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Y box-binding protein-1 binds preferentially to single-stranded nucleic acids and exhibits 3′→5′ exonuclease activity

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ABSTRACT

We have previously shown that Y box-binding protein-1 (YB-1) binds preferentially to cisplatin-modified Y box sequences. Based on structural and biochemical data, we predicted that this protein binds single-stranded nucleic acids. In the present study we confirmed the prediction and also discovered some unexpected functional features of YB-1. We found that the cold shock domain of the protein is necessary but not sufficient for double-stranded DNA binding while the C-tail domain interacts with both single-stranded DNA and RNA independently of the cold shock domain. In an *in vitro* translation system the C-tail domain of the protein inhibited translation but the cold shock domain did not. Both *in vitro* pull-down and *in vivo* co-immunoprecipitation assays revealed that YB-1 can form a homodimer. Deletion analysis mapped the C-tail domain of the protein as the region of homodimerization. We also characterized an intrinsic 3′→5′ DNA exonuclease activity of the protein. The region between residues 51 and 205 of its 324-amino acid extent is required for full exonuclease activity. Our findings suggest that YB-1 functions in regulating DNA/RNA transactions and that these actions involve different domains.

INTRODUCTION

Y box-binding protein-1 (YB-1), a transcription factor first identified by its ability to bind to the inverted CCAAT box (Y box), has been implicated in target gene transcription, cell proliferation (1) and cisplatin resistance (2). In previous reports we demonstrated that YB-1 is overexpressed in human cancer cell lines resistant to cisplatin (3). We also found that decreased YB-1 in cells increased sensitivity of cells to cisplatin and that YB-1 binds to cisplatin-modified DNA and interacts with proliferating cell nuclear antigen (PCNA) (4). These findings suggest that YB-1 acts at a damage recognition step in the DNA repair pathway.

YB-1 consists of three domains: a glycine-rich N-terminal domain, a highly conserved nucleic acid-binding domain, and a C-tail domain containing alternating regions of basic or acidic amino acids (1,5–7). No function has yet been assigned to the N-terminal region. The tail domain is thought to function as either a nucleic acid-binding or a protein–protein interaction domain. So far, YB-1 has been shown to interact with PCNA (4), JC virus T antigen (8), AP-2 (9), RelA tat (10) and Purα (11) through the C-tail domain. Thus, interaction of YB-1 with cellular or viral transcription factors can modulate gene regulation in a number of ways. However, just how YB-1 participates in interactions with various nucleic acids to contribute to cell function is unknown.

YB-1 has been shown to contain a unique DNA-binding domain, the cold shock domain (CSD), which is highly conserved in prokaryotes and eukaryotes (1,6,7). This domain is found in prokaryotic cold shock protein (CSP), which functions as an RNA chaperone. CSP destabilizes the secondary structure of RNA, controlling translation (12). Proteins constituting the family containing the CSD have been shown to not only bind to the Y box but also to single-stranded DNA, RNA and cisplatin-modified DNA (7,13–15).

To define the molecular mechanisms of YB-1 function we investigated the contribution of each domain to nucleic acid-binding properties. The capacity of each domain to modulate nucleic acid-binding activity was tested using purified glutathione *S*-transferase (GST) fusion proteins. Nucleic acid binding of YB-1 has been demonstrated in an *in vitro* system using purified *Escherichia coli*-produced YB-1. In this study we specifically analyzed the nucleic acid-binding properties of YB-1 in detail using single-stranded and double-stranded oligonucleotides with or without cisplatin modification, as

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well as various kinds of RNA. We found that the CSD itself failed to bind nucleic acids. As demonstrated by pull-down assay and immunoprecipitation, YB-1 can form a homodimer. Further, we found that the YB-1 protein has intrinsic 3′→5′ exonuclease activity. These findings help to explain the varied functions of YB-1.

MATERIALS AND METHODS

Cells and antibodies

MCF-7 cells were grown in Dulbecco's modified Eagle's medium (Nissui, Japan) supplemented with 10% fetal bovine serum. Anti-Flag monoclonal antibody (M2) and anti-Flag M2–agarose affinity gel were purchased from Sigma Chemical Co. Anti-hemagglutinin (HA)–peroxidase was purchased from Roche Molecular Biochemicals. Anti-YB-C antibody to the C-tail domain of YB-1 was generated as described previously (16).

Plasmid constructs

The plasmids, containing full-length GST–YB-1 cDNA and its five deletion mutants Δ1–Δ5, were described previously (4). To obtain GST–YB-1 Δ7 and Δ8, GST–YB-1 Δ3 was digested with *Sal*I, then the digested plasmid was self-ligated for GST–YB-1 Δ7 and the *Sal*I fragment was ligated to vector pGEX4T for GST–YB-1 Δ8. To construct GST–YB-1 Δ9, GST–YB-1 Δ8 was digested with *Eco*T14I and *Sma*I and self-ligated after filling-in with T4 DNA polymerase (MBI Fermentas). To construct HA–YB-1 to be expressed in *E.coli*, N-terminal HA-tagged YB-1 cDNA was cloned into ThioHis vector (Invitrogen); in this vector thioredoxin was deleted by digestion with *Nde*I and *Acc*65I. To construct Flag–YB-1, which is expressed in mammalian cells, N-terminal Flag-tagged YB-1 cDNA was cloned into vector pcDNA3 (Invitrogen). To obtain GST–p53, the full-length cDNA of human p53 was amplified from total RNA from human epidermoid cancer (KB) cells by reverse transcription–PCR using the primer pair 5′-CCATGGAG-GAGCCGCAGTCAGATCC-3′ and 5′-GAAGTGGAGAAT-GTCAGTCTGAGTCAGGCCC-3′. The PCR product was cloned into vector pGEM-T Easy (Promega). Then the cDNA fragment digested by *Not*I was gel-purified and cloned into vector pGEX-4T (Pharmacia Biotech).

Transient transfection and co-immunoprecipitation assay

MCF-7 cells were seeded in 6-well plates at a density of 1×10^5 cells/well. On the following day cells were transfected with 2 μg Flag–YB-1 expression plasmid with 6 μl of SuperFect according to the manufacturer's protocol (Qiagen). At 3 h following transfection cells were washed with phosphate-buffered saline (PBS) and the medium was replaced with fresh medium. After 48 h cells were washed twice with PBS and lysed in a binding buffer containing 50 mM Tris–HCl pH 8.0, 1 mM EDTA, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF). After standing for 30 min on ice lysates were centrifuged at 21 000 *g* for 10 min at 4°C. The supernatants (1 mg) were incubated with 5 μg/ml anti-Flag M2–agarose affinity gel for 30 min at 4°C and the beads washed three times with binding buffer. Immunoprecipitated samples and 50 μg supernatant were separated by SDS–PAGE and blotted onto Immobilon-P membrane filters (Millipore). The membranes were immunoblotted with anti-YB-1 polyclonal antibody or anti-Flag monoclonal antibody (M2) and developed by chemiluminescence using an ECL kit (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Expression of GST and HA fusion proteins and pull-down assay

GST fusion proteins and HA–YB-1 fusion protein were induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside as described previously (4). After *E.coli* was sonicated for 10 s in binding buffer (described above), soluble fractions were obtained by centrifugation at 21 000 *g* for 10 min at 4°C. Full-length GST–YB-1 or its deletion mutants immobilized on glutathione–Sepharose beads were washed three times with binding buffer and incubated with soluble HA–YB-1 fusion protein and 1 mM dithiothreitol (DTT) for 2 h at 4°C. The binding complex was washed three times with high salt binding buffer containing 50 mM Tris–HCl pH 8.0, 1 mM EDTA, 500 mM NaCl, 0.5% Nonidet P-40, 10% glycerol and 1 mM PMSF. After washing three times with the high salt binding buffer, pull-down samples were subjected to SDS–PAGE, immunoblotted with anti-HA–peroxidase and developed by chemiluminescence.

DNA gel-shift assay

Single-stranded oligonucleotides of the 32mer 5′-TCGATCGGG-GCGGGGCGATCGGGGCGGGGCGA-3′ and double-stranded oligonucleotides of the 25mer 5′-GGTGAGGCTGATTGG-CTGGGCAGGA-3′ were end-labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and purified from a 20% denaturing polyacrylamide gel in TBE (0.04 M Tris, 0.04 M sodium borate and 2 mM EDTA pH 8.0) buffer. Half of the amount of each prepared nucleotide was treated overnight with 0.3 mM cisplatin at 37°C and then purified by ethanol precipitation. Induced GST fusion proteins were eluted with 50 mM Tris–HCl pH 8.0, and 20 mM reduced glutathione according to the manufacturer's protocol (Amersham Pharmacia Biotech) and used directly for exonuclease assay (see below), a DNA gel shift assay and an RNA gel-shift assay (see below). For the DNA gel-shift assay, eluted GST fusion proteins and 4 ng radiolabeled oligonucleotides were mixed in reaction buffer containing 10 mM Tris–HCl pH 7.9, 20 mM NaCl and 10 mM DTT and incubated for 10 min at room temperature. Binding reactions were analyzed on 4% polyacrylamide gels in 0.5× TBE buffer, followed by autoradiography as described previously (4).

Exonuclease assay

The 5′-end-labeled single-stranded 32mer oligonucleotides were prepared as described above. To prepare 3′-end-labeled oligonucleotides, oligonucleotides of 5′-GATCTCGGGGCG-GGGCGATCGGGGCGGGGCGA-3′ and 5′-GATCTCGCC-CCGCCCCGATCGCCCCGCCCCGA-3′ were annealed. Then the 3′-end was labeled with [α-^{32}P]dCTP using the Klenow fragment for extension. The extended oligonucleotide was heat denatured at 94°C for 5 min, purified as described above, and used to test for exonuclease activity. The standard reaction mixture contained 50 mM Tris–HCl pH 7.5, 10 mM MgCl and 4 ng radiolabeled single-stranded oligonucleotides. The reactions were performed at 35°C for 1 h and terminated by adding a half volume of phenol. After vortex agitation and

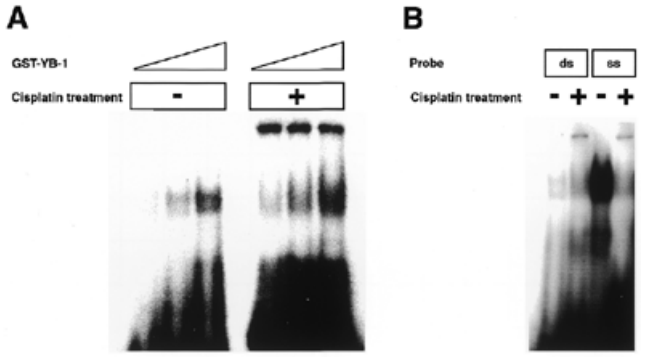

Figure 1. Effect of cisplatin treatment of DNA on binding by YB-1. (**A**) Increasing amounts (10, 30 and 90 ng) of YB-1 were incubated with double-stranded oligonucleotides (25mer) including the MDR1 Y box treated with (+) or without (–) cisplatin and used for DNA gel-shift assay. (**B**) Double- (25mer) or single-stranded (32mer) oligonucleotides treated with (+) or without (–) cisplatin were used for DNA gel-shift assay. The amount of YB-1 was ~30 ng. The gel was analyzed with a Fujix BAS 2000 bioimage analyzer (Fuji Photo Film).

brief centrifugation, reaction products were separated electrophoretically on a 20% polyacryamide–7 M urea gel.

RNA gel-shift assay

GST fusion proteins and 1 µg *in vitro* transcribed luciferase mRNA were incubated with or without the indicated RNase inhibitor. Binding reaction products were separated on 1 or 2% TBE–agarose gels and stained with ethidium bromide.

In vitro translation and luciferase assays

To prepare luciferase mRNA an *in vitro* transcription system (Promega) with luciferase control DNA was used according to the manufacturer's protocol. GST fusion proteins and antibody were added at various time points to the reaction mixtures, which included luciferase mRNA and rabbit reticulocyte lysates, and translation reactions were performed. Translation reactions were terminated by adding a 50-fold excess volume of reporter lysis buffer (Promega). Then 20 µl of translation reaction products and 40 µl of substrate from the Picagene kit (Toyoinki, Tokyo) were immediately mixed to determine luciferase activity as described previously (17,18).

RESULTS

YB-1 binds to both single- and double-stranded oligonucleotides

YB-1 is known to be a potent transcription factor, which can bind to double-stranded oligonucleotides containing an inverted CCAAT motif. We have previously shown that YB-1 preferentially binds to cisplatin-modified double-stranded DNA (4). To assess the binding activity of YB-1 to nucleic acids we prepared them treated with or without cisplatin. We have reproducibly observed that YB-1 preferentially binds to cisplatin-modified double-stranded DNA (Fig. 1A). When binding to single-stranded DNA was compared to binding to double-stranded DNA, YB-1 showed a higher affinity for single-stranded DNA than for double-stranded DNA (Fig. 1B). However, 20mer single-stranded DNA produced only a barely detectable band of protein–DNA complexes at the same YB-1 concentrations tested with the 32mer (data not shown).

The CSD is insufficient for DNA binding

YB-1 includes three domains (N-terminal domain, CSD and C-tail domain). To investigate the involvement of these three domains in DNA-binding activity, we produced a series of GST–YB-1 fusion proteins (Fig. 2A). These GST fusion proteins were expressed in bacteria and detected by staining (data not shown). Figure 2B shows that full-length YB-1 protein and the Δ1 and Δ2 mutants bound efficiently to the double-stranded Y box, while other GST fusion proteins did not. This indicated that the CSD was not sufficient for DNA binding; half of the C-tail domain adjacent to the CSD is required for appreciable DNA-binding activity. Further, these three fusion proteins displayed a higher affinity for the cisplatin-modified Y box than for its unaltered counterpart (data not shown). Next we analyzed single-stranded DNA binding. As predicted, YB-1 bound well to single-stranded DNA of 32mer length but not that treated with cisplatin (Fig. 1B). As shown in Figure 2C, full-length YB-1 and mutants Δ1 and Δ2, all containing the CSD, bound strongly to single-stranded DNA. A 32mer single-stranded DNA formed a faint but distinct band after incubation with Δ3, Δ7, Δ8 and Δ9, suggesting that the C-tail domain independently possesses single-stranded DNA-binding activity.

Two C-tail domains of YB-1 are involved in homodimerization

To examine the possibility that YB-1 might be a homodimer, Flag–YB-1 was expressed in MCF-7 cells. Cell extract was immunoprecipitated with anti-Flag antibody and immunoprecipitates were resolved by SDS–PAGE followed by immunoblotting with anti-YB-1 antibody (Fig. 3A, right). Specificity of the antibody and the molecular weight of Flag–YB-1 were confirmed by immunoblotting with anti-Flag antibody (Fig. 3A, left) or anti-YB-1 antibody (Fig. 3A, middle) using the same extracts. Endogenous YB-1 was co-immunoprecipitated by anti-Flag antibody when the Flag–YB-1 expression construct was transfected but not when empty construct was transfected.

We next determined whether YB-1 interacted with HA–YB-1 *in vitro* (Fig. 3B). HA–YB-1 was subjected to a pull-down assay in which the deletion series of YB-1 proteins was expressed as GST fusion proteins and immobilized on glutathione–Sepharose beads, followed by SDS–PAGE and immunoblotting with anti-HA antibody. We found that HA–YB-1 interacted strongly with GST–YB-1. Although GST, the N-terminal domain and the CSD did not interact with HA–YB-1, nearly equal amounts of HA–YB-1 bound to the other GST–YB-1 mutants. Taken together, these findings demonstrate that the C-tail domain modulates a specific interaction to form homodimers.

YB-1 is a 3′→5′ exonuclease

The amino acid sequence of YB-1 does not contain any sequence motif indicative of an enzymatic activity. However, single-stranded DNA is a good substrate for YB-1 binding. In a DNA gel-shift assay we noted that single-stranded DNA

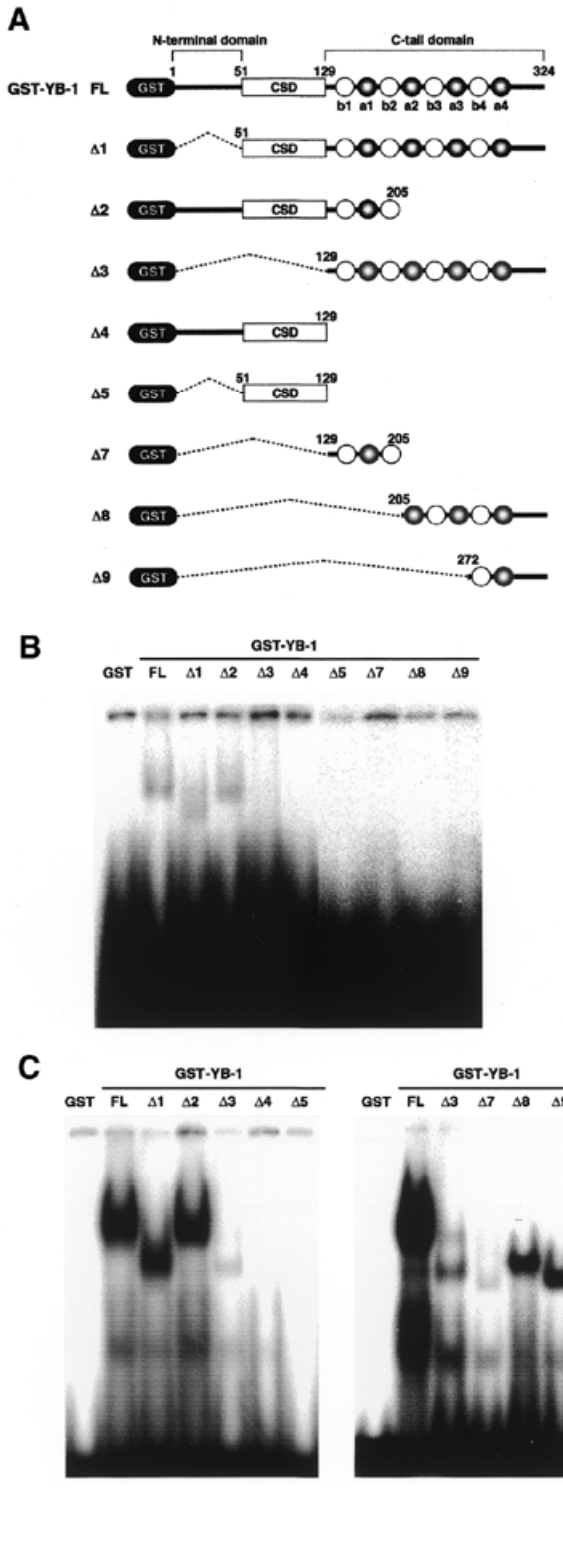

Figure 2. Preparation of GST–YB-1 mutants and double- and single-stranded DNA binding by YB-1. (**A**) Diagram of YB-1 protein and its derivatives. The CSD and the basic (b) and acidic (a) amino acid clusters are indicated for the wild-type YB-1 protein. (**B**) Approximately 100 ng GST fusion protein were incubated with the double-stranded target oligonucleotide (25mer) including the MDR1 Y box (18). (**C**) Approximately 100 ng fusion proteins were incubated with the oligonucleotide (32mer).

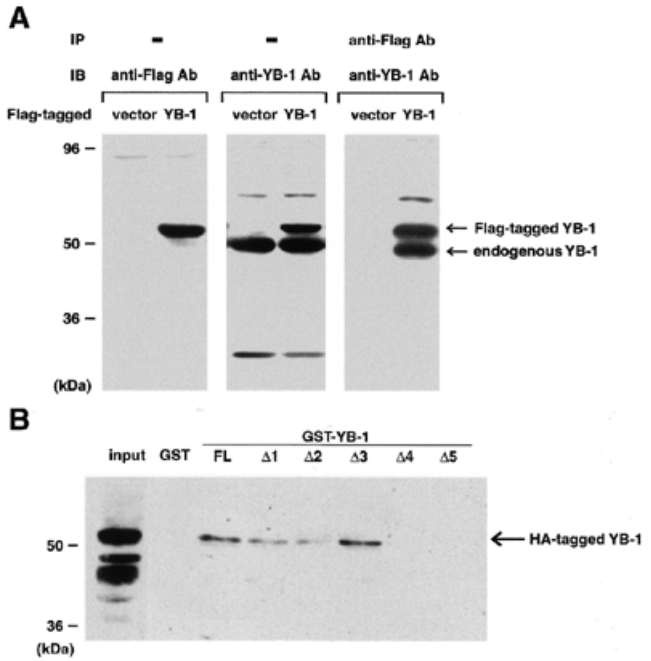

Figure 3. YB-1 interacts with YB-1. (**A**) *In vivo* binding of YB-1 and Flag–YB-1. MCF-7 cells were transfected with plasmids expressing Flag–YB-1 or Flag vector. Expression of transfected plasmid was confirmed by immunoblotting with either anti-Flag antibody (left) or anti-YB-1 antibody (middle). The same extracts were immunoprecipitated (IP) with anti-Flag antibody; bound endogenous YB-1 was visualized by immunoblotting with anti YB-1 antibody (right). (**B**) *In vitro* binding of GST–YB-1 and HA–YB-1. The full-length form of HA–YB-1 was expressed in bacteria. Extracts from cells were used for pull-down experiments. Equal amounts of GST–YB-1 and translated forms were bound to glutathione–agarose and incubated with extracts including HA–YB-1. After binding, glutathione–Sepharose beads were washed and boiled with sample buffer. Bound proteins were separated by SDS–PAGE, blotted and visualized by staining with an anti-HA-antibody.

incubated with YB-1 was degraded under certain reaction conditions (data not shown). To examine this activity in more detail, purified GST–YB-1 fusion proteins were incubated with a single-stranded DNA labeled at the 5′-end; the products were oligomers of gradually decreasing size (Fig. 4A, left). With 3′-end-labeled DNA the label appeared in the form of a mononucleotide (Fig. 4A, right). However, we could not detect any activity when a similar preparation of GST was used. To demonstrate that the activity was intrinsic to YB-1 we analyzed a series of GST–YB-1 fusion proteins. As seen in Figure 4B, 3′→5′ exonuclease activity was significant when full-length YB-1 and mutants Δ1, Δ2 and Δ3 were assayed. As a positive control purified GST–p53 was assayed, demonstrating 3′→5′ exonuclease activity. Enzymatic activity of YB-1 was comparable to that of p53. The active site was localized to the region between amino acids 51 and 205.

YB-1 is an RNA-binding protein that inhibits *in vitro* translation

Proteins including a CSD have been shown to bind to RNA (19). We next investigated whether YB-1 can bind and degrade RNA. *In vitro* transcribed luciferase mRNA was incubated with GST–YB-1 and analyzed by agarose gel electrophoresis.

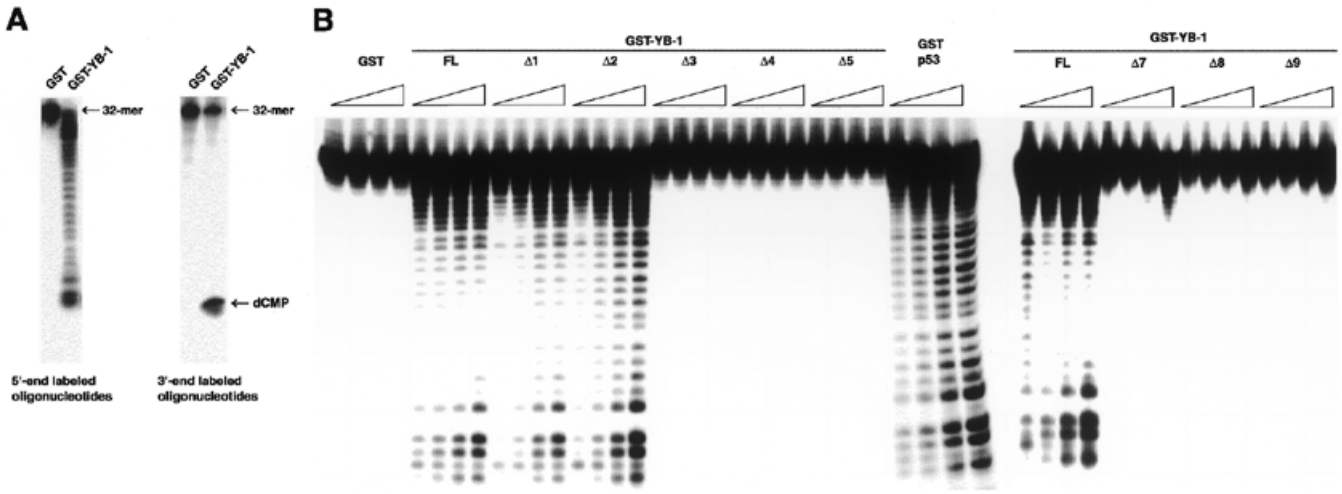

Figure 4. YB-1 is a 3′→5′ exonuclease. (**A**) Exonuclease activity upon 5′- or 3′-end-labeled substrates (32mer). Exonuclease activity was measured with 90 ng GST–YB-1 for 1 h at 35°C. Reaction products were analyzed on a 20% polyacrylamide gel containing 7 M urea. (**B**) 5′-End-labeled 32mer was incubated with increasing amounts of GST fusion protein (3, 10, 30 and 90 ng). GST–p53 was assayed as a positive control.

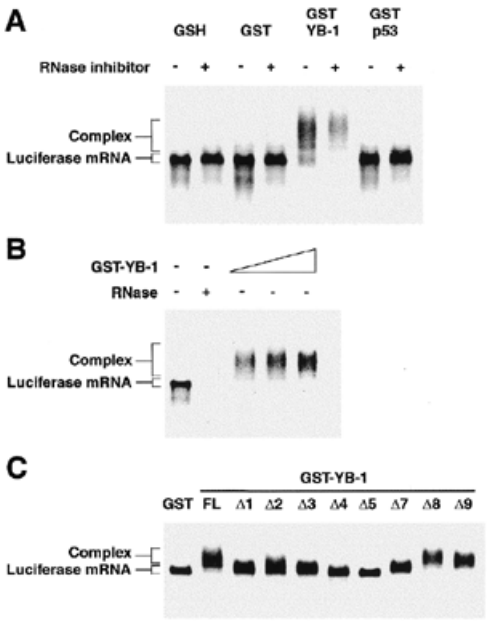

Figure 5. RNA binding of GST–YB-1 proteins. GST fusion proteins were tested for luciferase mRNA-binding activity. *In vitro* transcribed luciferase mRNA was used as probe. (**A**) Luciferase mRNA (0.3 µg) was incubated with 100 ng GST fusion protein. (**B**) Increasing amounts of GST–YB-1 (25, 50 and 100 ng) were used for mRNA binding. (**C**) A RNA gel-shift assay using full-length GST–YB-1 and mutants (100 ng) was performed with luciferase mRNA.

The band of RNA disappeared almost completely, while a slowly migrating, smeared band was observed when YB-1 only was added to the reaction (Fig. 5A). No degradation products were found, indicating that YB-1 has no ribonuclease activity. We believe that the appearance of the slowly migrating band was due to RNA binding by YB-1; in that case an electrophoretic mobility shift of the RNA–protein complex should be detectable in a conventional agarose gel. To confirm this possibility we investigated the dose-dependent effect of YB-1 in an RNA gel-shift assay. The intensity of the shifted band increased in a dose-dependent manner (Fig. 5B). Binding of YB-1 to RNA was observed when total RNA isolated from MCF-7 cells was used (data not shown). We performed an RNA gel-shift assay to map the RNA-binding domain of YB-1 using a series of GST–YB-1 fusion proteins. Significant RNA-binding activity was observed when full-length YB-1 and mutants Δ1, Δ2, Δ3, Δ7, Δ8 and Δ9 were used (Fig. 5C). These findings are consistent with the results shown for single-stranded DNA-binding activity in Figure 2. The C-tail domain interacts with RNA independently of the CSD.

In an attempt to identify the biochemical mechanism underlying the RNA-binding activity of YB-1, *in vitro* experiments were carried out to examine translation control activity (Fig. 6). As shown in Figure 6A, high luciferase activity was detected when GSH, GST or Δ5 was initially added to the reaction mixture. Luciferase activity was decreased by 15–40% of control activity when full-length YB-1 or Δ3 or Δ8 was added (Fig. 6A). This inhibitory effect was significantly reversed by addition of anti-YB-1 antibody (Fig. 6B). We confirmed that luciferase activity was not directly inhibited by addition of YB-1 (Fig. 6C). On the other hand, luciferase activity was similar to that of the control when YB-1 was added after 10 min incubation (Fig. 6D). These results show that the RNA-binding activity of YB-1 correlated well with the translation inhibitory activity.

DISCUSSION

The Y box protein YB-1 was first identified as a cellular constituent that bound to the Y box of the MHC class II promoter sequence (2). YB-1 has been shown to be highly conserved during the course of evolution (1,6,7). The nucleic

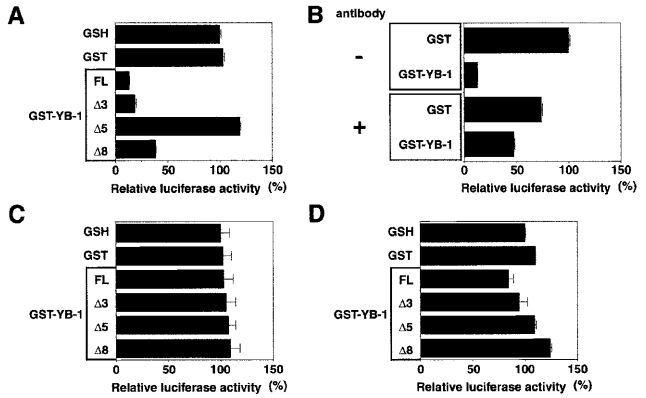

Figure 6. Effect of YB-1 on protein synthesis *in vitro*. Translation reactions were carried out with 20 μg/ml luciferase mRNA and 200 ng GST fusion proteins without antibodies (**A**) and in the presence of 1 μg of either rabbit IgG (–) or anti-YB-1 antibody (+) (**B**). GST fusion proteins were added either after the first 10 min of incubation (**D**) or at the end of incubation (**C**). Luciferase activities were measured as described in Materials and Methods.

acid-binding domain of YB-1 was favored to possess amino acid sequence homology with bacterial CSP and was called the CSD.

Increased expression of CSP in bacteria protects cells against low temperature stress (20). In eukaryotic cells, increased expression of YB-1 is associated with cell proliferation and transformation (1,21–23). YB-1 is distributed in both the nuclear and cytoplasmic compartments (22,24) and is involved in both transcription and translation of mRNA (25). We have previously shown that YB-1 is located mainly in the cytoplasm and then accumulates in the nucleus when cells are exposed to genotoxic stress (26). Determining whether YB-1 proteins are predominantly DNA-transacting or translational factors in eukaryotic cells is therefore important.

We have also shown that YB-1 is overexpressed in human cancer cell lines that are resistant to cisplatin, with cellular levels of YB-1 correlating with cisplatin sensitivity (16). Further, YB-1 preferentially binds cisplatin-modified DNA (4). These results indicate that in eukaryotes YB-1 is involved in multiple cellular functions through nucleic acid binding, reflecting the fact that the overall structure of YB-1 is different from that of bacterial CSP.

Nucleic acid binding of YB-1

To clarify the actions of the protein *in vitro* binding activities of YB-1 were analyzed using various substrates. Our data indicate that the CSD is necessary but not sufficient for binding to double-stranded DNA; the region adjacent to the boundary at the C-terminus of the CSD is required for high affinity DNA binding (Fig. 2B). Although similar results were obtained when both single-stranded DNA and RNA were used as substrates, the C-tail domain showed definite binding to single-stranded DNA and RNA in a CSD-independent manner (Figs 2C and 5C). This result is consistent with findings in previous reports (25,27). We confirmed that YB-1 binds preferentially to cisplatin-modified DNA, suggesting that YB-1 may be involved in DNA repair processes (Fig. 1).

Figure 3 shows that YB-1 monomers mutually associate *in vivo* and *in vitro* through the C-tail domain, suggesting that the C-tail domain is required for homomultimerization. As shown in Figure 2A, the C-tail domain consists of alternating regions of predominantly basic or acidic amino acids, each about 15–25 amino acids in length (19,28,29). This domain may function as a charge zipper to regulate interactions (30). The CSP consists of five β-strands that form a β-barrel structure (25,27). Bacterial CSP forms a dimer by hydrogen bonds linking the adjacent β4-strands and binds to single-stranded DNA or RNA (31). The β2-strand and β3-strand contain ribonucleoprotein 1 (RNP1) and RNP2 motifs, respectively (32). These strands, located in the N-terminal half of CSP, are well conserved in the CSD of YB-1. This region of the CSD may have affinity for the DNA groove and stabilize association with double-stranded DNA. The amino acid sequences of the β4-strand and β5-strand in the C-terminal region between the CSP and CSD of YB-1 differ between eukaryotes and bacteria (31,33), suggesting that the CSD of YB-1 cannot itself form a dimer. We observed that YB-1 did not interact with HA–YB-1 via the CSD. This may be the reason why YB-1 Δ5 cannot bind to nucleic acids. On the other hand, YB-1 bound to single-stranded DNA and RNA in a different manner compared with double-stranded DNA (Figs 2B and C and 5C). Because DNA is acidic, we suspect that the basic region of the C-tail domain might be the part of the protein that directly associates with single-stranded DNA and RNA. Interestingly, the mobility of the YB-1–nucleic acid complex is not dependent on the molecular size of YB-1, which may reflect conformational changes, including bending of DNA.

In *Xenopus* oocytes FRGY2, a CSD protein, forms ribonucleoprotein complexes and contributes to translation repression (34–36). To investigate whether YB-1 can bind to RNA, we analyzed RNA-binding activity using electrophoresis on an agarose gel containing ethidium bromide. This assay is simpler, faster and easier than the usual RNA gel-shift assay using polyacrylamide gel electrophoresis and labeled probes. YB-1 bound strongly to various RNAs, such as luciferase mRNA (Fig. 5), rRNA and tRNA (data not shown) Further, the density of YB-1 on a long RNA segment may be very high, since the YB-1–RNA complex migrated slowly in the agarose gel. p50 protein, the major core protein of mRNPs, has been identified as a Y box-binding protein based on a high degree of amino acid sequence homology (37–39). p50 protein has been shown to inhibit protein synthesis *in vitro* and *in vivo*.

Non-specific binding of YB-1 to RNA can protect the RNA from RNase digestion (data not shown). YB-1 can inhibit protein synthesis (Fig. 6A), but cannot do so once protein synthesis has been initiated (Fig. 6D), suggesting that attachment of YB-1 to RNA may interfere with binding of initiation factors or ribosome entry. The cellular quantity of YB-1 is a critical determinant of whether it simulates or inhibits translation (40). YB-1 antibody showed 25% inhibition of control activity on addition of GST alone (Fig. 6B), suggesting that our antibody may cross-react with rabbit YB-1 in reticulocyte lysate. In our system a large excess of YB-1 at a high YB-1:RNA ratio masks RNA from the translation machinery. Notably, the CSD itself has no RNA-binding and no translation inhibitory activities. Thus, YB-1 may regulate both transcriptional and translational events.

3′→5′ Exonuclease activity of YB-1

Wild-type p53, but not mutant p53, has recently been shown to exert intrinsic 3′→5′ exonuclease activity (41–43). The human DNA damage checkpoint protein hRAD9 has been shown to possess an intrinsic 3′→5′ exonuclease function (44). We therefore assayed p53 for cisplatin-modified DNA-binding activity. While p53 could bind weakly to single-stranded DNA, it could not bind to cisplatin-modified double- or single-stranded DNA (data not shown). We showed that YB-1 binds strongly to cisplatin-modified double-stranded DNA and weakly to cisplatin-modified single-stranded DNA (Fig. 1B). We have previously shown that YB-1 is an active participant in a variety of DNA repair processes (4). We therefore examined whether YB-1 acts as an exonuclease. We found that YB-1 possesses 3′→5′ exonuclease activity (Fig. 4A). Enzymatic activity of YB-1 was comparable to that of p53 (Fig. 4B); the active site of YB-1 for this activity was located between amino acids 51 and 205. The active site thus appeared to be related to the site of strong single-stranded nucleic acid binding (Fig. 2C). We tried to search the homologous legion between YB-1 and 3′–5′ exonuclease proteins including p53 (residues 80–280 of the 390 amino acids) and human Rad9 (residues 51–91 of the 391 amino acids), but we could not find any homologous regions. Further study will be required for the fine mapping of the active sites. We have previously shown that YB-1 interacts with PCNA, an essential protein in DNA repair (4). High mobility group protein 1 (HMG1) also binds preferentially to cisplatin-modified double-stranded DNA and interacts with the cell cycle regulator p53 (45,46). Both YB-1 and HMG1 are thought to be involved in cisplatin resistance. Nucleotide excision repair includes three phases: lesion recognition and local opening of the DNA helix; excision of the damaged region; and gap filling. Although a number of proteins have been identified as acting specifically in the first phase, both YB-1 and HMG1 could complement each other in regulating damage recognition. The 3′→5′ exonuclease activity of YB-1 may be involved in excision of the damaged region.

YB-1 is also phosphorylated and acetylated *in vitro* (data not shown). In *Xenopus* oocytes phosphorylated FRGY2 has been reported to maintain the stability of mRNA by means of high affinity binding (15,47). Thus, protein modification might regulate nucleic acid binding, dimerization and exonuclease activity.

Our findings suggest that YB-1 exerts a variety of biochemical activities involved in transcription, DNA damage recognition, DNA repair and translation. The present studies will provide additional clues in our attempt to understand the pleotropic DNA transactions of YB-1. However, more direct proof for an *in vivo* function of YB-1 must be obtained to substantiate our hypothesized dual role of YB-1 in binding to DNA and RNA.

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