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Unusual DNA binding characteristics of an *in vitro* translation product of the CCAAT binding protein mYB-1

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ABSTRACT

We have isolated a cDNA that encodes the murine CCAAT-binding protein mYB-1. The deduced amino acid sequence shows 95% identity with its presumed human homologue (hYB-1A) which was originally isolated as a protein that binds to the Y box of MHC class II genes. *In vitro* translated mYB-1 binds to CCAAT boxes of the MHCIIEα, HSVTK and mouse PCNA promoters but not to α-globin or human thymidine kinase CCAAT boxes. Interestingly, complexes formed between the *in vitro* translated protein and the various CCAAT boxes display the property of being competed more efficiently with self competitor DNA, regardless of the CCAAT box initially used as a probe. A similar phenomenon was observed in a cell extract of Con-A stimulated murine splenocytes when the same competition assays were performed. These results may reflect the generation of multiple forms of a particular CCAAT-binding protein, such as mYB-1, that display distinct, yet overlapping, DNA binding specificities.

INTRODUCTION

The CCAAT-box has been studied extensively as a transcription-enhancing element (1–5). Several CCAAT binding factors, including C/EBP and CTF/NF-1 have been identified and characterized (4, 6–13). C/EBP (first named CBP) was identified in rat liver according to its binding affinity to the CCAAT sequence in the HSVTK promoter (6). The same protein was purified subsequently as a common animal virus enhancer-binding protein (7). CTF is responsible for selective recognition of eukaryotic promoters that contain the sequence CCAAT (9), while NF-1 was first described as an adenovirus replication protein (14–16). These two factors were compared by Jones et al.(8) and suggested to be indistinguishable, both functionally and immunologically. C/EBP is distinguishable from CTF/NF-1 by its binding specificity and its thermal stability (4). In addition to these two very well characterized CCAAT-binding proteins, numerous others have been identified (23–26,33).

A DNA region containing an inverted CCAAT sequence called the Y-Box is a promoter element common to all major histocompatibility class II genes(17–20). The first Y box-binding protein characterized was designated NF-Y(21). Comparison of NF-Y with other CCAAT box-binding proteins suggested that NF-Y is distinct from C/EBP and CTF/NF-1 (22). Recently, two subunits of NF-Y were cloned and found to be homologous, in certain regions, to the yeast transcriptional activators HAP2 and HAP3 (23). Chodosh et al. (24) identified 2 CCAAT-binding proteins in human cells that they designated CP1 and CP2. Their relationship to other CCAAT-binding proteins in mammalian cells was not established although CP1 was demonstrated to be functionally similar to HAP2 and HAP3 (25). This suggests that CP1 may be the same as NF-Y (23).

An additional Y box-binding protein (hYB-1A) cDNA was sequenced and characterized (26). A DNA-binding protein cloned from human placenta, dbpB, which showed binding affinity to the EGF receptor enhancer (27), was found to be homologous to hYB-1A. More recently, two other Y box-binding proteins, homologous to hYB-1A, were cloned from Xenopus (28). The cDNA for YB-1 has also been cloned from cDNA libraries from rat using another CCAAT probe (29), from mouse using an interferon response element (30), and from human using probes for the MHC W box (31) and depurinated DNA (32). The latter three probes lacked the CCAAT consensus sequence, suggesting that the DNA recognition site of YB-1, like C/EBP, is not restricted to the CCAAT motif.

We isolated a cDNA for the murine homolog of hYB-1A as a gene whose expression is induced by interleukin 2 (IL-2) in mouse T lymphocytes (35). We show here unique binding characteristics of its *in vitro* translation product and provide possible explanations for this unusual behavior.

MATERIALS AND METHODS

Plasmids construction and oligonucleotides

The cDNA of the mouse Y box-binding protein (YB-1) was initially cloned from a lambda gt10 cDNA library of an IL 2-stimulated cloned T cell line, L2 (35). Since mouse YB-1

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cDNA has an internal EcoRI site, two EcoRI fragments of the insert, named B22.2A and B22.2B, were isolated and subcloned into Bluescript SK(−) and sequenced. For *in vitro* translation, two identical constructs containing a cDNA of mouse YB-1, pBC-113 and pBC-117, were made as follows: the entire coding sequence of the mouse YB-1 cDNA in the lambda gt10 clone was amplified by polymerase chain reaction (36). The primer for 5′-end of the cDNA sequence starts from the first codon after Methionine and ends at the tenth codon (5′AGC AGC GAG GCC GAG ACC CAG CAG CCG3′). The 3′-end primer includes two possible stop codons and two restriction enzyme cutting sites (NheI and NotI) for later subcloning (5′AGC GCT AGC GGC CGC AGC CGG CGT TTA CTC A3′). NotI digestion of the PCR product yielded a 5′ blunt end starting from the second codon of mouse YB-1 cDNA coding sequence and a NotI site at the 3′-end. This was then subcloned into pBβG5′ which is Bluescript SK(−) containing a 94 bp nucleotide inserted between the 5′ HindIII and EcoRI 3′ sites. This 94 bp nucleotide includes 78 bp of human β-globin mRNA 5′ untranslated region and the initiation codon. The purpose of this insertion will be described in the text. The construction strategy and final plasmid product are shown in Fig. 1.

Oligonucleotides used as probes or competitors for the binding assay are shown in Fig. 2. All of the oligonucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry on a DNA synthesizer manufactured by Applied Biosystems. Probes were end-labeled with [gamma-^{32}P]ATP. Both competitors and probes were purified by 20% TBE-PAGE.

DNA sequencing

CTAB plasmid minipreparations were used (37) and the DNA sequencing protocol was based on the Sanger method (38). The reagents used for DNA sequencing were from the Sequenase Kit of USB Co. (Cleveland, OH).

In vitro transcription and *in vitro* translation

RNA was made with an mCAP mRNA Capping Kit (Stratagene, La jolla, CA). One microgram of linearized RNase-free plasmid DNA was used for each transcription reaction and the transcribed RNA was translated at 30°C by using either the *In vitro* Translation Kit from Stratagene or RNase treated rabbit reticulocyte lysate from Promega (Madison, WI) without noticeable difference. The presence of the translation product (mYB-1 protein) was confirmed by SDS PAGE. Both RNA and protein products were kept at −80°C before use.

SDS PAGE

The method of Laemmli (39) was used. Five microliters of [^{35}-S] methionine (>1000Ci/mmol, Amersham, Co., Arlington Heights, IL) incorporated translation product was mixed with equal volume of 2× loading buffer (4% SDS, 20% glycerol, 160 mM Tris-HCl pH 6.8, 0.2 M DTT, and 0.002% bromophenol blue), boiled for 5 min, separated on a 12.5% SDS-polyacrylamide gel, and analyzed by autoradiography.

Preparation of cell extract

The cell extract of murine splenocytes was made by the method of Dignam et al. (40) except the use of lysis buffer was omitted. The splenocytes were collected by separating them from RBC using density gradient centrifugation through ficoll hypaque (1.090 g/ml). Splenocytes at a concentration of 6×10^6/ml in DMEM culture medium (35) containing 500 ng/ml of Con-A were incubated in 5% CO_2-37°C for 72 hours prior to the extraction.

The protein concentration of the cell and nuclear extract were quantitated using a Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA). The extracts were kept at −80°C before use.

Gel retardation assay

One microliter of mYB-1 *in vitro* translation product or two micrograms of nuclear extract was added to a binding mixture consisting of 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 1 microgram of poly[dI-dC].poly[dI-dC] (Sigma) and the indicated amount of

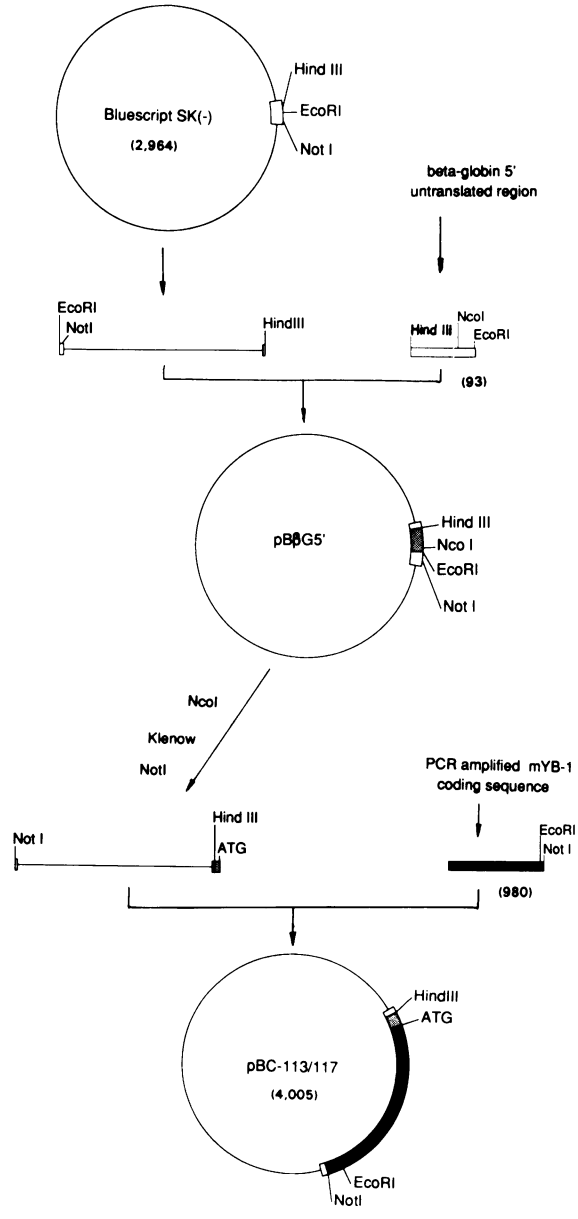

Fig. 1. Preparation of an mYB-1 construct for *in vitro* expression. To improve *in vitro* translation, 78 nucleotides corresponding to the β-globin mRNA untranslated sequence were placed 5′ to the mYB-1 coding sequence. Two constructs (pBC-113 and pBC-117) were isolated and tested in *in vitro* transcription and translation assays.

oligonucleotide (as competitor) at room temperature. The binding reaction was initiated by addition of 5000 cpm of probe (0.44 fmol). One microliter of 0.02% bromophenol blue was added 30 min later. The binding complexes were separated by 4.5% polyacrylamide gel electrophoresis at 4°C in buffer containing 25 mM Tris-190 mM glycine-1 mM EDTA, at a constant current of 30 mA per 20cm×20cm gel (41−43).

For competition assays, increasing amounts of competitor were added to the *in vitro* translation product or cell extract prior to the addition of probe. One nanogram of competitor is equivalent to a 100-fold excess of the amount of radiolabeled probe (5000 cpm, 0.45 fmol).

RESULTS

Sequence of mouse YB-1 and its similarity with human YB-1A

Mouse YB-1 cDNA was isolated from a lambda gt10 library prepared from IL-2 stimulated cloned T cells (35). The cDNA clone contains the entire 966 bp of coding sequence, 28 bp of 5′-untranslated region and 491 bp of 3′-untranslated region including a poly A tail (the sequence was submitted to EMBL, accession number X57621). The coding region of this mYB-1 cDNA has 95% homology with the coding sequence of human YB-1 (26) and 96.6% homology in the putative amino acid sequence. The mYB-1 cDNA contains 21 more bp at the 3′ end of the coding region that encodes 7 more amino acids than human YB-1A at the carboxyl terminus. There are deletions of three proline codons near the amino terminus of mYB-1. Furthermore, the six Arg-rich regions in human YB-1A showed no difference to those in mYB-1 except for the conversion of one amino acid from Gly^{200} to Ala^{200} in the third Arg-rich region.

Construction of pBC-113 and pBC-117

We used the cDNA sequence described above as a template to transcribe mYB-1 RNA. But the RNA made from *in vitro* transcription of this cDNA could not be translated by *in vitro* translation systems. A similar problem has been observed with other cloned cDNA constructs (personal communication, Dr. Thomas Kadesch). Since insufficient length of 5′ untranslated sequences can cause recognition and binding failure of ribosomes, we inserted 78 bp of human β-globin 5′-untranslated region (44,45) adjacent to the initiation codon of the mouse YB-1 cDNA. The preparation of the construct and the final plasmid construct (pBC-113 and pBC-117) are described in Fig. 1.

In vitro translation product of pBC-113/117

The mYB-1 *in vitro* translation product from either pBC-113 or pBC-117 has an apparent molecular weight of 42,000 daltons as determined by SDS-PAGE (Fig. 3). This agrees with the calculated molecular weight of 41,610 daltons. The observation of a single labeled protein after *in vitro* translation also indicated that there was a very low level of translation from endogenous mRNA in the rabbit reticulocyte lysate. The *in vitro* translation control was made by adding the same volume of DEP-treated water into the translation reaction mixture instead of mYB-1 transcript.

MHCIIEα	5′ ctgaaacatttttctgATTGGttaaaagttgag	3′
	3′ gactttgtaaaaagacTAACCaattttcaactc	5′
HSVTK	5′ cccagcgtcttgtcATTGGcgaattcgaaca	3′
	3′ gggtcgcagaacagTAACCgcttaagcttgt	5′
m-PCNA	5′ ccacgggtacgATTGGtccttgaggagagg	3′
	3′ ggtgcccatgcTAACCaggaactcctctcc	5′
α-GLOBIN	5′ ggcggcgctcATTGGctggcgcggagcccg	3′
	3′ ccgccgcgagTAACCgaccgcgcctcgggc	5′
β-GLOBIN	5′ gtgtgagcagATTGGcccttaccagggtgt	3′
	3′ cacactcgtcTAACCgggaatggtcccaca	5′

Fig. 2. Oligonucleotides used in binding assays. The inverted CCAAT boxes are in capital letters.

Self- and cross-competition of mYB-1 binding to CCAAT-containing sequences

Competition experiments were performed by adding increasing amounts of unlabeled probe to the binding mixture containing *in vitro* translated mYB-1, prior to the addition of a fixed amount of ^{32}P-labeled self or different probe. The results shown in Fig. 4 are representative of at least three experiments. For self-competition, MHCIIEα, HSVTK and mouse PCNA showed similar dose response curves (Fig. 4A). Results of the cross-competition experiments indicated that each of the sequences competed better for self (80−90% inhibition) than for others (10−40% inhibition) (Fig 4B).

A representative gel shift analysis in which five different inverted CCAAT sequences competed for mYB-1 binding of the MHCIIEα probe is shown in Fig. 4C. The mYB-1 protein showed specific binding to the inverted CCAAT sequence of MHCIIEα (Fig. 4, lane 7). Two binding complexes (A and B) were resolved. The higher band (complex A) represents specific binding, whereas the lower band (complex B) represents a background binding since it was also present in binding mixtures containing the *in vitro* translation control (no RNA; Fig. 4, lane 6). This lower band results from the binding of probe to an endogenous protein in the reticulocyte lysate. The background binding in the translation control with probe of MHCIIEα was much stronger than that in the mYB-1 translation product. It is likely that the presence of mYB-1 diminishes the concentration

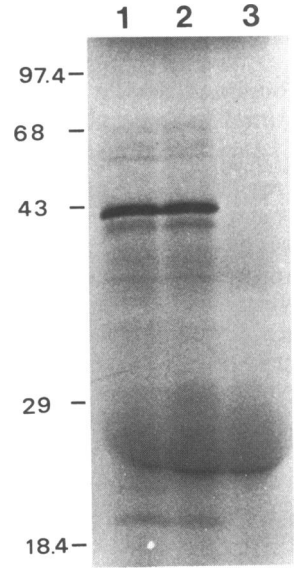

Fig. 3. SDS-PAGE of mYB-1 *In vitro* translation product. Molecular size markers in kDa are shown on the left. Lanes 1 and 2 depict the mYB-1 *in vitro* product from pBC-113 and pBC-117. Lane 3 shows the result of *in vitro* translation negative control.

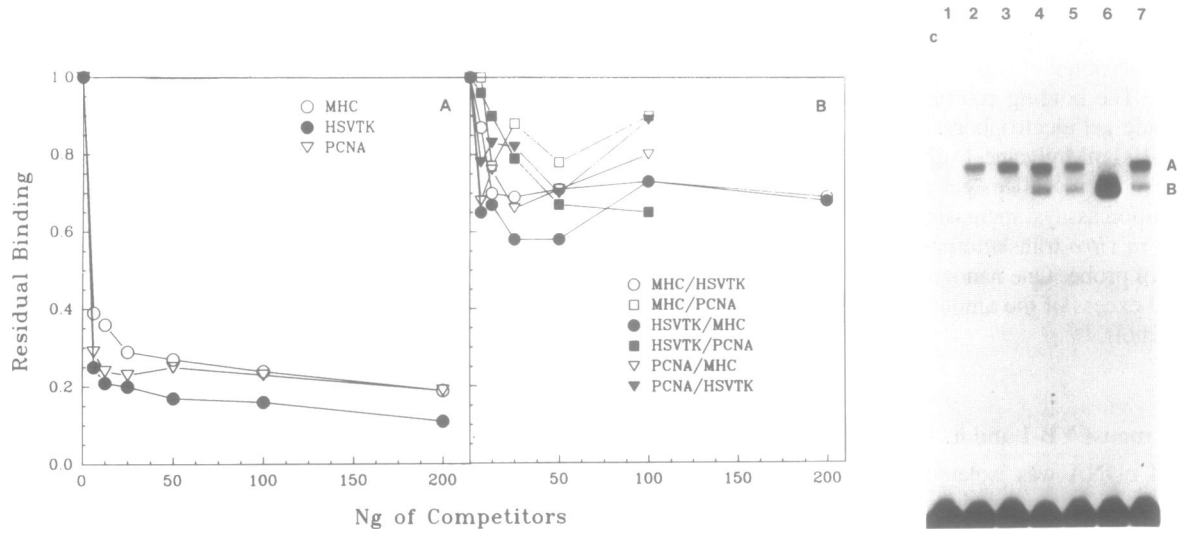

Fig. 4. Competitive binding characteristics of CCAAT containing sequencesfor the mYB-1 *in vitro* translation product. Indicated amounts of unlabeled competitors (one nanogram of competitor is equal to 45 fmol) were added to the incubation mixture prior to the addition of one microliter of mYB-1 *in vitro* translation product. The incubation period was initiated by the subsequent addition of labeled probe (0.45 fmol). Therefore, 1 ng of competitor is equal to a 100-fold excess of the amount of radiolabeled probe used. Panel A shows the dose response curves of self-competition. Different amounts of competitor were added up to 200 nanograms as shown. Panel B shows dose response curves of cross-competition. The symbols corresponding to each probe and competitor are illustrated in the panels. In panel B, the name of probes and competitors are written as probe/competitor. In panel C, 50 ng of each competitor were used in the binding reaction. Lane 7 shows the mYB-1 *in vitro* translation product binding to MHCIIEα without competitor. Competitors used in lanes 1 to 5 were: MHCIIEα, HSVTK, mPCNA, α-globin and β-globin. Lane 6 shows the background binding resulting from *in vitro* translation control binding to labeled MHCIIEα oligonucleotide.

of free probe below the dissociation constant of the background binding, thereby reducing the amount of complex B. Single-stranded non- specific oligonucleotide was also able to reduce this background binding (data not shown). Specific binding (complex A) to the MHCIIEα probe was competed effectively with 50 ng (5000 fold excess) of homologous competitor (lane 1); other oligonucleotides containing CCAAT sequences in the same molar excess showed very weak to no competition (lanes 2–5).

A similar unusual binding behavior of a CCAAT binding-protein in Con-A stimulated splenocytes

The unique binding behavior of mYB-1 *in vitro* translation product could have resulted from the artifactual generation of multiple forms of a single protein during *in vitro* translation or it could be an intrinsic property of the protein. To determine if an endogenous protein has similar binding properties, homologous and heterologous competition assays using MHC and PCNA CCAAT sequences were also performed with extacts from Con-A stimulated murine splenocytes (Fig. 5). Splenocyte extract forms multiple complexes with CCAAT sequences. Two of the complexes are shown in Fig 5. Complex 2 has a similar electrophoretic mobility as the mYB-1 *in vitro* translation product (data not shown) while complex 1 migrates more slowly. For complex 1, the MHC probe is a more efficient competitor in both self- and cross- competition assays. Therefore, the CCAAT binding protein in complex 1 appears to have a higher affinity for the MHC CCAAT sequence than for the PCNA CCAAT sequence. Conversely, for complex 2, self competition is more efficient for each probe than is cross competition. Thus the binding characteristics for complex 2 are the same as those found for mYB-1 *in vitro* translation product.

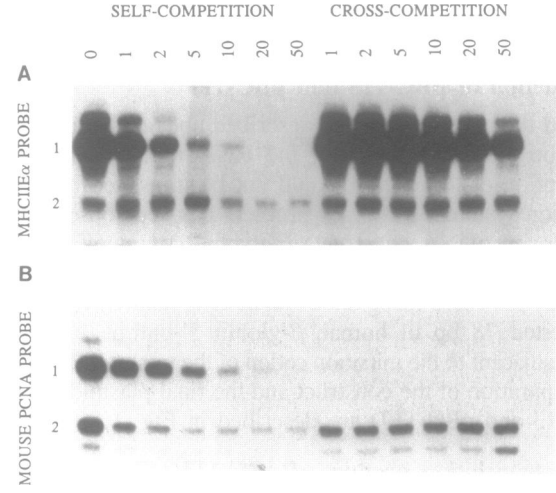

Fig. 5. Competitive binding characteristics of MHCIIEα and mouse PCNA CCAAT sequences for Con-A stimulated murine splenocyte derived complexes. Panel A: Binding of the MHC probe to Con-A stimulated splenocyte cell extract was competed by MHC (self) or mPCNA (cross) oligonucleotide. The number above each lane shows the amount of competitor used. 1 ng of competitor is equal to a 100-fold excess of the amount of radiolabeled probe used. Two binding complexes (1 and 2) are indicated. Panel B: Binding of the mPCNA probe to Con-A stimulated splenocytes was competed by mPCNA (self) or MHC (cross) oligonucleotide. The amount of competitor used is the same as that described in Panel A.

DISCUSSION

Two CCAAT-binding proteins have been identified that interact with the MHCIIEα Y box: NFY and YB-1. Dorn et al. (22) investigated the binding specificity of NFY for the MHC promoter CCAAT sequence relative to that found in several other

promoters. By competition assays, they ranked the relative affinity of NF-Y for the various CCAAT sequences as follows: MHCIIEα > α-globin > MSV > HSVTK > β-globin. They also identified a second binding activity that they designated as NFY*. This latter activity bound to an HSVTK probe more strongly than to an MHC probe as demonstrated by competition assays (22). Didier et al. isolated a cDNA clone for a CCAAT binding protein from a lambda gt11 human cDNA library by recognition of its binding activity using the Y box of MHC class II gene as a probe (26). This clone was designated hYB-1A and was considered different from NFY.

Subsequent to the original cloning of the cDNA for human YB-1 (26), cDNA clones of this protein have been isolated from human (27,31,32), mouse (30,35), rat (29) and Xenopus (28) cDNA libraries using a variety of different probes. The fact that some of these probes did not contain CCAAT boxes (30–32) and that the protein has a strong avidity for single-stranded or depurinated DNA could suggest that YB-1 is not truly a CCAAT binding transcription factor, but is instead involved in DNA repair (31). However, Tafuri and Wolfe (28) showed that Xenopus YB-1 stimulates transcription. Furthermore, some transcription factors such as C/EBP are known to bind to CCAAT boxes as well as other sequences (7). In addition, some transcription factors such as NF-1, are known to have other functions such as to facilitate DNA replication (14–16). Thus, the physiological function of YB-1 remains to be elucidated.

In this paper, we report the binding characteristics of the mouse YB-1 protein (mYB-1) for CCAAT containing probes. The cDNA clone was isolated as a gene that was responsive to IL-2 stimulation of T lymphocytes (35). The sequence of the cDNA and the protein encoded by it are 95% and 96.6% identical with those of the human YB-1A. *In vitro* translation of the protein encoded by this cDNA yielded a single product of the correct size (Fig. 3) that binds to several CCAAT sequences including MHCIIEα gene. These data indicate that identification of our clone as mouse YB-1 is correct.

The binding properties of the *in vitro* translation product of mYB-1 were examined by competition and binding assays. The relative binding affinities as assessed by the intensity of the specific signal in the gel shift assays were as follows: MHCIIEα > HSVTK > mPCNA >> β-globin with no binding to α-globin or human thymidine kinase CCAAT box probes (not shown). The observation that YB-1 can bind to some, but not all, CCAAT containing probes indicates that the protein has some sequence specificity, and does not bind exclusively to damaged DNA (31,32). Interestingly, all of the probes to which YB-1 binds have at least pairs of A nucleotides on one of the strands. The probe with the highest affinity for YB-1, MHCIIEα, is also the most AT rich suggesting that YB-1 may recognize non-CCAAT AT rich sequences.

Extensive titration of the competitors for the binding of the MHCIIEα probe indicated that no more than 40% of the binding could be competed, even by the best heterologous competitor, HSVTK (Fig. 4B and 4C). In contrast, more than 80% of the radioactive binding could be prevented by competition with a homologous competitor (Fig. 4A and 4C). The consistent observation that self competition is much more efficient for three probes (MHCIIEα, HSVTK and mPCNA) than cross competition and the fact that an *in vitro* translation product was used to obtain these data, rather than a nuclear extract, indicate that this is an intrinsic property of the protein. There are at least three possible explanations for this unusual binding behavior: 1) during or after *in vitro* translation, post-translational modification occurs to some, but not all, of the nascent proteins, thereby producing chemically distinct forms of mYB-1; 2) a factor in reticulocyte lysates can interact with mYB-1 to modulate its binding characteristics; or 3) during *in vitro* translation, differential folding occurs resulting in two conformations (isoforms) which have different binding characteristics. Because the predicted pI of mYB-1 is 11.4, it is difficult to determine if two different forms exist by two-dimensional electrophoresis. Affinity chromatography using the various oligonucleotides may be useful in separating the two binding forms if the eluting conditions do not alter the conformation of the protein.

The similar binding behavior of one of the CCAAT binding-proteins in Con-A stimulated splenocytes suggested that the unusual binding characteristics of the mYB-1 *in vitro* translation product are not due to experimental artifact. However, further studies are necessary to show whether the protein in Con-A stimulated splenocytes exhibiting the same binding behavior is *in vivo* mYB-1 protein. Antibodies to mYB-1 are being prepared to confirm the identity of this complex and to investigate the possibility of post-translational modification. Since several different CCAAT-binding proteins exist in a single cell, it will also be important to determine if there are multiple forms for those CCAAT-binding proteins. The existence of multiple forms would add another layer of complexity to the interaction of binding proteins with DNA and to the regulation of transcription.

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