding reactions with 2 and 4 μL, respectively, of protein extract (0.5 μg/μL total protein) from bacterial cells that contain the vector plasmid without the MBP-1 cDNA; lanes 4 and 5, dual protein binding reactions (as in Figure 6) with TBP and the same concentration of protein extracts as in lanes 2 and 3. The arrow shows the specific TBP-bound DNA shift, and the asterisk shows the nonspecific DNA binding activity.

nonspecific DNA binding activity present in these partially purified extracts. Commercially available purified TBP binds DNA in the absence of any poly[d(I-C)] in the binding reaction (Figure 6, lane 4) as per the manufacturer (Promega). Therefore, simultaneous protein binding reactions for Figure 6 were done both in the presence (lane 5) and in the absence (lane 6) of poly[d(I-C)]. When comparing lanes 3 and 6, the composition of the binding reactions differs only in the absence and presence of purified TBP, respectively. In the absence of poly[d(I-C)], there is a supershift obtained (lane 6), and this is due to the simultaneous binding of MBP-1 and TBP to the 50 bp P2 promoter fragment. The presence of poly[d(I-C)] is not optimal for DNA binding by purified TBP, so no supershift for dual protein binding is seen in the presence of poly[d(I-C)] in lane 5. Mithramycin, berenil, and distamycin (1 mM) are able to inhibit this supershift caused by dual protein binding (lanes 8, 10, and 12) and to some extent the single protein-bound shift (lanes 7−12). The effect of these minor groove binding ligands differs in the competition for minor groove binding as seen in Figure 6, and this can be explained and correlated with the differing DNA recognition properties for these ligands (see Discussion). Berenil is more effective than distamycin and mithramycin in terms of specifically inhibiting the formation of protein−DNA complexes.

Berenil can specifically inhibit both the single protein shift and the dual protein-bound supershift (Figure 8B) at concentrations as low as 0.1 mM (lane 3) and 0.2 mM (lane 4). In a control experiment, berenil is ineffective in a competitive electrophoretic mobility shift assay with the major groove binding NFkB−DNA binding activity (Figure 8A). The experiments here show that both MBP-1 and TBP bind the P2 promoter region simultaneously and do not compete with each other for DNA binding under *in vitro* conditions. These observations lead us to conclude that the minor groove of the c-*myc* P2 promoter region is the common recognition motif for simultaneous binding by both the general transcription factor TBP and MBP-1, a negative regulator of c-*myc* expression.

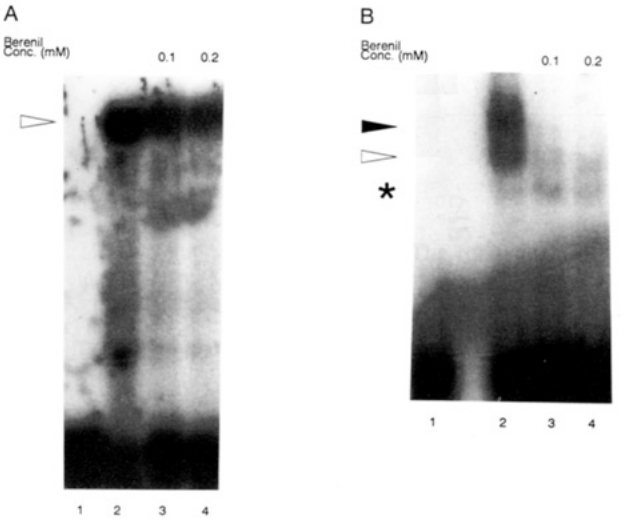

FIGURE 8: Berenil inhibits the DNA binding activity of both MBP-1 and TBP but not NFkB. (A) Lane 1, DNA control (as in Figure 4A); lane 2, binding reaction with NFkB; lanes 3 and 4, NFkB binding reaction with 0.1×10^{-3} and 0.2×10^{-3} M berenil, respectively. (B) Lane 1, DNA control (as in Figure 1); lane 2, binding reaction with recombinant MBP-1 and TBP; lanes 3 and 4, MBP-1 and TBP binding reaction with 0.1×10^{-3} and 0.2×10^{-3} M berenil, respectively. The solid arrow shows the dual protein-bound DNA shift, the open arrow shows the single protein-bound shift, and the asterisk shows the nonspecific protein-bound shift.

DISCUSSION

MBP-1 Binds in the Minor Groove of the c-myc P2 Promoter. This paper presents evidence that MBP-1 binds in the minor groove surface of the c-*myc* P2 promoter TATA box motif. One line of evidence supporting this conclusion is the major and minor groove alteration experiments. These experiments utilize a well-characterized approach (Starr & Hawley, 1991) which takes advantage of the dissimilarity of major groove surface for A-T and I-C base pairs and the dissimilarity of minor groove surface for G-C and I-C base pairs. The hydrogen acceptors and donors for the G-C to I-C base substitution are identical in the major groove but differ in the minor groove. The converse applies for the A-T to I-C substitution. The TATA box motif of the c-*myc* promoter is flanked by several G-C base pairs, so the sequence of the MBP-1 binding site was optimal for this strategy. MBP-1 binding was unaffected when the major groove was altered and inhibited when the minor groove was altered. This finding has several important implications as discussed below.

Some studies have linked the minor groove recognition property of a protein to its ability to bend the DNA upon binding (Suck *et al.*, 1988; White *et al.*, 1989; Giese *et al.*, 1992). Since many of the sequence-specific DNA binding proteins recognize the major groove at their binding site (Suzuki, 1993), bending of the DNA may well be necessary to accommodate for minor groove binding. A recent example which supports this correlation is TBP, which binds the minor groove and induces bending upon DNA binding (Horikoshi *et al.*, 1992). It has now become of interest to determine whether MBP-1 binding induces any such conformational change. Although the predominant effect of A-T to I-C substitutions is to change the groove characteristics, it is possible that there is an effect on the overall flexibility of the DNA due to these base changes. That such an effect might occur implies that the change in flexibility of the DNA did not alter minor groove recognition by the proten to any detectible degree.

Minor Groove Binding Ligands Inhibit MBP-1−DNA Binding. The second line of evidence supporting our conclusion that MBP-1 binds in the minor groove comes from the ability of minor groove binding ligands to compete for DNA binding. DNA binding by the well-characterized major groove binding protein NFkB (Baldwin & Sharp, 1988) is not inhibited by ligand concentrations higher than those that effectively compete out DNA binding by MBP-1 and TBP (Figures 4 and 5). Each of the ligands used in this study differs slightly in the mechanism of their minor groove recognition.

Distamycin binding to DNA induces a cooperative structural transition that can be transmitted to over about 100 bp (Hogan *et al.*, 1979). Most of the recent studies indicate that distamycin recognizes potential binding sites more by the shape of the DNA than by the sequence (Churchill *et al.*, 1990) and requires at least four consecutive A-T base pairs (Zimmer & Wahnert, 1986). In a mithramycin dimer bound to its 6 bp minor groove binding site, the central cytidine is reported to adopt an A-DNA sugar pucker and glycosidic torsion angle (Sastry & Patel, 1993). Mithramycin and distamycin have been used to compete for the binding of proteins in the major groove as well, such as Sp1 (Snyder *et al.*, 1991) and homeodomain peptides (Dorn *et al.*, 1992).

However, in the same competition studies of major groove binding peptides (Dorn *et al.*, 1992), berenil was shown to be ineffective in competing for the binding of peptides in the major groove. Also, Oakley *et al.* (1992) have shown that netropsin does not affect the binding of the major groove binding protein GCN4. Detailed studies of DNA-bound berenil have shown that berenil is closely isohelical with the floor of the minor groove (Hu *et al.*, 1992; Lane *et al.*, 1992; Yoshida *et al.*, 1990). Several NMR studies have confirmed that binding of berenil does not have any structural or conformational effect on the DNA.

Thus, each of the minor groove binding ligands used in this study differs in its minor groove recognition properties. Mithramycin and distamycin are not as highly specific for minor groove recognition as berenil is. In order to correctly interpret the results from these ligand competition assays, an appropriate set of control assays is necessary. The control assays used as a basis for comparison were competition assays with each ligand and the use of both a previously characterized, minor groove binding protein (TBP) and a major groove binding protein (NFkB). Additional minor groove binding ligands (Hoechst dye 33258 and netropsin) were also used in similar competition assays to arrive at the same result (data not shown). The fact that the nonspecific DNA binding activity, which occurs in the absence of nonspecific target DNA in the binding reactions, is not altered by berenil (Figure 6, lanes 3, 6, and 10; Figure 8B), but is slightly affected by distamycin and mithramycin (Figure 6, lanes 3, 6, 8, and 12), agrees with the above discussion on DNA structural implications of ligand binding and DNA recognition. Berenil−DNA binding does not affect major groove binding proteins (Dorn *et al.*, 1992, and Figure 8A) but can affect minor groove binding proteins at concentrations as low as 0.1 mM (Figure 8B). This finding also supports the previous structural studies which had concluded

that there is no conformational change on the DNA due to berenil binding.

MBP-1 and TBP Simultaneously Bind in the Minor Groove. Based on the fidelity of base pair recognition due to the hydrogen donors and acceptors on the groove surfaces, it was proposed that six potential recognition sites occur in the major groove as compared to three in the minor groove of the DNA (Seeman *et al.*, 1976). These interactions allow proteins to differentiate between all four base pairs in the major groove and only three in the minor groove. An increasing number of specific and nonspecific minor groove binding proteins have been reported in the past several years including HMG family proteins (Reeves & Nissen, 1990; Giese *et al.*, 1992; Thanos & Maniatis, 1992), nuclear scaffold protein SATB1 (Dickinson *et al.*, 1992), TBP (Starr & Hawley, 1991; Lee & Horikoshi, 1991), DNase I (Suck *et al.*, 1988), *Escherichia coli* IHF protein (Yang & Nash, 1989), and now MBP-1. In dual protein−DNA binding conditions, the presence of the minor groove binding ligands competes away the supershifted dual protein−DNA complex more effectively than the single protein−DNA complex (Figure 6, lanes 6, 8, 10, and 12, and Figure 8B). This demonstrates that the minor groove of the c-*myc* P2 promoter can allow MBP-1 and TBP to bind together and the simultaneous protein binding to the same minor groove can be effectively inhibited by minor groove binding ligands. Thus, this is the first evidence of the simultaneous recognition of the same DNA minor groove by two different proteins.

Biological Significance of Minor Groove Recognition by MBP-1. The c-*myc* P2 promoter is the stronger promoter for the c-*myc* protooncogene and accounts for 80% of the c-*myc* gene expression. This study has important implications on the efficiency of transcriptional initiation from the P2 promoter, since the TATA box motif is the binding site for the general transcription factor TBP. Binding of TBP occurs in the absence of other proteins in vitro and is an early step in the assembly of a multiprotein preinitiation complex at the promoter. The stabilization of this higher order complex has been attributed largely to the ability of TBP to bend the DNA upon minor groove binding (Horikoshi *et al.*, 1992). Both MBP-1 and TBP are about the same size and can simultaneously bind on the same face of the c-*myc* P2 promoter. Since MBP-1 has been shown to negatively regulate the c-*myc* promoter activity (Ray & Miller, 1991), the results from this paper provide an insight into the mechanism by which MBP-1 exerts its effect.

A model for the regulation of the c-*myc* P2 promoter by MBP-1 can be suggested, involving the simultaneous binding to the minor groove by both MBP-1 and TBP. Lieberman and Berk (1994) have recently shown that a transcriptional activator can stabilize the DNA−protein interactions of TAFs (transcription cofactors in the TFIID complex) and produce a change in the DNase I digestion pattern upstream and downstream in addition to the TBP footprint over the TATA box. It can be hypothesized that the binding of MBP-1 along with TBP to the TATA box on the c-*myc* P2 promoter can disrupt or prevent the assembly of other factors required for efficient transcription. It will be very important to study the molecular effect of this binding in terms of binding of other transcription factors, the base contacts, as well as TBP contacts that are affected. We are currently addressing relevant questions that arise as a result of this study.

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