The Y-box Motif Mediates Redox-dependent Transcriptional Activation in Mouse Cells*

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We show here that the OxyR response element (ORE) in the bacterial oxyR promoter can also function as a redox-dependent enhancer in mammalian cells. Fusion of ORE to an SV40 basal promoter driving chloramphenicol acetyltransferase (CAT) expression confers H_2O_2 inducibility to expression of the cat gene in mouse Hepa-1 hepatoma cells. Nuclear extracts from these cells contain DNA-binding proteins that specifically interact with ORE DNA, cannot be competed by cognate oligonucleotides to AP-1 or NF-κB, and are constitutively expressed, since treatment with H_2O_2 causes no detectable changes in binding activity or DNA-protein interaction. Recombinant cDNA clones that express ORE-binding proteins were isolated from a mouse hepatoma expression library and found to be representatives of two different members of the murine Y-box family of transcription factors. Canonical Y-box and ORE oligonucleotides compete with each other for binding to Y-box proteins in gel shift assays and antibodies to FRGY2, a Xenopus Y-box protein, supershift both Y-box and ORE DNA-protein complexes. In addition, antisense oligonucleotides to mouse YB-1 mRNA abolish induction of ORE-mediated cat expression by H_2O_2 and luciferase reporter constructs containing ORE, or the Y-box from the human MHC class II HLA-DQ gene, exhibit identical dose-dependent H_2O_2 inducibilities, which can be abolished by addition of 2-mercaptoethanol to the culture medium. These results suggest that the Y-box proteins may be an integral component of a eukaryotic redox signaling pathway.

Reactive oxygen intermediates, including superoxide anion, hydrogen peroxide, and hydroxyl radical, are generated during normal aerobic metabolism by the incomplete reduction of oxygen to water and are crucial for many physiologic processes, such as the respiratory burst of phagocytic cells. Reactive oxygen intermediates also pose a continuous risk of cell injury to aerobic organisms, a risk that is greatly elevated by exposure to environmental oxidants such as ionizing radiation, heavy metals, redox active chemicals, and hyperoxia, that increase their production (1–4). Reactive oxygen intermediates react with DNA, proteins, lipids, and cellular membranes, causing behavioral, cytotoxic, and mutagenic damage (5–8). With an increased understanding of the pathways leading to oxidant injury and of the protective role of antioxidant compounds, it is apparent that endogenous antioxidant mechanisms exist in a balance with each other and with endogenous oxidants and that perturbation of this balance contributes to the pathogenesis of many human diseases, including pulmonary oxygen injury, cancer, aging, and degenerative diseases (1–4).

Much of our current understanding of genetically determined enzymatic antioxidant defenses derives from work in enteric bacteria, where approximately 80 different proteins controlled by two different regulons are induced by oxidative stress (9). One of these regulons is stimulated by superoxide radicals and is controlled by the product of the oxyR gene (14–17). The OxyR protein is an autoregulatory, DNA-binding, redox-sensitive transcriptional activator of genes coding for peroxide-inactivating enzymes such as catalase (katG), NADPH-dependent alkalinehydroperoxidase (aphFC), and many others (18–20). The promoters of the genes regulated by OxyR show little sequence relatedness, and consequently there is a poor definition of consensus sequence elements for OxyR-binding DNA motifs (21–24).

Our understanding of the regulation of antioxidant defenses in eukaryotic systems is far less defined than in prokaryotes. Many genes are induced by reactive oxygen species (25–28), including transcription factors like those in the AP-1 (29–32) and NF-κB (33–35) families, but regulons resembling those controlled by soxRS and oxyR have not been described, although their existence has long been suspected. We report here that the bacterial OxyR-binding motif functions as a redox-dependent transcriptional enhancer in murine cells. This function results from the interaction of the ORE motif with a member of the Y-box family of DNA- and RNA-binding proteins.

EXPERIMENTAL PROCEDURES

Cell Lines, Transfections, and Growth Conditions—Mouse Hepa-1 hepatoma cells (36) and NIH3T3 cells were routinely maintained as monolayer cultures in α-MEM (Life Technologies, Inc.) supplemented with 5% fetal bovine serum. For transient transfection experiments, the bacterial chloramphenicol acetyltransferase (cat) gene and the firefly luciferase gene were used as reporters. Approximately 10 μg of the appropriate plasmid was transfected into semiconfluent Hepa-1 cells grown in 25-cm² tissue culture flasks by standard calcium phosphate techniques (37, 38) or as more recently optimized (39). To control for variations due to differences in transfection efficiency, all cultures were co-transfected with 1 μg of plasmid pCMV/gal (Clontech), which expresses the bacterial β-galactosidase gene under the control of the

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1 The abbreviations used are: ORE, OxyR response element; BrdUrd, bromodeoxyuridine; MEM, minimum Eagle's medium; PBS, phosphate-buffered saline; 2-ME, 2-mercaptoethanol; CAT, chloramphenicol acetyltransferase; bp, base pair(s); DTT, dithiothreitol; DCF, 2,7′-dichlorofluorescein; DA-DCFH, 2,7′-dichlorofluorescein diacetate; MHC, major histocompatibility complex.
cysteomegalovirus immediate-early enhancer and promoter. Expression of β-galactosidase under regulation by this enhancer is independent of treatment to the cells. Twelve to sixteen hours after transfection, the cells were washed with fresh medium and 1-2 h later were trypsinized and seeded into 6- or 12-well tissue culture plates. Five to six hours later, when the cells had recovered from trypsinization, the medium was replaced with fresh medium. Cells were washed with phosphate-buffered saline (PBS) and treated with PBS in PBS for 45 min. Control cells were mock-treated with PBS lacking H$_2$O$_2$. After treatment, cells were fed with α-MEM supplemented with 5% fetal bovine serum or with the same medium containing 140 μM 2-ME. Cell extracts were prepared 18 h later by three cycles of freeze-thawing and expression of CAT, lucerase, and β-galactosidase activities were determined. CAT activity was measured by the phase extraction method (40) using 0.2 μCi of [14C]chloramphenicol (Amersham Corp.) as the substrate. Chloramphenicol conversion to acetylated forms was 1–25%, well within the linear range of the assay (40). Luciferase and β-galactosidase activities were determined with the Promega luciferase assay system, following the manufacturer's specifications. Luciferase activities were measured in a Turner 20e luminometer. Data were normalized for differences in transfection efficiency by determination of the relative amount of chloramphenicol converted to acetylated forms or of arbitrary light units per unit of β-galactosidase. All determinations were done in duplicate plates, and experiments were repeated at least three times; the values shown are the means ± S.E.

For transient expression of ORE constructs in the presence of anti-Y-box or untreated controls, two different oligonucleotides, the following oli843 (mousebox, see Fig. 4) antisense oligonucleotides were used as phosphorothioated derivatives to block mRNA translation in vivo: Y1: (5′)CATGGTTGAGGTGATGGTGAT(-18). The numbers in parentheses indicate the coordinates relative to the ATG initiation codon (or sequences indicated above). Probes were synthesized with the TNT kit from Promega, using 1 μCi of recambinant plasmid DNA as the transcription template and following the manufacturer's instructions. Protein samples (30–50 μg of cell extract or 5 μl of a 50-μl in vitro translation reaction) were separated in 10% or 15% SDS-polyacrylamide gels and transferred to Immobilon by electroblotting. Nonspecific binding sites were scored by phosho-image analysis (Fig. 3). Y-Box Mediates Transcriptional Activation

Fig. 4) antisense oligonucleotides were used as phosphorothioated derivatives to block mRNA translation in vivo:

For analysis of their oxidant status, cells were grown on microscope coverslips at 37°C in the absence or presence of 25 μM H$_2$O$_2$ in PBS for 45 min, and the other half was treated only with PBS. Following treatment, all coverslips were washed with fresh PBS and incubated in complete medium for an additional 1 h. Thereafter coverslips were washed in PBS, incubated for 10 min at 37°C with 2,7'-dichlorofluorescein diacetate, washed again with PBS, and examined by fluorescent microscopy. The excitation and emission wavelengths for fluorescence measurements were 495 and 535 nm, respectively.

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Plasmids and Oligonucleotides—The plasmid pOxyR-72 was constructed by ligation of the 51-bp ORE oligonucleotide probe (specific activity: 1 × 10$^6$ dpm/μg) of the sequences indicated above. Probes were labeled with T4 polynucleotide kinase in the presence of (γ−32P)ATP. After incubation, samples were analyzed in nondenaturing 4% polyacrylamide gels and identified by exposing the gel to x-ray film. The complex A bands were excised from the gel and elutedoblotted onto NA 45 paper (Schleicher and Schuell). DNA was eluted from the paper by incubation in 1× NaI, 10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA at 56°C for 1 h, extracted with an equal volume of phenol-chloroform (1:1) and ethanol precipitated. The DNA was dissolved in 3 μl water and analyzed on a 4% polyacrylamide gel for 30 min, and analyzed in denaturing 15% polyacrylamide-urea gels.

For Southern analyses, nuclear and cytosolic extracts from H$_2$O$_2$-treated and untreated cells were prepared as described (43, 44), and proteins from coupled in vitro transcription-translation assays were synthesized with the TNT kit from Promega, using 1 μCi of recombinant plasmid DNA as the transcription template and following the manufacturer's instructions. Protein samples (30–50 μg of cell extract or 5 μl of a 50-μl in vitro translation reaction) were separated in 10% or 12.5% SDS-acrylamide gels and transferred to Immobilon membrane (Millipore). After transfer, membranes were washed in Tris-HCl, pH 7.8, 60 mM KCl, 1 mM MgCl$_2$, 1 mM DTT), and proteins were denatured by immersing the membranes in 6 M guanidine hydrochloride in buffer B. Denatured protein renaturation was accomplished by sequentially washing the membranes for 5 min in four successive 2-fold serial dilutions of the denaturing solution in buffer B, followed by two 5-min washes in buffer A. In some experiments, denaturation in 6 M guanidine hydrochloride and graded renaturation were omitted. Nonspecific binding sites were blocked by incubation for 1 h at room temperature in 5% non-fat milk in buffer B. Probes were prepared by labeling each strand of a double-stranded oligonucleotide separately at the 5′ end with T4 polynucleotide kinase in the presence of [γ−32P]ATP and allowing the strands to anneal. For maximum binding efficiency, probes were cotranslated with T4 DNA ligase. Binding of end-labeled, catenated DNA to immobilized Y-box probe was performed in a high ionic strength buffer B, containing 120 mM KCl and 2 × 10$^6$ dpm/μl probe. After binding, the membranes were washed three times in the buffer of a common composition, blocked, and exposed to x-ray film.

For Western blots, proteins were transferred to Immobilon, and the blot was blocked with 2% goat serum, 2% non-fat milk in 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1% Tween 20, 0.1% sodium dodecyl sulfate, and incubated for 1 h at room temperature. A rabbit anti-Xenopus FRY2 antibody (a kind gift from Dr. Alan Wolfe) was used at a 1:4,000 dilution. Detection was by the ECL chemiluminescence detection kit of Amersham Corp.

For UV-cross-linking, 50 pmol of ORE noncoding strand was annealed to an equimolar amount of a 10-mer oligonucleotide complementary to the 3′ end of the template. The BrdU substituted coding strand was synthesized by incubation with the Klenow fragment of E. coli DNA polymerase in the presence of 50 μM each Br-dUTP, dATP, dGTP, and 50 μCi of [α−32P]dCTP (3,000 Ci/mmoll for 1 h at 37°C.

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Approximately 10 ng of probe (1 \( \times 10^6 \) dpm) were incubated with 20 \( \mu \)g of nuclear extract protein from untreated cells. Binding mixtures were exposed to 12 \( J/\text{m}^2 \) of 254-nm UV light or left unexposed. Complexes were digested with 2 units of DNase I in the presence of 5 \( \mu \)M MgCl\(_2\) for 20 min at room temperature; one-third of three \( \times \) SDS-polyacrylamide gel electrophoresis buffer was added, the samples were heated to 100°C for 5 min and analyzed on 10% SDS-polyacrylamide gels.

Cloning and DNA Sequence Analyses—A Hepa-1 mRNA expression library in \( \lambda gt11\) (46) was screened by recognition site techniques (46–48) using as a probe a radiolabeled, cateden ORE (mean of four monomer copies), in the presence of a 1,000-fold excess of mutant ORE oligonucleotide. The two clones obtained after three screening cycles were plaque-purified, grown to high titer, and the DNA sequenced by cycle sequencing as described previously (49). Sequence analysis was done using the DNA analysis programs version 7.3 (Genetics Computer Group). The two Y-box clones found in these studies were termed oxi843 and oxi17. oxi843 was assembled from two overlapping clones that contained sequences coding for amino acid residues 1-141 and 48–311, respectively. Because of its identity with the known murine YB-1 protein, the sequence of oxi843 has not been submitted to GenBank™. oxi17 is contained in a single 2.4-kilobase pair \( \lambda gt11 \) insert, and its sequence has been submitted to GenBank™ with the accession number L35549.

RESULTS

The ORE Motif Functions as a Peroxide-responsive Eukaryotic Enhancer—To establish whether the bacterial oxyR promoter (hereafter referred to as the ORE motif) could function as a peroxide-responsive eukaryotic enhancer we constructed a pOxyRCAT reporter plasmid that joined the 51-bp wild-type ORE sequence (16) to a minimal enhancerless SV40 early promoter driving the bacterial cat gene. \( \text{H}_2\text{O}_2 \) treatment of mouse hepatoma Hepa-1 cells transiently transfected with this plasmid stimulated CAT activity 3–4-fold over the untreated control (Table I). In contrast, a control with a mutant pOxyRCAT reporter gave 2-3-fold lower values in untreated cells, and \( \text{H}_2\text{O}_2 \) treatment caused only a minor increase in activity that did not even attain the unstimulated levels observed with the wild-type construct. These findings were essentially identical in the two murine cell lines, Hepa-1 hepatoma and NIH3T3 fibroblasts, that were tested. It must be emphasized that these results were obtained from 18 determinations (six experiments done in triplicate) for the Hepa-1 cells and from four determinations (two experiments done in duplicate) for the NIH3T3 cells and that, however modest, the differences between treated and untreated cells are highly significant (\( p = 0.0006 \)) for the transfections with pOxyRCAT and not significant (\( p = 0.8 \)), for Hepa-1, and \( p = 0.3 \) for NIH3T3 cells for the transfections with pmOxyRCAT (Table I). Thus, we felt that the differences observed were convincing enough to warrant further investigation.

The actual extent of peroxide-induced oxidative stress in the Hepa-1 cells used in the rest of these experiments was estimated by measuring the uptake and oxidation of the fluorescent probe 2,7′-dichlorofluorescein diacetate (DA-DCFH). DA-DCFH is a reliable indicator of intracellular oxidant state (50–54), since it is taken up by cells and deacetylated by esterases, releasing free dichlorofluorescein (DCFH). DCFH is readily oxidized by reactive oxygen intermediates to form the fluorescent product 2,7′-dichlorofluorescein (DCF) (54–57). Cells grown under reducing conditions, i.e. Dulbecco’s modified Eagle’s medium supplemented with 25 \( \mu \)M 2-mercaptoethanol, displayed a mean DCF fluorescence per cell significantly lower than that of cells grown in unsupplemented medium. In both instances, fluorescence was nearly doubled after a 10-min treatment with 300 \( \mu \)M \( \text{H}_2\text{O}_2 \) (Table II), indicating that \( \text{H}_2\text{O}_2 \) causes a significant change in the level of intracellular oxidants.

Muirne DNA-binding Proteins Recognize the ORE Motif—The above results suggested that the oxyR element might function as an oxidant-dependent enhancer that was recognized by transcriptional activators. To test this hypothesis and to analyze the effect of oxidant status on the binding of this putative factor to the ORE, replica plates of Hepa-1 cells were grown in normal medium or in medium supplemented with 25 \( \mu \)M 2-ME. One-half of each culture was treated with \( \text{H}_2\text{O}_2 \), and the other half was left untreated. Nuclear protein extracts were analyzed by gel retardation, using a radiolabeled 51-bp ORE oligonucleotide (18–20) as the probe. Extracts were prepared and analyzed in the presence or absence of 20 \( \mu \)M DTT, since binding of reox-sensitive transcription factors to their cognate sequences is often affected by the presence of reducing agents in the binding reactions (18–20, 29–35). Three major and two minor DNA-protein complexes were detected. The two minor complexes, labeled D and E in Fig. 1A, appeared to be affected by the presence of DTT in extracts and binding reactions, but were often weak or undetectable. We have not analyzed these two complexes further. The three major complexes, labeled A, B, and C in Fig. 1A, were found in extracts from cells treated with \( \text{H}_2\text{O}_2 \) as well as in untreated cells, and their formation was independent of the oxidant status of extracts or binding reactions. The relative abundance of these three complexes varies from extract to extract, and in general, we observed that the more of the A complex was formed, the less of complexes B and C could be found, and vice versa, pointing at the possibility that complex A may result from the interaction of complexes B and C. In Fig. 1A, there is an apparent decrease of complex A oxidant-treated cells; these decrease was not reproducible and seems to be due to differences from extract to extract. Positive and negative binding controls were run with other DNA motifs. As expected, peroxide treatment activated the DNA-binding activity of NF\( \kappa \)B and failed to activate the nuclear translocation of the Ah receptor (data not shown).

The specificity of the DNA-protein interactions detected was examined in greater detail by using an excess of various unla-

### Table I

| Plasmid          | Cell line | Relative CAT activity | \( + \text{H}_2\text{O}_2 \) | \( - \text{H}_2\text{O}_2 \) | \( p \) value |
|------------------|-----------|-----------------------|-----------------------------|-----------------------------|--------------|
| pOxyRCAT Hepa-1  | NIH3T3    | 1.0 ± 0.05            | 4.2 ± 0.9                   | 0.0006                      |
| pmOxyRCAT Hepa-1 | NIH3T3    | 0.4 ± 0.10            | 0.9 ± 0.5                   | 0.8                        |
|                  | NIH3T3    | 0.3 ± 0.10            | 0.6 ± 0.2                   | 0.3                        |

### Table II

| Growth medium | Mean fluorescence per cell | \( + \text{H}_2\text{O}_2 \) | \( - \text{H}_2\text{O}_2 \) |
|---------------|--------------------------|-----------------------------|-----------------------------|
| DMEM          | 19,000 ± 700             | 32,300 ± 1,600*             |
| DMEM + 2-ME   | 13,900 ± 600*            | 26,700 ± 1,000*             |

*Value is significantly greater than value of cells not treated with \( \text{H}_2\text{O}_2 \) (\( p < 0.05 \)).

*Value is significantly smaller than that of similarly treated cells grown in the absence of 2-ME (\( p < 0.05 \)).
In both cases, one-half of each culture was treated with 300 nM KatG in PBS, and the other half was mock-treated with PBS. In addition, nuclear extracts were prepared in the absence or presence of 20 mM DTT. The positions of the five complexes with ORE probes are indicated at the left. B, competition of ORE binding by different oligonucleotides. Binding reactions were prepared in the presence of 1 mM DTT with extracts from untreated cells grown in the absence of 2-ME. Prior to addition of the labeled ORE probe, extracts were preincubated for 5 min with a 300-fold excess of the indicated oligonucleotides (see the "Experimental Procedures" for the complete sequences): AhpC, the 46-base pair-long OxyR binding sequence in the bacterial ahpFC promoter; KatG, the 51-base pair-long OxyR binding sequence in the bacterial katG gene promoter; NF-κB, the consensus NF-κB binding motif; AP-1, the consensus AP-1 binding site; and OxyR, the same 51-mer OxyR promoter sequence present in the probe.

Fig. 1. Characterization of ORE-binding proteins in Hepa-1 cells. A, gel retardation analyses of ORE-binding nuclear proteins. Nuclear extracts (30 μg) used were from Hepa-1 cells grown in the absence (−2ME) or in the presence (+2ME) of 2-mercaptoethanol. In both cases, one-half of each culture was treated with 300 nM H$_2$O$_2$ (+) in PBS, and the other half was mock-treated with PBS (−). In addition, nuclear extracts were prepared in the absence (−) or presence (+) of 20 mM DTT. The positions of the five complexes with ORE probes are indicated at the left. B, competition of ORE binding by different oligonucleotides. Binding reactions were prepared in the presence of 1 mM DTT with extracts from untreated cells grown in the absence of 2-ME. Prior to addition of the labeled ORE probe, extracts were preincubated for 5 min with a 300-fold excess of the indicated oligonucleotides (see the "Experimental Procedures" for the complete sequences): AhpC, the 46-base pair-long OxyR binding sequence in the bacterial ahpFC promoter; KatG, the 51-base pair-long OxyR binding sequence in the bacterial katG gene promoter; NF-κB, the consensus NF-κB binding motif; AP-1, the consensus AP-1 binding site; and OxyR, the same 51-mer OxyR promoter sequence present in the probe.

Fig. 2. Methylation interference analyses. A, methylation interference. To maximize any possible differences in binding characteristics between oxidative and reducing environments, DNA binding reactions were prepared with extracts from cells grown under the most divergent conditions. The nuclear extract from untreated cells (U) was from cells grown in the presence of 2-ME and prepared with 20 mM DTT; the nuclear extract from H$_2$O$_2$-treated cells (H) was from cells grown without 2-ME and prepared without DTT. In addition, binding reactions were run in the presence (U) or absence (H) of 20 mM DTT. Results of both coding and noncoding strands shown. C, control with free probe. The solid arrows point to the three G residues in the coding strand whose methylation interferes strongly with binding; the open arrow points to the G residue in the noncoding strand that shows less pronounced methylation interference. B, partial sequence of the binding motif in the oxyR promoter. The sequence shows the three G residues in the coding strand (closed stars) and the single G residue in the noncoding strand (open star) that form contacts with the binding protein.

Unlabeled ORE DNA was able to compete for its own binding effectively, while an excess of oligonucleotides containing the canonical binding sites for AP-1 or NF-κB had no effect on ORE binding (Fig. 1B). Remarkably, binding to ORE was readily competed by the OxyR binding sites in the bacterial katG and ahpFC promoters (Fig. 1B), indicating that the binding characteristics and specificity of the mammalian protein resembled very closely those of bacterial OxyR.

Methylation Interference and Cross-linking Analyses—The contacts between the ORE sequence and protein in complex A were defined by methylation interference. No differences in methylation interference patterns were detected between extracts prepared from H$_2$O$_2$-treated or untreated cells. Methylation at three G residues precluded protein binding in the coding strand in both cases (Fig. 2A). Methylation interference was apparent at one G residue in the noncoding strand; however, the effect was less distinct than that caused by methylation in the coding strand (Fig. 2A). Weaker methylation interference was also observed at several G and A residues both in the coding and the noncoding strands, suggesting that the DNA-protein contacts may extend over a relatively large region of the DNA. Interestingly, however, the three major G residues contacted by the mouse protein (indicated by stars in Fig. 2B) are a subset (three out of five) of the residues described for contact by the bacterial OxyR protein with its own promoter (21, 22).

The size of the murine ORE-binding protein was determined by Southwestern blot analyses of cytosolic and nuclear protein extracts from H$_2$O$_2$-treated or untreated cells. A protein with an apparent molecular mass of 46 kDa was detected in cytosolic extracts by interaction with a catenated ORE probe; in addition, two other minor species with apparent sizes of 33 and 55 kDa, respectively, were observed (Fig. 3A). In the nuclear fraction, proteins with masses of 33–35, 46, 50, and 55 kDa were all very prominent (Fig. 3A). As with the gel retardation experiments, there were no differences between binding activities in H$_2$O$_2$-treated or untreated cells. The binding specificity between fractionated proteins and the wild-type ORE probe was confirmed by probing with a mutant ORE, which did not bind under the conditions used to any of the proteins detected with the wild type (Fig. 3A).

UV cross-linking analyses with a BrdUrd-substituted probe verified the physical interaction between the ORE and a DNA-
binding protein. A major DNA-protein complex was detected that included a protein species with an apparent size of 46 kDa (Fig. 3B). UV cross-linking of this complex could be almost abrogated by competition with an excess of unlabeled ORE oligonucleotide, but not by an excess of an oligonucleotide containing the NFκB consensus binding site (Fig. 3B) or a mutant ORE or an AP-1 binding site (data not shown). No cross-linking was observed in the absence of UV irradiation (Fig. 3B) or when a mutant ORE was used as the probe (not shown). The intensity of the cross-linked band was considerably lower when the noncoding strand was substituted with BrdUrd, suggesting that this strand is less likely to make stable contact with the protein (data not shown). Since only the 46-kDa polypeptide can be cross-linked to ORE, we conclude that, of the six polypeptides detected by Southwestern analysis, the one at 46-kDa is the one more likely to form a stable interaction with ORE.

The ORE-binding Proteins Are Members of the Y-box Family of Transcription Factors—To clone and identify ORE-binding mouse proteins, a Hepa-1 cDNA expression library was screened for binding of radiolabeled, catenated ORE probe (46–48). To ensure binding specificity to any putative positive clones, screenings were performed in the presence of a vast excess of mutant ORE oligonucleotide. We obtained two sets of positive clones which, upon further restriction enzyme mapping and nucleotide sequence analyses (not shown), proved to encode overlapping regions of two different cDNAs. Comparison to the GenBank™ data base revealed that these cDNAs were related to known members of the Y-box family of transcription factors (58–63). Fig. 4 shows an alignment of the assembled amino acid sequences of the two sets of clones with their closest human and mouse homologues. The Oxi843 clone encodes an open reading frame closest to mouse (Musybox) and human (Humdbbp2) YB-1 proteins; it is 13 amino acids shorter at the carboxyl end than either of these, and it differs by only 3 amino acids in the remainder of the sequence. The sequence encoded by the Oxi17 clone resembles the human (Humdbbp2) YB-2 protein more closely, although it has an apparent deletion of 69 amino acids from residues 191 to 260. These similarities held true when representatives of YB-1 and YB-2 proteins from other species (chicken, rat, frog) were used for the comparisons (data not shown).

The Y-box Protein Forms Sequence-specific Complexes with ORE—Gel shift analyses were used to establish whether the complexes between Y-box protein and ORE were the result of sequence-specific interactions. Unlabeled ORE, as well as a 45-residue oligonucleotide containing the Y-box and flanking regions of the human MHC class II HLA DQ α-2 gene, efficiently competed binding of the labeled ORE probe to crude nuclear extracts from untreated cells. Both oligonucleotide competitors were able to compete with equal effectiveness for the C complex, but the Y-box oligonucleotide competed less readily for the B complex (Fig. 5A). Conversely, formation of complexes between the Y-box protein and its cognate oligonucleotide was competed by ORE, as well as by Y-box oligonucleotide.
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Fig. 5. A Y-box protein recognizes the ORE. A, competition gel retardation. Prior to incubation with the ORE probe (OxyR) or the Y-box motif probe (Yb) nuclear extracts (5 μg) were incubated in the presence of increasing amounts (25-, 100-, and 300-fold excess) of competitor oligonucleotides with the wild-type ORE motif (OxyR), the mutant ORE sequence (mOxyR), or the Y-box motif (Yb). B, antibody supershift. Nuclear extracts were incubated with the indicated probes for 10 min and 1 μl of rabbit preimmun (PI) or immune (Im) serum was added and incubation continued for another 10 min. The double-headed arrow indicates the position of the supershifted band. C, Western and Southwestern analyses. oxi843 plasmid DNA was transcribed and translated in vitro; 1/6 of the product was separated in 12.5% SDS-acrylamide gels (O), along with 30–50 μg of nuclear (N) and cytosolic (C) extracts from untreated Hepa-1 cells. For chemiluminescence detection, immune rabbit anti-Xenopus FRGY2 serum was used at a 1:4,000 dilution (lanes 1–3). For Southwestern analyses, the ORE probe was used as described in the legend to Fig. 3A. Lanes 4–6 were denatured in 6 M guanidine hydrochloride and subjected to graded renaturation; lanes 7–9 were the same ones used for the Western blot and were analyzed for ORE binding without denaturation and renaturation treatments.

Fig. 6. Transient expression of ORE constructs in the presence of anti-Y-box mRNA antisense oligonucleotides. Cells were grown for at least three generations (72 h) in medium containing oligonucleotides at the indicated concentrations. Thereafter, they were seeded, transfected with pOxyRCAT and control pCMVβgal plasmids, and analyzed for transient expression. Y1, Y2, Y3, and Yc were described under “Experimental Procedures.” Ym refers to an equimolar mix of Y1, Y2, and Y3. Antisense oligonucleotides were kept in the medium throughout transfection and subsequent growth period prior to the assay. The values shown are the mean ± S.E. of two experiments done in duplicate.

H₂O₂ treatment: 0 300 μM

Antisense: – Ym Y1 Y2 Y3 Yc

Western and Southwestern analyses were used to analyze the relationship between the protein encoded by the OXi843 clone and the proteins in the DNA-protein complexes detected in extracts from Hepa-1 cells (Fig. 3A). In Western blots, immune anti-Xenopus antiserum clearly detected a major protein at approximately 46 kDa from nuclear and cytosolic Hepa-1 extracts as well as from the in vitro translation product of oxi843 (Fig. 5C, lanes 1–3). In a Southwestern blot of the same proteins, the ORE probe showed strong binding to the same 46-kDa protein (Fig. 5C, lanes 4–6), and, in addition, to the nuclear proteins in the 33- to 55-kDa size range observed previously. When the blot used for the Western analysis (Fig. 5C, lanes 1–3) was probed directly for ORE binding, omitting the denaturation and graded renaturation steps, only the 46-kDa protein (Fig. 5C, lanes 1–3) and the 33-kDa proteins gave strong signals, while the other additional proteins did not bind the probe (Fig. 5C, lanes 7–9). These results strongly suggest that oxi843 codes for the 46-kDa protein detected in nuclear and cytosolic cell extracts and that this protein is the one more likely to form stable complexes with ORE.

Y-box Proteins Play an Essential Role in the Oxidative Stress Response—To analyze the possible role of Y-box proteins in the transcriptional response to peroxide treatment, antisense oligonucleotides to oxi843 mRNA were tested for their capacity to block H₂O₂ induction of CAT expression directed by the pOxyRCAT reporter plasmid. Three different 21-mer phosphorothioated oligonucleotides, spanning overlapping regions of the translational initiation site, were used individually and in combination. A 21-mer with a random rearrangement of the same nucleotide sequence was used as a control. Because the ORE-binding proteins are expressed constitutively, it was necessary to grow the cells for three to four generations in the presence of antisense oligonucleotides to eliminate intracellular levels of Y-box proteins. The antisense oligonucleotides were added directly to the culture medium at concentrations ranging from 2.5 to 50 nM. Activation of CAT expression by H₂O₂ treatment was blocked by a 2.5 nM mixture of all three antisense oligonucleotides (Fig. 6, Ym). When tested individually, all three oligonucleotides significantly decreased the extent of activation, although to different levels. In particular, the Y1 oligonucleotide, that is centered over the initiation codon, almost completely abolished the effect of H₂O₂ at the higher of the two.
concentrations used (50 nM) (Fig. 6). In contrast, the control oligonucleotide had little if any effect on CAT activation, even at 50 nM (Fig. 6, Yc). We did not use antisense oligonucleotides to ox217, the other Y-box clone isolated, that is more closely related to the human YB-2 DBPA2 gene, because the sequence information available for this clone is incomplete, stopping 13 amino acids short of the initiation site in its human homologue.

The effect of the Y-box protein on H$_2$O$_2$-induced CAT expression might be the result of an idiosyncratic phenomenon affecting the OxyR response element and having no biological relevance in eukaryotic systems. To address this question, the effect of peroxide treatment on the transient expression of a luciferase reporter plasmid containing the Y-box sequences from the human MHC class II HLA DQ $\alpha$-2 gene was determined. Expression of his reporter plasmid, termed pYbLuc, was compared with expression directed by a similar pOxyRLuc reporter plasmid carrying the OxyR response element instead of the Y-box. As controls we used reporter constructs with mutated Y-box and ORE, respectively. Luciferase induction after H$_2$O$_2$ treatment showed identical dose-response curves, reaching a plateau by 150 $\mu$M H$_2$O$_2$ for the two wild-type constructs, whereas there was no effect on expression directed by the mutated response elements (Fig. 7A). The effect of H$_2$O$_2$ was observed only if, after treatment, cells were grown in unsupplemented culture medium; for both constructs, inclusion of 140 $\mu$M 2-ME in the medium abolished induction by peroxide, also decreasing by more than 10-fold the basal expression level (Fig. 7B). These results strongly suggest a role for the Y-box and Y-box proteins in eukaryotic redox signaling.

FIG. 7. Redox effects on the transient expression of Y-box reporter plasmids. A, H$_2$O$_2$, dose response. Relative luciferase activity, normalized to the value in untreated cells, is shown as a function of H$_2$O$_2$ concentration for reporter plasmids containing Y-box (pYbLuc) and ORE (pOxyRLuc) motifs and their corresponding mutant controls. Although the relative values can almost be superimposed, the absolute values for pOxyRLuc were always higher by a factor of 3–4-fold than the corresponding values for pYbLuc. B, 2-ME block. After treatment with 50 $\mu$M H$_2$O$_2$, transfected cells were cultured in unsupplemented medium (stippled) or in medium containing 140 $\mu$M 2-ME (filled). Luciferase activity values are shown relative to those obtained in cells that were not treated with either H$_2$O$_2$ or 2-ME.

**DISCUSSION**

Our results show that the promoter of the bacterial oxyR gene functions in eukaryotic cells as an oxidative stress-responsive enhancer. At least one of the murine trans-acting factors that recognize this promoter has been cloned and is a member of the Y-box family of transcriptional activators. We show that the Y-box motif and Y-box proteins participate in redox-dependent gene expression based on three different criteria. First, peroxide-dependent activation of the reporter gene is blocked by antisense oligonucleotides to YB-1 mRNA. Second, a reporter gene carrying the Y-box motif responds to peroxide treatment as effectively as the reporter with the bacterial OxyR promoter. Third, the reducing agent 2-ME added to the culture medium abolishes the effect of peroxide on expression.

The Y-box transcription factors comprise a family of DNA- and RNA-binding proteins of molecular sizes ranging from 36 to 56 kDa, conserved throughout evolution, from E. coli to humans (58–60, 62–64). Their DNA binding domain spans an 80-amino acid region near the NH$_2$ terminus, with a high degree of relatedness to the prokaryotic cold shock response proteins CSPB and CSPA (65–67). It could be argued that the Y-box transcription factors are not sequence-specific DNA-binding proteins, yet the evidence to the contrary is overwhelming. In eukaryotes, the cold shock domain plays an essential role in sequence-specific DNA binding. Its recognition element is the Y-box, an inverted CCAAT motif contained within the sequence GATTGGC common to the promoter of many genes (Fig. 8), including all the MHC class II genes, HSP70, histone H$_2$B, c-jun, PCNA, herpes simplex virus thymidine kinase, and DNA polymerase $\alpha$, as well as DNA elements in which the CCAAT motif is absent (see Refs. 60 and 63 for a review). More recently, a transcriptionally functional Y-box has been found in the promoter of the cystic fibrosis CFTR gene (68). In the case of the ORE used in our experiments, an imperfect Y-box is present in two motifs, ATAGGT and ATT-GCT, adjacent to the three G residues involved in methylation interference. One of these motifs is identical to the sequence ATAGxG, and the other matches it in 5-out-of-6 residues. This sequence has recently been suggested as a potential consensus motif for oxidized OxyR contacts (23, 24). Similar imperfect Y-box motifs can be found in the ahpF C and katG promoters (Fig. 8). In gel retardation experiments, canonical Y-box oligonucleotides compete ORE binding and vice versa, and anti-FRGY2 antibodies supershift the ORE complexes, indicating that binding is likely to involve these imperfect Y-box elements.

The cold shock domain also mediates the binding of Y-box proteins to mRNA molecules in ribonucleoprotein storage particles (60, 61, 63, 69); no clear evidence exists of other physiologic functions for these proteins. Our results strongly suggest that one likely role of Y-box proteins involves redox-dependent gene activation. In this context, the experiments with 2-ME-treated cells are particularly revealing. Addition of 2-ME to the culture medium not only eliminates almost completely the peroxide effect but also decreases expression to levels well below those found in untreated Hepa-1 cells (Fig. 7B), suggesting that even in the absence of H$_2$O$_2$ treatment, these cells are under...
Y-box Mediates Transcriptional Activation

MHC Class II

Xenopus hsp70

Xenopus TFIIIA

HSV tk

HSV tk*

Sea urchin H2B

Rat testis H2B

Consensus

E. coli oxyR

E. coli AhpFC

E. coli katG

FIG. 8. Alignment of Y-box elements and OxyR-binding promoters. References for the different Y-box sequences are given in the text. HSV tk* refers to a linker-scanning mutant of the HSV-1 tk gene promoter Y-box that has greater transcriptional activation activity than the wild type (71).

considerable oxidative stress, which is relieved by the presence of 2-ME. We would predict that the activity of the Y-box proteins might depend on the oxidant status of the cell, and by extension, on cell lineage and density, culture conditions, medium, and many other factors. Preliminary experiments using other cell lines suggest that this may in fact be the case.

Little is known as to the activation mechanisms that regulate Y-box protein activity. Many recent studies indicate that redox cycling at cysteine residues regulates in part the DNA binding activity of several mammalian transcription factors (29–35, 70). Although other amino acids may be capable of redox cycling, the lack of cysteine residues in Y-box proteins (Fig. 4 and Refs. 58–63) is further indication that the actual redox cycling at cysteine residues regulates in part the DNA binding activity of these proteins. If this were the case, Y-box proteins could be constitutively bound to their response element regardless of the proximal status of the cell, serving as a scaffold for the assembly of other factors, including the putative redox-sensing protein. Such a scaffolding role has been proposed for the region of alternating acidic and basic amino acid modules in the carboxyl-terminal third of the protein (60, 63). This hypothesis would explain the absence of apparent changes in methylation interference patterns of treated and untreated extracts. Alternatively, methylation interference may be inadequate to detect subtle changes in DNA-protein contacts which might activate transcription.

In prokaryotes, the OxyR protein functions as a sensor, as well as a transcriptional activator/silencer that binds DNA and responds to hydroxyl radicals. In eukaryotes, the redox-sensing mechanism, transcriptional activation, and DNA binding may be functions performed by different proteins. There is little sequence relatedness between OxyR and Y-box proteins, indicating that they are unlikely to be evolutionarily related. It is striking, however, that some of the elements of the regulatory pathway that governs the response to oxidative stress appear to be shared by prokaryotic and eukaryotic organisms.

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