The Transcriptional Regulatory Protein, YB-1, Promotes Single-stranded Regions in the DRA Promoter

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YB-1 is a member of a newly defined family of DNA-and RNA-binding proteins, the Y box factors. These proteins have been shown to affect gene expression at both the transcriptional and translational levels. Recently, we showed that YB-1 represses interferon-γ-induced transcription of class II human major histocompatibility (MHC) genes (1). Studies in this report characterize the DNA binding properties of purified, recombinant YB-1 on the MHC class II DRA promoter. The generation of YB-1-specific antibodies further permitted an analysis of the DNA binding properties of endogenous YB-1. YB-1 specifically binds single-stranded templates of the DRA promoter with greater affinity than double-stranded templates. The single-stranded DNA binding sites of YB-1 were mapped to the X box, whereas the double-stranded binding sites were mapped to the Y box of the DRA promoter, by methylation interference analysis. Most significantly, YB-1 can induce or stabilize single-stranded regions in the X and Y elements of the DRA promoter, as revealed by mung bean nuclease analysis. In concert with the findings that YB-1 represses DNA transcription, this study of YB-1 binding properties suggests a model of repression in which YB-1 binding results in single-stranded regions within the promoter, thus preventing loading and/or function of other DNA-specific transactivating factors.

YB-1 is a member of a recently defined family of DNA-binding proteins, the Y box factors, also known as the cold shock domain factors (2, 3). These proteins represent a multigene family identified in a number of eukaryotic and prokaryotic organisms. Members of this family include human YB-1, dbpA, dbpB, NSEB-1, and BP-8; frog FRGY1, FRGY2, YB3, p56, and p54; rat EF1A; mouse MUSY1, MSY1, and avian EF1A, RSV-EF1, chkbYB-1; bovine yEF1Aα1; and bacterial cspA and cspB (3–7). Y box proteins are highly conserved, with 97% amino acid homology between rat EF1A and human YB-1. These factors have been shown to regulate gene expression at both the transcriptional and translational levels and several have been suggested to have roles in DNA repair as well as DNA and RNA condensation. Two closely related Y box proteins, YB-1 and EF1A, have been shown to regulate transcription. EF1A can activate transcription through the Rous sarcoma virus long terminal repeat (8, 9), while YB-1 has been shown to activate transcription through the HIV, HTLV1, JCV promoters (10, 37). Recently, we showed that YB-1 represses transcription of human major histocompatibility (MHC) class II genes (1). Several other Y box proteins (FRGY2, FRGY1, MSY1) were shown to repress translation and protein expression by sequestering mRNA in gametes (11–13) and somatic cells (14). Y box proteins have a broad specificity for nucleic acids, binding double-stranded DNA, depurinated double-stranded DNA, single-stranded DNA and RNA (3–6, 8, 12, 13, 15–18). Studies to date indicate that the Y box factors are likely to affect gene expression by various different mechanisms.

YB-1 was originally cloned by screening a human B cell expression library using a double-stranded oligonucleotide probe spanning the human DRA X and Y elements (15). These authors found that YB-1 binding was strong to the Y box and weaker to the X box. Binding to the Y box was dependent on the inverted CCAAT sequence in the Y box. The X and Y elements are highly conserved in murine and human MHC class II promoters and are necessary for basal, as well as interferon-γ (IFN-γ)-induced transcription. We have since shown that YB-1 represses MHC class II gene expression (1). Transfection of cells with YB-1 expression vector repressed endogenous IFN-γ-induced class II mRNA and protein expression, as well as IFN-γ-induced class II DRA promoter-driven reporter gene expression.

Sequences within eukaryotic promoters have been described that are sensitive to reagents that cleave single-stranded DNA and have been implicated in transcriptional regulation, including the promoters of the c-myc, β-globin, platelet-derived growth factor A, epidermal growth factor receptor, and decorin genes (19–23). In addition, eukaryotic single-stranded DNA-binding proteins have recently been described such as FSB, STR, MF3, p70, ERDP-1 (20, 21, 24, 25) and are implicated in transcriptional regulation. FSB specifically binds single-stranded PUSE, the far upstream element of the c-myc promoter, and transactivates PUSE-CAT reporter constructs (24). In addition, activation of the c-myc gene correlates with the induction of single-stranded sequence in FUSE in vivo.

In this study, the binding properties of YB-1 on the MHC DRA promoter are characterized. We demonstrate that YB-1 specifically binds single-stranded templates of the DRA promoter with much greater affinity than the double-stranded template. Through methylation interference analysis the contact points of YB-1 in the DRA promoter are identified. The possible mechanisms by which YB-1 represses DRA transcription are explored. Mung bean nuclease analysis reveals that YB-1 can stabilize single-stranded regions within the X and Y elements of the DRA promoter. We propose a model of tran...
scriptional repression in which YB-1 binding results in single-stranded regions in the DNA promoter and as a consequence prevents the binding and/or function of the X and Y box trans-activating factors.

MATERIALS AND METHODS

Recombinant Human YB-1—The EcoRl fragment of the human YB-1 cDNA was ligated into the pSP65 vector (15). The recombinant YB-1 (designated rYB-1) was generated by incubating the GST/ YB-1 beads with thrombin (Sigma) at 1 unit/20 µl of a 50% bead slurry in 50 mM Tris (pH 7.4), 150 mM NaCl, 2.5 mM CaCl₂, for 1 h at 27°C. GST was eluted from the beads with 5 mM reduced glutathione, 50 mM Tris (pH 8.0) for 15 min at 27°C. Antibodies—Polyclonal antibody to YB-1 antiserum was generated by multiple immunizations of a New Zealand White rabbit with 100–200 µg of YB-1. Its specificity was determined initially by enzyme-linked immunosorbent assay followed by Western blot analyses, using rYB-1 as an antigen in both assays. IgG fractions of the antiserum, as well as preimmune sera, were purified over protein A/protein G columns (Pierce) according to the manufacturer’s protocol.

Western Blot—Western blots were performed as described (35). Membranes were developed by chemiluminescence following the ECL protocol (Amersham Corp.).

Whole Cell and Nuclear Extracts—The human B cell line, Raji, the cervical carcinoma line cell, HeLa, and the promyelocytic leukemia cell, U937, were obtained from the ATCC and maintained under recommended conditions. Nuclear extracts were prepared as described previously (26).

Cell extracts were prepared by lysing cells in 10 mM Hepes (pH 8.0), 1 mM EDTA, 7 mM β-mercaptoethanol, 50 mM KCl, 0.4% Nonidet P-40, 1 mM peptatin, 1 mM leupeptin, 1 µM E-64, and 100 µM phenylmethylsulfonyl fluoride (Boehringer Mannheim). The lysates were centrifuged for 30 min at 15,000 × g at 4°C, and the supernatants were used in EMSA reactions. Protein concentrations for whole cell and nuclear extracts were determined by the Bio-Rad protein assay (Bio-Rad).

Electrophoretic Mobility Shift Assay—EMSA assays were carried out as described previously.2 Probes used in the study are illustrated in Fig. 1. Single-stranded oligonucleotides were end-labeled by T4 polynucleotide kinase (New England Biolabs, Beverly, MA), annealed to the opposite strand, and either gel-purified or purified over Nencoll columns (DuPont NEN). The probe (1 × 10⁶ cpm) was incubated with rYB-1 or cell extract and 2 µg of poly(dI-dC) in 10 mM Tris (pH 7.6), 1 mM EDTA, 0.1% Triton X-100, 5% glycerol, 80 mM NaCl, 4 mM MgCl₂, and 10 mM dithiothreitol, at 4°C for 20 min. In select experiments, the same sequence spanned by the X + Y oligonucleotide (Fig. 1) was excised from the plasmid pUC.XY5O. This excised fragment is designated X + Y oligo. pUC.XY50 was constructed by cloning the X + Y oligonucleotide into the XbaI site of the plasmid pUC18.8 X + Y oligo was isolated by a BanHI/HindIII digest and labeled by a fill-in reaction using Sequenase 2, DNA polymerase (U. S. Biochemical Corp.). It was of interest to note that while purified rYB-1 bound DNA probes in EMSA assays, whole cell bacteria lysate significantly inhibited DNA binding by rYB-1. This could be due to bacterial repressor proteins or competitive binding of rYB-1 by bacterial DNA or RNA. For supershift experiments, antibody was preincubated with the EMSA reaction mix for 30 min at 4°C prior to the addition of probe. To determine the specificity of DNA binding, 100-fold molar excess of unlabeled homologous or heterologous DNA was added to the EMS reaction for 15 min at 4°C prior to the addition of probe.

Sequencing and Footprinting—Methylation interference was performed as described previously (30) with minor modifications. Oligonucleotides spanning either the X or Y elements of the DNA promoter (Fig. 1) were end-labeled on one strand with T4 polynucleotide kinase, annealed to the opposite strand, methylated with dimethyl sulfate for 1.5–1.5 min at 27°C, and purified over Nencoll columns. To form single-stranded probes, the double-stranded, methylated probe was digested with NlaIII and immediately to ice. Double-stranded or single-stranded probes were incubated with rYB-1, as described above for EMSA, at the maximum concentrations of YB-1 necessary to form a complex with the specific probe being used. Bound and free fractions of probe were separated by nondenaturating gel electrophoresis, recovered from the gel by electroelution, extracted with phenol/chloroform, and eluted with piperidine. Samples were separated by electrophoresis in a 12% polyacrylamide urea gel.

Mung bean nuclease was used to detect single-stranded regions in double-stranded probes. For mung bean nuclease, an 187-base pair probe (Fig. 1, WXT) spanning the DNA promoter was generated by restriction enzyme digest of the plasmid, 5’-3152 DNA-ATCAT described previously (31). The plasmid was digested with XhoI, end-labeled with T4 polynucleotide kinase, and digested with BstYI to generate a 187-base pair, single end-labeled probe. This fragment was purified by polyacrylamide electrophoresis and electroelution. The probe (4 × 10⁵ cpm) was incubated with rYB-1 (1 µg probe in the presence of 1 µg of poly(dI-dC) in the EMS buffer in a 10-µl reaction at 37°C for 20 min. The volume of the reaction was expanded five times and 0.10 volume of both a 10 × mung bean nuclease buffer (New England Biolabs) and 10 mM ZnSO₄ were added. Mung bean nuclease reactions were carried out at saturating concentrations of mung bean nuclease activity, as determined by previous titrations of mung bean nuclease on the WXT probe. One unit of mung bean nuclease (New England Biolabs) was added to the reaction and incubated at 37°C. Ten-µl aliquots of the reaction were stopped at different time points by incubation for 20 min at 37°C in 240 ml of stop buffer (100 mM Tris (pH 8.0), 100 mM NaCl, 20 mM EDTA, 0.1% SDS, 100 µg/ml proteinase K, 200 µg/ml glycyld). The samples were then precipitated and separated on a 6% polyacrylamide urea, wedge gel. Sequencing reactions (32) of the WXT probe were run simultaneously with the mung bean nuclease reactions.

RESULTS

YB-1 was originally cloned by its binding to a probe containing the DNA X (comprising of X1 and X2) and Y proximal promoter elements, of which all are required for MHC class II transcription (15). We have since shown that YB-1 can repress both endogenous DR protein and mRNA levels, as well as reporter-driven, reporter gene transcription (1). In order to examine the mechanism of YB-1-mediated repression, we produced recombinant YB-1 protein (designated rYB-1) and characterized its binding activity on the DNA promoter. We also used the rYB-1 to generate YB-1-specific antibodies to identify endogenous YB-1.

Recombinant Human YB-1—rYB-1 was generated as described under “Materials and Methods.” Bacteria were transformed either with pGEK2T alone or pGEK2T-YB-1 and induced with isopropyl-β-D-thiogalactoside to express GST or the GST/YB-1 fusion protein (Fig. 2; lanes 2 and 3). GST/ YB-1 was purified by binding to glutathione-Sepharose beads (lanes 2 and 3). Thrombin treatment of the GST/YB-1-beads generated a predominant cleavage product of apparent molecular mass of 33–34 kDa (lane 5). The expected molecular mass of rYB-1 is 35.6 kDa.
Fig. 2. Purification of recombinant YB-1. Samples of lysates from bacteria induced with isopropyl-β-D-thiogalactopyranoside to express GST (lane 2), the GST/YB-1 fusion protein (lane 3), GST/YB-1 purified on glutathione-Sepharose beads (lanes 4 and 6), and YB-1 cleaved from GST/YB-1 beads with thrombin (lane 5) and molecular weight markers (M) were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue.

Detection of Endogenous YB-1 in Nuclear Extracts—rYB-1 was used to generate YB-1-specific antisera. Western blot analysis of whole sera demonstrates the specificity of the anti-YB-1 antisera (Fig. 3A). This antisera detected the rYB-1 protein migrating at approximately 33–34 kDa. No cross-reactivity was seen with GST or two other recombinant DNA-binding proteins, NF-YB and hXBP, known to bind to the DRA promoter. The IgG-enriched anti-YB recognized a band of approximately 45 kDa in nuclear extracts of two human cell lines, Raji and HeLa (Fig. 3B). This is consistent with the reported molecular mass of dbpB of 42 kDa in HeLa nuclear extracts (33). At higher concentrations of polyacrylamide, this band resolved to a doublet (data not shown). An additional high molecular weight band was consistently observed (70 kDa) in these extracts and may represent a new member of the Y box family of proteins. The difference between the expected molecular mass and the observed molecular mass of YB-1 (36 and 45 kDa, respectively) may be due to post-translational modifications. Treatment of these extracts with calf intestinal alkaline phosphatase did not affect the migration pattern of these bands (data not shown), indicating that these proteins are not likely to be phosphorylated.

YB-1 Preferentially Binds Single-stranded DNA—For ease of discussion, each probe is referred to as DS (double-stranded) or SS (single-stranded); the SS designation followed by a number 1 indicates the sense strand, whereas a number 2 indicates the antisense strand. EMS analysis shows rYB-1 binding to DS and SS oligonucleotide probes spanning the X and Y elements of the DRA promoter (Fig. 4). rYB-1 bound with the greatest affinity to X + YSS1 (Fig. 4, lanes 2–7). Slower migrating complexes were formed at high concentrations of rYB-1. These resolved to a faster migrating species as the YB-1 concentration was titrated down. These slower migrating complexes may represent multimers of YB-1, as reported for high concentrations of FRGY1 and FRGY2 (13), or binding to multiple sites on the probe. At 5 ng of rYB-1, significant complex formation is still observed on X + YSS1. GST, purified as a negative control, showed no DNA binding (data not shown).

In contrast to strand 1, rYB-1 bound to X + YSS2 and to X + YDS with much lower affinities (Fig. 4, lanes 8–19). To rule out the possibility that YB-1 binding on the double-stranded probe was due to contaminating single-stranded species, the double-stranded probe was isolated directly from a plasmid (Fig. 4, lanes 20–24). Binding to this probe was extremely weak, indicating that YB-1 binds poorly to double-stranded DNA containing the X and Y elements. Preferential YB-1 binding to single-stranded DNA is consistent with the ability of other Y box proteins (dbpB, NSEP-1, BP-8, and cspB) to bind single-stranded DNA (5, 16, 17, 34).

To determine the specificity of rYB-1 binding, reactions were carried out with 100-fold molar excess cold homologous or heterologous single-stranded oligonucleotides (Fig. 5A, lanes 1–3). Unlabeled homologous X + YSS1 DNA competed the complex formation, whereas an oligonucleotide spanning strand 1 of an unrelated DNA, the W element of the DRA promoter, did not. Similarly, other single-stranded, heterologous oligonucleotides, including strand 1 of the DRA octamer element, strand 2 of the W element, and strand 1 of the myb binding site of the c-myc promoter did not compete for rYB-1 binding (data not shown).

EMS analysis using whole cell extracts from the U937 cell line incubated with the X + YSS1 probe resulted in a complex that co-migrated with rYB-1 (Fig. 5A, lanes 1 versus 4). In addition to this band, a slower migrating complex was observed. Both complexes were specifically competed by homologous but not heterologous probe, as was rYB-1 (Fig. 5A, lanes 5 and 6).

The anti-YB-1 antisera was used to identify the endogenous YB-1 in U937 extracts. Addition of YB-1-specific antibodies to the rYB-1 EMS reaction resulted in a slower migrating, super-shifted complex (Fig. 5B, lanes 3, 5, 7, and 9). No such complex was observed either with the negative control antibodies (lanes
Fig. 4. YB-1 forms a stronger complex on single-stranded DNA with a greater affinity than double-stranded DNA. YB-1 binding properties were analyzed on single-stranded and double-stranded X + Y probes by EMS. Increasing concentrations of rYB-1 (500–5 ng) were incubated with X + Y probes and separated on nondenaturing gels. Probes were: X + Y single strand, strand 1 (X + YSS1), X + Y single strand, strand 2 (X + YSS2), X + Y double strand, annealed oligonucleotides (X + YDS), and X + Y double strand probe isolated from plasmid (X + YDS-re). Putative YB-1 multimers are marked by the solid arrow and monomers by the asterisk.

4, 6, 8, and 10) or with antibodies in the absence of rYB-1 (lane 1). The supershifted band in the U937 extract is of similar migration as rYB-1 and is antibody-specific (compare lanes 11, 13, and 15 versus 12, 14, and 16). Its formation was also dependent on the concentration of antibody used.

When single-stranded probes spanning either the X box or the Y box were used, it was found that rYB-1 bound preferentially to XSS1 and to YSS2 (Fig. 6). Titration of rYB-1 on these probes shows that complex formation was lost at 40 ng of rYB-1 on YSS2 whereas a strong complex formation was observed at this concentration on XSS1. This may reflect a stronger affinity of YB-1 for XSS1 than YSS2. Both XSS1 and YSS2 complexes were also competed by an homologous cold competitor, but not by an unrelated probe, indicating specificity of binding (data not shown). rYB-1 also formed a weak complex with XDS and YDS (Fig. 6, lanes 21–24).

YB-1 Single-stranded Binding Maps to X2 and Double-stranded Binding to Y—Methylation interference analysis was used to identify nucleotides involved in YB-1 binding to both double-stranded and single-stranded sequence spanning the X box or the Y box of the DRA promoter. Piperidine cleavage of methylated single-stranded probes generates an A/G sequence ladder as compared with only a G ladder following cleavage of double-stranded probes (for example see Fig. 7A, lanes 1 and 4 versus 7 and 10).

Fig. 5. A, endogenous YB-1 binding to single-stranded DNA is specific. rYB-1 (lanes 1–3) and whole cell U937 extract (lanes 4–6) were incubated in the presence or absence of 100-fold molar excess homologous (X + Y) or heterologous (W) competitor DNA in an EMS assay. The asterisk marks putative monomeric rYB-1 and endogenous YB-1. The solid arrowhead marks the slower migrating complex in the U937 extract. B, anti-YB-1 supershifts endogenous YB-1. rYB-1 (lanes 2–10) or U937 whole cell extract (lanes 11–17) were preincubated with 5, 2, 1, or 0.5 μl of anti-YB-1 (I) or negative control (C) antibodies for 30 min at 4°C. The reactions were incubated with the X + XSS1 probe in an EMS assay. Monomeric YB-1 is marked by an asterisk, and the specific, supershifted antibody-YB-1-probe complex is noted with an open arrowhead.

Fig. 6. YB-1 preferentially binds XSS1 and YSS2. rYB-1 was incubated in concentrations ranging from 750 to 40 ng with the following single-stranded probes: XSS1 (lanes 1–5), YSS1 (lanes 6–10), XSS1 (lanes 11–15), or YSS2 (lanes 16–20) and double-stranded probes: XSS1 (lane 21) and YDS (lane 24) in EMS reactions. The asterisk marks putative monomeric rYB-1.

Methylation interference analysis identified single-stranded YB-1 binding sites on both strands of the X box in the X2 element, with the strongest protection on XSS1 (Fig. 7A, lanes 1–6, summarized in C). This strand bias is consistent with the greatest binding affinity of YB-1 for XSS1 observed by EMS (Fig. 6). Four times more rYB-1 was required to generate a
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A

\[
\begin{align*}
\text{X}_{\text{SS1}} & : & F & B & F \\
\text{X}_{\text{SS2}} & : & F & B & F \\
\text{Y}_{\text{DS1}} & : & F & B & F \\
\text{Y}_{\text{DS2}} & : & F & B & F
\end{align*}
\]

B

\[
\begin{align*}
10' & : & r\text{YB-1} & - & + \\
30' & : & r\text{YB-1} & - & + \\
1 \text{ hr} & : & r\text{YB-1} & + & + \\
2 \text{ hr} & : & r\text{YB-1} & + & +
\end{align*}
\]

C

Methylation Interference

\[
\begin{align*}
\text{MBN Sensitivity} & : & \text{X1} & \text{X2} & \text{Y} \\
-138 & : & \text{W} & \text{Y} & \text{X1} & \text{X2} & \text{W}
\end{align*}
\]

Fig. 7. A, methylation interference analysis of YB-1 binding. Methylation interference was carried out as described under “Materials and Methods” using \text{Y}_{\text{DS1}}, \text{Y}_{\text{DS2}}, \text{X}_{\text{SS1}}, \text{X}_{\text{SS2}} probes. Free and bound fractions are marked as F and B, respectively. Strong interference and hypersensitive sites are marked by large closed and open arrowheads, respectively, whereas weaker patterns are marked by smaller arrows. The positions of the bands in the probe are denoted by the nucleotide base number (see Fig. 7C). B, YB-1 promotes single-stranded regions in the X and Y elements of the DRA promoter. 1 \mu g of YB-1 was incubated with the WWXY probe in EMS conditions, followed by incubation with the single-strand-specific mung bean nuclease for increasing periods of time at 37°C. Hypersensitive sites are marked by open circles. C, summary of YB-1 interaction on the DRA promoter. YB-1 contact points on single-stranded DNA (closed arrows) and YB-1-induced hypersensitive sites (open arrows) on the DRA promoter, as determined by methylation interference analysis, are illustrated. YB-1-induced single-stranded sites on a double-stranded probe, as determined by mung bean nuclease analysis, are indicated by open circles in the lower panel. The W, X, Y, and octamer elements are noted by shaded boxes.
methylated interference pattern on X. In contrast to the results with the X probes, no interference pattern was observed on the Y probes, nor Y probes, despite rYB-1 interaction with Y seen by EMS (Fig. 6). One obvious explanation is that methylation of G residues did not interfere with protein binding.

The EMS results in Fig. 6 demonstrate that rYB-1 binds Y, although with a lower affinity than Y seen (lanes 23 and 24). Previously, double-stranded Y box binding sites have been reported for the Y box protein EF1 (8, 9). In those reports, methylation interference analyses were carried out using chick embryo nuclear extracts (or fractions of) that most likely contained other Y-binding proteins, such as NF-YA and NF-YB. Purified, recombinant YB-1 is used here to address this concern and to define YB-1 double-stranded Y box binding. Incubation of rYB-1 with Y resulted in a stretch of hypersensitive G and A residues on both strands of the probe (Fig. 7A, lanes 7–12). This observation suggests that YB-1 preferentially binds methylated G and A residues in the context of the CCAAT sequence.

YB-1 Promotes Single-stranded Regions in the DRA Promoter. Due to the high affinity of YB-1 for single-stranded DNA, we examined the possibility that YB-1 could induce or stabilize single-stranded regions within the DRA promoter region using mung bean nucleases. Mung bean nuclease is a single-stranded DNA nuclease, with a much lower affinity for double-stranded DNA than the single-stranded nuclease, S1. Mung bean nuclease analysis of the DRA promoter supports the results from the methylation interference analysis. Incubation of a double-stranded DRA probe with mung bean nuclease at 37 °C for 10 min with or without rYB-1 did not result in the appearance of a mung bean nuclease-sensitive region (Fig. 7B, lanes 1 and 2). However, incubation of the probe with mung bean nuclease and rYB-1 for longer time periods resulted in regions of enhanced sensitivity around the X and Y boxes relative to the control reactions in the absence of rYB-1 (Fig. 7B, lanes 4, 6, and 8 versus 3, 5, and 7). The presence of YB-1 in these reactions accelerated or enhanced the appearance of these sites. The nuclease-sensitive sites around the Y box coincide with the hypersensitive sites seen around the Y box by methylation interference (summarized in Fig. 7C), indicating that YB-1 either enhances melting of this region or traps the DNA in a melted configuration. The mung bean nuclease-sensitive sites in the X box border the YB-1 single-strand contact points determined by methylation interference (Fig. 7C). Although the probe used in the mung bean nuclease assay spans 187 base pairs, the YB-1 enhanced single-stranded regions upstream of the TATA box (marked by the asterisk in B) cluster primarily around the X and Y boxes, suggesting these elements may play a role in the configuration of the promoter.

DISCUSSION

Previous experiments have shown that YB-1 can suppress DRA promoter function in IFN-γ-inducible cell lines. This manuscript explores the possible mechanism by which this occurs. The most important finding is that YB-1 induces or stabilizes a single-stranded region in the Y box. This region coincides with the binding site of YB-1 on a double-stranded probe, as determined by previous DNA binding assays (15). Based on these findings we propose that YB-1 binds to double-stranded sequences flanking the Y box which may be prone to single-strandedness and induces or stabilizes the single strand configuration.

The CCAAT box per se in the Y element binds the NF-Y/CBF family of proteins. Previously, we have shown that the in vivo occupancy of NF-Y is the most critical step in the assembly of the proteins binding the proximal elements (35). Mutations in the CCAAT sequence prevented in vivo occupancy of adjacent X1 elements as determined by genomic footprinting. Taken together we propose that YB-1-induced single-strandedness around the CCAAT element may interfere with the binding of NF-Y/CBF to the CCAAT box and disrupt the assembly of this proximal promoter.

We have also identified YB-1 binding sites by methylation interference analysis within the X1 element once this sequence has been made single-stranded. The mung bean nuclease data also demonstrate that YB-1 can induce or stabilize single-stranded sites within the X element on a double-stranded probe. The mung bean nuclease-sensitive sites directly flank the methylation interference contact sites. This suggests that YB-1 may also bind to double-stranded sequences in or around the X element and induce or stabilize a single-stranded region. This structural change and/or occupancy of the X2 element may additionally prevent binding of X box transactivating proteins, e.g. hXBP, X2BP, and RFX (17, 29, 36, 37).

Single-stranded DNA binding has been described for several Y box proteins (4, 5, 17, 18). The binding of two other Y box proteins, NSEP-1 and BF-F1, to single-stranded DNA that is pyrimidine-rich has been interpreted to indicate binding to triple helix DNA or H-DNA in the human c-myc and β-globin promoters, respectively. The reagents used here, as well as in these other studies, cannot distinguish between H-DNA and single-stranded DNA. There are several CT-rich stretches in the DRA promoter flanking the X box and the Y box; however, these are most likely too short to form H-DNA. In addition, the binding sites determined by methylation interference are not CT-rich. Alternatively, it is possible that the nucleosome-sensitive sites in the c-myc promoter to which NSEP-1 binds are in fact single-stranded DNA, as opposed to H-DNA.

In addition to our study, two studies have shown that another Y box protein, FRY2, represses gene expression. The mechanism has been elegantly determined and appears to be mediated by binding to and preventing translation of mRNA (11). In these studies, mRNA levels were either maintained at a steady state or increased. YB-1, however, appears to be acting at the level of the DR promoter, as opposed to sequestering mRNA, in that DBR mRNA levels are reduced, and the inhibition is specific to the MHC class II promoter-CAT constructs, whereas the heat shock 70- and thymidine kinase-CAT constructs are not affected. Although we have shown here that...
YB-1 promotes single-stranded DNA, we cannot rule out that YB-1 may also interact with RNA repressing translation.

An additional contribution of this study is the generation of recombinant YB-1- and YB-1-specific antibody. This antibody recognizes not only rYB-1, but has allowed us to identify endogenous YB-1 in whole cell extracts as well as in nuclear extracts. This will allow us to further characterize YB-1 under varying conditions.

We have shown that YB-1 is a transcriptional repressor and propose that this repression is accomplished by promoting single-stranded regions in the Y and X elements. Significant multimer formation was observed with rYB-1, as has been reported for other Y box proteins (13). It is possible that one dimer or multimer of YB-1 spans the X and Y elements, with one unit binding strand 1 in the X box while the other unit binds strand 2 in the Y box (Fig. 8). In this situation YB-1 may not only prevent binding of the necessary transactivating factors to X and Y, but may also introduce a contortional constraint in this region of the promoter.

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