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Identification of two novel mouse nuclear proteins that bind selectively to a methylated c-Myc recognizing sequence

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ABSTRACT

The c-Myc recognizes the sequence CACGTG (Blackwell, T. K., Kretzner, L., Blackwood, E.M., Eisenman, R. N., and Weintraub, H. (1990) Science 250, 1149–1151), and its binding is inhibited by methylation of the core CpG (Prendergast, G. C. and Ziff, E. B. (1991) Science 251, 186–189). We identified two novel nuclear proteins, MMBP-1 and MMBP-2, that bound specifically and under physiological salt condition to the c-Myc binding motif of which cytidine in the CpG sequence was methylated. MMBP-1 was about 42 kD and MMBP-2 was about 63 kD. MMBP-1 was found in specific cells, while MMBP-2 was found in all the cell lines tested, suggesting that MMBP-1 may modulate the role of MMBP-2 in tissue specific manner. We propose that the two proteins play a role in the regulation of c-Myc function through stabilizing or destabilizing the methylation state of the c-Myc binding motif.

INTRODUCTION

The expression of mammalian genes often correlates with the methylation state of CpG sequences in the promoter region (*1*). The promoter regions of the house keeping genes are undermethylated in all cells. In contrast, the tissue specific genes are usually methylated at the 5 position of cytidines in CpG sequences, except in the tissues in which they are expressed (*2*). Usually, *in vitro*-methylated genes are inactive when transfected into cells (*3*). During the cell differentiation, some of the tissue specific genes become demethylated in their promoter region. The inhibition of DNA methylation followed by DNA replication can passively demethylate the genes. A possibility of positive demethylation without DNA replication is also reported (*4, 5*). In either mechanism, at least one of the proteins that participates in the demethylation step(s) must recognize either the hemi- or fully methylated CpG in a sequence specific manner. At present, several proteins that bind methylated CpG have been identified in vertebrates (*6–11*). MeCP-1 (*6*) and MeCP-2 (*7*) specifically bind to the methylated CpG. Both proteins, however, have no sequence specificity except the density of methylated CpG in the DNA. Although MDBP binds the methylated DNA in a sequence specific manner, its specificity is low: it also binds to sequences that are not methylated (*8–10*). MDBP-2 binds the hormone responsive promoter region of vitellogenin gene of chick (*11*). The binding region of MDBP-2 is demethylated prior to estrogen-induced transcription of the gene (*12*). Partial sequencing of MDBP-2 revealed that the protein is a member of the histone H1 family and that it has no sequence specificity in binding to methylated DNA (*13*). MDBP-2, as well as MeCP-1 and MeCP-2, inhibits the *in vitro* transcription (*13–16*). So far, except for MDBP, no protein that recognizes methylated CpG in a sequence specific manner has been identified.

C-Myc is a nuclear proto-oncogene product that plays an important role in controlling proliferation and differentiation of the cells (*17*); however, the mechanism of c-Myc action remains unknown. C-Myc function as a transcription factor with its partner Max (*18, 19*). It is a DNA-binding protein which recognizes a so-called E-box sequence containing a CpG sequence in the core (*20, 21*). Two other transcription factors, upstream stimulatory factor (USF) (*22*) and transcription factor E3 (TFE3) (*23*), the members of Myc family, also recognize the same E-box sequence as c-Myc binding motif that contains a CpG sequence in the center. USF and TFE3 recognize both unmethylated and methylated cytidine in the core CpG sequence in the c-Myc binding motif (*21*). On the other hand, methylation of the CpG sequence in the motif inhibits the binding of c-Myc protein or its complex with Myn, a murine homologue of Max (*21, 24*). This suggests that the function of c-Myc is regulated, at least in part, through the methylation state of the CpG. In the present study, we identified two novel proteins that recognize the methylated c-Myc binding motif in a sequence specific manner. The proteins that bind to the methylated c-Myc binding motif may provide a clue to understand the mechanism by which c-Myc controls gene expression.

MATERIALS AND METHODS

Cells and cell culture

Mouse myogenic cell line C2C12 was kindly provided by Dr Nabeshima at National Institute of Neuroscience, Japan. Fibroblast cell lines C3H/10T1/2 and 3T6-Swiss albino, and

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PCC4 AG derived from testis were obtained from Japanese Cancer Research Resources Bank. Mouse erythroleukemia (MEL) strain 11A2 (*25*) was generously provided by Dr Watanabe at Tokyo University.

C2C12, C3H/10T1/2, 3T6-Swiss albino and PCC4 AG were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. MEL was cultured in ES medium containing 2% fetal calf serum (*25*).

C2C12 cells were cultured in plastic dishes coated with 0.1% gelatin and differentiated into myotubes by changing the medium to Cosmedium (CosmoBio) (Ichikawa and Yoshimi, unpublished results). All the cells were maintained at 37°C under 5% CO_2 atmosphere.

Preparation of nuclear extract and its fractionation by heparin-agarose chromatography

The nuclear extracts from different types of cell lines were prepared as described by Dignam *et al.* (*26*), with a slight modification as follows. All the solutions contained 1 mM DTT. The high salt extract of isolated nuclei was centrifuged in a Beckman TL 100.2 rotor at 4°C for 10 min at 100,000 rpm. After dialyzing against 20 mM Hepes buffer (pH 7.4) containing 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT and 20% (v/v) glycerol (buffer A), aliquots of extracts were frozen in liquid nitrogen and stored at −80°C until use.

The crude nuclear extract was loaded onto a heparin-agarose (Sigma) column, equilibrated with buffer B (50 mM Tris−HCl (pH 8.0), 0.2 mM EDTA, 1 mM DTT, 20% (v/v) glycerol) containing 0.01 M KCl. Protein bound to the column was eluted in a stepwise manner with 0.05, 0.1, 1.0 M KCl in buffer B (*27*). The eluate was dialyzed against buffer A and used for the binding studies.

Oligonucleotides

The oligonucleotides used in this study were synthesized with an Applied Biosystem model 381A DNA synthesizer using β-cyanoethyl-diisopropyl phosphoramidites;

5′ TCAGA CCACG TGGTC GGGTG TTCCT GA 3′ (myc1)
5′ TCAGA CCAmCG TGGTC GGGTG TTCCT GA 3′ (myc1m)
5′ TCAGG AACAC CCGAC CACGT GGTCT GA 3′ (myc2)
5′ TCAGG AACAC CCGAC CAmCGT GGTCT GA 3′ (myc2m)
5′ AGCCA AGTGC TACCG CGTGT GGCTG CCA 3′ (MyoD1)
5′ AGCCA AGTGC TACmCG mCGTGT GGCTG CCA 3′ (MyoD1m)
5′ TGGCA GCCAC ACGCG GTAGC ACTTG GCT 3′ (MyoD2)
5′ TGGCA GCCAC AmCGmCG GTAGC ACTTG GCT 3′ (MyoD2m)

where mC indicates 5-methyl deoxycytidine. The underlines indicate the E-box sequence (CANNTG) in each oligonucleotide. As the E-box of the 'MyoD' did not have a CpG sequence in the core, two CpG out side the E-box were methylated. After synthesis, the oligonucleotides were purified by HPLC using C18-reverse phase cartridge, eluted with 6.75−40% acetonitrile gradient in 0.1 M triethanolamine acetate (pH 7.2). The c-Myc recognition sequence ('myc') was taken from ref. 21. The sequence 'MyoD' was the 5′ (−276∼ −249, nucleotide numbers from the initiation site) upstream region of the genomic sequence of MyoD gene (*28*).

Equal moles (∼35 pmoles) of complementary strands were phosphorylated with T4 polynucleotide kinase in 10 μl of 50 mM Tris−HCl (pH 8.0) buffer containing 10 mM $MgCl_2$ and 10 mM DTT, in the presence of [γ-^{32}P]ATP (Amersham International, >185 TBq/mmol) or unlabeled ATP. To anneal the oligonucleotides, the mixture was 10-fold diluted with 50 mM NaCl in 10 mM Tris−HCl (pH 8.0) and 1 mM DTT, and then boiled for 10 min. Then the mixture was cooled slowly to room temperature in a water bath that was preheated to 65°C. The labeled and annealed oligonucleotides were passed through a Sephadex G-50 (fine) column, equilibrated with 10 mM Tris−HCl (pH 8.0) buffer containing 1 mM EDTA, to remove unincorporated radioactivities.

UV-light crosslinking of radiolabeled oligonucleotides to DNA binding protein

Crude nuclear extract (∼6 μg protein) or heparin fractionated extracts that were recovered from the same amount of nuclear extract (thus representing an equivalent amount) were preincubated at room temperature for 10 min in 20 μl of 12 mM Hepes (pH 7.4) buffer containing 1.5 mM Tris−HCl, 60−300 mM KCl, 1 mM EDTA, 0.36 mM PMSF, 0.6 mM DTT, 12% (v/v) glycerol, 1% (w/v) Triton X-100 and 3 μg poly(dI-dC), with or without unlabeled oligonucleotides in double stranded

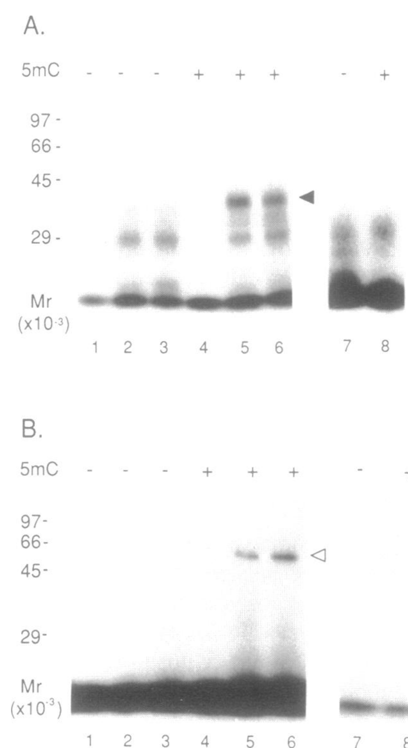

Figure 1. Detection of sequence specific and methylation dependent binding of the nuclear proteins. Crude nuclear extract (∼6 μg) prepared from proliferating C2C12 cells was incubated either in low (60 mM KCl, panel A) or high (300 mM KCl, panel B) salt concentrations with labeled oligonucleotides (2 ng, ∼40,000 cpm). The mixture was UV crosslinked for 0.5 min (lanes 1 and 4), 10 min (lanes 2 and 5) or 30 min (lanes 3 and 6−8), and then was subjected to SDS-PAGE (12% gel) and autoradiographed as described in the MATERIALS AND METHODS. The labeled oligonucleotides used were c-Myc binding motif ('myc', lanes 1−6) or the 5′ upstream sequence (−276∼ −249 from the initiation site) of MyoD gene ('MyoD', lanes 7 and 8). The 5 position of cytidine in CpG sequence in oligonucleotides was either unmethylated (5mC '−', lanes 1−3 and 7) or fully methylated (5mC '+', lanes 4−6 and 8). The closed and the open arrowheads indicate the 42 (MMBP-1) and the 63 kD (MMBP-2) protein bands that bound the methylated 'myc', respectively. The positions of molecular weight (Mr) are indicated. The band at 30 kD recognized both methylated and unmethylated form of 'myc', and was not further pursued.

form (50-fold molar excess). Radiolabeled oligonucleotide probe in double stranded form (2.0 ng, about 20,000 cpm/ng; see above) was added, and the mixture was incubated for additional 30 min at 30°C. The mixture on ice was then irradiated at a distance of 12 cm from UV germicidal bulbs (10W×4). Each sample received an equal volume of 2× sample buffer for SDS-PAGE (130 mM Tris−HCl (pH 6.8), 4% (w/v) SDS, 0.2% (v/v) 2-mercaptoethanol, 0.1 mg/ml bromophenol blue), and was boiled for 5 min. Subsequently, the samples were electrophoresed on a 12% polyacrylamide gel (*29*). After the electrophoresis the gel was dried and subjected to autoradiography with a Kodak intensifying screen at −80°C.

Gel mobility shift assay

The samples were incubated as in the section for the 'UV-light crosslinking' assay. The mixtures, with or without UV-light irradiation, were electrophoresed on a 4% polyacrylamide gel (acrylamide:*bis*-acrylamide=30:0.4, w/w) in 0.13×TBE buffer with the electrophoresis buffer of 0.13×TBE at 15 V/cm at 4°C (*30*). TBE (1×) consisted of 89mM Tris-base, 89mM boric acid and 25mM EDTA, pH 8.3. After the electrophoresis the gel was dried and subjected to autoradiography.

Other methods and reagents

Protein concentration was determined as described by Smith *et al.* (*31*), with bovine serum albumin as a standard.

DMEM and ES media were purchased from Nissui Seiyaku (Tokyo, Japan), and fetal calf serum was from Gibco (Paisley, Scotland). Cosmedium was obtained from CosmoBio (Tokyo, Japan) and gelatin was from Iwaki Glass (Tokyo, Japan). The reagents for the synthesis of oligonucleotides were purchased from Applied Biosystems (Foster City, CA), except that 5-methyl deoxycytidine β-cyanoethyl-diisopropyl phosphoramidite was from Pharmacia (Uppsala, Sweden). T4 polynucleotide kinase was obtained from Toyobo (Osaka, Japan) and poly(dI-dC) was from Pharmacia (Uppsala, Sweden). The other reagents used were guaranteed grade.

RESULTS

Methylated DNA binding proteins that recognize c-Myc binding motif in a nuclear extract

To search for DNA binding proteins that distinguish methylated from unmethylated CpG in a sequence specific manner, we prepared two types of double stranded oligonucleotides with one or two methylated CpG sequences. One oligonucleotide corresponded to the c-Myc recognition site ('myc') reported by Prendergast *et al.* (*24*) and the other one corresponded to an unrelated control sequence, the upstream sequence of the mouse MyoD genomic gene that contained a MyoD binding site ('MyoD') (*28*). The radiolabeled oligonucleotides, either unmethylated or methylated on both strands (=fully methylated) were mixed with a nuclear extract of C2C12, UV-light irradiated, and after SDS denaturation electrophoresed on a polyacrylamide gel. Two specific bands were crosslinked to the fully methylated 'myc' oligonucleotide (Fig. 1). The 42 kD (panel A, indicated by a closed arrowhead) and the 63 kD proteins (panel B, indicated by an open arrowhead) specifically recognized the fully methylated 'myc' (lanes 5 and 6). These radiolabeled bands appeared in time-dependent manner by UV-light irradiation, (they were not detected in the samples crosslinked for 0.5 min (lanes 1 and 4)). The two proteins that were crosslinked to the methylated 'myc' were not seen when either fully methylated or unmethylated 'MyoD' were used as a probe (Fig. 1, panels A and B, lanes 7 and 8). Therefore, the two proteins bound not only methylation-specific, but also in a sequence-specific manner.

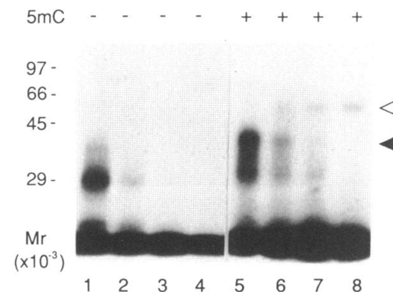

Figure 2. Effect of KCl concentration on the binding activity of 'myc' to the nuclear extract. The labeled 'myc' and the nuclear extracts prepared from C2C12 cells were incubated and UV-crosslinked (30 min) in 60 mM (lanes 1 and 5), 120 mM (lanes 2 and 6), 200 mM (lanes 3 and 7) and 300 mM (lanes 4 and 8) KCl, and the mixtures were subjected to analysis as in Fig. 1. The probes were either unmethylated (5mC '−', lanes 1−4) or fully methylated (5mC '+', lanes 5−8) at CpG. The closed and the open arrowheads indicate MMBP-1 and MMBP-2, respectively.

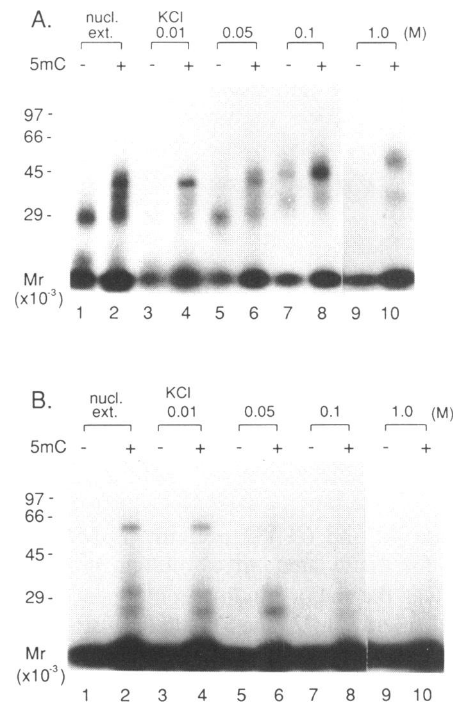

Figure 3. Binding activities of the heparin-agarose fractionated nuclear extract to 'myc'. The crude nuclear extract ('nucl. ext.') was loaded onto a heparin-agarose column at 0.01 M KCl and was eluted with 0.05, 0.1, 1.0 M KCl. Each fraction, an equivalent amount of nuclear extract (see MATERIALS AND METHODS), was tested for the binding activity either to the fully methylated (5mC '+', lanes 2, 4, 6, 8 and 10) or to the unmethylated (5mC '−', 1, 3, 5, 7 and 9) 'myc' in low (60 mM KCl, panel A) and high (300 mM KCl, panel B) salt concentrations. The condition of UV-crosslinking was identical to that in Fig. 2.

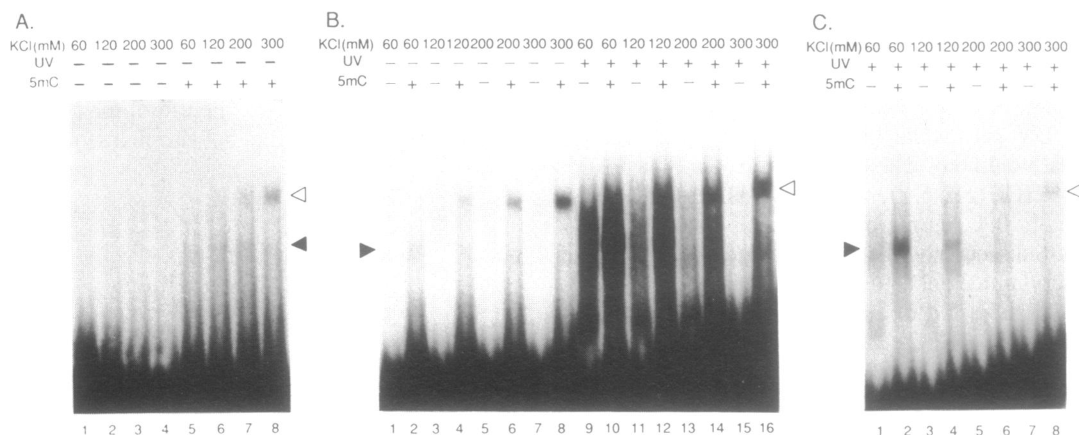

Figure 4. Gel mobility shift assay of MMBP-1 and MMBP-2. The labeled 'myc' and the crude nuclear extract (panel A) or the flow-through fraction from a heparin-agarose column, which was an equivalent amount of nuclear extract (panel B and C), were incubated in 60mM, 120mM, 200mM and 300mM KCl, and the mixtures were subjected to analysis as described in the MATERIALS AND METHODS. The probes 'myc' were either unmethylated (5mC '−') or fully methylated (5mC '+'). Panel C shows the short exposure of lanes 9−16 of panel B. The closed and the open arrowheads indicate MMBP-1 and MMBP-2, respectively.

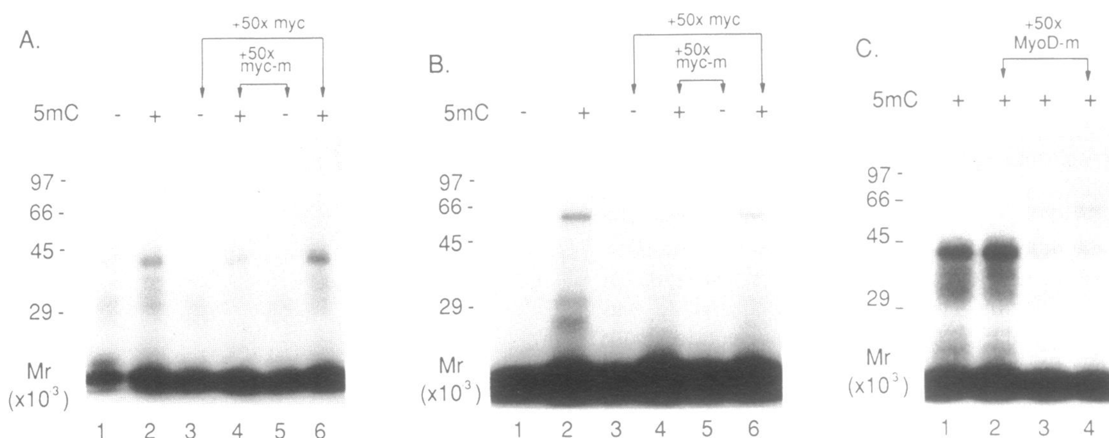

Figure 5. Reduction of the binding activities of MMBP-1 and MMBP-2 to the radio-labeled methylated 'myc' in the presence of excess amount of non-labeled methylated 'myc'. The effect of the presence of excess amounts of non-labeled 'myc' on the binding of the proteins was examined in low (60 mM KCl, panel A, and panel C lanes 1 and 2) or high (300 mM KCl, panel B, and panel C lanes 3 and 4) salt concentration. Binding to labeled 'myc' was competed with a 50-fold molar excess of unlabeled unmethylated (panels A and B, lanes 3 and 6) or fully methylated (panels A and B, lanes 4 and 5) 'myc', or unlabeled fully methylated 'MyoD' (panel C, lanes 2 and 4). The labeled 'myc' were either unmethylated (5mC '−', panels A and B, lanes 1, 3 and 5, and panel C, lanes 1 and 3) or fully methylated (5mC '+', panels A and B, lanes 2, 4 and 6, and panel C, lanes 2 and 4). The binding and UV-crosslinking conditions were identical as in Fig. 2.

We named the 42 and 63 kD proteins as MMBP-1 and -2, respectively, for methylated 'myc' binding protein.

MMBP-1 was the major binding component in 60 mM KCl (Fig. 1, panel A and Fig. 2 lane 5), and MMBP-2 was the major binding component in 300 mM KCl condition (Fig. 1, panel B and Fig. 2, lane 8). Importantly, binding of both MMBP-1 and MMBP-2 to the fully methylated 'myc' was detected in 120 mM KCl concentration (Fig. 2, lane 6), which approximates physiological salt concentrations. Hence it is likely that both proteins can recognize the c-Myc binding motif *in vivo*.

Fractionation of nuclear extract on a heparin-agarose column

Nuclear extract from proliferating C2C12 cells was fractionated on a heparin-agarose column and used for the UV-crosslinking experiments (Fig. 3). Most of the MMBP-1 as well as the MMBP-2 that bound preferentially to the fully methylated 'myc' were recovered in the flow-through fraction (0.01 M KCl). About 65% and 85% of the binding activities of MMBP-1 and MMBP-2, respectively, were recovered to this fraction, (Fig. 3, panels A and B, lanes 2 and 4). As shown in Fig. 3, lanes 5−10, some bands other than the 42 and the 63 kD proteins that bound specifically to the fully methylated 'myc' were detected. Those proteins, however, were not found by UV-crosslinking when crude nuclear extract was used (see Figs 1 and 2), and therefore, were not further pursued in this study.

Gel mobility shift assay of MMBP-1 and MMBP-2

We tried to detect MMBP-1 and MMBP-2 bands by gel mobility shift assay. When the radiolabeled fully methylated 'myc' and crude nuclear extract of C2C12 or the flow-through fraction from a heparin-agarose column were incubated, two shifted bands were detected (Fig. 4, panel A, lanes 5−8 and panel B, lanes 2, 4,

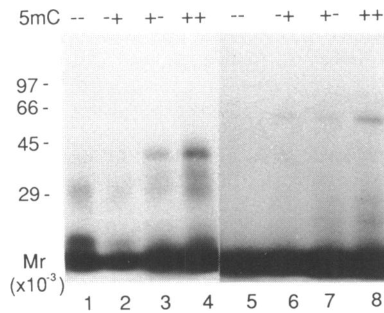

Figure 6. The binding activities of the proteins to the hemimethylated 'myc'. The binding activities of the proteins were tested in low (60 mM KCl, lanes 1–4) or high (300 mM KCl, lanes 5–8) salt concentration. The labeled 'myc' used were unmethylated (myc1/myc2 '−', lanes 1 and 5), hemimethylated (myc1/myc2m '−+', lanes 2 and 6, or myc1m/myc2 '+−', lanes 3 and 7) or fully methylated (myc1m/myc2m '++', lanes 4 and 8). The binding and UV-crosslinking experiments were performed as in Fig. 2.

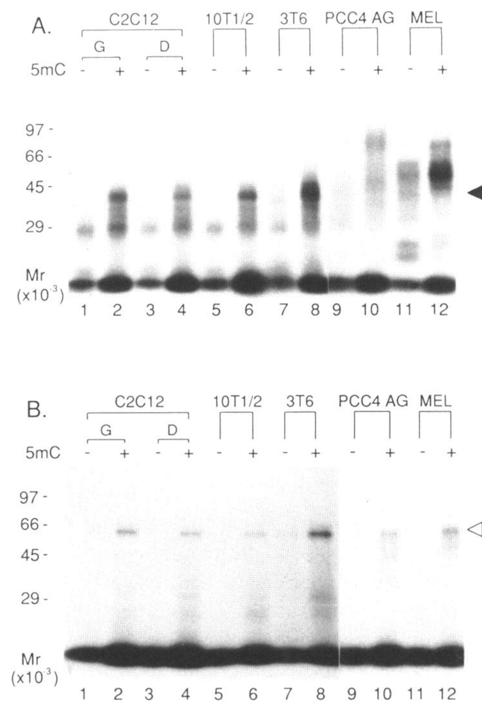

Figure 7. Cell type specific expressions of the binding activities of MMBP-1 and MMBP-2. Panels A and B show cell type specific expression of the methylated 'myc' sequence dependent binding of MMBP-1 and MMBP-2 in low (60 mM KCl) and high (300 mM KCl) salt concentrations, respectively. The nuclear extracts were prepared from growing C2C12 cells (lanes 1 and 2, 'G') and differentiated C2C12 cells (lanes 3 and 4, 'D'), C3H/10T1/2 (lanes 5 and 6, '10T1/2'), 3T6-Swiss albino (lanes 7 and 8, '3T6'), PCC4 AG (lanes 9 and 10), and MEL (lanes 11 and 12). The labeled 'myc' was unmethylated (5mC '−', the lanes of odd numbers) or fully methylated (5mC '+', lanes of even numbers). The nuclear extract of about 6 μg was used for each of the experiments. The binding and UV-crosslinking experiments were done as in Fig. 2. The close and the open arrowheads indicate MMBP-1 and MMBP-2, respectively.

6 and 8). The appearance of the bands depended on the methylation of 'myc' and on the salt concentration in the incubation buffer. The band with low mobility increased its intensity as the KCl concentration was raised from 60mM to 300mM. And, the high mobility band was detected when the mixture was incubated in 60mM or 120mM KCl (panel A, lanes 5 and 6, and panel B, lanes 2 and 4), and was not seen when incubated in 300mM KCl (panels A and B, lane 8). About 100% and 60% of the activities of the low and high mobility bands were recovered to the flow-through fraction, respectively. Gel mobility shift assay after UV-light crosslinking without SDS-denaturation again demonstrated two shifted bands (panel B, lanes 9–16 and panel C). The crosslinked bands with low and high mobilities increased and decreased their intensities as the KCl concentration was raised from 60mM to 300mM, respectively (panel C, lanes 2, 4, 6 and 8), indicating that the bands are MMBP-1 and MMBP-2. The mobility of each UV-crosslinked band was identical to that of the low or high mobility band appeared without UV-light irradiation (panel A, lanes 5–8 and panel B, lanes 2, 4, 6 and 8). From the property of the mobilities, together with the properties that the binding activities depended on the salt concentration and were recovered to the flow-through fraction from a heparin-agarose column, we concluded that the two bands seen in the gel mobility shift assay were MMBP-1 and MMBP-2.

The binding specificity of MMBP-1 and MMBP-2

As we would expect for sequence-specific binding, excess of non-labeled fully methylated 'myc' (Fig. 5, panels A and B, lane 4) but not excess of non-labeled unmethylated 'myc' (panels A and B, lane 6) competed with labeling of both MMBP-1 and MMBP-2 bands. Furthermore, non-labeled fully methylated 'MyoD' did not compete with labeling of both MMBP-1 and MMBP-2 bands (panel C), indicating that MMBP-1 and MMBP-2 were not recognizing just a methylated CpG. These results support our notion that MMBP-1 and MMBP-2 proteins preferentially bind to the methylated 'myc' sequence.

The hemimethylated oligonucleotide (myc1m/myc2, '+−') could bind MMBP-1 about half as well as fully methylated 'myc' (compare Fig. 6 lanes 3 and 4). In contrast, 'myc' that was hemimethylated in opposite strand (myc1/myc2m, '−+') scarcely bound the protein (lane 2). Thus, MMBP-1 recognizes a strand specific methylation in the 'myc' sequence. In contrast to MMBP-1, either of the two different hemimethylated 'myc' sequences bound equally well to MMBP-2, albeit at an about 2-fold reduced level of fully methylated 'myc' (Fig. 6 lanes 6–8).

Cell type specificity of the binding activities of the two proteins

Figure 7 (panels A and B) shows cell type specific expressions of the binding activities of the proteins to methylated 'myc'. MMBP-1 was detected in specific cells only, e.g. in fibroblasts (C3H/10T1/2 and 3T6-Swiss albino) and myogenic cells (C2C12) both in proliferating and differentiated stages, but not in cell lines derived from testis (PCC4 AG) and from blood cells (MEL) (Fig. 7, panel A). In contrast, MMBP-2 was detected in all the cell lines tested (Fig. 7, panel B). Thus, MMBP-2 seems to be ubiquitously expressed.

DISCUSSION

The promoter regions are demethylated in many of the tissue (cell) specific genes when they are active. We hypothesized that specific proteins exist that recognize these promoter sequences in i) methylation dependent and ii) sequence specific manners. Some of these proteins may participate directly or indirectly in the demethylation system. As a first step to test this hypothesis, we searched for the proteins that recognize the methylated CpG in

a specific sequence. We found two novel proteins that bound the c-Myc binding motif ('myc') containing a pair of methylated CpG. The two proteins, MMBP-1 and MMBP-2, recognized the identical 'myc' sequence under physiological salt concentration. The binding activity of MMBP-1 detected in gel mobility shift assay was lower than that of UV-crosslinked one (compare Fig. 4, panel B, lanes 1−8 and 9−16). This may be because the labeled oligonucleotide was dissociated from MMBP-1 during the electrophoresis. This may happen when MMBP-1 binds to and dissociates from the methylated 'myc' very quickly even though MMBP-1 binds to the oligonucleotide in high affinity in the binding buffer, or when MMBP-1 binds weakly to the methylated 'myc'. Granting MMBP-1 binds weakly to the motif, it does not mean that MMBP-1 is unimportant *in vivo*. These two proteins are different from the known methylation-dependent binding proteins, such as MeCP-1 (*6, 14, 15*), MeCP-2 (*7, 16*), MDBP (*8−10*) and MDBP-2 (*11, 13*), in several points. First, MMBP-1 and MMBP-2 have different molecular weight than the known proteins. The reported molecular weights of MeCP-1, MeCP-2, MDBP and MDBP-2 are 120, 84, 80−120 (estimated from the value $s_{20,w}$=6.1) and 21K, respectively (*6−8, 13*). Second, the binding sequence, 'myc', used in the present study is different from the consensus sequences recognized by those four proteins (*6, 11, 13*). Third, in contrast to the MeCP and MDBP-2 proteins, which have no sequence specificity in binding (*7, 13, 16*), both of the two proteins described here bound in a sequence specific manner, since the proteins could not recognize the methylated 'MyoD' motif. From those results we concluded that the methylation dependent binding proteins, MMBP-1 and MMBP-2, are novel proteins.

C-Myc, which is believed to be linked to proliferation and differentiation (*6*), is a DNA binding protein and functions as a transcription factor (*17−21*). In the present study we could not detect the binding activity of c-Myc protein to the 'myc' oligonucleotide. The expression levels of endogenous c-Myc may be too low for the detection. At present, two other transcription factors, USF and TFE3 are known to recognize the identical E-box sequence that contains a CpG in the core (*22, 23*). USF and TFE3, however, bind both methylated and unmethylated c-Myc binding motif (*21*), while c-Myc binds only to the unmethylated sequence (*21, 24*). Therefore, USF or TFE3 cannot be identical to the two methylation dependent binding proteins described in this study, which bound specifically to the fully methylated or hemimethylated motif. The binding of the two proteins to the methylated c-Myc binding motif may facilitate or prevent the methylated site from demethylation. We therefore propose that these two proteins may control the function of c-Myc protein by regulating its binding to DNA through stabilizing or unstabilizing the methylated form. Further characterization of the two proteins may hopefully shed light on the function and regulation of the c-Myc protein.

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