Binding Capacity of Human YB-1 Protein for RNA Containing 8-Oxoguanine†

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ABSTRACT: 8-Oxoguanine (8-oxo-7,8-dihydroguanine) is generated in the cellular nucleotide pool as well as in nucleic acids, by the action of oxygen radicals produced in cells. 8-Oxoguanine has the potential to pair with both cytosine and adenine, and thus, the persistence of this base in messenger RNA would cause translational errors. To prevent such an outcome, organisms should have mechanisms for preventing the misincorporation of 8-oxoguanine-containing nucleotide into RNA and for removing 8-oxoguanine-containing RNA from processes of translation. We now report that mammalian Y box-binding protein 1 (YB-1 protein) possesses the activity to bind specifically to RNA containing 8-oxoguanine. On incubation with a purified preparation of YB-1 protein, 8-oxoguanine-containing RNA forms stable complexes with the protein while normal RNA scarcely forms such a complex. Using a series of deletion mutants which produce altered forms of YB-1 protein lacking some parts of the sequence, domains of the protein necessary for RNA binding were identified. *Escherichia coli* cells expressing normal or truncated forms of YB-1 protein with the binding capacity acquire resistance against paraquat, a drug that induces oxidative stress in cells, whereas cells with truncated proteins lacking such an activity do not. YB-1 protein may disturb the bacterial system in recognizing oxidatively damaged RNA, thus exerting a dominant negative effect on cell growth. We propose that YB-1 protein may discriminate the oxidized RNA molecule from normal ones, thus contributing to the high fidelity of translation in cells.

Oxygen radicals produced through normal cellular metabolism damage biologically important substances, such as nucleic acids and proteins. More than 20 different types of oxidatively altered purine and pyrimidine bases have been detected in nucleic acids (*1*, *2*). Among them, an oxidized form of a guanine base, 8-oxoguanine,[1] appears to be the most important since it causes mispairing during DNA synthesis (*3*−*5*). This oxidized guanine base can pair with both cytosine and adenine with an almost equal efficiency, and as a result, transversion mutations are induced (*6*−*9*). Studies using *Escherichia coli* mutator mutants revealed that cells are equipped with elaborate mechanisms for counteracting such deleterious effects of 8-oxoguanine. The MutM protein removes 8-oxoguanine paired with cytosine in DNA, while the MutY protein removes adenine paired with 8-oxoguanine (*10*−*13*). Oxidation of guanine also proceeds in the form of a free nucleotide, and an oxidized form of dGTP, 8-oxo-dGTP, is a potent mutagenic substrate for DNA synthesis. The MutT protein of *E. coli* hydrolyzes 8-oxo-dGTP to monophosphate, thereby preventing misincorporation of 8-oxoguanine into DNA (*14*). The concerted actions of these proteins lead to a high fidelity of DNA replication.

An oxidized form of GTP, 8-oxoGTP, is produced in the cellular nucleotide pool and can be incorporated into RNA (*15*). In *E. coli*, this could also be prevented by the action of the MutT protein, since this protein has the potential to hydrolyze 8-oxoGTP as efficiently as does 8-oxo-dGTP (*15*). In mammalian cells, sanitization of the nucleotide pool appears to occur in a somewhat different manner. MTH1 protein, the mammalian counterpart of MutT protein, is less capable of hydrolyzing 8-oxoGTP, and its potential to hydrolyze 8-oxoGTP is almost 2% of that for 8-oxo-dGTP (*16*). Mammalian cells apparently possess another enzyme(s) that acts specifically on 8-oxoGTP (unpublished result). Even though misincorporation of 8-oxoguanine into RNA is prevented by such mechanisms, oxidation of guanine residues would occur *in situ*. Since RNA is mostly single-stranded, oxidation of guanine in RNA may occur more frequently than that in DNA, in which their bases are protected by hydrogen bonding. The persistence of the oxidized bases in mRNA would lead to synthesis of a large amount of

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[1] Abbreviations: 8-oxoguanine, 8-oxo-7,8-dihydroguanine; 8-oxo-GTP, 8-oxo-7,8-dihydroguanosine 5′-triphosphate; 8-oxo-dGTP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate; 8-oxo-dGTPase, 8-oxo-7,8-dihydrodeoxyguanosine triphosphatase; 8-oxoGMP, 8-oxo-7,8-dihydroguanosine 5′-monophosphate; 8-oxo-dGMP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-monophosphate; 8-oxoGDP, 8-oxo-7,8-dihydroguanosine 5′-diphosphate; 8-oxo-dGDP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-diphosphate; DTT, dithiothreitol; TLC, thin-layer chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; GST, glutathione *S*-transferase.

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anomalous proteins which would be hazardous to the cell.

One way to avoid such a catastrophe is to discriminate 8-oxoguanine-containing RNA from normal RNA and to prevent the former from entering cellular translational machinery. Polynucleotide phosphorylase (Pnp) protein of *E. coli* can bind specifically to 8-oxoguanine-containing RNA, and a stable RNA−protein complex is formed (*17*). This protein may function to prevent oxidatively damaged mRNA molecules from entering the translation process. In mammalian cells, polynucleotide phosphorylase activity has not been detected; hence, such a role may be carried out by other proteins. In search of such a protein, we noted that YB-1, a human protein with multiple regulatory activities, might play such a role. YB-1 was originally identified as a transcription factor, which binds to the Y box, the CCAAT sequence present in the promoter of MHC class II and some other genes (*18*, *19*). This protein is a major core protein of messenger ribonucleoprotein, and overproduction of YB-1 in mammalian cells inhibits their protein synthesis (*20*). Moreover, the protein has a weak RNA binding capacity, and addition of excess amounts of a purified preparation of YB-1 protein to the *in vitro* translation system decreases the rate of protein synthesis (*20*, *21*). These findings prompted us to determine if YB-1 protein has the potential to specifically bind to 8-oxoguanine-containing RNA.

EXPERIMENTAL PROCEDURES

Nucleic Acids, Enzymes, and Chemicals. [^{32}P]-8-OxoGTP, [^{32}P]-8-oxoGMP-labeled poly(8-oxoG·A), and [^{32}P]UMP-labeled poly(U·A) were prepared, as described previously (*17*). Competitor poly(8-oxoG·A) and poly(U·A) were prepared using radioactive nucleoside triphosphates with very low levels of specific radioactivity (5×10^4 dpm/nmol) as substrates. The concentrations of the RNAs were determined using the specific activities and were represented as nucleotides. *E. coli* RNA polymerase, RNase A, yeast tRNA, and paraquat were purchased from Sigma.

Plasmids, Bacterial Cells, and YB-1 Proteins. Plasmids containing GST and the full-length or deleted YB-1 cDNAs have been described elsewhere (*22*). *E. coli* DH5α [*supE44*, Δ*lacU169 (φ80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA*] cells were transformed by one of these plasmids and used in these experiments. Bacteria with the plasmid containing YB-1 cDNA were grown at 30 °C unless otherwise specified. The existence of a large amount of YB-1 protein is toxic for cell growth. The GST and GST−YB-1 fusion proteins were purified, as described previously (*21*). Briefly, cells were grown at 30 °C, and formation of YB-1 protein was induced by the addition of 1 mM IPTG, followed by incubation for an additional 2 h. Bacterial cells were collected by centrifugation and sonicated in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% glycerol, 0.5% Noridet P40, 1 mM DTT, and 0.5 mM PMSF. After centrifugation, the supernatant was applied to a glutathione−Sepahrose 4B column. Proteins were eluted with 100 mM Tris-HCl (pH 8.0), 20 mM reduced glutathione, and 120 mM NaCl, and each of the fusion proteins was identified by Coomassie Blue staining of 10% SDS−PAGE.

RNase Protection Assay. Twenty picomoles of labeled copolymers, poly(U·A) and poly(8-oxoG·A), was preincubated with or without 60 pmol of purified GST−YB-1 protein fusion in 10 μL of a reaction mixture containing 0.1 M Tris-HCI (pH 7.4), 5 mM $MgCl_2$, and 0.4 mM DTT at 0 °C for 10 min. After preincubation, 1 μL of RNase A (0.4 mg/mL) was added to 10 μL of the reaction mixture, and the mixture was further incubated at 30 °C. At the indicated times, 0.5 μL of the reaction mixture was withdrawn and spotted onto a TLC plate, PEI-cellulose F (Merck), and the plate was developed in 1 M LiCl. Radioactive spots were monitored using a Fuji BAS2000 image analyzer.

Gel Shift Assay. For the standard assay, 10 pmol of ^{32}P-labeled polynucleotides was preincubated with various amounts of purified protein preparation (10−30 pmol) in 5 μL of a reaction mixture containing 0.1 M Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.4 mM DTT for 10 min on ice, and then incubated with or without RNase A at 30 °C for 10 min. A solution (2 μL) containing 1% bromophenol blue, 25% glycerol, and 50 mM EDTA was added to the reaction mixture, and the samples were analyzed by native 5% PAGE. Electrophoresis was carried out at 10 V/cm for 2 h in 90 mM Tris-borate (pH 8.0)/2 mM EDTA buffer. Gels were dried, and radioactive bands were monitored, as described above.

Sensitivity to Paraquat. Spot Assay. E. coli DH5α cells harboring various plasmids were incubated at 30 °C in L broth for full growth. The total numbers of bacterial cells in cultures were adjusted to a concentration of 3×10^9 cells/mL. In general, bacterial cells expressing GST−YB-1 fusion proteins grow more slowly than cells expressing GST protein alone. Three microliters of dilutes, containing approximately 2×10^4 cells, was spotted on L plates containing 20 mg/L thymine and various amounts of paraquat. The plates were incubated overnight at 37 °C.

Determination of Colony-Forming Potential. Fully grown cultures prepared at 30 °C were diluted to an appropriate concentration, and 0.1 mL of dilutes was spread on plates containing 20 mg/L thymine and various amounts of paraquat. The plates were incubated at 37 °C overnight (for plates without paraquat) or for 2 days (for plates containing paraquat). The relative colony-forming potential was determined by dividing the number of colonies formed on plates with paraquat by the number of colonies on plates without paraquat.

Other Procedures. Protein concentrations were determined using Bio-Rad protein assay kits with bovine serum albumin as a standard (*23*). Other procedures were as described previously (*16*).

RESULTS

Binding of YB-1 Protein to 8-Oxoguanine-Containing RNA. When poly(8-oxoG·A), in which the 8-oxoGMP moiety was labeled with ^{32}P, was incubated with an excess amount of RNase A, all radioactive materials were converted to nucleotides. However, when the labeled poly(8-oxoG·A) was preincubated with a purified preparation of GST−YB-1 fusion protein and then exposed to the same amount of RNase, more than half of the 8-oxoguanine-containing polyribonucleotide remained as a polymer, even after prolonged incubation (Figure 1). Under the same conditions, poly(U·A) was completely digested.

Specific Binding of YB-1 Protein to 8-Oxoguanine-Containing RNA. Protection of 8-oxoguanine-containing

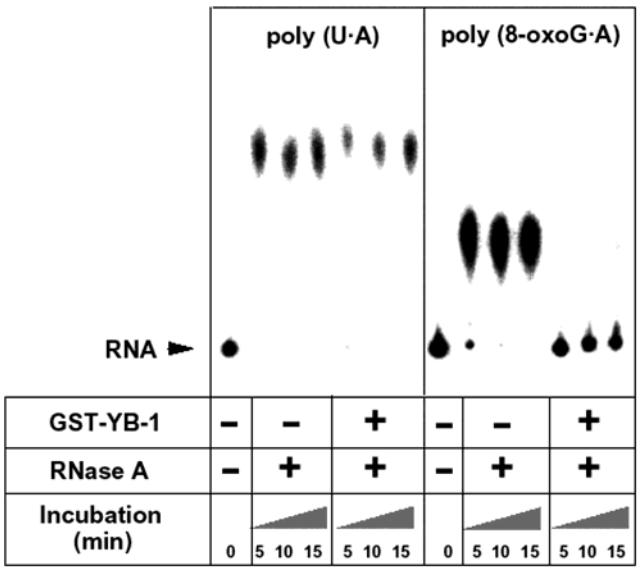

FIGURE 1: RNase protection assay for 8-oxoguanine-containing polyribonucleotide. An arrowhead indicates the origin where the polynucleotides remain.

RNA from nuclease digestion may exist because of binding of RNA to the YB-1 protein. To examine this possibility, a gel shift assay was carried out. Labeled poly(8-oxoG·A) was incubated with GST−YB-1 fusion protein or GST protein, and then the mixtures were subjected to gel electrophoresis (Figure 2A). On incubation of the material with GST−YB-1 fusion protein, several bands appeared in the upper region of the gel, thus indicating formation of complexes of the protein with labeled polyribonucleotide. Significant amounts of complexes remained even after treatment with an excess amount of RNase A. In the case of GST protein, such a band was absent in the upper region of the gel and all the material was degraded with RNase A. Therefore, YB-1 protein apparently has the potential to bind poly(8-oxoG·A).

To determine if this binding is specific for RNA containing 8-oxoguanine, poly(U·A) was examined regarding binding capacity. As shown in Figure 2B, only a small amount of material was detected at the position where protein−nucleic acid complexes migrate, when poly(U·A) was incubated with GST−YB-1 fusion protein in the absence of RNase A. These faint binding signals disappeared when the complexes were treated with RNase A.

In the next experiment, various types of RNAs were added to the reaction mixture as competitors for complex formation (Figure 3). When a 10-fold excess of 8-oxoguanine-containing RNA was added to the reaction mixture, the level of formation of the complex by the labeled probe decreased to 10% of the level of control (no addition). On the other hand, addition of a 20-fold excess of yeast tRNA or poly(U·A) had no appreciable effect on complex formation. Thus, the binding of YB-1 protein is indeed specific for 8-oxoguanine-containing RNA. The mobility pattern of the complexes is somewhat different from that obtained with the previous experiment. In this regard, it may be worth noting that 10 pmol of the protein, one-third of that in the previous experiment, was used in this experiment.

Domains of YB-1 Protein Required for Binding. YB-1 protein consists of three major domains, namely, the alanine/proline-rich N-terminal domain, the highly conserved nucleic acid-binding domain, and the C-terminal domain containing alternating regions of basic or acidic amino acids (*24*−*27*). To determine which domains are required for specific binding to 8-oxoguanine-containing RNA, a series of deletion mutant proteins lacking one or two of the domains were examined for binding capacity (Figure 4). A truncated protein Δ1, which lacks the N-terminal region, still possessed a high level of binding capacity. When the most C-terminal half of protein was removed (for Δ2), most of the binding capacity was lost. No binding activity was detected with the central region or the C-terminal region alone (for Δ3 and Δ5). It seems

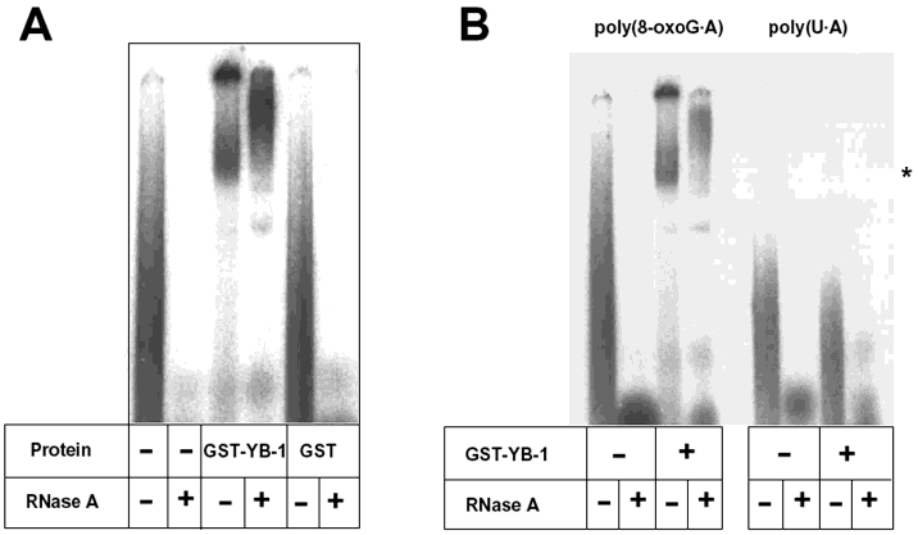

FIGURE 2: Gel shift assay to detect binding complexes. (A) Binding of YB-1 protein to 8-oxoguanine-containing polyribonucleotide. In 5 μL of the reaction mixture, 10 pmol of ^{32}P-labeled poly(8-oxoG·A) was preincubated with 30 pmol of purified GST−YB-1 fusion protein or GST protein at 0 °C for 10 min and then treated with or without 40 μg/mL RNase A at 30 °C for 10 min. After RNase A treatment, the reaction mixtures were analyzed, using 5% PAGE. (B) Specificity of YB-1 protein for formation of binding complexes. In 5 μL of the reaction mixture, 10 pmol of ^{32}P-labeled polymers, poly(U·A) or poly(8-oxoG·A), was preincubated with 30 pmol of a purified preparation of GST−YB-1 fusion protein at 0 °C for 10 min and then treated with or without 40 μg/mL of RNase A at 30 °C for 10 min. The reaction mixtures were analyzed, using 5% PAGE. An asterisk indicates the binding signal for the complex formed by poly(U·A) and the purified protein in the absence of RNase A.

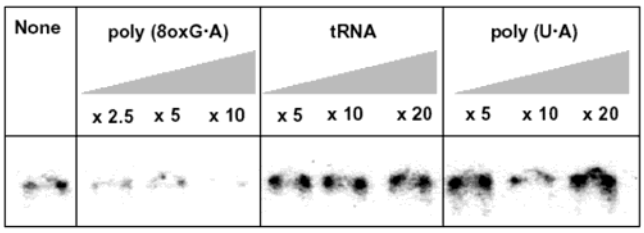

FIGURE 3: Competition for binding to 8-oxoguanine-containing polyribonucleotide with various RNAs. Ten picomoles of purified YB-1 protein was preincubated with a 2.5−20-fold excess of competitor RNAs (25−200 pmol) at 0 °C for 10 min, and then 10 pmol of ^{32}P-labeled poly(8-oxoG·A) was added. The reaction mixtures were incubated at 0 °C for 10 min, followed by an additional incubation with 4 μg/mL RNase A at 30 °C for 10 min. After incubation, the reaction mixtures were analyzed, using 5% PAGE, and radioactive bands were monitored, as described above.

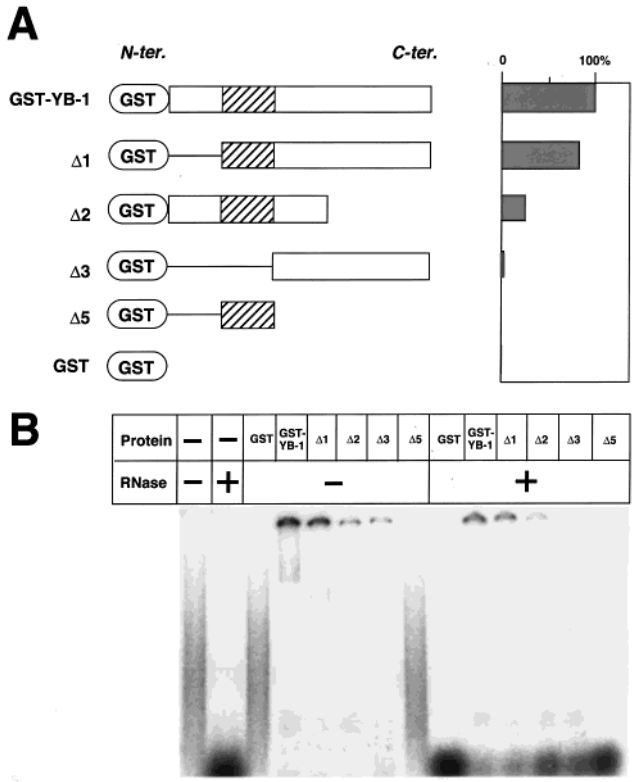

FIGURE 4: Determination of the domains required for specific binding to 8-oxoguanine-containing polyribonucleotide. (A) Schematic illustration of various forms of proteins and their specific binding activities (percent). Hatched boxes represent cold shock domains (CSDs). Specific binding activities were determined by monitoring the amounts of the binding complexes after treatment with RNase A. The specific binding activity of full-length YB-1 protein is expressed as 100%. (B) Gel shift assay to detect binding complexes. Ten picomoles of ^{32}P-labeled polymers was preincubated with or without 30 pmol of purified preparations of various forms of the protein at 0 °C for 10 min and then incubated with or without 40 μg/mL RNase A at 30 °C for 10 min.

that both central and C-terminal regions are required for proper binding.

Effects of Paraquat on E. coli Cells Producing Various Forms of YB-1 Protein. Paraquat induces oxidative stress in metabolically active cells (*28*), and growth of *E. coli* cells under aerobic conditions is inhibited by this agent. Therefore, it was of interest to determine how various forms of YB-1 protein affect aerobic growth of *E. coli* cells. In an experiment, the result of which is shown in Figure 5, cultures of

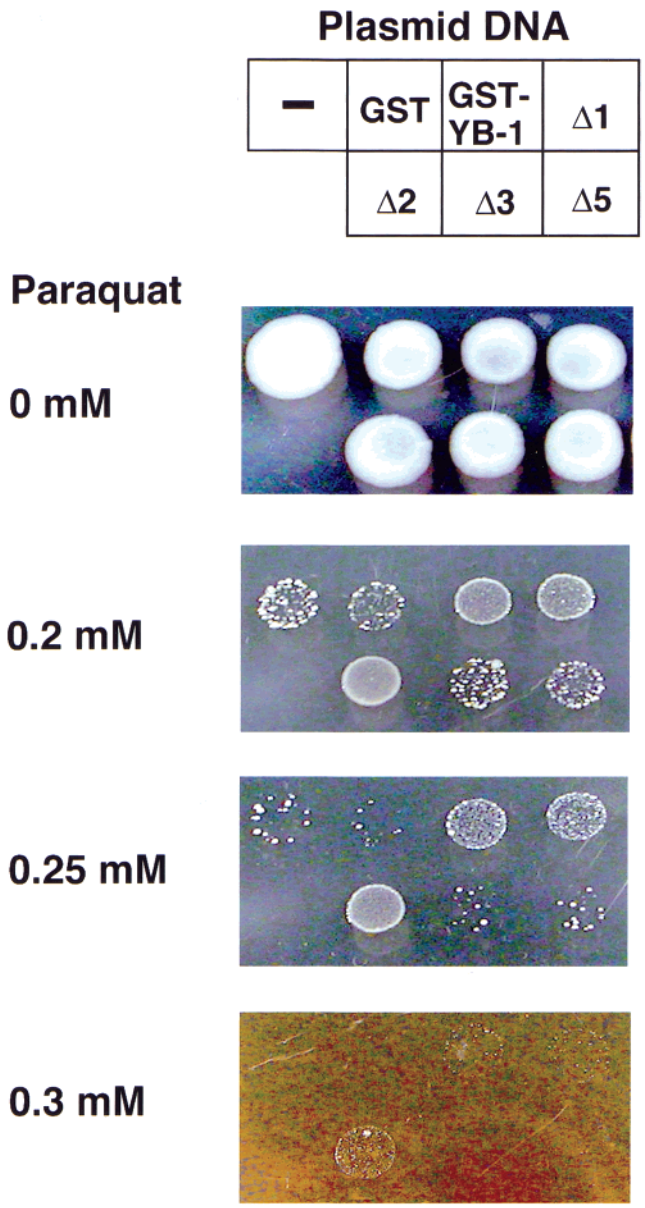

FIGURE 5: Spot assay to monitor the growth inhibitory effects of paraquat. *E. coli* DH5α cells carrying various types of plasmids were grown in L broth overnight at 30 °C. Cell suspensions containing 2 × 10^4 cells were spotted on L plates containing 20 mg/L thymine and various amounts of paraquat, and the plates were incubated overnight at 37 °C.

E. coli cells harboring plasmids which produce various types of YB-1 protein were spotted on plates containing different levels of paraquat. In the presence of 0.25 mM paraquat, there is little growth of *E. coli* cells without YB-1 protein. Under the same conditions, cells carrying YB-1 protein grow and those producing Δ1 and Δ2 showed similar degrees of growth. On the other hand, growth of cells with Δ3 and Δ5 was strongly inhibited in the presence of the same dose of the drug. It is remarkable that the specific binding capacity for binding of YB-1 protein to 8-oxoguanine-containing RNA and the potential of the protein to afford cell growth in the presence of paraquat are well-correlated.

The colony-forming potential of these cells was further determined by plating the cells on agar medium containing various doses of paraquat. Since the effect of paraquat is bacteriostatic, numbers of detectable colonies on plates

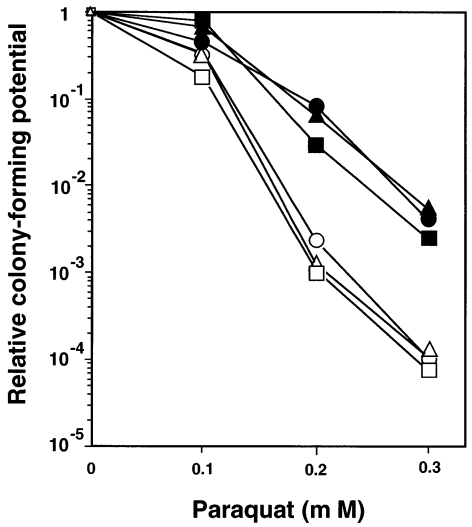

FIGURE 6: Colony-forming potential of transformants on paraquat-containing plates. Relative colony-forming abilities were determined by dividing the numbers of colonies formed on plates containing paraquat by those formed on plates without paraquat: (○) GST, (●) GST−YB-1, (■) Δ1, (▲) Δ2, (□) Δ3, and (△) Δ5.

containing paraquat increased gradually with longer incubations (*17*). Thus, the number of colonies on paraquat plates was determined at a fixed time, namely, incubation for 2 days at 37 °C. As shown in Figure 6, bacterial strains can be divided into two groups, one being relatively resistant to paraquat and the other exhibiting increased sensitivity. Cells carrying GST−YB-1 fusion protein, Δ1, and Δ2 belong to the former group, while cells with Δ3, Δ5, and GST belong to the latter. In this case, too, a clear distinction was made, in which the groupings were essentially the same as those obtained using the spot test.

DISCUSSION

Guanine residues in nucleic acids may be attacked by active oxygen species, and this *in situ* oxidation would occur more frequently in RNA molecules which are mostly single-stranded than in double-stranded DNA, in which the bases are protected by hydrogen bonding. Moreover, once the guanine bases in RNA are oxidized, they become unrepairable. This is in contrast to the case of DNA, in which most oxidized guanine bases could be excised and replaced with intact bases through actions of the DNA repair machinery (*10*−*13*). The persistence of the oxidized guanine bases in mRNA would lead to errors in translation, as was shown by suppression of the *lacZ* system (*15*). When these situations are considered, it may be important for organisms to be equipped with a certain mechanism that recognizes RNA containing 8-oxoguanine and eliminates such from the cellular translation system.

In the study presented here, we examined this problem by determining if mammalian YB-1 protein has the capacity to bind RNA carrying 8-oxoguanine. YB-1 protein was first identified as a cellular constituent that binds to the CCAAT box, the Y box, of the MHC class II promoter sequence to regulate the rate of transcription of the gene (*18*). The protein is a major core protein of messenger ribonucleoprotein (*20*) and possesses RNA binding activity (*21*). Overproduction of YB-1 protein in COS cells blocks protein synthesis, and addition of the purified YB-1 protein to the *in vitro* translation system severely reduces its protein-synthesizing capacity (*20*, *21*). These results suggested that YB-1 protein may be involved in regulation of both transcription and translation processes. It was also revealed that YB-1 interacts with several cellular components, including PCNA, p53, and hnRNA K (*22*, *29*, *30*). Indeed, levels of expression of YB-1 protein are associated with various biological phenomena, including cell proliferation and transformation (*24*, *31*−*33*). These findings prompted us to determine if YB-1 protein has the capacity to discriminate oxidatively damaged RNA from normal RNA.

YB-1 protein consists of three major domains: the alanine/proline-rich N-terminal domain, the highly conserved nucleic acid-binding domain, and the C-terminal domain containing alternating regions of basic or acidic amino acids (*24*−*27*). The highly conserved domain, located in the central region, is found in related proteins in both prokaryotes and eukaryotes and is termed the "cold shock domain" (CSD) (*24*, *26*, *27*). Proteins containing CSD bind to the Y box and also to single-stranded DNA and RNA (*27*, *34*−*36*). On the other hand, the C-terminal domain is thought to interact with several cellular and viral proteins, including PCNA, JC virus T antigen, AP-2, RelA tat, and Purα (*22*, *37*−*40*). In the study presented here, we found that the full-length YB-1 protein as well as truncated proteins Δ1 and Δ2, all containing CSD and the C-terminal region, are capable of binding to 8-oxoguanine-containing RNA. On the other hand, Δ3 and Δ5 constructs containing CSD or the C-terminal region alone do not exhibit specific binding capacity. These results indicate that both the CSD and the C-terminal domain are essential for exerting proper binding to RNA containing 8-oxoguanine.

The Pnp protein of *E. coli* specifically binds to 8-oxoguanine-containing RNA, and mutants defective in this enzyme are hyperresistant to paraquat (*17*). Pnp protein has been defined as a component of the RNA degradosome, which is involved in mRNA turnover (*41*, *42*). Messenger RNA turnover in *E. coli* is initiated by a series of endonucleolytic cleavages primarily initiated by RNase E, and followed by processive 3′−5′ exonucleolytic degradation by polynucleotide phosphorylase and/or RNase II. Taking advantage of this bacterial system, we asked if expression of the mammalian binding protein would render *E. coli* cells resistant to the oxidative stress caused by paraquat. When YB-1 protein is expressed in *E. coli* cells, 8-oxoguanine sites in RNA may be occupied by mammalian protein, and as a result, RNA degradosome cannot function. In the study presented here, we found that YB-1 protein indeed affords bacterial cells resistance to paraquat. The unique paralleled effects of bacterial Pnp and mammalian YB-1 proteins on oxidative stress of the cell imply that there are similar mechanisms in the two systems.

Evidence presented in this paper implies that YB-1 protein may be involved in sequestering oxidatively damaged RNA from normal cellular processes. However, further studies are necessary for better defining the roles of YB-1 protein *in vivo*. Construction of mice defective in YB-1 protein would be useful for investigating this problem, and studies on this line are in progress in this laboratory.

Oxidative damage to nucleic acids may be a prominent feature of vulnerable neurons in mammals. In *in situ* immunostaining analysis, a large amount of 8-oxoguanine

was found in the cytoplasm of neurons of patients with Alzheimer's disease but not in normal subjects. The oxidized base appears to be predominantly associated with RNA, since immunoreactions were inhibited by preincubation with RNase but not with DNase (*43*). This notion was confirmed in ultrastructural examinations in which most of the immunoreactivity was associated with ribosomes in the endoplasmic reticulum (*44*). Similar results were obtained in cases of Down's syndrome and Parkinson's disease, while in the case of Parkinson's disease, both RNA and DNA were oxidatively damaged (*45*, *46*). One of the interesting features is that oxidation of RNA is the earliest event in both Alzheimer's disease and Down's syndrome, namely, that it occurs prior to disease progression and lesion formation (*44*, *46*). It will be of interest to determine if there is any alteration in YB-1 function in the nervous system of patients with these diseases.

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