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Cloning and characterization of a c-*myc* intron binding protein (MIBP1)

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ABSTRACT

The cDNA for a c-*myc* intron 1 binding protein 1 (MIBP1) in the rat was isolated from λgt11 and λZAPII cDNA libraries. Sequencing of the cDNA clones revealed a long ORF which encoded a putative protein of 2437 amino acid residues. This protein has two widely separated zinc finger regions, each of which carries C2H2 motifs. When expressed in *E.coli* as a fusion protein, part of the MIBP1 showed sequence-specific binding to the target sequence, i.e., a 9-bp sequence in the rat c-*myc* intron 1. MIBP1 is most likely the rat counterpart of human MHC binding protein-2 (MBP-2/HIV-EP2), based on the 86% similarity in nucleotide sequence and 93% similarity in amino acid sequence. Northern blotting revealed a high level of MIBP1 mRNA in the brain.

INTRODUCTION

c-*myc* is known to be involved in oncogenesis and cell immortalization (1,2). The c-*myc* protein is a sequence-specific DNA binding protein, which acts as a hetero-oligomer with Max, and is thought to regulate transcription (3).

c-*myc* is a typical immediate early gene, i.e., its expression is rapidly and transiently elevated just after cells are exposed to growth stimuli (4,5). The level of c-*myc* mRNA seems to be controlled by three separate processes; initiation of transcription, blockage of transcript elongation and mRNA degradation. Early studies suggested that *cis*-acting regulatory elements were present in the exon 1–intron 1 region of the gene, which might be the binding site for transcriptional factors (6). Elongation of c-*myc* transcript is blocked at the exon 1–intron 1 region and this blockage of transcription seems to be a major mode of regulation of this gene during cell differentiation (7), although the precise mechanism of this transcription-blockage is unknown. Some protein may bind to a sequence in this region, and signal RNA polymerase II either stop or proceed.

Other findings also suggest that the intron 1 region is involved in regulation of expression of the gene. In some Burkitt lymphomas and plasmacytomas, breakpoints have been found within exon 1 or intron 1. Disruptions or point mutations in this region in Burkitt lymphomas (8,9) seem to activate a cryptic promoter in intron 1 (10,11), resulting in deregulation or overexpression of c-*myc* (12,13). All of these observations suggest that the exon 1–intron 1 region of c-*myc* is important in the regulation of its expression. In light of these facts, protein binding sites have been sought in this region (the 5′-portion of intron 1) and several sites were identified which may regulate expresison of the gene (14,15 and our unpublished data).

c-*myc* expression varies among tissues. Its mRNA level is very low in normal liver, but is increased in hepatocarcinoma and regenerating liver of the rat (5,16,17). These observations prompted us to look for factors that negatively regulate expression of c-*myc* in normal liver. We have detected several protein-binding sites in the 5′-region of intron 1 of c-*myc* using liver nuclear extract of rat by gel shift, DNase I footprinting and methylation interference (data not shown) analyses. In an effort to characterize c-*myc* regulation by the proteins that bind to these sites, we isolated a cDNA clone which coded for one of these proteins by the southwestern method. cDNA clones which covered a complete coding region of this protein were obtained by further screening.

MATERIALS AND METHODS

Gel shift assay and DNase I footprinting using nuclear extract

Nuclear extract from liver of 5-week-old Sprague-Dawley rats was prepared as described by Gorski *et al.* (18). Rats were sacrificed under ether anesthesia by exsanguination from neck. Probe, specific competitor (nucleotides 458 to 580, nucleotide of the major transcription initiation, P2, numbered 1; see ref. 19) and non-specific, unrelated competitor (−532 to −453) DNA's were prepared by PCR using template plasmid pMP4

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which carried upstream exon 1 and part of intron 1 of rat c-*myc* (19). One of the primers was labeled at the 5′-end with ^{32}P by T_4 polynucleotide kinase and $\gamma^{32}P$-ATP (16), and used to amplify the target sequence to obtain the probe. Binding reactions were carried out for 15 min on ice in 20 ml of 15 mM HEPES-KOH, pH 7.6, 50 mM KCl, 0.05 mM EDTA, 12 % glycerol, 1 mM DTT, 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl-fluoride (PMSF, Sigma) and 2 mg of poly(dA-dT):poly(dA-dT) (Pharmacia) (18). The complexes formed were analyzed either by gel electrophoresis in 0.25×TBE or by DNase I footprinting as described by Lichtsteiner *et al.* (20).

Southwestern and Southern screening

A λgt11 liver cDNA library of Sprague-Dawley rat (Clonetech) was used for Southwestern screening (21). Concatemer of one of the protein binding sites in intron 1 (TAGTTGGGGTA-GGCCGGGGC; nucleotides 510 to 529) was used as a probe. A λZAPII brain cDNA library of Sprague-Dawley rat (Stratagene) was used for further screening to obtain longer cDNA by Southern hybridization. Inserts of the λZAPII clones were converted to plasmids by methods suggested by the supplier. Appropriate DNA segments were excised from plasmids, labeled by the random priming method (Multiprime DNA Labeling System, Amersham) and used as probes for hybridization. Standard methods were followed for other procedures of cloning and DNA analysis (22).

Sequencing

Nucleotide sequences were determined by an automated DNA sequencer (A.L.F. DNA sequencer, Pharmacia). A Delta-*Taq* DNA sequencing kit (USB), Circum*Vent* DNA sequencing kit (New England Bio-Labs) or AutoRead sequencing kit (Pharmacia) was used for sequencing reactions. 5′-Fluorescein-labeled primers were made with a DNA synthesizer with FluorePrime (Pharmacia) and purified as specified by the supplier. Sequence data were assembled and analyzed with PC GENE program (Intelligenetics).

Northern blot analysis

RNA of various rat tissues was extracted by the acid guanidinium thiocyanate−phenol−chloroform (AGPC) method (23). Total RNA, 5 μg per lane, was applied to formaline−agarose gel, separated by electrophoresis, blotted onto nylon membrane and hybridized as described previously (5). A fragment near the 5′-end of the coding region (A in Fig. 2) was excised from plasmid p111, purified by gel electrophoresis, and labeled by the random priming method for use as a probe.

Methylation interference analysis of β-gal-MIBP1 fusion protein

β-Galactosidase−MIBP1 fusion protein was expressed using an *E.coli* clone harboring λ3a-21 (Fig. 2). IPTG-induced bacterial cells were collected and suspended in a solution containing 25mM HEPES-KOH, pH 7.6, 40mM KCl, 0.1mM EDTA, 10% glycerol, 1mM DTT and 0.1mM PMSF (18). Cells were disrupted by sonication, and centrifuged to collect the supernatant. This crude extract was further purified by precipitation with a 33% saturation of ammonium sulfate. The pellet was dissolved, dialyzed against the suspending solution, and used for analysis.

A probe, an 89-bp fragment of nucleotides 491 to 579 in rat c-*myc* intron 1, was prepared by PCR using labeled primers as described above. Competitor fragments were also made by PCR using unlabeled primers. Binding reactions and gel electrophoresis were carried out as described for the assay of nuclear extract. Methylation interference analysis was performed as previously described (24).

RESULTS

Protein-binding sites in c-*myc* intron 1

We have searched for possible protein-binding sites in the exon 1−intron 1 region of rat c-*myc* by gel shift and DNase footprinting experiments using rat liver nuclear extract. Several sites were identified by these analyses. One of which, a gel shift assay of a 123-bp region in the 5′-portion of c-*myc* intron 1 is shown in Figure 1. This region included sequence elements which are suggested to regulate c-*myc* expression in mouse or human cells (14,15). The gel shift assay revealed at least 7 distinct bands, suggesting the formation of specific complexes between the probe and nuclear proteins of rat liver. Many of the bands disappeared with competition from the same sequence, but not with the unrelated sequence (Fig. 1b). Thus, these complex formations were sequence-specific.

DNase footprinting analysis revealed that distinct regions within this DNA fragment were protected by nuclear proteins (Fig. 1c). Prominent protection was observed at 522 to 535 on the upper (non-coding) strand and at 518 to 545 on the lower (coding) strand. Several hypersensitive sites were also observed both at protected regions and at positions distal to them, which suggested that there were more than two protein-binding sites on this fragment. Methylation interference analysis suggested that the protected region shown in Figure 1b could be divided into two sub-regions (518−524, 525−545), each of which accepting a distinct protein to form complexes with different mobilities in gel shift analysis (data not shown).

Cloning of MIBP1 cDNA

Analyses of protein binding to the 5′-portion of intron 1 using the nuclear extract suggested that there is one definite binding site near nucleotides 510 to 530. Therefore, we screened a λgt11 rat liver cDNA expression library by the southwestern method using a concatemer of this intron region as a probe, following the procedures of Vinson *et al.* (21). A single cDNA clone, λ3a-21, which carried a 2.4-kb insert, was obtained by screening 2×10^6 phages. Nucleotide sequence determination of this clone revealed a small open reading frame (ORF, 237 amino acids) which had two C2H2 zinc finger motifs located side by side, at the 5′-terminal side of the insert. We referred the gene for this ORF as MIBP1. However, there were several reasons why this clone was apparently chimeric. 1. There were three *Eco*RI sites within the insert, and a stop codon was found just after the second *Eco*RI site. 2. Northern blotting revealed mRNAs of different sizes when fragments of the 5′- or 3′-side of the second *Eco*RI site were used as probes.

We then tried to obtain a non-chimeric, full-length cDNA by hybridization screening. For this purpose, a rat brain cDNA library was used because preliminary Northern blot analysis indicated a high level of MIBP1 mRNA expression in the brain. Probes C and D (Fig. 2), which were located outside the zinc finger motifs, were used for plaque hybridization. Fifteen positive clones were identified by this screening. The length and sequences (300−400 bases) of both ends of the insert of each clone were determined. The entire sequence of the longest clone, pd1, was then determined using internal primers, some of which were

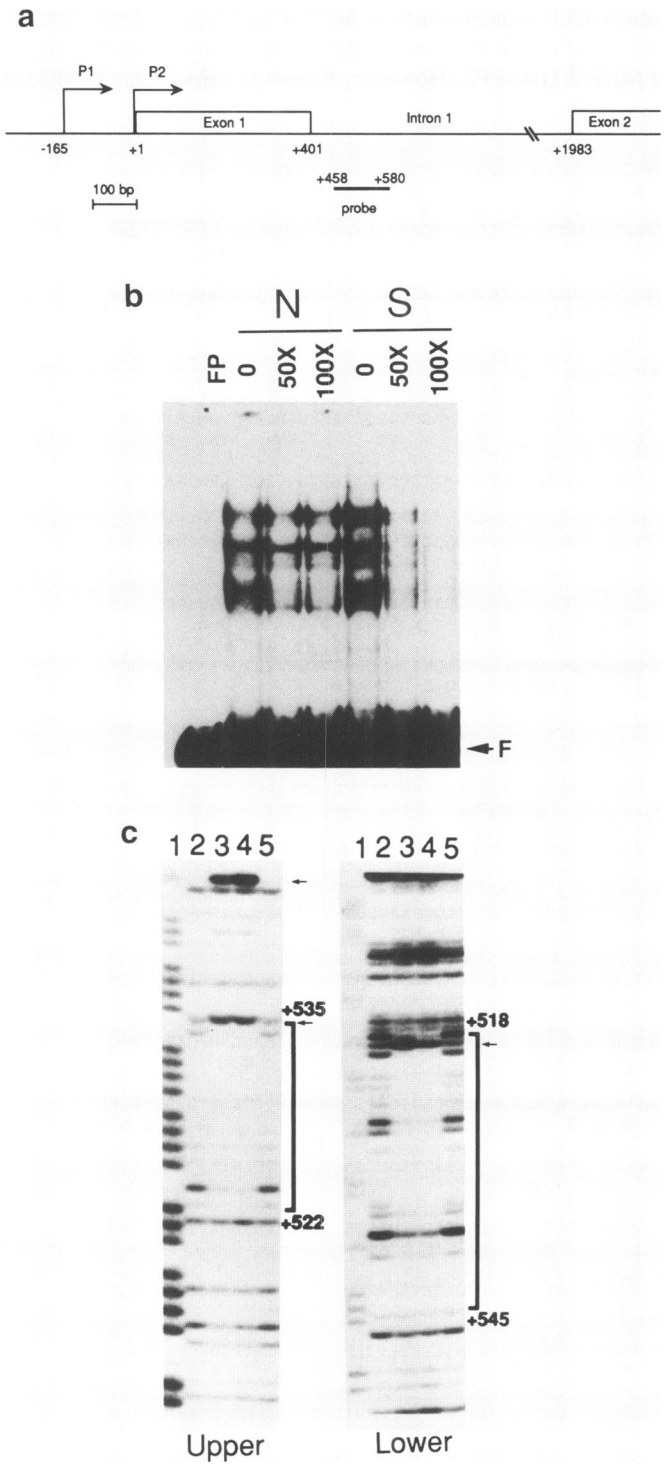

Figure 1. Binding of rat liver nuclear proteins to c-*myc* intron 1. A 123-bp fragment in intron 1 (**a**) was used as a probe to study protein binding sites within this region. Minor and major promoters are indicated as P1 and P2, respectively. (**b**) Gel shift assay. F, free probe. FP, probe alone incubated without nuclear extract. Competitors (N, non-specific; S, specific) are indicated above the top line, while their amounts (expressed as multiples of the probe) are indicated below the line. (**c**) DNase I footprinting. Vertical bars at the side of the autoradiogram indicate maximal regions of protection. Sites that became hypersensitive with protein binding are marked by arrows. Products of A/G reactions in the Maxam–Gilbert chemical cleavage method were loaded in lane 1. The labeled probes were treated with DNase I in the absence (lanes 2, 5), or presence of 10 μg (lane 3) or 20 μg (lane 4) of normal rat liver nuclear extract.

synthesized according to the end-sequences of each clone. A long ORF was found, which continued to the 5′-end of pd1, suggesting that this reading frame extended further upstream. Therefore, we re-screened the same cDNA library using probe B, which was located at the 5′-end of pd1 (Fig. 2). Twenty-two clones were obtained, and each clone was assembled as described above. The p111 clone carried the longest insert and extended to the 5′-end. Clone p128 covered the 3′-end including poly(A) signal and a poly(A) stretch. Thus, a total of 37 clones which constituted a contig of 9.8 kb were obtained (Fig. 2).

Nucleotide sequence of MIBP1 cDNA and protein

The entire nucleotide sequence of MIBP1 cDNA was obtained as a composite of sequences of pd1, p111 and other clones. The entire region covered by the pd1 and p111 plasmids was sequenced on both strands. Figure 3 shows the amino acid sequences of MIBP1 protein (nucleotide sequence deposited to DDBJ, accession no. D37951). We reasoned that the initiation codon was at nucleotides 767–769, because an in-frame stop codon was found 24 bp upstream from this initiation codon and the sequence around this ATG agreed with Kozak's rule (25). The termination codon of this ORF was found at nucleotides 8075–8077. The putative MIBP1 protein consisted of 2437 amino acids and its expected molecular weight was about 267 kd.

The MIBP1 protein has two zinc finger cluster regions in the N-terminus (from codons 191 to 241) and in the C-terminus (from codons 1792 to 1842) of the protein, each cluster containing two C2H2 zinc finger motifs. A nuclear localization signal and the acidic region were found at codons 935–941 and downstream of the second zinc finger cluster, respectively. Several serine/threonine-rich regions were also found scattered throughout this cDNA.

Homology

The homology of MIBP1 to other sequences was examined against GenBank, EMBL and SWISSPROT databases using the PC-GENE program. AGIE-BP1 (rat angiotensinogen gene-inducible enhancer-binding protein 1, ref. 26) and AT-BP1 (rat α1-antitrypsin promoter binding protein 1, ref. 27) were almost identical to the C-terminal region of MIBP1, and are likely to be partial clones of MIBP1 cDNA. The AGIE-BP1 sequence corresponded to the region from 5332 to 9106 of MIBP1 cDNA. The AT-BP1 clone was shorter than AGIE-BP1.

In addition, the MIBP1 sequence was highly homologous to human MBP-2/HIV-EP2 (MHC binding protein-2/human immunodeficiency virus type 1-enhancer binding protein 2) (28, 29). Rat MIBP1 and human MBP-2/HIV-EP2 were 86% and 93% similar in nucleotide sequence and amino acid sequence,

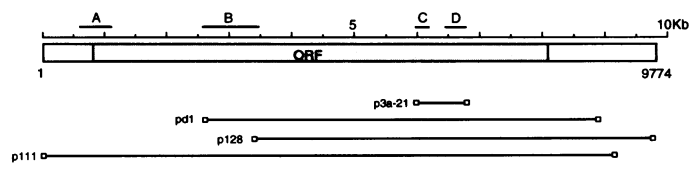

Figure 2. Gross structure of MIBP1 cDNA. λ3a-21 was obtained by southwestern screening. Fragments A was used as a probe for Northern blotting, and fragments B, C and D were used as probes for hybridization screening. Some representative clones are indicated below.

respectively, when aligned by the GAP program from UWGCG (30). Therefore, MIBP1 is most likely the rat counterpart of human MBP-2/HIV-EP2. Figure 3 shows the alignment of the amino acid sequences of rat MIBP1 and human MBP-2/HIV-EP2. Zinc finger motifs of MIBP1 were exactly the same as those of MBP-2/HIV-EP2. However, there was a discrepancy between

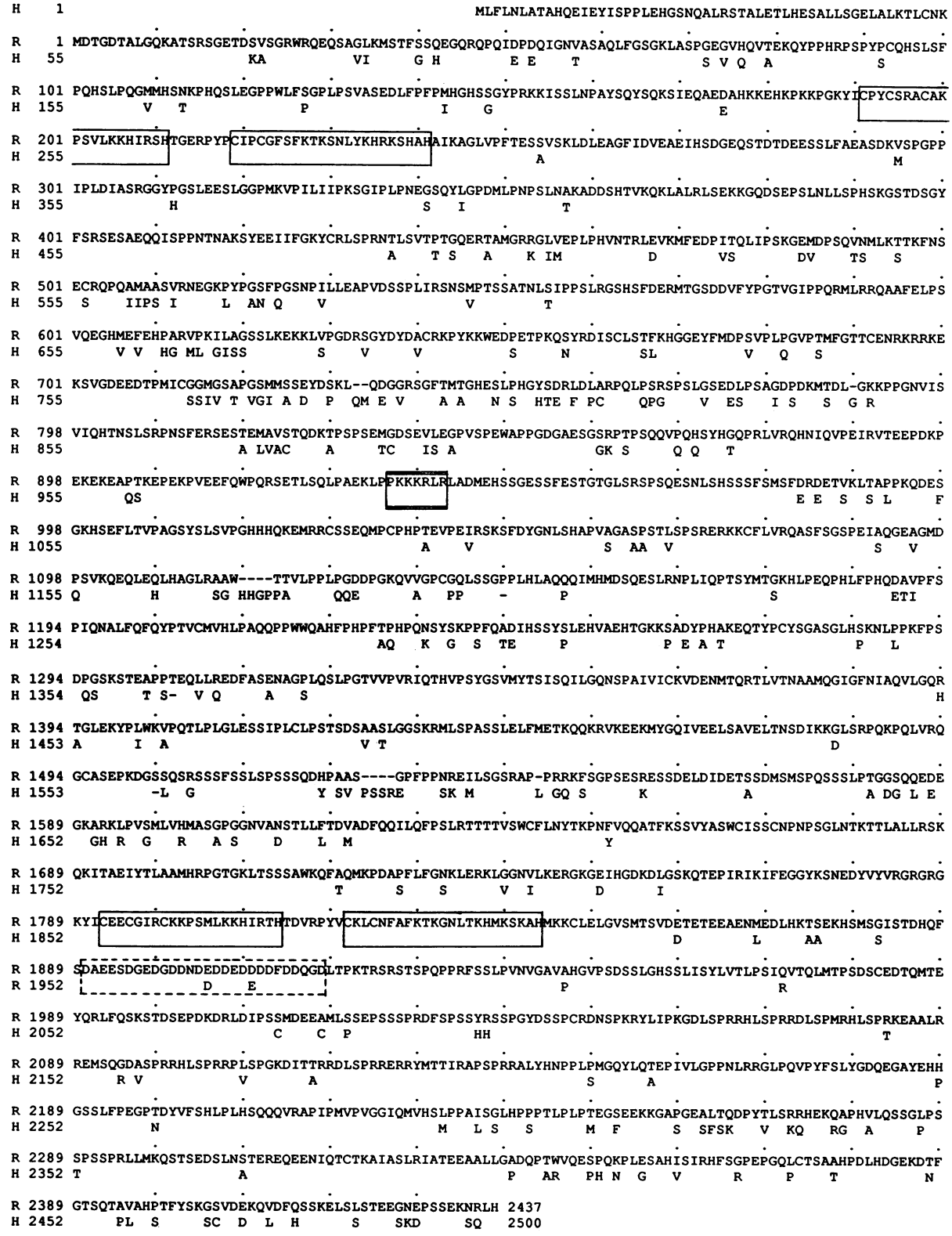

Figure 3. Comparison of amino acid sequences of rat and human MIBP1/MBP-2 . The entire sequence of the rat MIBP1 protein is shown at the top. Only amino acids in the human MBP-2 protein which are different from those of MIBP1 are shown. Gaps are hyphenated. Zinc finger motifs, acidic region and nuclear localization signal are boxed with plain, broken and double lines, respectively.

the two amino acid sequences regarding the position of the initiation methionine. The initiation codon of rat MIBP1 corresponded to the second ATG in human MBP-2. As Van't Veer *et al.* have noted (28), the second ATG of MBP-2/HIV-EP2 might be the actual initiation codon. The fact that the nucleotide sequence near the second ATG agreed with Kozak's sequence supports this conclusion.

MIBP1 also showed a 50 % similarity in amino acid sequence to human PRDII-BF1 (Interferon β promoter binding protein; also known as MBP-1/HIV-EP1) (31). The two widely separated zinc finger cluster regions in these two proteins were highly homologous. All four zinc finger motifs were similar between rat MIBP1 and human PRDII-BF1, with 85−100 % identity. The nuclear-localization signal and the acidic region were similar. Both proteins also contained serine/threonine-rich regions. Therefore, the MIBP1 protein (MBP-2/HIV-EP2 protein in human) and the PRDII-BF1/MBP-1/HIV-EP1 protein belong to the two-handed zinc finger protein family.

MIBP1 mRNA expression

Expression of MIBP1 mRNA in various tissues was examined by Northern blot analysis. As shown in Figure 4, MIBP1 mRNA was detected as a single band near 10kb. The same single band was also observed when a much longer fragment (from position 646 to 8887) was used as a probe (data not shown). The amount of MIBP1 mRNA was very low in the liver, but high in the brain. In other tissues, e.g. spleen, kidney and muscle, the expression was moderate. No significant change in the level of MIBP1 mRNA was observed in regenerating liver (data not shown).

DNA binding of MIBP1 protein

We next examined the binding specificity of MIBP1 protein to DNA by gel shift analysis using the λ3a-21 clone. A protein, *E.coli* β-galactosidase at the N-terminus, fused to 271 amino acids of the second zinc finger cluster near the C-terminal region of MIBP1 (corresponding to codon 1735−2005), appended by C-terminal 5 amino acids of unknown origin, is expected to be expressed under the control of the *lac* promoter-operator.

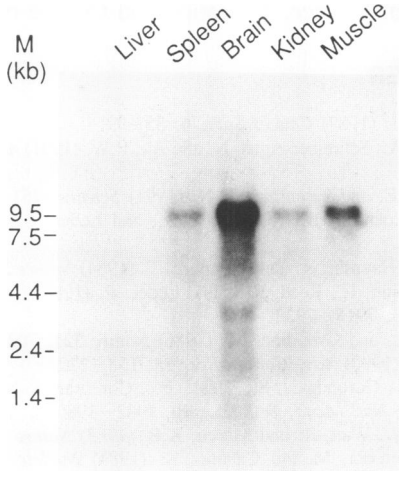

Figure 4. Northern blot analysis. Total RNA from liver, spleen, kidney, brain and muscle were used. Fragment A of Figure 2 was used as a probe.

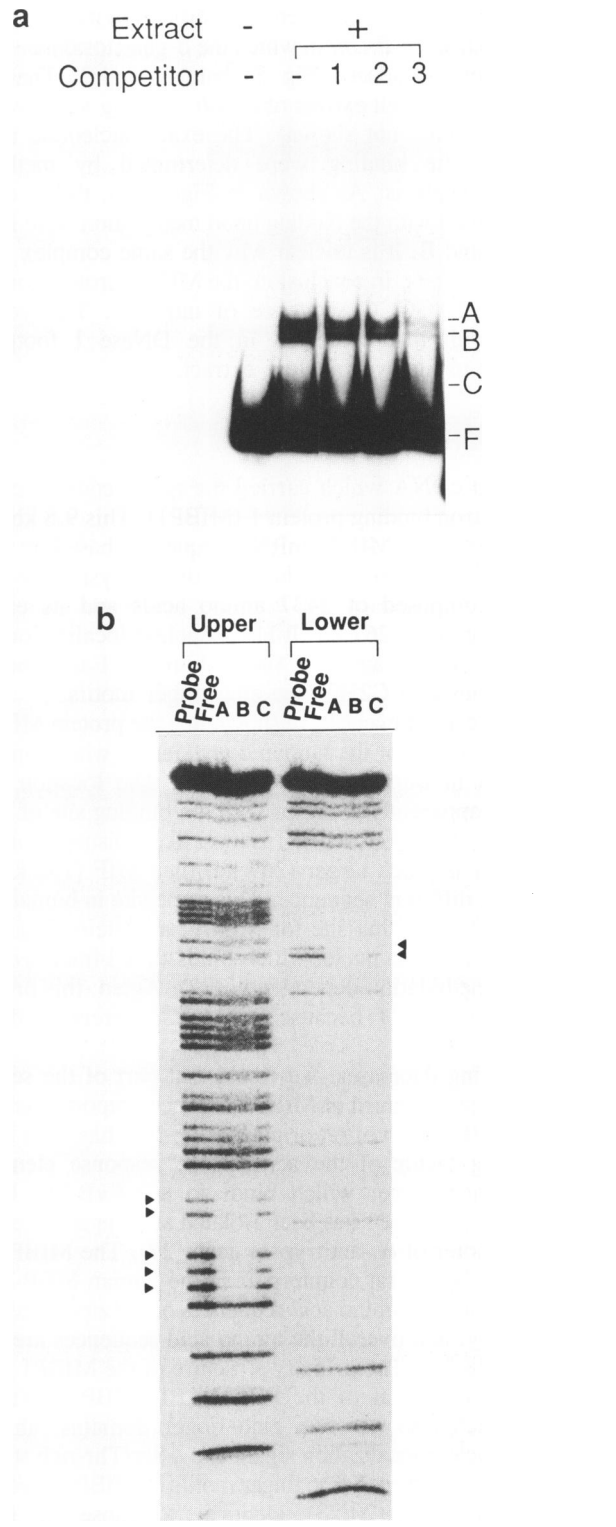

Figure 5. Binding activity of the MIBP1 protein. (**a**) Gel shift analysis was performed without competitor and with a 100-fold molar excess of competitor fragments. Two fragments of similar lengths from c-*myc* exon 3 (competitor 1, nucleotides 6634 to 6765; competitor 2, nucleotides 6799 to 6916) were used as competitors with unrelated sequences. Unlabeled probe fragments (competitor 3) served as a specific competitor. Two specific bands are marked A and B, while the non-specific band is marked C. F denotes free probe. (**b**) Methylation interference analysis of bands A, B and C. Arrows indicate G-residues recognized by MIBP1.

By gel shift analysis, sequence-specific activity was detected in the cell extract of *E.coli* in which the β-galactosidase−MIBP1 fusion protein was induced (Fig. 5a, bands A and B). These bands were absent when cell extract of *E.coli* carrying λgt11 was used in the assay (data not shown). The exact nucleotide residues involved in the binding were determined by methylation interference analysis. As shown in Figure 5b, the G residues which interfered with the binding upon methylation were identical in bands A and B. It is unclear why the same complex showed two different bands. In conclusion, the MIBP1 protein could bind to the GGGTAGGCC sequence of intron 1. This sequence coincided with that protected in the DNase I footprinting experiments using liver nuclear extract.

DISCUSSION

We obtained cDNA which carried the entire coding region of the c-*myc* intron binding protein 1 (MIBP1). This 9.8 kb cDNA covered most of the MIBP1 mRNA sequence, based on the size of the message (10 kb) in Northern blotting analysis. The MIBP1 protein is composed of 2437 amino acids and its expected molecular weight is 267 kd. It has a nuclear localization signal and two widely separated zinc finger domains. Each zinc finger domain carries two C2H2-type zinc finger motifs.

Zajac-Kaye and Levens have shown that the protein MIF binds to a specific region of the human c-*myc* intron where mutations are frequent in Burkitt lymphoma (15). The location of this binding site apparently overlaps with the binding site of MIBP1 reported here. However, MIBP1 is most likely distinct from MIF, since MIBP1 is much larger (267 kd) than MIF (138 kd), and recognizes a different sequence. This same site in human c-*myc* seems to be the binding site for MDBP, a protein ubiquitously found in mammalian nuclei which binds to a 14mer sequence, sometimes methylation-dependently (32). Again, this protein is distinct from MIBP1 because it has a different recognition sequence.

By searching databases, we found that part of the sequence (the C-terminal one-third of MIBP1) has been reported as AGIE-BP1 or AT-BP1, both of rat origin. AGIE-BP1 has been isolated as a binding factor of the acute-phase response element of angiotensinogen gene, which binds to a NFκB-like binding sequence (26). AT-BP1 has been isolated as a negative regulator for the promoter of α_1-antitrypsin gene (27). The MIBP1 gene is thought to be the rat counterpart of the human MBP-2/HIV-EP2 gene, since the amino acid sequences of the zinc finger motif match exactly, and overall the amino acid sequences are highly conserved (28,29). The primary structure of the MIBP1 protein is also similar to that of the PRDII-BF1/ MBP-1/ HIV-EP1 protein, which also has two zinc finger domains, an acidic domain, a nuclear localization signal and a Ser/Thr-rich structure (31). The two C-terminal zinc finger motifs of MIBP1 were 100% (21/21) and 86% (20/23) identical to those of PRDII-BF1/MBP-1/HIV-EP1.

We also demonstrated that a bacterially synthesized fusion protein which carries the C-terminal zinc finger domain of MIBP1 is able to bind sequence-specifically to a motif (GGGTAGGCC) which is found in intron 1 of rat c-*myc*. The C-terminal zinc finger clusters of AT-BP1, AGIE-BP1, MBP2 and PRDII-BF1 have been shown to bind to NFκB-like motifs. Particularly strong bindings of these proteins to the MHC-1 promoter motif (GGGG-ATTCCCC) have been reported (26−28,31). Considering all of these results, we can conclude that proteins in this family can bind to the $GGGN_{(4-5)}CC$ sequence as a minimal requirement. In the case of PRDII-BF1, the binding specificity of the N-terminal zinc finger cluster is the same as that of the C-terminal (31). The binding activity of the N-terminal zinc fingers of MIBP1 has not yet been examined, since expression of this portion of the protein in *E.coli* has been unsuccessful to date.

The $GGGN_4CC$ motif is also found at a similar position in the 5′-region of intron 1 of human c-*myc*, suggesting that MIBP1 is important in the regulation of c-*myc* regardless of the species. Both AGIE-BP1 and human MBP-2 suppress transcription of the corresponding targets, i.e., angiotensinogen gene and MHC class I genes, respectively (26,28). In view of these findings, MIBP1 may also serve as a negative regulator of c-*myc*, possibly by competing with NFκB-like factor(s) in the binding to the target sequence. The level of MIBP1 mRNA is high in the brain or muscle, but low in normal liver, while both tissues consist of non-dividing cells and show low c-*myc* expression. Therefore, there is no simple correlation between the MIBP1 mRNA level and c-*myc* expression. Perhaps, post-translational modification plays an important role in the function of MIBP1. There are also other possible explanations. Liver cells have the capacity to acutely express c-*myc*. mRNA immediately after exposure to growth stimuli, as in the case of regenerating liver after partial hepatectomy (5). Brain and muscle cells are inert with respect to growth, and remain quiescent for their entire life. Thus, MIBP1 is not a quickly responding transcriptional regulator of c-*myc*. Instead, it may maintain the quiescent state of the cell as a long-term regulator of c-*myc*. Moderate expression of MIBP1 mRNA in the spleen or kidney may be explained by the heterogeneous cell population in these organs. Now that a cDNA clone for MIBP1 is available, it will be possible to analyze whether and how MIBP1 is involved in the regulation of c-*myc*.

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