MOLECULAR AND CELLULAR BIOLOGY, Mar. 1996, p. 1035–1046 Vol. 16, No. 3
0270-7306/96/$04.00+0

Physical and Functional Sensitivity of Zinc Finger Transcription Factors to Redox Change

XIAOSU WU, NANETTE H. BISHOPRIC, DARYL J. DISCHER, BRIAN J. MURPHY, AND KEITH A. WEBSTER*

Department of Cell and Molecular Biology, SRI International, Menlo Park, California 94025

Received 6 November 1995/Accepted 15 December 1995

Redox regulation of DNA-binding proteins through the reversible oxidation of key cysteine sulfhydryl groups has been demonstrated to occur in vitro for a range of transcription factors. The direct redox regulation of DNA binding has not been described in vivo, possibly because most protein thiol groups are strongly buffered against oxidation by the highly reduced intracellular environment mediated by glutathione, thioredoxin, and associated pathways. For this reason, only accessible protein thiol groups with high thiol-disulfide oxidation potentials are likely to be responsive to intracellular redox changes. In this article, we demonstrate that zinc finger DNA-binding proteins, in particular members of the Sp-1 family, appear to contain such redox-sensitive -SH groups. These proteins displayed a higher sensitivity to redox regulation than other redox-responsive factors both in vitro and in vivo. This effect was reflected in the hyperoxidative repression of transcription from promoters with essential Sp-1 binding sites, including the simian virus 40 early region, glycolytic enzyme, and dihydrofolate reductase genes. Promoter analyses implicated the Sp-1 sites in this repression. Non-Sp-1-dependent redox-regulated genes including metallothionein and heme oxygenase were induced by the same hyperoxic stress. The studies demonstrate that cellular redox changes can directly regulate gene expression in vivo by determining the level of occupancy of strategically positioned GC-binding sites.

There is compelling evidence for both direct and indirect pathways for the regulation of gene expression by changes in cellular redox state. Hypoxic and hyperoxic stresses can activate or repress the transcription of certain genes by pathways that probably involve protein kinases (5, 16, 18, 24, 28, 52, 68, 70, 82, 84). The response to severe oxidative stress may involve an additional effect in which redox-sensitive factors can be directly activated or inactivated through the oxidation of sulfhydryl residues. The binding of factors AP-1, Sp-1, Egr-1, NF-κB, v-rel, c-myb, E2, IRE-BP, p53, and USF to nucleic acid is reduced or lost when critical cysteine residues are oxidized or alkylated (1, 3, 4, 33–35, 37, 48, 53, 60, 66, 78, 89). HoxB5, a member of the mammalian homeodomain gene family, is an example of a factor that is activated by oxidation (30). In the case of AP-1, a cellular DNA-repair protein that may regulate the redox equilibrium has been described previously (90). Oxidative inactivation of USF has been shown to correlate directly with transcriptional activity in an in vitro assay (60). It has been proposed that the reactive cysteines may constitute redox-sulfhydryl switches which directly regulate gene expression (35, 60). In support of this, factor Sp-1 in rat liver appears to become progressively oxidized during aging, resulting in the reversible loss of binding activity by an in vitro assay (3). To date, there have been no reports to demonstrate that oxidizing agents or redox stresses can directly mediate transcription factor binding or gene expression in situ.

Zinc finger proteins are one of the largest classes of DNA-binding proteins (6, 62). They are prime targets for redox regulation since they all contain essential cysteine residues as part of the metal-binding, DNA-intercalating fingers (22, 41). The best-characterized zinc finger transcription factor is Sp-1 (13, 41). Sp-1 is a member of an extended family of DNA-binding proteins that have three zinc finger motifs and bind to GC-rich DNA (6, 13, 69). Sp-1 is ubiquitously expressed in mammalian tissues, although with widely different abundances (63). It binds with different affinities to DNA sites that have variations of the preferred consensus sequence, and it contributes to the transcriptional regulation of numerous genes (31, 44, 46, 49). Sp-1 factors have been shown to play a critical role in the transcriptional regulation of a class of genes that do not contain classical TATA or CAAT box elements in their proximal promoters. These include a number of so-called housekeeping genes such as glycolytic enzymes, dihydrofolate reductase (DHFR), thymidylate synthase, and adenine deaminase (74), as well as some nonhousekeeping genes (10, 27, 91) and erythroid-specific genes (12, 51).

In this paper, we show that Sp-1 factors are redox responsive in vitro and in vivo and that the hyperoxidative transcriptional repression of selected endogenous genes can be related to oxidative inactivation of Sp-1 binding.

(A preliminary report of this work has been published in abstract form [88a].)

MATERIALS AND METHODS

Cell culture, nuclear extracts, and gel mobility shift assay. HeLa cells were cultured in basal medium with Earle's salts (Gibco/BRL) supplemented with 10% fetal calf serum, 1% nonessential amino acids, penicillin (100 U/ml), and streptomycin (100 U/ml) at 37°C under 5% CO_2. C_2C_{12} myogenic cells (92) were cultured in Dulbecco modified Eagle medium supplemented also with 10% fetal calf serum and penicillin-streptomycin (100 U of each per ml) at 37°C under 10% CO_2. Nuclear extracts were prepared from confluent plates as described previously (25, 86) except that dithiothreitol (DTT) was excluded from all extraction buffers. For redox modification of extract proteins in vitro, 8 μg of nuclear extract in buffer was pretreated with diamide (Sigma Chemical Co.), *N*-ethylmaleimide (NEM), or DTT at 22°C for 30 min as indicated in the figure legends. For redox modification in situ, cells at 60 to 80% confluence were treated with L-buthionine-[*S*,*R*]-sulfoximine (BSO; Sigma) for 18 to 20 h followed by diamide for 30 to 60 min as indicated.

Sequences of the oligonucleotide probes (sense strands) were as follows: SRE,

* Corresponding author. Mailing address: Department of Cell and Molecular Biology, SRI International, 333 Ravenswood Ave., Menlo Park, CA 94025. Phone: (415) 859-6495. Fax: (415) 859-3342. Electronic mail address: cardiol@unix.sri.com.

5′ CAACACCCAAATATGGCT 3′; AP-1, 5′ AGGAGCCGCAAGTGACTCA GCGCGG 3′; Egr-1, 5′ ATTCGATCGCGGGGGCGGGAGC 3′; high-affinity Sp-1, 5′ ATTCGATCGGGGCGGGGCGAGC 3′; low-affinity Sp-1, 5′ ATTCG ATCGGGGCGGAGAGAGC 3′; and NF-κB, 5′ AGTTGAGGGGACTTTCCC AGGC 3′. Gel-purified double-stranded oligonucleotides were end labeled with [^{32}P]ATP by using T4 polynucleotide kinase (Promega Biotec) and [γ-^{32}P]ATP (NEN DuPont). Equal amounts of radioactive probe (1.5×10^4 to 2.5×10^4 cpm) were added to binding reaction mixtures that contained 8 μg of nuclear extract protein in 20 μl of a buffer containing 4 mM Tris (pH 7.8), 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 60 mM KCl, 30 mM NaCl, 0.1 mM EDTA, and 1 μg of poly(dI-dC) (Pharmacia). Reaction mixtures were incubated for 15 min at 22°C before separation on nondenaturing 6% polyacrylamide gels at 4°C (86). Antibodies were preincubated with nuclear extracts for 2 h at 4°C, and competitor DNAs were preincubated at 22°C for 30 min before being mixed with the labeled oligonucleotide. Proteins were determined by Bio-Rad assay. Stock solutions (1,000×) of BSO and diamide were made in water.

Plasmids and antibodies. pSp-1CAT and pSVSp-1-CAT are described in reference 14, pAP-1-Col-CAT was a gift from M. Karin (University of California, San Diego) (23), and pSVHCA-CAT and β-actin-CAT were gifts from L. Kedes (University of Southern California, Los Angeles) (87). These constructs are described in detail in Fig. 4a. pEno_{628}-luc was a gift from C. Peterson (University of Arkansas, Little Rock) (75), p$DHFR_{184}$-CAT was a gift from J. Azizkhan (Cancer Cell Unit, Roswell Park Memorial Institute, Buffalo, N.Y.) (74), pHOΔ33SX2-CAT was a gift from J. Alam (Louisiana State University Medical Center, New Orleans) and contains the distal heme oxygenase (HO) inducer-dependent enhancer fused to the proximal promoter −33 bp upstream from the transcription start site (described in reference 2), and pMTIIa-CAT has been described previously (56). Truncated and mutated metallothionein (MT) and β-enolase promoters were constructed by using oligonucleotides to prime PCRs with the respective parent plasmids (pMT-CAT and pEno_{628}-luc). The PCR primers, based on published sequences (42, 59), were as follows: Eno wild type (WT), 5′ TTTCTCGAGGCCCAGTGTGAAGGGGGGAGGATGGAGAGA AAGAGAGGCGGGGCTGGCT; Eno (mutant), 5′ TTCTCGAGGCCCAGTG TGAAGGGGGGAGGATGGAGAGAAAGAGAAATGGGGCTGGCT; Eno, 5′ TTTAAGCTTCTTGGGATGTCTTCGCTGGAG (antisense); MT IIA (wild type [WT]), 5′ TTTCTCGAGGCGGGGCGTGTGCAGGCACGCCCG GGGCGGGGC; MT IIA (mutant [M]), 5′ TTTCTCGAGGCGGGGCGTGTG CAGGCACGAATGGGGCGGGGC; MT IIA, 5′ TTTAAGCTTGGGACTTG GAGGAGGCGTGGTGGAGTGCAG (antisense). All primers contained *Xho*I cloning sequences on the 5′ end and *Hin*dIII sequences on the 3′ end. PCR conditions were as follows: 30 s at 94°C, 30 s at 50°C, and 15 s at 72°C. The PCR products were inserted into the multiple cloning site of pGL2BV (Promega Biotec). All sequences were verified by sequence analysis with Sequenase version 2.0 (Amersham). The sequences and mutations are shown in Fig. 7a. Antibodies directed against DNA binding site peptides of Sp-1, AP1, Egr-1, and NF-κB were purchased from Santa Cruz Biotech, Inc., Santa Cruz, Calif.

Cell transfections and treatments. For quantitative PCR (QPCR) RNA analyses, subconfluent HeLa cells were transfected with 20 μg (per 100-mm plate) or 10 μg (per 60-mm plate) of the indicated plasmid construct by the standard calcium phosphate technique as described previously (7, 8, 86). Two to three days after transfection, the culture medium was replaced with fresh medium with or without 50 μM BSO. After a further 24 h, cells were treated with diamide or 10 μg of actinomycin D per ml. The cells were harvested 0, 2, and 4 h after adding diamide or actinomycin D. For transient expression assays, C_2C_{12} cells at 40% confluence were transfected as described above. Two days after transfection, the medium was replaced with Dulbecco modified Eagle medium containing 0.5% horse serum, and the cultures were treated with BSO and H_2O_2, as indicated in the figure legends, after a further 3 to 5 days. Luciferase activity in cell extracts was assayed with a Promega Biotec assay kit exactly as recommended by the manufacturer and an LKB BioOrbit 1250 luminometer; chloramphenicol acetyltransferase (CAT) enzyme activity was assayed as described previously (87). For RNase protection assays, the same protocols were used except that cells were harvested as described below for Northern (RNA) blots.

Transcriptional analyses. (i) QPCR. Following transfections and treatments as described above or in the figure legends, total RNA was isolated by solubilizing cell pellets in 4.0 M guanidinium thiocyanate and pelleting through CsCl as described previously (84). Reverse transcription was performed in a reaction mixture containing 2.5 μg of total RNA, 600 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories), and 0.5 mM deoxynucleoside triphosphates (dNTPs) in 50 mM Tris-HCl (pH 8.3)–75 mM KCl–10 mM DTT–3 mM $MgCl_2$–50 mg of oligo(dT) per ml–1 U of RNasin per μl for 1 h at 37°C. Primers for PCR were as follows: CAT primers TGCTCATC CGGAATTCCGTATGGC (sense) and GGCGAAGAAGTTGTCCATATTG GC (reverse strand), producing a 250-bp product; and internal control β-actin TGGAGAAGAGCTATGAGCTGCCTG (sense) and GTGCCACCAGACAG CACTGTGTTG (reverse strand), producing a 210-bp fragment. The PCR (57) combined 5 μl of the reverse transcription (2.5 μg of initial RNA) with 45 μl of PCR buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$) containing 50 pmol of both primer pairs, 0.2 mM dNTPs, 0.2 μCi of [α-^{32}P]dCTP, and 2.5 U of *Taq* polymerase (Promega Biotec). The templates were amplified for 20 cycles (30 s at 94°C, 30 s at 65°C, 1 min at 72°C), separated on 2% agarose gels, and transferred to Hybond-N nylon membranes (Amersham). Autoradiographs and ethidium-stained gels were analyzed as described below and normalized with respect to β-actin. To check for contaminating chromosomal DNA, RNA samples were subjected to 20 PCR cycles without reverse transcription. No product could be detected in these samples, demonstrating that contaminating chromosomal DNA did not interfere with the assay (data not shown). The validity and accuracy of the QPCR method were verified by subjecting each primer set and RNA sample to a range of PCR cycles (from 15 to 35) and by varying the input RNA (from 0.5 to 5 μg). As shown in Fig. 4b, there was a linear relationship between input RNA and PCR product for each transfected promoter when the samples were subjected to 20 cycles of amplification. By these procedures, the intra-assay percent coefficient of variation [%CV = (standard deviation/mean) × 100] calculated from 22 PCR analyses and seven separate samples was 13.4.

(ii) Northern analysis. C_2C_{12} cells were grown to 80% confluence as described above, the medium was replaced with Dulbecco modified Eagle medium and 0.5% horse serum, and the cells were incubated for a further 3 to 5 days. Cultures were treated with 50 μM BSO for 18 to 20 h and then exposed to an atmosphere of 50% O_2–5% CO_2–45% N_2 for 12 h or to 2 mM H_2O_2 for 4 h. Total RNA was isolated by solubilizing cells in 6 M guanidinium thiocyanate as described above. Agarose gels, blotting, and hybridizations were all as described previously (84). cDNA probes including pyruvate kinase (83), β-enolase (gift from C. Peterson) (75), DHFR (gift from P. Berg, Stanford University, Stanford, Calif.), HO (gift from R. Tyrrell, University of Bath, Bath, United Kingdom), and MT-1 and 18S rRNA (both from the American Type Culture Collection) were labeled by random priming (Prime-It kit; Stratagene) to 10^8 cpm/μg of DNA. Autoradiographs were analyzed with a Lynx 4000 Molecular Biology Workstation (Applied Imaging Corp., Santa Clara, Calif.) as described previously (85). DNA loading on gels was monitored by ethidium staining and by probing with the 18S rRNA probe as the control.

RNase protection assay. Luciferase mRNA from transfected β-enolase reporter constructs was analyzed by RNase protection with an assay kit purchased from Promega Biotec. pGEM-luc (Promega Biotec) was linearized with *Eco*RV and used as the template to transcribe a 413-bp antisense RNA probe with T7 RNA polymerase and [^{32}P]CTP. The assay procedure was essentially as described by the manufacturer: 10 μg of RNA was hybridized overnight with 3×10^5 cpm of probe at 42°C, after which the samples were digested for 1 h with 10 U of RNase I at 21°C, separated by electrophoresis on a 6% acrylamide denaturing gel, dried, and exposed to X-ray film (Kodak).

Measurement of cellular thiol. Cellular thiols were measured spectrophometrically as described previously (39, 80) with 10 mM (final concentration) Ellman's reagent (Sigma). For nonprotein thiols, monolayers were rinsed three times with cold phosphate-buffered saline and treated with cold 2.5% trichloroacetic acid containing 0.1 mM EDTA. Cells were scraped off the dishes, and the suspension was centrifuged at 12,000 × *g* for 5 min. Thiol levels were measured in the supernatant. For total soluble thiol, monolayers were rinsed and scraped into 0.1 mM Tris chloride (pH 7.8). Cells were disrupted by three freeze-thaw cycles, and insoluble material was removed by centrifuging at 12,000 × *g* for 5 min. Thiols were measured in the supernatant as described above. In our hands, 2.5% trichloroacetic acid did not significantly precipitate purified glutathione (GSH) (Sigma); therefore, it is assumed that the nonprotein thiol assay represents a measure of cellular GSH.

RESULTS

The relative sensitivities of selected transcription factors to oxidation were measured by exposing HeLa cell nuclear extracts to various concentrations of thiol-reactive agents, prior to mixing with the specific oligonucleotide and analyzing by gel mobility shift. Figure 1a compares the effects of diamide (thiol oxidizing), DTT (thiol reducing), and NEM (thiol alkylating) on the binding of nuclear proteins to oligonucleotides containing the SRE, AP-1, and Sp-1 consensus sequences. Under the conditions used here, SRF binding was not affected by diamide at any concentration tested, AP-1 binding was eliminated by 5 mM but not 2 mM diamide, and Sp-1 binding was substantially reduced with 2 mM diamide and eliminated by 5 mM diamide. In agreement with previous reports (1, 37, 75), the effects of diamide on both AP-1 and Sp-1 binding were fully reversible by treatment with DTT. Treatment with high concentrations of NEM eliminated the binding of all three proteins. These results suggest that transcription factor Sp-1 is more sensitive to oxidative inactivation, or is less abundant, than AP-1 proteins in these nuclear extracts.

To further elucidate this effect, nuclear extracts were treated with lower diamide concentrations and the levels of binding to

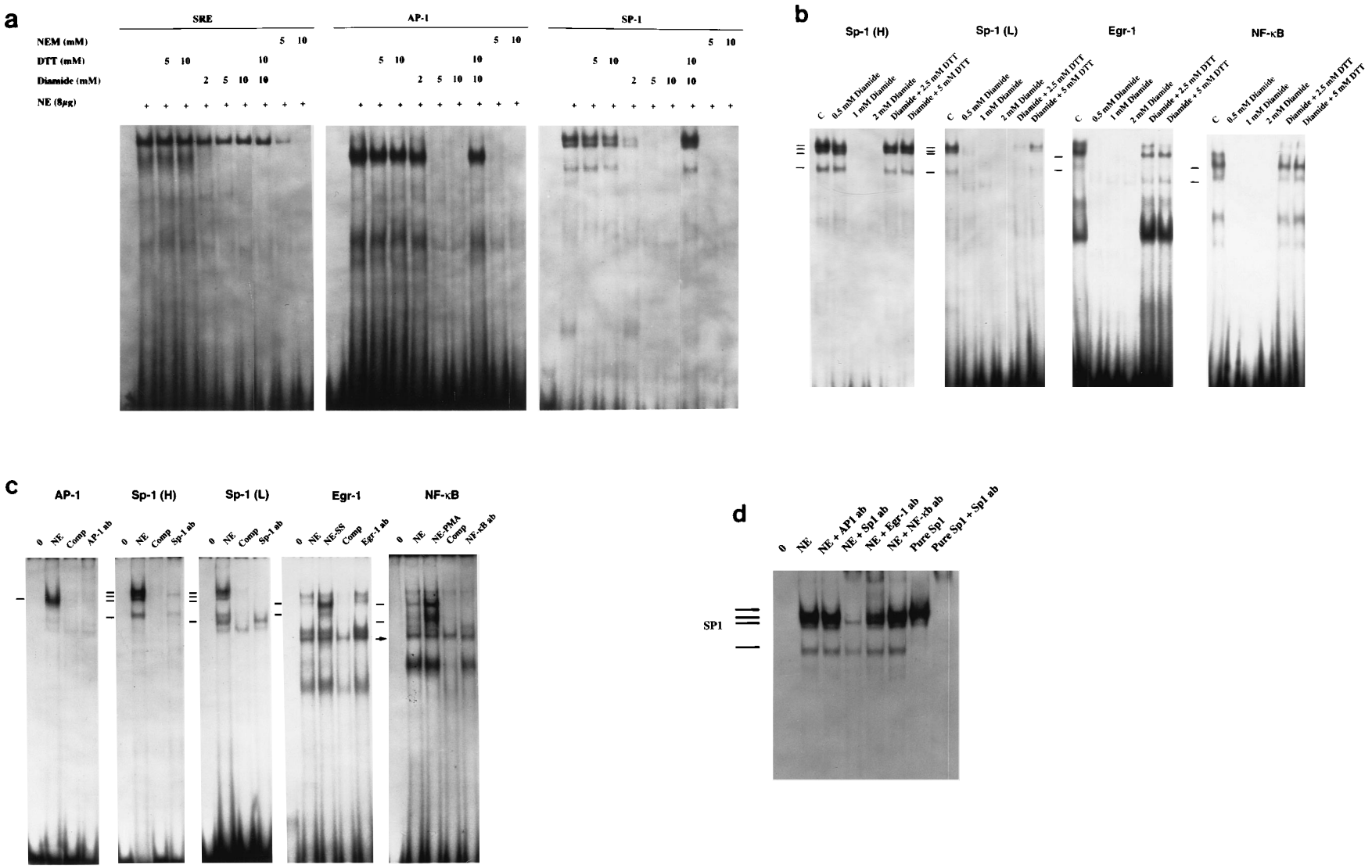

FIG. 1. Effects of sulfhydryl-modifying agents on transcription factor binding in vitro. (a) Nuclear extracts (NE) were prepared from HeLa cells as described in Materials and Methods. For each panel, lanes are as follows: lane 1, no nuclear extract; lanes 3 and 4, nuclear extract was incubated with 5 or 10 mM DTT for 1 h before mixing with the probe; lanes 5 to 7, nuclear extracts were incubated with 2, 5, or 10 mM diamide for 30 min at 22°C before mixing with the probe; lane 8, nuclear extract was incubated with 10 mM diamide for 30 min and then dialyzed against extraction buffer containing 10 mM DTT for 1 h; lanes 9 and 10, extracts were incubated with 5 or 10 mM NEM for 1 h. (b) Nuclear extracts and binding reaction mixtures were as described for panel a. Probe sequences, including high (H)- and low (L)-affinity Sp-1, Egr-1, and NF-κB, are given in Materials and Methods. For binding to Egr-1 and NF-κB DNA probes, cells were serum stimulated by culturing them in 0.1% serum for 24 h and then treating them with 20% fetal calf serum for 3 h (Egr-1) or exposed to 100 nM phorbol myristate acetate for 20 h (NF-κB) before preparing nuclear extracts. Probe-specific complexes are indicated by bars to the left of each panel. (c) Specific antibodies (4 μg) or 100-fold excess unlabeled probe (Comp) was added to the extracts and incubated for 1 h at 4°C before adding ^{32}P-labeled oligonucleotide. For Egr-1 and NF-κB panels, NE-SS and NE-PMA refer to nuclear extracts from cells stimulated with serum or phorbol myristate acetate, respectively, as described above. Bars indicate specific complexes; the arrow indicates nonspecific binding. (d) In lanes 3 to 6, nuclear extracts were incubated with each specific antibody as described above and then mixed with a labeled oligonucleotide containing the high-affinity Sp-1 binding site. In lanes 7 and 8, the same Sp-1-specific oligonucleotide was mixed with 100 ng of purified Sp-1 protein (Promega Biotec) without (lane 7) or with (lane 8) Sp-1-specific antibody.

oligonucleotides containing both high- and low-affinity Sp-1 sites, Egr-1, and NF-κB consensus sequences were compared (Fig. 1b). Under the conditions described for Fig. 1b, neither SRF nor AP-1 binding was affected (not shown). Binding of Sp-1 to the high-affinity site was slightly reduced by treatment with 0.5 mM diamide and eliminated by 1 mM diamide. Binding to the low-affinity site was eliminated by 0.5 mM diamide and was not so readily reversed by treatment with DTT [compare lanes 5 and 6, panel Sp-1 (H), with the corresponding lanes in panel Sp-1 (L)]. Predictably, these results demonstrate that occupancy of the low-affinity Sp-1 binding site requires higher concentrations of reduced Sp-1 and is therefore more redox sensitive than binding to the high-affinity site. Egr-1 and NF-κB proteins were induced by serum stimulation or phorbol ester treatment, respectively, as described in the figure legend, and DNA binding was analyzed exactly as described for Sp-1, with the appropriate oligonucleotides. The binding of proteins to Egr-1- and NF-κB-specific probes was eliminated by treating extracts with 0.5 mM diamide. In both cases, bands representing multiple protein-probe complexes were eliminated. The loss of multiple complexes through oxidation of factors Egr-1 and NF-κB has been described previously, although the identity of the multiple bands is not clear (37, 78). Most of the bands appear to be probe specific (see below), and the oxidation is also probe specific, since proteins that complexed with SRE and AP-1 were not affected by the treatments described for Fig. 1b. In all cases, the effects were reversible by treating oxidized extracts with DTT.

To verify the specificities of protein complexes in these analyses, specific antibodies directed against each factor binding site were reacted with the nuclear extracts before mixing with the specific probe. The results are shown in Fig. 1c and d, where probe-specific bands are indicated to the left of each panel. A single band was detected with the AP-1 probe, and this was eliminated by antibody blocking or excess cold probe (self-competition). Four specific bands were detected with the Sp-1 consensus sequence, and all of these were reduced or eliminated by interaction with the antibody and exhibited self-competition. The binding of two proteins to the Egr-1 oligonucleotide was enhanced by serum induction. These bands were both blocked by antibody reaction or self-competition. In different experiments, variable numbers of higher-mobility complexes formed with the Egr-1 probe. These complexes did not interact with the antibody but were usually inhibited by excess cold Egr-1 probe. These smaller complexes may be nonspecific cross-reacting proteins or proteolytic fragments of the Egr-1 protein with modified binding characteristics. Two specific bands were also generated with the NF-κB-specific probe; both were blocked by antibody or self-competition, and a third, nonspecific band was routinely present but was not changed by antibody, self-competition, or oxidation (indicated by the arrow in Fig. 1c).

The specificities of the antibodies for each specific probe complex were confirmed by premixing each antibody with nuclear extract and then performing binding reactions with the individual probes. Figure 1d shows an example with the Sp-1 complex: α-Sp-1 antibody reacted with Sp-1-probe-specific nuclear proteins or with purified Sp-1. Sp-1-specific binding was not affected by α-AP-1, α-Egr-1, or α-NF-κB-specific antibodies.

It was shown previously that redox modulation of transcription factor USF could eliminate the transcription that was driven by a USF-dependent promoter in vitro (60). This form of oxidative inactivation has not yet been directly demonstrated in situ. To determine whether a similar oxidative stress would have the same effect in situ, HeLa cells were treated with a combination of BSO, a specific inhibitor of γ-glutamylcysteine synthetase, and diamide. During these treatments, cellular thiol levels were reduced (Table 1), and presumably some of the more reactive cysteine residues on intracellular proteins will be oxidized. Following the redox stress, nuclear extracts were made under nonreducing conditions and protein binding was assayed by gel mobility shift as described above. In Fig. 2a, the in situ redox responses of nuclear proteins binding to SRE, AP-1, and high-affinity Sp-1 consensus oligonucleotides are compared. Neither SRF nor AP-1 binding was affected by these treatments in situ, but Sp-1 binding was reduced 60% by treatment with diamide alone (1 h) and essentially eliminated by the combination of BSO and diamide at the concentrations shown. The binding was fully recovered when the extracted proteins were treated with DTT. To ensure that Sp-1 oxidation did not occur in vitro during extract preparation, BSO-treated cells were exposed to 1 mM diamide and immediately processed for nuclear extractions. This treatment did not affect nuclear extract complexing (data not shown). These results suggest that Sp-1 was selectively and reversibly oxidized by diamide in vivo. In two of six experiments, AP-1 binding was also reduced by BSO and diamide treatment; Fig. 2a shows an example in which there was no response.

TABLE 1. Thiol levels in HeLa cells following treatments with oxidizing agents[a]

Treatment	Thiol (nmol/10^6 cells)	
	Nonprotein (%)	Total soluble (%)
Control	12.6 ± 0.7 (100)	39.1 ± 2.3 (100)
BSO (18 h)	3.5 ± 0.3 (27)	21.8 ± 2.4 (56)
BSO + diamide (1 h)	1.8 ± 0.1 (14)	20.0 ± 2.4 (51)
BSO + diamide (2 h)	1.8 ± 0.2 (14)	17.0 ± 1.4 (43)
BSO + diamide (4 h)	1.7 ± 0.1 (13)	15.3 ± 2.2 (39)
BSO + diamide (0.4 mM) (1 h)	1.4 ± 0.1 (11)	8.7 ± 0.8 (22)
BSO + 1 mM H_2O_2 (4 h)	1.6 ± 0.3 (13.5)	14.8 ± 3.2 (40)

[a] Thiol measurements were made spectrophotometrically as described in Materials and Methods. For nonprotein thiol determinations, cellular proteins were precipitated with trichloroacetic acid before the thiol assay. For total soluble thiol, cells were freeze-thawed three times before thiol assay. BSO, where indicated, was used at a concentration of 50 μM, and treatments were for 18 h prior to the addition of diamide. The diamide concentration was 200 μM except where otherwise indicated.

To confirm these results, HeLa cells were exposed to a series of increasing concentrations of diamide for 30 min and extracts were again tested for binding to specific oligonucleotides as described above. These results are shown in Fig. 2b. Consistent with the results shown in Fig. 2a, binding of extract proteins to the AP-1 consensus oligonucleotide was not affected by any of the treatments. The binding of protein to the high-affinity Sp-1 oligonucleotide was reduced by 50% with 0.4 mM diamide ($n = 2$). Binding to the low-affinity site was reduced 60% by 0.2 mM diamide and 85% by 0.4 mM diamide ($n = 2$). The effects of these treatments on the binding of proteins to the Egr-1 and NF-κB consensus oligonucleotides are shown in the right-hand panels of Fig. 2b. Consistent with the in vitro studies (Fig. 1b), factor binding to Egr-1 and NF-κB sites was sensitive to oxidation in situ. Both of the upper-band Egr-1 complexes were reduced by treatment with >200 μM diamide. NF-κB-related binding was also reduced or eliminated by 200 μM diamide. The BSO-diamide treatment did not affect nonspecific binding to the NF-κB probe, as indicated by the arrow. All of these effects were fully reversible by treating the in situ redox-stressed extracts or cells with DTT (not shown). Treatment of cells with BSO and up to 0.4 mM diamide as described for Fig.

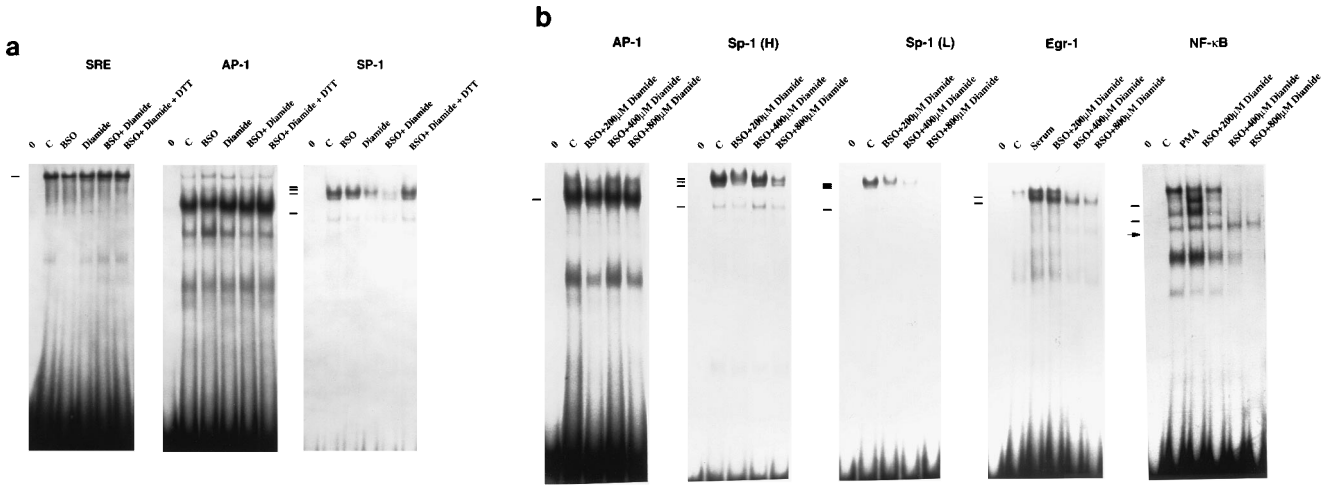

FIG. 2. Effects of sulfhydryl-modifying agents on transcription factor binding in situ. (a) Immediately prior to the extraction of nuclear proteins, HeLa cells were untreated (lane 2) or treated with 50 μM BSO for 18 h (lane 3), 1 mM diamide for 1 h (lane 4), 50 μM BSO for 18 h followed by 1 mM diamide for 1 h (lane 5), or BSO plus diamide as in lane 5 followed by dialysis against 10 mM DTT for 1 h (lane 6). Nuclear extracts and binding reaction mixtures were exactly as described in the legend to Fig. 1. (b) HeLa cells were either untreated or treated with BSO (50 μM, 18 h) followed by diamide at the indicated concentration for 30 min. Cells were serum or phorbol myristate acetate stimulated for binding to Egr-1 and NF-κB probes, respectively, as described in Materials and Methods. Nuclear extracts, binding reaction mixtures, and gel electrophoresis were as described in Materials and Methods and in the legend to Fig. 1.

2 did not significantly affect cell viability as determined by growth curve analyses on treated cultures; however, higher concentrations of diamide were toxic (data not shown). These results suggest a redox sensitivity series as follows: zinc finger factors = NF-κB > AP-1 > SRE.

Since stable DNA complexing may affect the susceptibility of Sp-1 cysteine sulfhydryl groups to oxidation in situ, we compared the effects of treating nuclear extracts with diamide before or after mixing with the high-affinity Sp-1 probe. These results are shown in Fig. 3. Although a small protective effect was apparent in the prebound sample, as revealed by slightly increased binding, binding in both samples was reduced >50% by 0.2 mM diamide and eliminated by 2 mM diamide. Knoepfel et al. (48) recently reported that the oxidation of Sp-1 by 10 mM H_2O_2 in vitro at 4°C was prevented by prebinding the extract to DNA. Therefore, the accessibility of the binding site thiol groups to oxidizing agents is probably dynamic and may be regulated by both the temperature and time of interaction as well as by the type of oxidizing agent.

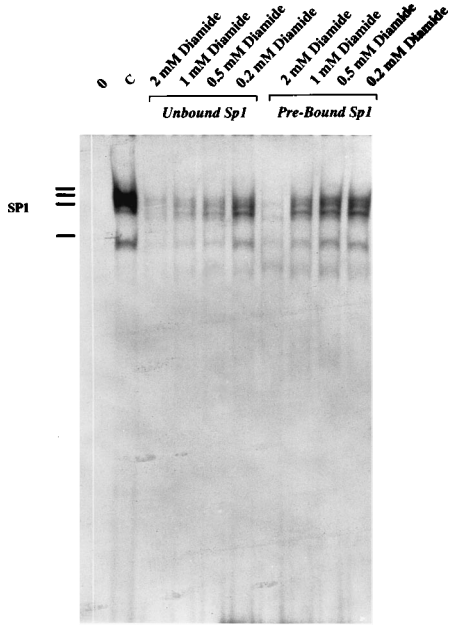

FIG. 3. Effect of DNA binding on the redox sensitivity of Sp-1. The conditions for unbound Sp-1 lanes were as described for Fig. 1a. For prebound Sp-1 lanes, nuclear extracts from untreated HeLa cells were preincubated with labeled high-affinity Sp1 oligonucleotide probe for 30 min before treatment with diamide as described for Fig. 2. Binding reaction mixtures were exactly as described in the Fig. 1 legend.

If transcription factors become oxidized and inactive under conditions of redox stress in vivo, it should be possible to demonstrate reduced transcription from promoters that are dependent on such factors for expression. To test this, we compared the effects of the mildest effective oxidative stress, determined from the binding studies, on transcription of the CAT gene in HeLa cells following transfection with Sp-1-dependent and -independent promoters. The plasmid constructs used are illustrated in Fig. 4a. pSp-1-CAT contains the simian virus 40 (SV40) early promoter with six Sp-1 sites, including both high- and low-affinity sites, ligated to the CAT gene. Transcription is dependent on Sp-1 binding to the promoter and is reduced 80% by mutation of the low-affinity site and 95% by mutation of both the low- and medium-affinity sites 1 and 2 (14). pSVSp-1-CAT contains both the SV40 early promoter and the 72-bp enhancer repeats, and transcription of CAT is also dependent on Sp-1 binding (14). Control promoters without Sp-1 binding sequences included plasmids pAP-1-Col-CAT (23) and pSVHCA-CAT. The pAP-1-Col-CAT construct contains the collagenase promoter truncated to −73 bp, and transcription is dependent on the binding of AP-1 to a single site centered at −69 bp. pSVHCA-CAT is the same as pSVSp-1-CAT except that the early SV40 promoter Sp-1 sites were replaced to −43 bp with the human cardiac actin promoter containing no Sp-1 sites.

Transcription was quantitated by QPCR as described in Materials and Methods. Figure 4b shows that there was a linear relationship between input RNA and PCR product for each transfected probe when the described conditions for reverse transcription and amplification were used. Figures 4c and d

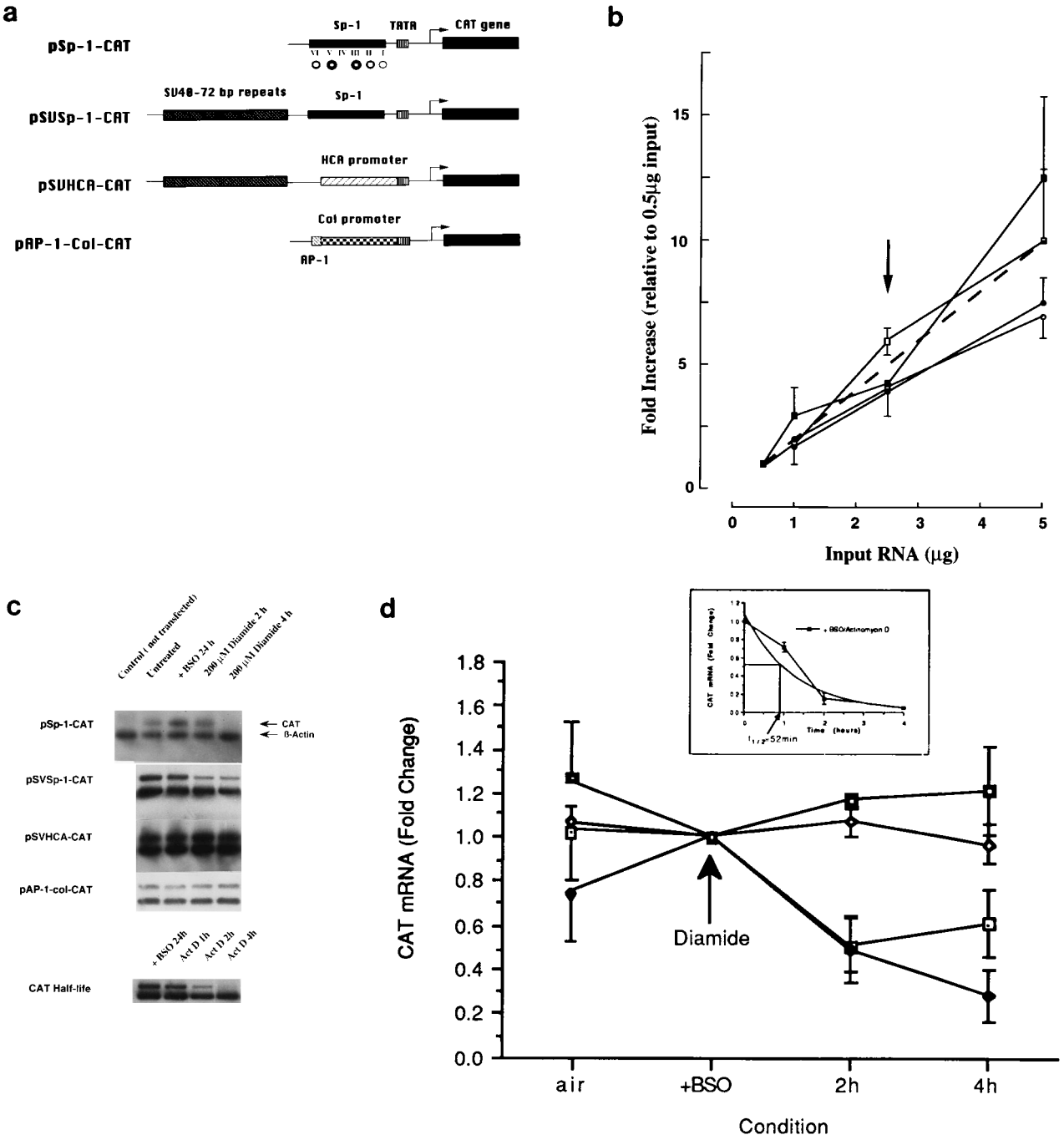

FIG. 4. Redox regulation of Sp-1-dependent transcription in situ. (a) Promoter constructs used to identify Sp-1-specific regulation. pSp-1-CAT and pSVSp-1-CAT are described in reference 14. The thickness of the circle under each Sp-1 site represents the relative binding affinity of the site as described in reference 14. pAP-1-Col-CAT has no Sp-1 sites and was a gift from M. Karin (University of California, San Diego) (23); pSVHCA-CAT has no Sp-1 or AP-1 sites and was a gift from L. Kedes (University of Southern California, Los Angeles). HCA, human cardiac actin. (b) Linear range of QPCR. PCRs were run as described in Materials and Methods with the indicated amounts of input RNA. Error bars (standard errors of the means) were from at least three separate experiments; errors of <10% are omitted. Arrow indicates input RNA (2.5 μg) that was used for all experiments described for panels c and d below. Open circles, solid circles, open squares, and solid squares represent pSp-1-CAT, pSVHCA-CAT, pAP-1-Col-CAT, and pSVSp-1-CAT, respectively. Dashed line indicates theoretical values for an ideal linear response. (c) Representative blots of CAT-primed PCR products. HeLa cells, 40 to 50% confluent, were transfected with the indicated plasmid as described in Materials and Methods. Individual plates were untreated or treated with 50 μM BSO and 0.2 mM diamide or 10 μg of actinomycin D (Act D) per ml as described in Materials and Methods. The cells were harvested 0, 2, and 4 h after addition of diamide (or actinomycin D). RNA was extracted from cell pellets and used for reverse transcription PCR. (d) Quantitation of transcripts during treatments with diamide or actinomycin D. The data shown in panel c were quantitated as described in Materials and Methods ($n = 4$). All values were normalized to the β-actin signal. The half-life of β-actin mRNA in the presence of actinomycin D was estimated to be 10.5 h (not shown). Solid diamonds, open diamonds, solid squares, and open squares represent pSP-1-CAT, pAP1-Col-CAT, pHCA-CAT, and pSVSp-1-CAT, respectively.

show representative blots and quantitation, respectively, of reverse transcription QPCR-amplified CAT mRNA. Following an increase in the CAT message transcribed from pSP-1-CAT after 24 h of exposure to BSO, there was a progressive decline of CAT transcripts in cells transfected with either pSp-1-CAT or pSVSp-1-CAT after the cultures were exposed to 200 μM diamide. The time required for a 50% decrease in message level was 120 min for pSp-1-CAT. The CAT transcript level from pSp-1-CAT continued to decline over the full 4 h, whereas the decay from pSVSp-1-CAT was less at the later time point. This difference may be related to redox-insensitive transcription driven by the SV40 72-bp enhancer repeats in pSVSp-1-CAT. The half-life of the CAT message, as determined with actinomycin D, was 52 min (Fig. 4d, inset). The level of CAT mRNA in cells transfected with pAP-1-Col-CAT and pSVHCA-CAT did not change during exposure to the same conditions. These results demonstrate that, under our experimental conditions, transcript levels supported by the Sp-1-dependent promoters were specifically reduced by the treatment with BSO and diamide. The absence of any effect on the CAT message transcribed from non-Sp-1-dependent promoters demonstrates that diamide treatment per se did not increase the degradation rate of the CAT mRNA. Therefore, the effects are compatible with the gel mobility shift data in demonstrating an inhibition of Sp-1 binding to and transcription from the Sp-1-CAT and SVSp-1-CAT promoters and subsequent degradation of the preexisting CAT mRNA.

The effects of BSO, diamide, and H_2O_2 treatments on cellular thiol levels are shown in Table 1. Nonprotein thiols and total soluble thiols were reduced 73% and 44%, respectively, by treatment with 50 μM BSO for 18 h. Subsequent treatment with 200 μM diamide or 1 mM H_2O_2 resulted in further reductions by 13% and 5 to 9% for nonprotein and total soluble thiols, respectively. The thiol levels remained relatively stable over 4 h.

To determine whether direct oxidative inactivation of Sp-1 and related zinc finger transcription factors of the type described above may contribute to a physiological response to severe oxidative stress, we analyzed the effects of oxidative stress on the endogenous transcript levels and promoter functions of two classes of redox-regulated genes: (i) DHFR, pyruvate kinase M (PKM), and β-enolase (the promoters of these genes lack classical TATA or CAAT box elements but contain a 5′-proximal Sp-1 binding site[s] that is essential for efficient transcription) and (ii) HO and MT (these gene promoters contain TATA motifs and upstream elements that confer a positive transcriptional response to oxidative stress [19, 38, 79]). Previous studies have shown that the latter two genes are induced by severe oxidative stress caused by exposure to diamide, diethyl maleate, or H_2O_2, all of which change the redox potential of the cells by depleting GSH stores. Responses of the former genes to oxidative stress have not been reported.

Figure 5 shows the effects of oxidative stress on the transcript levels of PKM, β-enolase, DHFR, HO, and MT. These experiments were carried out in myogenic C_2C_{12} cells to permit expression of the muscle-specific PKM and β-enolase gene promoters. There were small inductions of all transcripts by the mild hyperoxia that was imposed by culturing the cells for 18 h under elevated (50%) oxygen with or without BSO. These inductions may involve activation of antioxidant response elements in the HO and MT genes (19, 79). We have not identified a mechanism for the inductions of glycolytic enzyme genes by the milder stress; however, previous studies (24, 45, 58), including our own experiments (not shown), suggest that protein kinase pathways are activated by this level of oxidative stress and could be involved in these inductions. PKM and

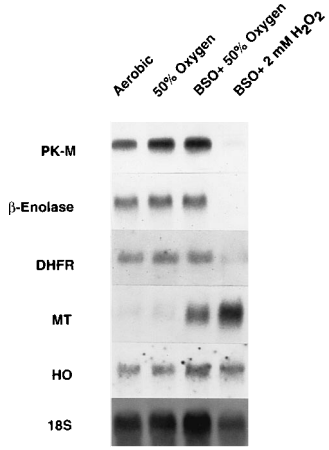

FIG. 5. Responses of transcript levels to oxidative stress. C_2C_{12} cells were cultured as described in Materials and Methods. Treatments were as follows: lane 1, aerobic culture; lane 2, 50% O_2–40% N_2–10% CO_2; lane 3, as in lane 2 except that cells were pretreated for 20 h with 50 μM BSO; lane 4, cells were treated for 20 h with 50 μM BSO followed by 2 mM H_2O_2 for 4 h. Northern blot procedures and probes are described in Materials and Methods.

β-enolase transcripts dropped abruptly following treatment of the cells with BSO and H_2O_2. Glucose-metabolizing enzyme mRNAs are characteristically unstable; half-lives of 1 h, 45 min, 40 min, and 30 min have been reported for pyruvate kinase (20), lactate dehydrogenase (40), glucokinase (71), and phosphenolpyruvate carboxykinase (36), respectively. The decreased transcript levels are consistent with an inhibition of de novo transcription and subsequent mRNA degradation, similar to that shown in Fig. 4c for Sp-1-CAT transient expression. The half-life for DHFR mRNA is about 6 h (50); therefore, the decay following inhibition of transcription will be correspondingly slower than that of the glycolytic enzyme transcripts. Consistent with this, there was only a two- to threefold decline in DHFR transcripts during the 4-h treatment after normalizing to 18S rRNA. Although mRNA stability may be affected by these treatments, the decline in transcript levels is consistent with an inhibition of transcription of PKM, β-enolase, and DHFR genes by H_2O_2 in BSO-treated cells. In stark contrast to this, HO and MT gene transcripts were both strongly induced by the same BSO-H_2O_2 treatment. The transcript levels were elevated by 3.1- and 24-fold, respectively ($n = 2$), again after normalizing to the 18S rRNA probe. These inductions of HO and MT gene transcript levels with 2 mM H_2O_2 demonstrate that the oxidative repression of the former set of genes by the same oxidative stress is at least partially selective and not a consequence of general toxicity to the transcriptional machinery. H_2O_2 concentrations of 10 mM or more (fivefold higher than that used here) were reported to be optimal for the induction of HO and MT in hepatoma cells (19). The 18S rRNA probe in the Northern blot shown in Fig. 5 indicates slight overloading of lane 3 and underloading of lane 4 in this experiment. In parallel experiments (not shown), treatment of cells with a combination of BSO (18 h) and 200 μM diamide (as described above for Fig. 4) mediated an 85% loss of β-enolase transcripts and fivefold induction of MT after 2 h.

To determine whether these endogenous gene transcript changes could be related to promoter function, we tested the effects of the same oxidative stress on transient expression from promoter-reporter constructs. Figure 6 shows that, while the transient expression from β-enolase and DHFR gene promoters was repressed by oxidative stress, HO and MT-IIA

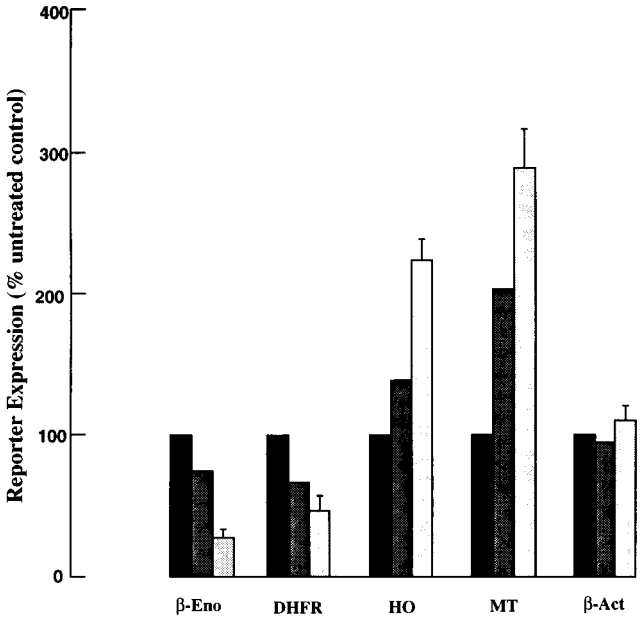

FIG. 6. Effect of oxidative stress on transient expression in C_2C_{12} cells. Plasmid constructs and the procedures for transfections, treatments, and expression are described in Materials and Methods. β-Eno is $pEno_{628}$-luc, DHFR is $pDHFR_{184}$-CAT, HO is pHOΔ33SX2-CAT, MT is pMT-IIa-CAT, and β-Act is β-actin-CAT. Treatments were as follows: black bars, untreated; darkly shaded bars, 50 μM BSO for 18 h followed by 0.5 mM H_2O_2 for 20 h; lightly shaded bars, 50 μM BSO for 18 h followed by 1.0 mM H_2O_2 for 20 h. Error bars represent standard errors of the means from three separate experiments.

gene promoters were induced; reporter constructs that contained the β-actin promoter were not significantly affected. These results support a role for proximal promoter elements in the transcriptional response to this oxidative stress.

To further define the responsive elements, β-enolase and MT promoters were truncated to −101 and −98 bp upstream from the respective transcription start sites. As shown in Fig. 7a, both promoters contain Sp-1 binding sites at the extreme 5′ ends (underlined in the figure). The MT promoter contains a TATA element at position 5′ −30, but β-enolase (and PKM) gene promoters have GATA sequences at this position (Fig. 7a, boldface type). In gel mobility shift assays, we found that oligonucleotides containing the β-enolase–PKM GATA element did not bind purified TFIID and nuclear extract proteins that bound to the element were not inhibited by a TATA box consensus sequence oligonucleotide (data not shown). Therefore, this element and its binding proteins are distinct from the TATA complex and are more likely related to previously described GATA elements (12, 51, 55). Protein binding to the β-enolase–PKM Sp-1-like element is shown in Fig. 7b. Four bands were inhibited by an excess of unlabeled self-oligonucleotide and eliminated or reduced by premixing with an α-Sp-1 binding site antibody as described in Materials and Methods. Purified Sp-1 bound to the site, and the binding was not affected by a control (α-AP-1) antibody (lane 5). The gel mobility shift pattern was very similar to that seen with the consensus Sp-1 oligonucleotide shown in Fig. 1 and 2, and the Sp-1-binding oligonucleotide described for Fig. 1 [SP-1 (H)] was as effective as self in gel mobility shift competition assays (not shown). It can be concluded that the complex which binds to the β-enolase–PKM proximal GC element contains Sp-1 or Sp-1 like protein(s). The right-hand panel of Fig. 7b shows that mutation of the GC box eliminated all four binding complexes. Results similar to these were obtained with oligonucleotides corresponding to the MT-IIA Sp-1-GC site (shown in Fig. 7a) and its corresponding mutated oligonucleotide (data not shown).

Transient expression from transfected constructs and the effects of oxidative stress are shown in Fig. 7c. The truncated β-enolase promoter was still potently repressed by BSO-H_2O_2 treatment. Mutation of the β-enolase Sp-1 site reduced expression to <10% of the native promoter, and the residual weak

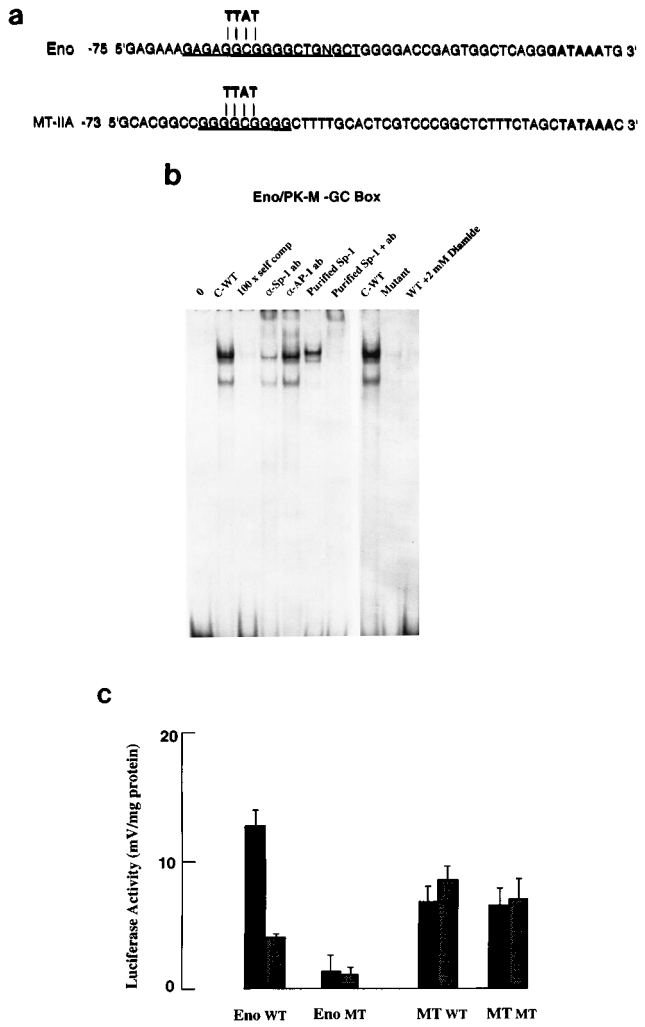

FIG. 7. Effect of Sp-1 binding site mutations on oxidative repression of truncated β-enolase and MT promoters. (a) Sequences of β-enolase and MT-IIA proximal promoters. Underlined bases indicate the 18-bp sequence common to both β-enolase and PKM promoters and the Sp-1 binding sites. Mutations introduced into Sp-1 binding sites are indicated. TATA and GATA boxes are indicated by boldface letters. (b) Nuclear extractions from C_2C_{12} cells and gel mobility shift assays were as described in Materials and Methods. In lanes 2 to 6, nuclear extracts were mixed with a normal (C-WT) oligonucleotide corresponding to bp −54 to −78 of the β-enolase promoter as described for panel a. Antibody, competition, and purified Sp-1 reactions were as described for Fig. 2. In lane 9, an oligonucleotide with the mutated bases shown in panel a was reacted with nuclear extract. In lane 10, the nuclear extract was pretreated with 2 mM diamide as described for Fig. 1 before mixing with the WT β-enolase oligonucleotide. (c) Transient expression of the truncated and mutated β-enolase and MT-IIA promoters described for panel a. WT, normal truncated promoter; MT, mutated truncated promoter. Transfected C_2C_{12} cells were treated and analyzed as described in Materials and Methods. Treatments were as follows: black bars, untreated; shaded bars, 50 μM BSO for 18 h followed by 2 mM H_2O_2 for 9 h. Error bars represent standard errors of the means (n = 3).

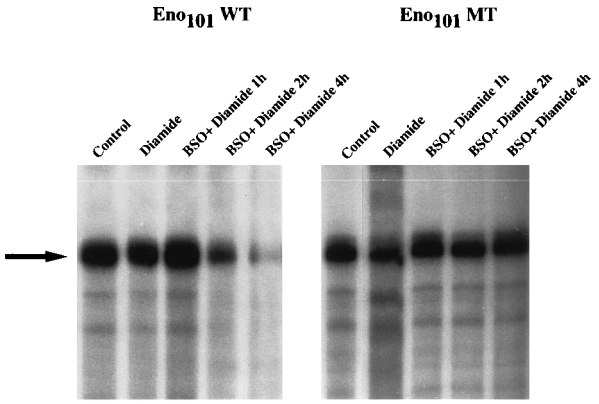

FIG. 8. RNase protection assay of transcripts from oxidatively stressed cells transfected with truncated β-enolase promoter constructs. Transfections, treatments, and the RNase protection protocols are described in Materials and Methods. The arrow indicates the expected 368-bp protected luciferase RNA fragment. The figure is representative of two experiments.

expression was no longer redox sensitive. In contrast, mutation of the MT Sp-1 site had a minimal impact on promoter function, presumably because of the intact TATA element, and neither the native nor the mutated promoter was strongly redox sensitive. These experiments implicate the β-enolase–PKM proximal Sp-1 binding site as a target for oxidative inactivation of promoter function by BSO-H_2O_2 and diamide treatments.

To further define the role of Sp-1 binding in the redox response of the β-enolase promoter, cells transfected with the truncated promoters were stressed with BSO and diamide as described for Fig. 4 and the reporter luciferase RNA was analyzed by an RNase protection assay. Figure 8 shows the time course of transcript changes directed by native and mutated promoters. Transcripts from the native promoter declined precipitously over 4 h of treatment with BSO-diamide, but there was no change in the luciferase RNA levels transcribed from the mutated promoter that lacked the Sp-1 binding site. A similar decay of luciferase transcripts occurred when $pEno_{628}$luc-transfected cells were treated with either H_2O_2 or diamide in the presence of BSO (not shown).

DISCUSSION

A series of previous reports described the reversible inactivation of transcription factor binding to DNA by the oxidation of sulfhydryl groups in vitro (see references in the Introduction). While the strategic location and in vitro redox responsiveness of cysteine residues in a number of transcription factors suggest that they could be targets for transcriptional regulation by redox, the effects have not previously been demonstrated in situ. Here we confirm previous reports that described the reversible loss of zinc finger binding by oxidizing agents in vitro (3, 37, 48) and extend these studies by demonstrating that the same agents can reversibly oxidize and inactivate transcription factor binding in situ. Furthermore, the relative sensitivity of factors to oxidation in vitro was maintained in situ. By analyzing a series of genes and their promoters, we provide evidence that Sp-1-dependent transcription is selectively sensitive to oxidation. Both diamide and H_2O_2 inhibited transcription from Sp-1-dependent SV40, β-enolase, and DHFR promoters but not from MT or HO or promoters in which the Sp-1 sites were replaced or mutated. Although the binding studies demonstrated a correlation between transcription repression and loss of Sp-1 binding, it is possible that oxidation could interfere with Sp-1 transcriptional activation by another mechanism(s) possibly in addition to the direct effects on DNA binding.

In HeLa cells, there was a close correlation between Sp-1 binding in situ and transcriptional repression of SV40 promoters. Reporter transcripts generated from these promoters decreased by 50% over 2 h following treatment of the transfected cells with BSO and 200 μM diamide. This effect correlated with an approximately 60% reduction of low-affinity Sp-1 binding (Fig. 2b). Transcription from the early SV40 promoter has previously been shown to be dependent on Sp-1 binding to the low-affinity binding site (14). The same conditions did not affect SRF or AP-1 transcription factor binding or dependent transcription and did not irreversibly damage the cells. Treatment of cells with BSO alone did not reduce the binding of Sp-1 even though cellular thiol was significantly reduced (Table 1). Under these conditions, it is possible that DNA-protein complexing protects binding site sulfhydryls so that equilibration with the more reactive soluble thiols is repressed. Diamide appeared to penetrate the DNA complex (Fig. 3), but extended exposure to BSO was found to be toxic, so it was not possible to test for a time-dependent equilibration of Sp-1 (data not shown). In what may be an analogous condition, Ammendola et al. (3) recently reported that most of the Sp-1 in aged rat liver, where reactive oxygen levels are elevated, is oxidized and inactive. Short-term (<24 h) BSO treatment caused an apparent increase in Sp-1-dependent transcription from pSp-1-CAT but not from pSV-Sp-1-CAT. This was not related to any apparent change in DNA binding (Fig. 2a and b and 4b and c) or of the sensitivity of binding to diamide treatment in vitro (data not shown). We have not determined the mechanism for this effect.

The results suggest that some zinc finger sulfhydryl groups may have relatively high thiol-disulfide oxidation potentials, within a range that would allow direct redox changes to regulate gene expression (11, 35, 66). Intracellular thiol measurements (Table 1) demonstrated that BSO and 200 μM diamide or 1 mM H_2O_2 treatments caused a 60% reduction of total cellular thiols, some of which were protein associated. Therefore, a broad spectrum of protein thiols is likely to be oxidized by these conditions, including cysteine residues in the AP-1 transcription factor. The observation that Sp-1 was more sensitive than AP-1 in vitro suggests that (i) Sp-1 thiol groups are more reactive in HeLa cell nuclear extracts than are AP-1 thiol groups, (ii) Sp-1 protein is less abundant than AP-1 in the nuclear extracts, or (iii) Sp-1 binds to its site with lower affinity than AP-1. Additionally, the higher response of Sp-1 in vivo may indicate a more reactive or accessible thiol within the DNA–Sp-1 protein complex compared with that in the AP-1–DNA complex.

Decreases in the endogenous transcript levels of PKM, β-enolase, and DHFR genes by severe oxidative stress correlated with a repression of the individual promoters as measured by reporter gene expression and reporter RNA accumulation, the effects on DHFR being somewhat less than those on the glycolytic enzymes (Fig. 6 through 8). All three genes lack proximal promoter TATA box elements and appear to rely on alternative Sp-1 sites for efficient transcription. Sp-1 complexes have been shown previously to act as surrogate TATA motifs to direct initiation of polymerase type 2 genes in TATA-less promoters (10, 12, 27, 51, 74, 91). Therefore, Sp-1 binding exerts an overriding influence on the regulation of transcription of these genes. This was supported by the dramatic loss of transcription caused by mutating the Sp-1 site in the β-enolase promoter (Fig. 7). A similar dependence on Sp-1 binding was

previously shown for transcription of the DHFR promoter (74). The redox sensitivity of these highly Sp-1-dependent promoters (PKM, β-enolase, DHFR), the sensitivity lost in the same promoter element by Sp-1 mutation, and the lack of oxidative repression of Sp-1-independent promoters (HO, MT) support a direct role for Sp-1 in the hyperoxidative regulation of these endogenous genes. It is possible that other TATA-less genes will be similarly redox regulated; in particular, many erythroid-specific gene promoters, including the erythropoietin receptor, myeloid integrin CD11b, and erythroid-specific pyruvate kinase (12, 51), lack TATA elements but contain conserved Sp-1 and GATA-1 binding sites. In these promoters, both Sp-1 and GATA-1 binding factors are zinc finger proteins and may be redox responsive. More subtle effects of oxidative stress would be predicted for the numerous other promoters in which the regulatory role of Sp-1 is less pronounced than it often is in the TATA-less condition.

The studies may be relevant to numerous other metalloprotein-regulated cellular reactions (81), although they do not necessarily apply to other redox stresses, for which the responses will depend on the nature of the stress as well as on the intracellular sites of generation of reactive oxygen species (17, 29, 35, 88). Although diamide specifically targets thiol groups, these groups may also be targets for more general oxidizing agents (17, 29, 64). The reactivity of a thiol group is determined by its accessibility and the microenvironment within the protein, including adjacent amino acids. Thiol groups with metal associations are among the strongest nucleophiles in the cell (11, 72). It has been demonstrated previously that the treatment of nuclear extracts with H_2O_2, the zinc chelator thionein, or the GC-DNA-binding antibiotic mithramycin could specifically prevent binding of protein to Sp-1 sites (48, 61, 93). Mithramycin treatment paralleled the redox effects described here in the selective inhibition of DHFR and collagen-α1 gene transcription (9, 65). We have also found that the phylloquinone-oxidizing agent menadione (80) reduced the binding of Sp-1 and AP-1 to their respective oligonucleotide binding sites (not shown).

Oxidative stresses as severe as those described here are probably associated with some pathophysiological conditions. In particular, inflammatory, immune, and acute-phase responses involve a range of redox-regulated steps (19, 67). Reperfusion injury in susceptible tissues such as heart, kidney, and brain occurs when previously ischemic tissue is reoxygenated (32, 54). The initial cytotoxicity is probably caused by the generation of reactive oxygen associated with reoxygenation of hypoxic tissue and subsequently by polymorphonuclear leukocyte accumulation, activation of membrane-bound NADPH-dependent oxidase systems, and triggering of inflammation (17, 67). Hypoxic tissue becomes sensitized to oxidative stress through at least two mechanisms: (i) hypoxia can cause a precipitous fall in cellular GSH through the suppression of GSH-synthesizing enzymes (15), and (ii) hypoxia mediates increases of endothelial xanthine dehydrogenase and xanthine oxidase activities (76). The combination of decreased redox buffering with enhanced generation of superoxide probably amplifies reperfusion-related cytotoxicity. Satriano et al. (65) demonstrated that an activated NADPH oxidase system or xanthine oxidase-hypoxanthine couple was much more potent than 100 μM H_2O_2 in stimulating a redox stress response in mesangial cells. Oxidative inactivation of transcription factor binding of the type described in this article could be part of the overall response to this type of severe oxidative stress.

Redox mechanisms were important determinants in the development of early life forms, including, presumably, their transcription-regulatory pathways (21, 47). Coupled with the postulated early appearance of metalloprotein regulatory complexes and, in particular, zinc fingers, possibly as the first nucleic acid-binding proteins (6, 22, 26), it is possible that redox regulation of the Sp-1 family of DNA-binding factors was an important early transcriptional control pathway that has persisted through evolution. Direct redox regulation of DNA-binding proteins may still be an active component of gene regulation in eukaryotes, as it is in prokaryotes (21, 43, 73, 77), even though eukaryotic cells have evolved strongly redox-buffered intracellular environments.

The imposition of extreme stress on cells and organisms usually results in the arrest of DNA synthesis and cell division and the suspension of housekeeping functions. Therefore, part of this response may include the transcriptional repression of glycolytic enzyme, DHFR, and other genes through common Sp-1 targets, coupled with a preferential induction of stress response genes, as part of a general survival strategy.

ACKNOWLEDGMENTS

This work was supported by grants HL44578 (K.A.W.) and HL49891 (N.H.B.) from the NIH and by the Cigarette and Tobacco Surtax of the State of California through the Tobacco-Related Disease Research Program of the University of California, grant 4RT-0134 (K.A.W.).

REFERENCES

1. **Abate, C., L. Patel, F. J. Rauscher, and T. Curran.** 1990. Redox regulation of Fos and Jun DNA-binding activity in vitro. Science **249:**1157–1161.
2. **Alam, J.** 1994. Multiple elements within the 5′ distal enhancer of the mouse heme oxygenase-1 gene mediate induction by heavy metals. J. Biol. Chem. **269:**25049–25056.
3. **Ammendola, R., M. Mesuraca, T. Russo, and F. Cimino.** 1994. The DNA-binding efficiency of Sp1 is affected by redox changes. Eur. J. Biochem. **225:** 483–489.
4. **Anderson, M. T., F. J. T. Staal, C. Gitler, and L. A. Herzenberg.** 1994. Separation of oxidant-initiated and redox-regulated steps in the NF-κB signal transduction pathway. Proc. Natl. Acad. Sci. USA **91:**11527–11531.
5. **Bauskin, A. R., I. Alkalay, and Y. Ben-Neriah.** 1991. Redox regulation of a protein tyrosine kinase in the endoplasmic reticulum. Cell **66:**685–696.
6. **Berg, J. M.** 1992. Sp1 and the subfamily of zinc finger proteins with guanine-rich binding sites. Proc. Natl. Acad. Sci. USA **89:**11109–11110.
7. **Bergelson, S., R. Pinkus, and V. Daniel.** 1994. Intracellular glutathione levels regulate Fos/Jun induction and activation of glutathione S-transferase gene expression. Cancer Res. **54:**36–40.
8. **Bishopric, N. H., B. Sato, and K. A. Webster.** 1992. Positive regulation of the skeletal α-actin gene by Fos and Jun. J. Biol. Chem. **270:**2567–2573.
9. **Blume, S. W., R. C. Snyder, R. Ray, S. Thomas, C. A. Koller, and D. M. Miller.** 1991. Mithramycin inhibits Sp1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo. J. Clin. Invest. **88:**1613–1621.
10. **Boisclair, Y. R., A. L. Brown, S. Casola, and M. M. Rechler.** 1993. Three clustered Sp1 sites are required for efficient transcription of the TATA-less promoter of the gene for insulin-like growth factor-binding protein-2 from the rat. J. Biol. Chem. **268:**24892–24901.
11. **Cappel, R. E., and H. F. Gilbert.** 1988. Thiol/disulfide exchange between 3-hydroxy-3-methyglutaryl-CoA reductase and glutathione. J. Biol. Chem. **263:**12204–12212.
12. **Chen, H., H. L. Pahl, R. J. Scheibe, D. Zhang, and D. G. Tenen.** 1993. The Sp1 transcription factor binds the CD11b promoter specifically in myeloid cells in vivo and is essential for myeloid-specific promoter activity. J. Biol. Chem. **268:**8230–8239.
13. **Courey, A. J., D. A. Holtzman, S. P. Jackson, and R. Tjian.** 1989. Synergistic activation by the glutamine-rich domains of human transcription factor Sp1. Cell **59:**827–836.
14. **Courey, A. J., and R. Tjian.** 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell **55:**887–898.
15. **Cowan, D. B., R. D. Weisel, W. G. Williams, and D. A. Mickle.** 1993. Identification of oxygen regulatory elements in the 5′ flanking region of the human glutathione peroxidase gene. J. Biol. Chem. **268:**26904–26910.
16. **Cowan, D. B., R. D. Weisel, W. G. Williams, and D. A. G. Mickle.** 1992. The regulation of glutathione peroxidase expression by oxygen tension in cultured human cardiomyocytes. J. Mol. Cell Cardiol. **24:**423–433.
17. **Cross, A. R., and O. T. G. Jones.** 1991. Enzymic mechanisms of superoxide production. Biochim. Biophys. Acta **1057:**281–298.

18. **Czyzyk-Krzeska, M., D. A. Bayliss, E. E. Lawson, and D. E. Millhorn.** 1992. Regulation of tyrosine hydroxylase gene expression in the rat carotid body by hypoxia. J. Neurochem. **58:**1538–1546.
19. **Dalton, T., R. D. Palmiter, and G. K. Andrews.** 1994. Transcriptional induction of the mouse metallothionine-1 gene in hydrogen peroxide-treated Hepa cells involves a composite major late transcription factor/antioxidant response element and metal response promoter elements. Nucleic Acids Res. **22:**5016–5023.
20. **Decaux, J., B. Antoine, and A. Kahn.** 1989. Regulation of the expression of the L-type pyruvate kinase gene in adult rat hepatocytes in primary culture. J. Biol. Chem. **264:**11584–11590.
21. **Demple, B., and C. F. Amabile-Cuevas.** 1991. Redox redux: the control of oxidative stress responses. Cell **67:**837–839.
22. **Desjarlais, J. R., and J. M. Berg.** 1992. Toward rules relating zinc finger protein sequences and DNA binding site preferences. Proc. Natl. Acad. Sci. USA **89:**7345–7349.
23. **Devary, Y., R. A. Gottlieb, L. F. Lau, and M. Karin.** 1991. Rapid and preferential activation of the c-jun gene during the mammalian UV response. Mol. Cell. Biol. **11:**2804–2811.
24. **Devary, Y., R. A. Gottlieb, T. Smeal, and M. Karin.** 1992. The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell **71:**1081–1091.
25. **Dignam, J. D., R. M. Lebovitz, and R. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
26. **Evans, R. M., and S. M. Hollenberg.** 1988. Zinc fingers: guilt by association. Cell **52:**1–3.
27. **Faber, P. W., H. C. J. van Rooij, H. J. Schipper, A. O. Brinkmann, and J. Trapman.** 1993. Two different, overlapping pathways of transcription initiation are active on the TATA-less human androgen receptor promoter. J. Biol. Chem. **268:**9296–9301.
28. **Firth, J. D., B. L. Ebert, C. W. Pugh, and P. J. Ratcliffe.** 1994. Oxygen-regulated control elements in the phosphoglycerate kinase and lactate dehydrogenase A genes: similarities with the erythropoietin 3′ enhancer. Proc. Natl. Acad. Sci. USA **91:**6496–6500.
29. **Frei, B.** 1994. Reactive oxygen species and antioxidant vitamins: mechanisms of action. Am. J. Med. **97**(Suppl. 3A)**:**5S–13S.
30. **Galang, C. K., and C. A. Hauser.** 1993. Cooperative DNA binding of the human HoxB5 (Hox-2.1) protein is under redox regulation in vitro. Mol. Cell. Biol. **13:**4609–4617.
31. **Gill, G., E. Pascal, Z. H. Tseng, and R. Tjian.** 1994. A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the $dTAF_{II}110$ component of the Drosophila TFIID complex and mediates transcriptional activation. Proc. Natl. Acad. Sci. USA **91:**192–196.
32. **Gottlieb, R. A., K. O. Burleson, R. A. Kloner, B. M. Babior, and R. L. Engler.** 1994. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J. Clin. Invest. **94:**1612–1628.
33. **Guehmann, S., G. Vorbrueggen, F. Kalkbrenner, and K. Moelling.** 1992. Reduction of a conserved Cys is essential for Myb DNA-binding. Nucleic Acids Res. **20:**2279–2286.
34. **Hainaut, P., and J. Milner.** 1993. Redox modulation of p53 conformation and sequence-specific DNA binding *in vitro*. Cancer Res. **53:**4469–4473.
35. **Hentze, M. W., T. A. Rouault, J. B. Harford, and R. D. Klausner.** 1989. Oxidation-reduction and the molecular mechanism of a regulatory RNA-protein interaction. Science **244:**357–359.
36. **Hod, Y.** 1994. The degradation of phosphoenolpyruvate carboxykinase (GTP) mRNA is regulated by cyclic AMP but not by dexamethasone. Arch. Biochem. Biophys. **308:**82–88.
37. **Huang, R., and E. D. Adamson.** 1993. Characterization of the DNA-binding properties of the early growth response-1 (Egr-1) transcription factor: evidence for modulation by a redox mechanism. DNA Cell Biol. **12:**265–273.
38. **Jaiswal, A. K.** 1994. Antioxidant response element. Biochem. Pharmacol. **28:**439–444.
39. **Jocelyn, P. C.** 1987. Spectrophotometric assay of thiols. Methods Enzymol. **143:**44–50.
40. **Jungmann, R. A., D. C. Kelley, M. F. Miles, and D. M. Milkowski.** 1983. Cyclic AMP regulation of lactate dehydrogenase. J. Biol. Chem. **258:**5312–5318.
41. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51:**1079–1090.
42. **Karin, M., and R. I. Richard.** 1982. Human metallothionine genes, primary structure of the metallothionine-II gene and a related processed gene. Nature (London) **299:**797–802.
43. **Kiley, P. J., and W. S. Resnikoff.** 1991. Fnr mutants that activate gene expression in the presence of oxygen. J. Bacteriol. **173:**16–22.
44. **Kim, S., U. S. Onwuta, Y. I. Lee, R. Li, M. R. Botchan, and P. D. Robbins.** 1992. The retinoblastoma gene product regulates Sp1-mediated transcription. Mol. Cell. Biol. **12:**2455–2463.
45. **Kindy, M. S., J. P. Carney, R. J. Dempsey, and J. M. Carney.** 1991. Ishemic induction of protooncogene expression in gerbil brain. J. Mol. Neurosci. **2:**217–228.
46. **Kingsley, C., and A. Winoto.** 1992. Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. Mol. Cell. Biol. **12:**4251–4261.
47. **Klausner, R. D., and J. B. Harford.** 1989. Cis-trans models for post-transcriptional gene regulation. Science **246:**870–872.
48. **Knoepfel, L., C. Steinkuhler, M. Carri, and G. Rotilio.** 1994. Role of zinc-coordination and the glutathione redox couple in the redox susceptibility of human transcription factor Sp1. Biochem. Biophys. Res. Commun. **201:**871–877.
49. **Kriwachi, R. W., S. C. Schultz, T. A. Steitz, and J. P. Caradonna.** 1992. Sequence-specific recognition of DNA by zinc-finger peptides derived from the transcription factor Sp1. Proc. Natl. Acad. Sci. USA **89:**9759–9763.
50. **Leys, E. J., G. F. Crouse, and R. E. Kellems.** 1984. Dihydrofolate reductase gene expression in cultured mouse cells is regulated by transcript stabilization in the nucleus. J. Cell Biol. **99:**180–187.
51. **Max-Audit, I., J. Eleouet, and P. Romeo.** 1993. Transcriptional regulation of the pyruvate kinase erythroid-specific promoter. J. Biol. Chem. **268:**5431–5437.
52. **Maxwell, P. H., C. W. Pugh, and P. J. Ratcliffe.** 1993. Inducible operation of the erythropoietin 3′ enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. Proc. Natl. Acad. Sci. USA **90:**2423–2427.
53. **McBride, A. A., R. D. Klausner, and P. M. Howley.** 1992. Conserved cysteine residue in the DNA-binding domain of the bovine papillomavirus type 1 E2 protein confers redox regulation of the DNA-binding activity *in vitro*. Proc. Natl. Acad. Sci. USA **89:**7531–7535.
54. **McCord, J. M.** 1987. Oxygen-derived free radicals: a link between reperfusion injury and inflammation. Fed. Proc. **46:**2401–2406.
55. **Molkentin, J. D., D. V. Kalvakolanu, and B. E. Markham.** 1994. Transcription factor GATA-4 regulates cardiac muscle-specific expression of the alpha-myosin heavy-chain gene. Mol. Cell. Biol. **14:**4947–4957.
56. **Murphy, B. J., K. R. Laderoute, R. J. Chin, and R. M. Sutherland.** 1994. Metallothionine IIA is up-regulated by hypoxia in human A431 squamous carcinoma cells. Cancer Res. **54:**5808–5810.
57. **Neubauer, A., B. Neubauer, and E. Liu.** 1991. Polymerase chain reaction based assay to detect allelic loss in human DNA. Nucleic Acids Res. **18:**993–998.
58. **Olah, Z., S. Komoly, N. Nagashima, F. Joo, U. R. Rapp, and W. B. Anderson.** 1991. Cerebral ischemia induces transient intracellular redistribution and intranuclear translocation of the raf proto-oncogene product in hippocampal pyramidal cells. Exp. Brain Res. **84:**403–410.
59. **Peshavaria, M., and I. N. M. Day.** 1991. Molecular structure of the human muscle-specific enolase gene (ENO3). Biochem. J. **275:**427–433.
60. **Pognonec, P., H. Kato, and R. G. Roeder.** 1992. The helix-loop-helix repeat transcription factor USF can be functionally regulated in a redox-dependent manner. J. Biol. Chem. **267:**24563–24567.
61. **Ray, R., S. Thomas, and D. M. Miller.** 1990. Mithramycin selectively inhibits the transcriptional activity of a transfected c-myc gene. Am. J. Med. Sci. **299:**203–208.
62. **Rhodes, D., and A. Klug.** 1993. Zinc fingers. Sci. Am. **268:**56–65.
63. **Saffer, J. D., S. P. Jackson, and M. B. Annarella.** 1991. Developmental expression of Sp1 in the mouse. Mol. Cell. Biol. **11:**2189–2199.
64. **Sanders, S. P., J. L. Zweier, P. Kuppusamy, S. J. Harrison, D. J. P. Bassett, E. W. Gabrielson, and T. J. Sylvester.** 1993. Hyperoxic sheep pulmonary microvascular endothelial cells generate free radicals via mitochondrial electron transport. J. Clin. Invest. **91:**46–52.
65. **Satriano, J. A., M. Shuldiner, K. Hora, Y. Xing, Z. Shan, and D. Schlondorff.** 1993. Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor-a and immunoglobulin G. J. Clin. Invest. **92:**1564–1571.
66. **Schenk, H., M. Klein, W. Erdbrugger, W. Droge, and K. Schulze-Osthoff.** 1994. Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-κB and AP-1. Proc. Natl. Acad. Sci. USA **91:**1672–1676.
67. **Schreck, R., P. Rieber, and P. A. Baeuerle.** 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NK-κB transcription factor and HIV-1. EMBO J. **10:**2247–2258.
68. **Semenza, G. L., P. H. Roth, H.-M. Fang, and G. L. Wang.** 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J. Biol. Chem. **269:**23757–23763.
69. **Seto, E., B. Lewis, and T. Shenk.** 1993. Interaction between transcription factors Sp1 and YY1. Nature (London) **365:**462–464.
70. **Shaul, P. W., W. B. Campbell, M. A. Farrar, and R. R. Magness.** 1992. Oxygen modulates prostacyclin synthesis in ovine fetal pulmonary arteries by an effect on cyclooxygenase. J. Clin. Invest. **90:**2147–2155.
71. **Sibrowski, W., and H. J. Seitz.** 1984. Rapid action of insulin and cyclic AMP in the regulation of functional messenger RNA coding for glucokinase in rat liver. J. Biol. Chem. **259:**343–346.
72. **Snyder, G. H., M. J. Cennerazzo, A. J. Karalis, and D. Field.** 1981. Electrostatic influence of local cysteine environments on disulfide exchange kinetics. Biochemistry **20:**6509–6518.

73. **Storz, G., L. A. Tartaglia, and B. N. Ames.** 1990. Transcriptional regulation of oxidative stress-inducible genes: direct activation by oxidation. Science **248:**189–194.
74. **Swick, A. G., M. C. Blake, J. W. Kahn, and J. C. Azizkhan.** 1989. Functional analysis of GC element binding and transcription in the hamster dihydrofolate reductase gene promoter. Nucleic Acids. Res. **17:**9291–9304.
75. **Taylor, J. M., J. D. Davies, and C. A. Peterson.** 1995. Regulation of the myoblast-specific expression of the human β-enolase gene. J. Biol. Chem. **270:**2535–2540.
76. **Terada, L. S., D. M. Guidot, J. A. Leff, I. R. Willingham, M. E. Hanley, D. Piermattei, and J. E. Repine.** 1992. Hypoxia injures endothelial cells by increasing endogenous xanthine oxidase activity. Proc. Natl. Acad. Sci. USA **89:**3362–3366.
77. **Toledano, M. B., I. Kullik, F. Trinh, P. T. Baird, T. D. Schneider, and G. Storz.** 1994. Redox-dependent shift of OxyR-DNA contacts along an extended DNA-binding site: a mechanism for differential promoter selection. Cell **78:**897–909.
78. **Toledano, M. B., and W. J. Leonard.** 1991. Modulation of transcription factor NF-κB binding activity by oxidation-reduction in vitro. Proc. Natl. Acad. Sci. USA **88:**4328–4332.
79. **Tyrrell, R. M., L. A. Appelgate, and Y. Tromvoukis.** 1993. The proximal promoter region of the human heme oxygenase gene contains elements involved in stimulation of transcriptional activity by a variety of agents including oxidants. Carcinogenesis **14:**1–6.
80. **Tzeng, W., T. Chiou, C. Wang, J. Lee, and Y. Chen.** 1994. Cellular thiols as a determinant of responsiveness to menadione in cardiomyocytes. J. Mol. Cell. Cardiol. **26:**889–897.
81. **Vallee, B. L., and D. S. Auld.** 1990. Zinc coordination, function, and structure of zinc enzymes and other proteins. Biochemistry **29:**5647–5659.
82. **Wang, G. L., and G. L. Semenza.** 1993. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc. Natl. Acad. Sci. USA **90:**4304–4308.
83. **Webster, K. A.** 1987. Regulation of glycolytic enzyme RNA transcriptional rates by oxygen availability in skeletal muscle cells. Mol. Cell. Biochem. **77:** 19–28.
84. **Webster, K. A., D. Discher, and N. H. Bishopric.** 1993. Induction and nuclear accumulation of fos and jun proto-oncogenes in hypoxia cardiac myocytes. J. Biol. Chem. **268:**16852–16859.
85. **Webster, K. A., D. J. Discher, and N. H. Bishopric.** 1994. Regulation of fos and jun immediate-early genes by redox or metabolic stress in cardiac myocytes. Circ. Res. **75:**361–371.
86. **Webster, K. A., and L. Kedes.** 1990. The c-*fos* cyclic AMP-responsive element conveys constitutive expression to a tissue-specific promoter. Mol. Cell. Biol. **10:**2402–2406.
87. **Webster, K. A., G. E. O. Muscat, and L. Kedes.** 1988. Adenovirus E1A products suppress myogenic differentiation and inhibit transcription from muscle-specific promoters. Nature (London) **332:**553–561.
88. **Werns, S. W., and R. Lucchesi.** 1990. Free radicals and ischemic tissue injury. Trends Physiol. Sci. **11:**161–166.

88a. **Wu, X., et al.** 1994. Zinc fingers are redox sensors during acute oxidative stress. Circulation **90:**I-250.

89. **Xanthoudakis, S., G. Miao, F. Wang, Y. E. Pan, and T. Curran.** 1992. Redox activation of Fos-Jun binding activity is mediated by a DNA repair enzyme. EMBO J. **11:**3323–3335.
90. **Xanthoudakis, S., G. G. Miao, and T. Curran.** 1994. The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. Proc. Natl. Acad. Sci. USA **91:**23–27.
91. **Xu, J., K. L. Thompson, L. B. Shephard, L. G. Hudson, and G. N. Gill.** 1993. T3 receptor suppression of Sp-1-dependent transcription from the epidermal growth factor receptor promoter via overlapping DNA-binding sites. J. Biol. Chem. **268:**16065–16073.
92. **Yaffe, D., and O. Saxel.** 1977. A myogenic cell line with altered serum requirements for differentiation. Differentiation **7:**159–166.
93. **Zeng, J., R. Heuchel, W. Schaffner, and J. H. R. Kagi.** 1991. Thionein can modulate DNA binding and transcription activation by zinc finger containing factor Sp1. FEBS Lett. **279:**310–312.