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Role of Cysteine Residues in Regulation of p53 Function

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Previous studies of p53 have implicated cysteine residues in site-specific DNA binding via zinc coordination and redox regulation (P. Hainaut and J. Milner, Cancer Res. 53:4469–4473, 1993; T. R. Hupp, D. W. Meek, C. A. Midgley, and D. P. Lane, Nucleic Acids Res. 21:3167–3174, 1993). We show here that zinc binding and redox regulation are, at least in part, distinct determinants of the binding of p53 to DNA. Moreover, by substituting serine for each cysteine in murine p53, we have investigated the roles of individual cysteines in the regulation of p53 function. Substitution of serine for cysteine at position 40, 179, 274, 293, or 308 had little or no effect on p53 function. In contrast, replacement of cysteine at position 173, 235, or 239 markedly reduced in vitro DNA binding, completely blocked transcriptional activation, and led to a striking enhancement rather than a suppression of transformation by p53. These three cysteines have been implicated in zinc binding by X-ray diffraction studies (Y. Cho, S. Gorina, P. D. Jeffrey, and N. P. Pavletich, Science 265:346–355, 1994); our studies demonstrate the functional consequences of the inability of the central DNA-binding domain of p53 to bind zinc. Lastly, substitutions for cysteines at position 121, 132, 138, or 272 partially blocked both transactivation and the suppression of transformation by p53. These four cysteines are located in the loop-sheet-helix region of the site-specific DNA-binding domain of p53. Like the cysteines in the zinc-binding region, therefore, these cysteines may cooperate to modulate the structure of the DNA-binding domain. Our findings argue that p53 is subject to more than one level of conformational modulation through oxidation-reduction of cysteines at or near the p53-DNA interface.

Mutation of p53 DNA, a tumor suppressor gene (24), is the most common feature of a wide variety of human cancers (2, 4, 25, 40, 53). Wild-type p53 is thought to suppress tumor formation by acting as a transcriptional regulator (13, 31, 46). The majority of tumor-associated p53 mutations affect amino acids in the central, highly conserved region of p53 that is responsible for the specific DNA binding function of p53 (3, 41, 56). Once bound specifically to DNA, p53 activates target genes through its N-terminal transactivation domain (14, 43, 45). A C-terminal oligomerization domain facilitates but is not absolutely essential for transactivation (55).

Recent experiments suggest that the wild-type p53 exerts its tumor suppressor effect by controlling the expression of genes involved in the cell cycle (11, 23, 59), in cell arrest after DNA damage (29), and in apoptosis (7, 34, 39, 48). Wild-type p53, but not transforming mutant p53, turns on the expression of a gene known as *WAF1* (11) or *CIP1* (23) coding for a 21-kDa protein that inhibits the activity of the cyclin-dependent kinases necessary for cell cycle progression. Likewise, wild-type p53, but not transforming mutant p53, activates expression of the *GADD45* gene, whose expression contributes to arrest of the cell cycle in the G_1 phase following DNA damage and allows DNA repair before DNA replication occurs (29). The *WAF1*/*CIP1* gene is also expressed in cells induced to undergo apoptosis by p53-dependent mechanisms (10). In addition, wild-type p53 is a transcriptional activator of the *bax* gene (39) encoding Bax, which acts as an accelerator of apoptosis. To arrest cells or trigger apoptosis at certain times but not at others, there must be differential regulation of specific target genes by wild-type p53. One mechanism for such differential regulation may be the nuclear accumulation of wild-type p53 which occurs in response to UV and ionizing radiation (35) or other DNA-damaging agents (17). Alternatively, the binding of p53 to regulatory DNA sequences may be influenced by differential modification of the protein through phosphorylation or oxidation-reduction.

Binding of p53 to target DNA is dependent on a wild-type conformation of the protein (22). Analysis with monoclonal antibodies indicates that the conformation of wild-type p53 protein differs from that of transforming mutant p53 (38). The conformation of wild-type p53 can be modulated by oxidation-reduction; reduction is required for optimal binding of wild-type p53 to target DNA (21, 28, 44). These observations suggest that specific cysteine residues are involved in the binding process. Cysteines are known to play an important role in the binding of a number of transcriptional regulatory proteins to DNA. In the case of transcriptional factors TFIIIA and SP1, key cysteine residues are responsible for the formation of zinc fingers essential for DNA binding (8, 12). By means of a different mechanism, oxidation-reduction of a single conserved cysteine residue in the DNA-binding domains of transcriptional factors fos and jun modulates binding of the heterodimer to target DNA (1). In examining the amino acid sequence of wild-type p53, we identified a sequence in p53 homologous to that downstream of a critical cysteine residue in the DNA-binding domain of fos. The sequence in fos is $CX_1NRRX_2LTX_1TLX_6L$, where X represents any amino acid; the sequence downstream of Cys-239 in p53 is $CX_4NRRX_2LTX_2TLX_6L$. The similarity of these two regions suggested that the redox state of p53 might be of critical importance in modulating its binding to DNA.

To examine the potential involvement of specific cysteines in p53 in DNA binding, transactivation, and suppression of cell transformation we made a series of mutant p53s. In each case, one of the twelve cysteine residues in murine p53 was changed to serine. We compared the biological activity of each of these

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mutant p53s with that of wild-type p53. We have found that there are three cysteines (Cys-173, Cys-235, and Cys-239) in p53 which are essential for suppression of transformation, transactivation, and in vitro DNA binding. Interestingly, these are the same three cysteines which, on the basis of X-ray crystallography, appear to be involved in binding a zinc ion (6). Additional cysteines, however, contribute to the suppression of transformation and transactivation functions of p53.

MATERIALS AND METHODS

Cells and baculovirus infections. Insect Sf9 cells were grown in Grace's medium supplemented with yeastolate and lactalbumin hydrolysate plus 10% fetal calf serum. Sf9 cells were infected with a high-titer recombinant baculovirus, nuclear polyhedrosis virus (NPV), containing wild-type p53 (52) in order to overexpress wild-type murine p53 or with recombinant baculovirus expressing the cysteine-mutant p53 proteins.

Primary rat embryo fibroblasts (REFs) (Fisher inbred rats, Whittaker Bioproducts, Inc.) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. NCI-H358 cells (human bronchioloalveolar carcinoma; American Type Culture Collection) were cultured in RPMI 1640 medium plus 10% fetal bovine serum, and HCT-116 cells were cultured in DMEM plus 10% fetal bovine serum.

Murine p53 plasmid DNA and site-directed mutagenesis of cysteine residues. Site-directed mutagenesis (32) was carried out on the pBS.KS+ plasmid containing wild-type murine p53 cDNA under the control of the Harvey murine sarcoma virus long terminal repeat (52). Each of the 12 cysteine residues was individually changed to a serine residue, since serine is structurally similar to cysteine but has a hydroxyl group in place of a sulfhydryl group. The Cys→Ser substitutions were confirmed by sequencing. The Cys-mutant p53 DNAs were moved into the baculovirus expression system as follows. The DNAs were recombined into *Autographa californica* NPV by using the pJVP10-Z shuttle vector. Sf9 cells were then transfected with cationic liposomes (Invitrogen) containing wild-type *Autographa californica* NPV DNA and the recombinant p53-pJVP10-Z DNA. Blue recombinant virus plaques were selected by using Bluo-gal as the substrate (54). Each recombinant virus coding for mutant p53 was purified through two rounds of plaque purification and assayed for p53 protein production.

Cell transformation assay. REFs at passage 4 were triply transfected on 6-cm-diameter plates with 2.5 μg of pSP72-ras (45) kindly provided by A. Levine, 2.5 μg of pBS-E1A (45), and 5 μg of pBS.KS+ containing wild-type p53 or Cys-mutant p53 in the presence of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammoniummethylsulfate (DOTAP) liposome transfection reagent (Boehringer-Mannheim). One hour prior to transfection, the DNA mixes were prepared in HEPES-buffered saline (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.1], 137 mM NaCl, 6 mM D-(+)-glucose, 5 mM KCl, 0.7 mM Na_2HPO_4) in a total volume of 100 μl. The DNA mixes were combined with 30 μl of DOTAP diluted with 70 μl of HEPES-buffered saline and incubated for 10 min at room temperature. DMEM with 10% fetal bovine serum and 2 mM glutamine (1 ml) was then added to each DNA-DOTAP mixture, and the diluted solution was pipetted onto a plate of REFs containing 4 ml of growth medium. The medium-plus-transfection mix was removed 18 h later, and the cells were washed twice with DMEM and refed with growth medium. After an additional 24 h, the cells were trypsinized and transferred to 10-cm-diameter plates. The cells were refed every 3 to 4 days for 12 to 14 days. Monolayers were stained with 0.05% Coomassie blue in 50% methanol and 10% acetic acid, and foci were counted.

CAT transactivation assay. Either HCT-116 cells or NCI-H358 cells on 6-cm-diameter plates were doubly transfected with 2 μg of PG_{13}-CAT kindly provided by B. Vogelstein (31) and 2 μg of pBS.KS+ containing wild-type p53 or Cys-mutant p53 in the presence of DOTAP as described above. The medium-plus-transfection mix was removed 16 to 18 h posttransfection, and the cells were washed with RPMI 1640 and refed with growth medium. The cells were harvested 24 h later by scraping in 250 mM Tris, pH 7.8, with 1 mM EDTA and were lysed by three freeze-thaw cycles. Protein concentrations of the lysates were determined by the Bradford assay (Bio-Rad), and equal amounts of protein were used for the chloramphenicol acetyltransferase (CAT) assay. Lysates containing 10 to 20 μg of protein were incubated with 0.5 mM acetyl coenzyme A and 0.1 μCi of D-threo[dichloroacetyl-1-^{14}C]chloramphenicol (Amersham) in CAT buffer for 4 h at 37°C. The products of the CAT assay were extracted with ethyl acetate, separated by chromatography on silica gel thin-layer chromatograms in chloroform-methanol (19:1) solvent, detected by exposure to Kodak XAR-5 film, and quantitated with NIH Image version 1.43.

Analysis of p53 protein production in transfected REF and NCI-H358 cells. The murine sarcoma virus promoter of the pBS.KS+ plasmid containing wild-type p53 DNA or the Cys-mutant p53 DNAs were replaced with the cytomegalovirus (CMV) promoter from pCMH6Kp53 (56) in order to enhance the expression of p53 protein in transfected cells. REF or NCI-H358 cells on 10-cm-diameter plates were transfected with 20 μg of wild-type or mutant p53-containing vector DNA in the presence of DOTAP as described above. The cells were harvested 26 to 28 h posttransfection by scraping in phosphate-buffered saline. Cell pellets were lysed in lysis buffer (100 mM Tris-HCl [pH 9.0], 150 mM NaCl, 0.5% Nonidet P-40 [NP-40], 80 μg of aprotinin per ml, 800 μg of leupeptin per ml) for 30 min at 4°C. After centrifugation at 18,000 rpm for 20 min in a Beckman JA-20 rotor at 4°C, p53 protein present in soluble lysates was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and probed with a mixture of monoclonal antibodies PAb 242, PAb 248, and PAb 421, each diluted 1:500 (see below).

Purification of wild-type p53 and cysteine-mutant p53 proteins. Extracts were prepared from recombinant baculovirus wild-type p53-infected or Cys-mutant p53-infected Sf9 cells at approximately 65 h postinfection by lysis in buffer containing 150 mM Tris-HCl (pH 9.0), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 1 mg of leupeptin (Sigma) per ml, and 1 mg of aprotinin (Sigma) per ml for 30 min at 4°C. The lysates were then centrifuged at 18,000 rpm for 20 min at 4°C in a Beckman JA-20 rotor with microadaptors, and the supernatant was used for protein purification. Wild-type or mutant p53s were purified by overnight binding at 4°C to monoclonal antibody PAb 421 chemically cross-linked to protein A-Sepharose (50). After successive washing of the beads with 50 mM Tris-HCl (pH 8.0)–0.5 M LiCl–1 mM EDTA followed by 20 mM Tris-HCl (pH 8.5)–1 mM EDTA–10% glycerol, p53 protein was eluted with decapeptide KKGQSTSRHK-amide (Multiple Peptide Systems, San Diego, Calif.) at a concentration of 0.1 mg/ml in elution buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.1% NP-40) (52). Wild-type p53 protein was also prepared as described above in solutions pretreated with Chelex-100 resin (Bio-Rad) in order to obtain purified protein free of exogenous divalent cations. Eluted p53 proteins were either stored in elution buffer or concentrated in a Centricon-30 microconcentrator (Amicon), washed several times with buffer containing 10 mM HEPES (pH 7.8), 5 mM KCl, 0.5 mM $MgCl_2$, and 10% glycerol, and stored at 4°C in this buffer until used in the gel mobility shift assay.

Preparation of ^{32}P-labeled target DNA containing a p53 consensus sequence. Two single-stranded complementary oligonucleotides containing the p53 consensus sequence described by Funk et al. (18) were synthesized, annealed, and then ligated into Bluescript KS cut with *Bam*HI and *Pst*I; the resulting pBS.KS.Shay plasmid was kindly provided by J. Stenger. The nucleotide sequence contained within the p53 consensus sequence in the center of the fragment was 5′-GGACATGCCCCGGGCATGTCC-3′ (18). ^{32}P-labeled target DNA was prepared from plasmid DNA cut with *Bam*HI and *Pst*I. The restriction fragment containing the p53 consensus sequence was purified and then end filled with Klenow fragment (Boehringer), unlabeled deoxynucleoside triphosphates, and [α-^{32}P]dGTP. The labeled DNA was put over a Nuc-Push column (Stratagene), ethanol precipitated, resuspended in Tris-EDTA, and stored at −20°C until used in the gel mobility shift assay.

Gel mobility shift assay of sequence-specific DNA binding by p53. The assay conditions were similar to those described by Hupp et al. (27). A typical reaction mixture (20 μl) contained 50 ng of purified wild-type p53, 2 ng of ^{32}P-end-labeled DNA with the consensus sequence described by Funk et al. (18), and 20 ng of Bluescript SK$^+$ DNA in DNA binding buffer (25 mM HEPES [pH 7.6], 50 mM KCl, 0.5 mg of bovine serum albumin per ml, 20% glycerol, 0.1% NP-40). Purified p53 protein was preincubated with DTT at a specified final concentration (usually 5 mM) for 20 min at room temperature prior to incubation with the target DNA plus Bluescript competitor DNA. After the addition of DNA, the reaction mixtures were incubated at room temperature for 30 min to allow for protein-DNA binding. During this time, a prechilled low-ionic-strength 4% polyacrylamide gel was preelectrophoresed at 100 V in running buffer containing 0.33× Tris-borate-EDTA and 0.1% Triton X-100 at 4°C. At the end of the incubation period, 10-μl samples were loaded onto the 4% polyacrylamide gel while the gel was running at 100 V. The samples were electrophoresed at 200 V for 1 h at 4°C. The gel was then dried and exposed to Kodak XAR-5 film overnight.

Blotting and immunodetection of mobility-shifted p53-DNA complexes on a Nytran plus membrane. After separation of the p53-DNA complexes from unbound DNA by gel electrophoresis, the gel was blotted onto a Nytran plus membrane (Schleicher & Schuell) overnight in Tris-borate-EDTA. The DNA-protein complexes were cross-linked onto the Nytran plus membrane in a UV Stratalinker 2400 (Stratagene) by using the "auto crosslink" setting (1200 μJ). The ^{32}P-labeled target DNA in the p53-DNA complexes was detected by exposing the Nytran plus membrane to XAR-5 film. The Nytran plus membrane was blocked, and the p53 protein in the p53-DNA complexes was immunodetected with a mixture of monoclonal antibodies, PAb 242, PAb 248, and PAb 421, diluted 1:500. Biotinylated goat anti-mouse immunoglobulin G and streptavidin-alkaline phosphatase were added and detected with BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium) and Nitro Blue Tetrazolium by using the protocol outlined in the Immunoselect system (Gibco/BRL).

Glutaraldehyde cross-linking and gel analysis of the oligomeric structure of p53 proteins. Approximately 550 ng of wild-type or Cys-mutant p53 protein was used in a total volume of 15 μl of elution buffer; 0.1 M HEPES, pH 7.8, was added to the protein to give a final concentration of 0.01 M. Glutaraldehyde (1%) in 0.01 M HEPES was added to the protein mixture to give a final glutaraldehyde concentration of 0.1%. Cross-linking was carried out at 37°C for 30 min in 1% SDS–0.5% β-mercaptoethanol–0.1 M phosphate buffer, pH 7.1. After incubation overnight at room temperature, an equal volume of 2× sample

buffer (40% glucose, 0.4× gel buffer, bromophenol blue) was added, and the samples were boiled for 2 min. The samples were analyzed on a 5% polyacrylamide–0.05% SDS gel (pH 7.1) with a 4% stacking gel (pH 5.5) and run in 0.05 M phosphate buffer (pH 7.1)–0.05% SDS (42, 57). The gel was blotted onto nitrocellulose (Schleicher & Schuell) overnight at 12 V, and p53 oligomers were immunodetected with monoclonal antibodies PAb 242, PAb 248, and PAb 421 and with the Immunoselect system (Gibco/BRL).

RESULTS

Reduction of p53 by DTT enhances sequence-specific DNA binding. Wild-type murine p53 protein expressed in insect cells infected with a recombinant baculovirus (52) was used in a standard gel mobility shift assay (16, 27). The DNA used in the assay was a ^{32}P-end-labeled 55-bp DNA fragment containing the consensus sequence described by Funk et al. (18) in the center of the fragment. In order to examine specific binding of purified p53 to DNA containing the p53 consensus sequence (consensus fragment), a 10-fold excess of competitor DNA (Bluescript SK$^+$) was included in the reaction mixture. Under these conditions, gel-shifted DNA was barely detectable (Fig. 1A, lane 2) and was visible only after autoradiography for long exposure times. Pretreatment of purified p53 protein with DTT resulted in a marked increase in binding of p53 to the consensus fragment in the presence of Bluescript competitor DNA. When p53 was titrated with increasing amounts of DTT, an optimal DTT concentration of 2 mM or higher was required for enhancement of DNA binding (Fig. 1A). The bound DNA exhibited a multiple banding pattern. The p53 protein-DNA binding was inhibited by specific unlabeled consensus fragment but not by nonspecific poly(dA-dT) DNA (Fig. 1B).

To demonstrate the presence of p53 protein in the multiple bands seen in the gel mobility shift assay, the protein-DNA complexes were transferred from the polyacrylamide gel onto a Nytran plus membrane and immunodetected with a mixture of monoclonal antibodies. The positions of the p53 protein molecules coincided with those of the labeled DNA bands detected by autoradiography of the same membrane (Fig. 2). The results demonstrate that p53 protein bound to the target DNA is responsible for its retardation. Evidence suggests that the fastest-migrating protein-DNA complex represents DNA bound to tetrameric p53, while the more slowly migrating forms represent DNA bound to octameric and higher oligomeric forms of p53. Comparison of the autoradiogram with the immunoblot indicates that target DNA binds preferentially to octameric p53 rather than tetrameric p53 at higher protein concentrations (Fig. 2). These findings imply that protein-protein interactions are involved in octamer-DNA binding.

Alkylation of free sulfhydryl groups in p53 abolishes binding of p53 to DNA. The marked effect of DTT on the gel mobility shift assay indicates that wild-type p53 must be in a reduced state in order to bind to DNA in a sequence-specific manner and that one or more cysteine residues may be involved in this process. Some transcriptional regulatory proteins contain a single critical cysteine residue that is sensitive to redox control (1, 19). Reduction of this cysteine residue in fos or jun by DTT or by Ref-1, a nuclear redox protein, stimulates the DNA binding activity of fos-jun heterodimers and jun-jun homodimers (58). In contrast, treatment of fos or jun with *N*-ethylmaleimide, an agent that alkylates free sulfhydryl groups, inhibits DNA binding (1). To determine if there is a similar mechanism operating in p53, we reduced purified wild-type p53 with DTT and then alkylated the protein with *N*-ethylmaleimide prior to DNA binding in the gel mobility shift assay. When p53 was pretreated with 2 mM DTT followed by 4 mM *N*-ethylmaleimide, there was a decrease in the ability of the protein to bind to the labeled consensus fragment (Fig. 3).

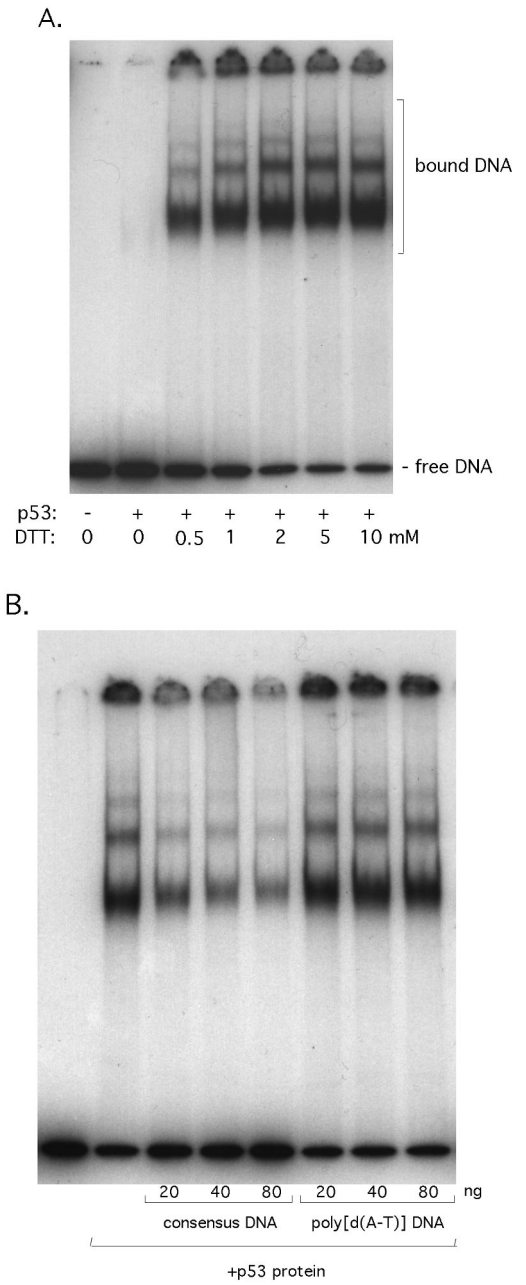

FIG. 1. (A) Stimulation by DTT of wild-type p53 protein binding to DNA containing a consensus sequence. Purified p53 protein (50 ng) was incubated with various concentrations of DTT (0.5 to 10 mM) for 20 min at room temperature prior to incubation with 2 ng of ^{32}P-labeled consensus DNA and 20 ng of unlabeled Bluescript DNA. Protein-DNA complexes were separated from unbound DNA by gel electrophoresis and visualized by autoradiography. (B) Inhibition of wild-type p53 binding to DNA containing a consensus sequence by specific competitor DNA (with the consensus sequence) but not by nonspecific poly(dA-dT) DNA. Purified p53 protein (50 ng) was incubated with 2 ng of ^{32}P-labeled consensus DNA, 20 ng of unlabeled Bluescript carrier DNA, and increasing amounts (20 to 80 ng) of unlabeled specific competitor DNA or nonspecific poly(dA-dT) DNA.

The data suggest that one or more cysteine residues in p53 are critical to the ability of p53 to bind DNA in a sequence-specific manner.

Binding of zinc by p53 is required for sequence-specific DNA binding. Specific cysteine residues in a number of transcrip-

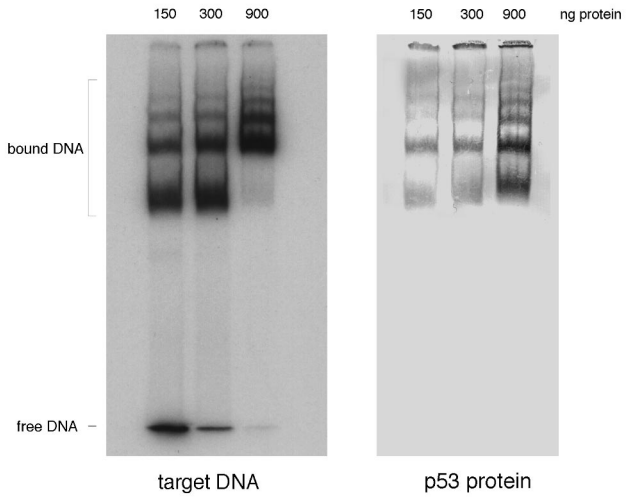

FIG. 2. Presence of p53 protein in the mobility-shifted target DNA bands. Increasing concentrations (150 to 900 ng) of wild-type p53 purified in the presence of Chelex-treated solutions were incubated with 2 ng of ^{32}P-labeled consensus DNA and 20 ng of unlabeled Bluescript DNA. The mobility shift gel was blotted onto a Nytran plus membrane. The bound target DNA on the blot was detected by autoradiography, and p53 protein on the same blot was immunodetected with monoclonal antibodies PAb 242, PAb 248, and PAb 421 followed by biotinylated secondary antibody and streptavidin-alkaline phosphatase.

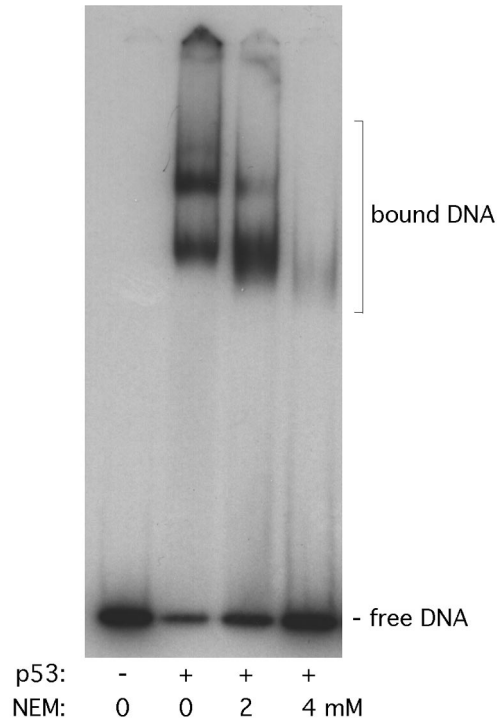

FIG. 3. Effect of alkylation of wild-type p53 protein on binding to target DNA. Wild-type p53 (50 ng) was incubated with 2 mM DTT followed by 2 or 4 mM *N*-ethylmaleimide (NEM) for 30 min prior to incubation with 2 ng of ^{32}P-labeled consensus DNA and 20 ng of unlabeled Bluescript DNA.

tional regulatory proteins have been shown to bind zinc and to form zinc finger motifs that are essential for binding of these proteins to target DNA (8, 12). On the basis of the sensitivity of p53 to the zinc chelator 1,10-phenanthroline (OP), Hainaut and Milner (20) have proposed the existence of two zinc-binding domains in each molecule of wild-type p53 and have noted the role of bound zinc in stabilizing the wild-type conformation of the protein. Crystals containing the central domain of p53 complexed with target DNA (6) indicate that one zinc ion is in fact associated with each monomer of p53.

To determine if the binding of zinc by wild-type p53 is necessary for recognition of target DNA in our system, we tested the effect of OP on the ability of p53 to bind and retard the labeled consensus fragment. The p53 protein used was purified but not concentrated and dialyzed. We have found that the extent of binding of p53 to DNA depends on the method of purification and that the protein has more binding activity before, rather than after, concentration and dialysis. Presumably, oxidation of the protein during concentration and dialysis interferes with DNA binding. When p53 was pretreated with 5 mM DTT followed by OP at a final concentration ranging from 0.32 to 5 mM, there was a marked decrease in the ability of p53 to bind to target DNA (Fig. 4A), in contrast to the significant binding of target DNA by p53 pretreated with DTT but not OP (Fig. 4A). Treatment of p53 with DTT noticeably retarded the migration of the protein-DNA complex in the gel. Thus, gel migration of the complex reflects the redox state of p53.

The ability of p53 to form oligomers was not noticeably altered as a result of chelation of the zinc ions. Analysis of OP-treated p53 protein by SDS-polyacrylamide gel electrophoresis after glutaraldehyde cross-linking indicates that OP does not affect the oligomeric forms of p53 at the OP concentrations used in the DNA mobility shift assay (Fig. 4B). Similar to the results seen in the gel mobility shift assay (Fig. 4A), non-DTT-treated p53 oligomers migrated farther in the SDS-polyacrylamide gel than DTT-treated p53 oligomers (Fig. 4B; compare lanes 1 and 2). OP did not shift the p53 oligomers to the electrophoretic forms associated with oxidation as long as DTT was still present (Fig. 4).

Reduction of p53 facilitates sequence-specific DNA binding in the absence of exogenous zinc. Although zinc ions associated with purified wild-type p53 are essential for binding of the protein to consensus DNA, the presence of bound zinc is not sufficient to prevent oxidation of the protein, as evidenced by the electrophoretic shift seen in the purified protein upon incubation with DTT (Fig. 4A). The mechanism by which DTT stimulates the binding of wild-type p53 to target DNA is not known. The reducing agent may be responsible for shifting the protein from an oxidized to a reduced state that in turn is required for sequence-specific DNA binding. Alternatively, the reducing agent may stimulate DNA binding by generating free sulfhydryl groups that subsequently coordinate exogenous zinc and thereby stabilize p53 in a conformation required for binding to DNA. In order for DTT-treated p53 to bind exogenous zinc, there must be zinc ions present in the p53-containing solutions. If binding of exogenous zinc by the reduced p53 protein is not required for DNA binding, then chelation of exogenous zinc ions should not affect the ability of p53 to bind to DNA in the presence of DTT.

To examine the relative contributions of zinc binding and protein reduction to the ability of p53 to bind to DNA, p53 protein was purified in the presence of Chelex-treated solutions and in the presence of non-Chelex-treated solutions. Wild-type p53 isolated in non-Chelex-treated solutions containing trace amounts of zinc retained the ability to bind to DNA, whereas p53 isolated in Chelex-treated solutions no longer bound to target DNA (Fig. 5). Apparently, trace amounts of zinc or other metals in the non-Chelex-treated solutions help maintain the DNA-binding ability of p53 during the purification process. Addition of $ZnCl_2$ alone at a final

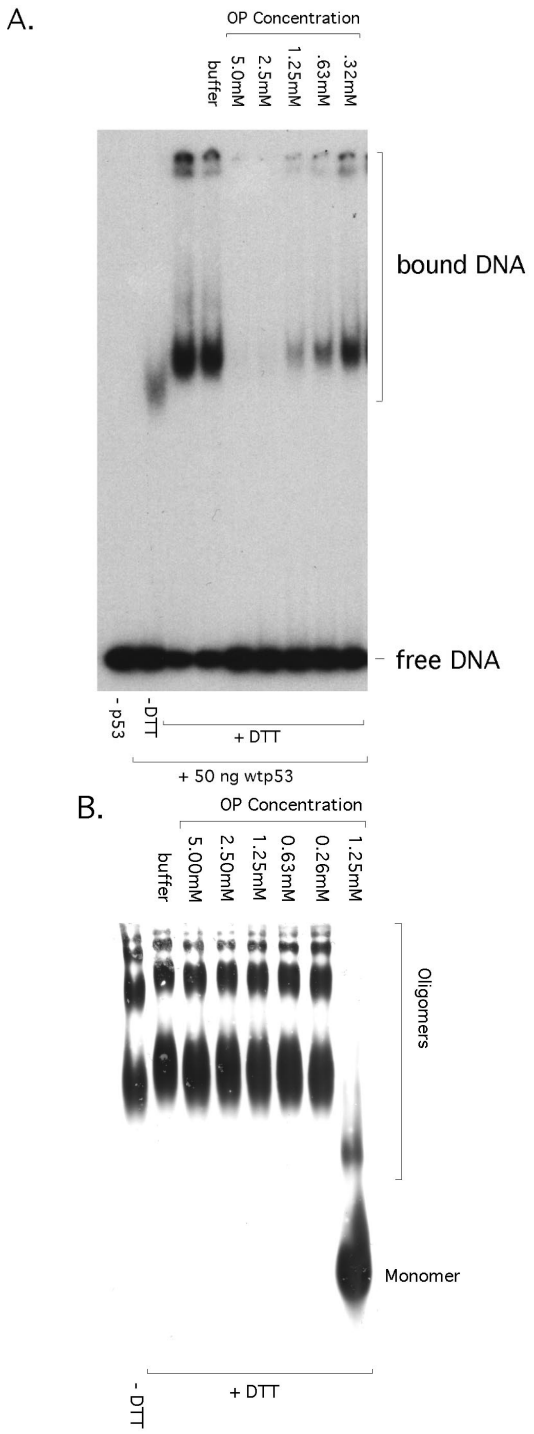

FIG. 4. Effect of OP on DNA binding by wild-type p53 protein. (A) Wild-type p53 protein (wtp53; 50 ng) was incubated with 5 mM DTT followed by decreasing amounts of OP (final concentrations ranging from 5.0 to 0.32 mM) prior to incubation with 2 ng of ^{32}P-labeled consensus DNA and 20 ng of unlabeled Bluescript DNA. The p53-DNA complexes were separated from unbound DNA by gel electrophoresis. (B) Wild-type p53 protein was incubated with decreasing amounts of OP (ranging from 5.0 to 0.26 mM), cross-linked with 0.1% glutaraldehyde, and analyzed by SDS-polyacrylamide gel electrophoresis. p53, not cross-linked with glutaraldehyde, was also run on the gel to indicate the position of the monomer (last lane on right). The p53 proteins were blotted onto nitrocellulose and immunodetected with monoclonal antibodies PAb 242, PAb 248, and PAb 421.

concentration of 0.125 mM, however, did not stimulate DNA binding of p53, whether purified in the Chelex-treated or non-Chelex-treated solutions (Fig. 5). In contrast, DTT changed the electrophoretic migration of the tetramer-DNA complexes and markedly stimulated DNA binding by p53 (Fig. 5) both in the presence and in the absence of exogenous zinc. These findings argue that zinc remains tightly bound to p53 throughout purification but is not sufficient for optimal DNA binding; both bound zinc and p53 reduction contribute to optimal DNA binding. DTT, therefore, enhances the binding of p53 to DNA by mechanisms independent of the interaction of p53 with exogenous zinc via free sulfhydryl groups. Reduction of cysteines not involved directly in zinc binding appears to be required for sequence-specific DNA binding.

Cys-173, Cys-235, and Cys-239 in p53 are required for suppression of transformation by *E1A* and *ras*. We noted an amino acid sequence in p53 ($CX_4NRRX_2LTX_2TLX_6L$ involving Cys-239) that is homologous to the sequence downstream of the critical redox-regulated cysteine residue ($CX_1NRRX_2LTX_1TLX_6L$) in the DNA-binding domain of fos. This sequence is 100% conserved in p53 from different species (51). A computer search of 23,000 sequences with PC-Gene identified only three proteins with this motif: p53, fos, and ATF. In view of this homology, the observed redox modulation of wild-type p53 (Fig. 1 and 5) suggested that Cys-239 might be involved in sequence-specific DNA binding. In order to study the role of Cys-239 and the other cysteine residues in the biological functions of p53, the 12 cysteines in murine p53 were individually changed to serines, since serine is structurally similar to cysteine but has a hydroxyl group in place of a sulfhydryl group. Of the 12 cysteines in murine p53, 9 are highly conserved, while Cys-40, Cys-293, and Cys-308 are not (51). The wild-type and Cys-mutant p53 proteins were compared with respect to their ability to suppress transformation of primary REFs by *E1A* and *ras*, their ability to bind to a consensus target sequence and transactivate the CAT gene in vivo, and their ability to bind to consensus DNA in vitro.

Cotransfection studies of REFs have previously shown that wild-type p53 suppresses transformation, while certain mutant p53 proteins enhance transformation (15). We have tested each of the 12 cysteine-to-serine substitution mutants for the ability to affect transformation of REFs. Control transfections were done with *E1A*, *ras*, and either the KS+ vector, wild-type p53, or p53A135V (which contains an alanine-to-valine mutation at position 135). The p53A135V mutant encodes a prototype transforming p53 protein (24, 37).

The numbers of transformed foci resulting from cotransfection of REFs with each of the cysteine-mutant p53 DNAs in conjunction with *E1A* and *ras* are shown in Table 1. Some of the Cys-mutant p53 proteins (p53C40S, p53C179S, p53C274S, p53C293S, and p53C308S) behave like wild-type p53 and suppress transformation by E1A and ras, while three mutants (p53C173S, p53C235S, and p53C239S) behave like p53A135V and strongly enhance transformation by E1A and ras. In addition, there is another class of cysteine mutants (p53C121S, p53C132S, p53C138S, and p53C272S) in which the mutant p53 proteins behave in an intermediate manner and enhance transformation moderately. In general, the mutants which no longer suppress transformation are those residing in the highly conserved regions II to V of p53 (51), although p53C274S is an exception in that it suppresses transformation as well as does wild-type p53. Differences in the abilities of the mutants to enhance or suppress transformation are not the result of differences in p53 protein level within the transfected cells. Analysis of extracts from REF cells transfected with p53 DNA under the control of the CMV promoter showed similar levels

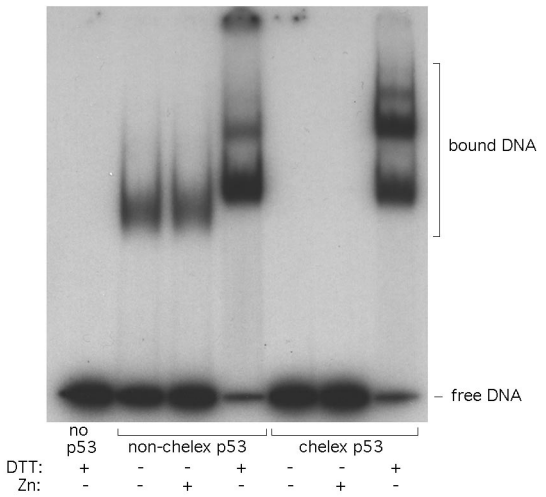

FIG. 5. Requirement of reduced conformation of wild-type p53 protein, but not exogenous zinc, for sequence-specific DNA binding. Wild-type p53 protein was purified in the presence of non-Chelex-treated solutions or Chelex-treated solutions. Purified p53 protein (100 ng) was incubated with 2 ng of ^{32}P-labeled consensus DNA and 20 ng of unlabeled Bluescript carrier DNA for 30 min at room temperature. The presence or absence of 0.125 mM $ZnCl_2$ or 5 mM DTT in the assay is indicated. The p53-DNA complexes were separated from unbound DNA by gel electrophoresis and detected by autoradiography.

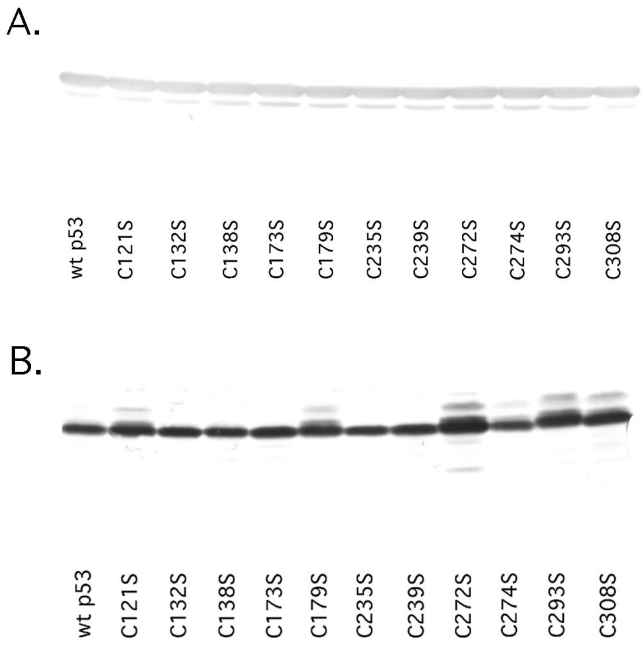

FIG. 6. Immunodetection of p53 protein expressed in REF cells (A) and NCI-H358 cells (B). Cells were transfected with wild-type p53 (wtp53) or Cys-mutant p53 DNA under the control of the CMV promoter. Cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose, and p53 was detected with a mixture of PAb 242, PAb 248, and PAb 421 and with the Immunoselect system (Gibco/BRL).

of p53 protein in cells transfected with wild-type p53 DNA or any of the cysteine mutant p53 DNAs (Fig. 6A).

Cys-173, Cys-235, and Cys-239 are essential for transactivation by p53. To examine the effect of the Cys→Ser substitutions on the binding of p53 to target DNA in vivo, we analyzed the cysteine-mutant p53s for transcriptional activation of PG_{13}-CAT (31), a construct containing 13 copies of a p53 consensus DNA-binding sequence linked to the CAT gene. Kern et al. (31) have shown in cotransfection studies that wild-type p53 protein activates the expression of CAT and that the extent of activation depends on the number of p53 DNA-binding sites. In contrast, most oncogenic p53 mutant proteins are unable to transactivate CAT (31), presumably because of an inability to bind to p53 DNA-binding sites (30).

TABLE 1. Results of cotransfection of REFs with wild-type or Cys-mutant p53 DNA plus *E1A* and *ras*

Plasmid DNA transfected	Mean ratio ± SD[a]
E1A+*ras*+p53wt[b]	0.28 ± 0.13
E1A+*ras*+p53A135V	2.55 ± 0.18
E1A+*ras*+p53C40S	0.33 ± 0.05
E1A+*ras*+p53C121S	1.47 ± 0.49
E1A+*ras*+p53C132S	1.73 ± 0.61
E1A+*ras*+p53C138S	1.70 ± 0.70
E1A+*ras*+p53C173S	2.37 ± 0.15
E1A+*ras*+p53C179S	0.59 ± 0.33
E1A+*ras*+p53C235S	3.00 ± 0.27
E1A+*ras*+p53C239S	2.89 ± 0.57
E1A+*ras*+p53C272S	1.35 ± 0.13
E1A+*ras*+p53C274S	0.36 ± 0.21
E1A+*ras*+p53C293S	0.76 ± 0.46
E1A+*ras*+p53C308S	0.60 ± 0.33

[a] The mean ratios are the average numbers of foci per plate of REF cells transfected with *E1A*, *ras*, and p53 DNA relative to the average numbers of foci per plate of REF cells transfected with *E1A*, *ras*, and KS+ DNA, with two plates per transfection. The mean ratios and standard deviations were obtained by averaging data from three different experiments, each one of which included the complete set of p53wt, p53A135V, and Cys-mutant p53 DNAs.

[b] p53wt, wild-type p53.

HCT-116 cells expressing low levels of endogenous p53 were cotransfected with PG_{13}-CAT and a vector containing either wild-type or mutant p53 DNA. The results are represented in a bar graph showing the percentage of conversion of [^{14}C]chloramphenicol to [^{14}C]mono-acetylchloramphenicol for each of the 12 cysteine-mutant p53s, as well as for the KS+ vector, wild-type p53, and p53A135V (Fig. 7A). It should be noted that in the presence of vector alone there was a significant basal level of CAT activity, presumably due to transactivation of PG_{13}-CAT by endogenous p53 protein.

The results show that those p53 Cys-mutant proteins (p53C40S, p53C179S, p53C274S, p53C293S, and p53C308S) which suppress transformation transactivated CAT as well as did wild-type p53. In contrast, the three cysteine-mutant p53s (p53C173S, p53C235S, and p53C239S) which markedly enhance transformation did not transactivate CAT above the level seen with vector alone, presumably because the mutant proteins do not bind to the consensus sites. In fact, the highly transforming Cys mutants consistently decreased the CAT activity to a level below that produced by vector alone (Fig. 7A), which is likely due to a dominant negative effect of the transforming p53 protein on the endogenous wild-type protein. In general, those Cys mutants (p53C121S, p53C132S, p53C138S, and p53C272S) that act in an intermediate fashion with respect to the suppression of transformation had an intermediate effect on the transactivation of CAT. The data suggest that transformation is enhanced by an inability of p53 to bind to and activate specific target regulatory sequences required for suppression of transformation.

The transactivation assays were repeated, using NCI-H358 cells which have no endogenous p53 function. It can be seen that in the NCI-H358 cells there was no baseline CAT activity

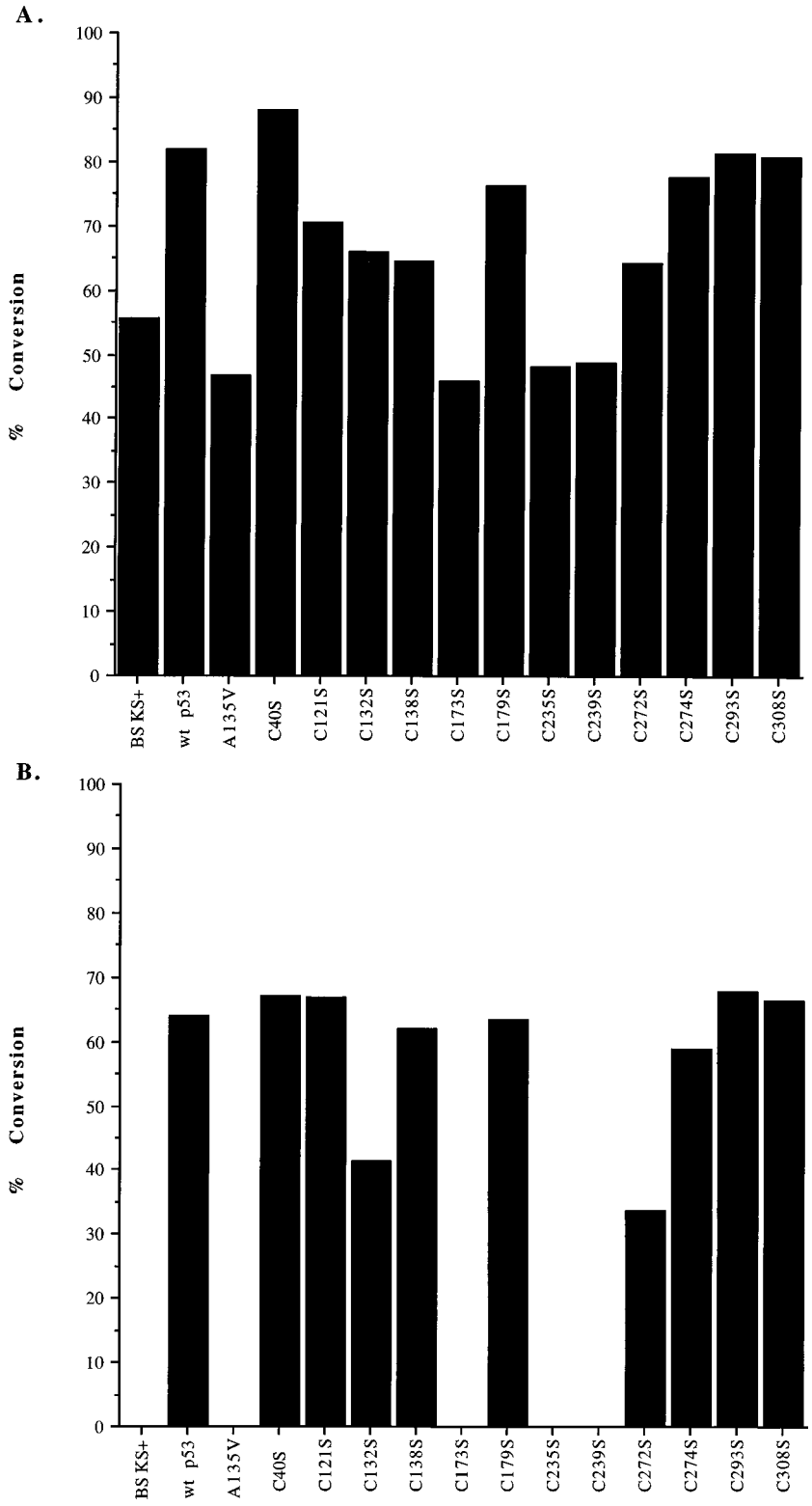

FIG. 7. CAT transactivation assay of cysteine-mutant p53 proteins expressed in HCT-116 cells (A) and NCI-H358 cells (B) cotransfected with PG_{13}-CAT and wild-type p53 (wt p53) or Cys-mutant p53 DNA. In each case, results from two independent experiments were similar and were averaged. The bar graphs show the percentages of conversion of [^{14}C]chloramphenicol to [^{14}C]mono-acetylchloramphenicol for the KS+ vector alone, wild-type p53, p53A135V, and each of the cysteine-mutant p53 proteins. The products were separated by chromatography on silica thin-layer chromatography plates, visualized by autoradiography, scanned, and quantitated by using NIH Image version 1.43.

detected when vector alone was used (Fig. 7B). The complete lack of CAT activity in the presence of the highly transforming Cys-mutant p53s (p53C173S, p53C235S, and p53C239S) is particularly evident (Fig. 7B). Analysis of extracts from NCI-H358 cells transfected with p53 DNA under the control of the CMV promoter showed similar levels of p53 protein in cells transfected with wild-type p53 DNA and in cells transfected with the Cys-mutant p53 DNAs (Fig. 6B).

Overall, similar results were obtained with both cell types, although the transactivation data from the NCI-H358 cells do not correlate as well with the transformation data (Table 1) as do the transactivation data from the HCT-116 cells. With respect to the highly transforming and suppressing p53 mutants, the amount of CAT activity in the NCI-H358 cells is highly correlated with the transformation suppression ability of the mutant p53 protein (Fig. 7B; Table 1). With respect to the intermediate p53 mutants, however, p53C121S and p53C138S transactivate CAT at a level similar to that of wild-type p53, while p53C132S and p53C272S transactivate CAT at a reduced level. These differences may be due either to endogenous wild-type p53 being present in HCT-116 cells (or REF cells) but absent in NCI-H358 cells, to different levels of other endogenous proteins that interact with or modify the mutant p53 proteins, or to different levels of p53 expression by the weak murine sarcoma virus and the strong CMV promoters.

Cys-173, Cys-235, and Cys-239 are involved in in vitro DNA binding by p53. To determine if the differential regulation of transcription in vivo by wild-type and Cys-mutant p53 proteins was the direct result of differential binding to DNA, we examined the in vitro binding of purified wild-type p53 protein and of each of the Cys-mutant p53 proteins to a consensus DNA sequence. The target DNA used in the mobility shift assays was a ^{32}P-end-labeled 55-bp fragment with the 5′-GGACATGC CCCGGGCATGTCC-3′ consensus sequence (18) in its center.

When assayed for in vitro DNA binding, the three highly transforming cysteine-mutant p53 proteins (p53C173S, p53C 235S, and p53C239S) exhibited a marked reduction in binding relative to that of wild-type p53 protein, both at room temperature and at 37°C (Fig. 8A), even in the presence of DTT. Although the binding is weak, a shift in migration of all three protein-DNA complexes after DTT treatment (Fig. 8) argues that the altered electrophoretic migration of p53 following reduction depends on more than one cysteine residue. The weak binding of the p53C173S, p53C235S, and p53C239S mutant proteins to DNA in vitro contrasts with their complete inability to transactivate PG_{13}-CAT in vivo and suggests that other factors, such as the extent of reduction of p53 or the interaction of p53 with coregulatory proteins, may play a role intracellularly. The other nine Cys-mutant p53s bind target DNA almost as well as does wild-type p53 at room temperature (Fig. 8B).

The ability of p53C274S to bind DNA, to suppress transformation, and to transactivate surprised us. X-ray crystallography has shown that the equivalent cysteine (Cys-277) in human p53 is located in the major groove of bound DNA; this cysteine is postulated to form a hydrogen bond with thymine or cytosine (6). Upon resequencing the p53C274S DNA used in the transformation and transactivation studies, we found that 10 of 10 independently isolated plasmid DNAs contained the Cys-to-Ser substitution at this site. In fact, it seems reasonable to assume that the hydroxyl group on the serine residue would be just as likely as the sulfhydryl group on the cysteine residue to form a hydrogen bond with thymine or cytosine in the consensus sequence.

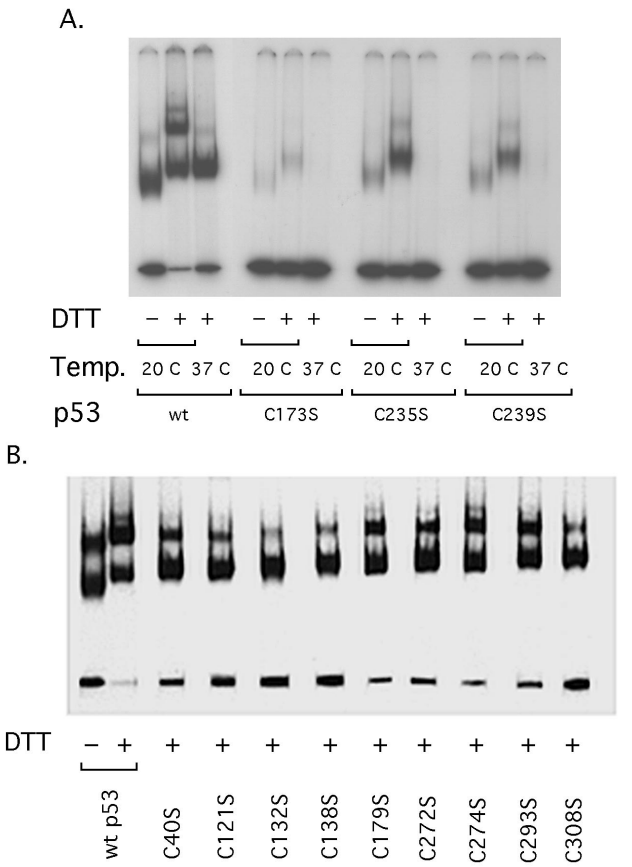

FIG. 8. In vitro sequence-specific DNA binding by wild-type p53 protein and the cysteine-mutant p53 proteins. The wild-type and mutant p53 proteins (200 ng each) were purified from recombinant baculovirus-infected Sf9 cells and incubated with 2 ng of ^{32}P-labeled consensus DNA and 20 ng of unlabeled Bluescript DNA for 30 min at room temperature (B) or at room temperature and 37°C (A) with or without prior DTT treatment. The p53-DNA complexes were separated from unbound DNA by gel electrophoresis and detected by autoradiography. (A) Comparison of the three transforming Cys-mutant p53 proteins with wild-type p53 protein (wt). (B) Comparison of the other Cys-mutant p53 proteins with wild-type p53 protein (wt p53).

Wild-type p53 and the Cys-mutant p53 proteins form tetramers and higher-order oligomeric structures. Both wild-type p53 protein and transforming mutant p53A135V protein purified from the baculovirus expression system exist as tetramers and multiples of tetramers (52). The formation of heterooligomers containing both mutant and wild-type p53s is thought to be one means by which transforming p53 inhibits the function of endogenous wild-type p53 and exerts a dominant negative effect (33). Assuming that this hypothesis is correct, those cysteine-mutant p53s which are highly transforming should retain the ability to form higher-order oligomeric structures.

To determine whether any of the Cys-mutant p53 proteins were compromised in their ability to oligomerize, the quaternary structure of each of the Cys-mutant p53 proteins was compared with that of wild-type p53 protein. The proteins were cross-linked with 0.1% glutaraldehyde to preserve oligomeric structure. All of the Cys-mutant p53 proteins retain the ability to form tetramers and higher-order oligomeric structures (octamers and dodecamers), similar to the case for wild-type p53 (Fig. 9). This finding suggests that the quaternary structure of p53 is not easily disrupted by these point mutations. The effect of the Cys→Ser mutations on either transac-

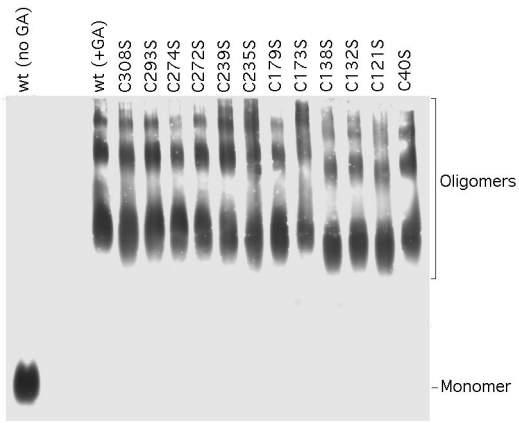

FIG. 9. Analysis of the oligomeric structure of wild-type p53 protein and the cysteine-mutant p53 proteins. The wild-type (wt) and mutant p53 proteins (550 ng each) were cross-linked with 0.1% glutaraldehyde (GA) at 37°C for 30 min, and the oligomers were then separated from one another by electrophoresis in a denaturing polyacrylamide gel in phosphate buffer. The gel was blotted onto nitrocellulose, and the p53 oligomers were immunodetected with a mixture of monoclonal antibodies, PAb 242, PAb 248, and PAb 421 followed by biotinylated secondary antibody and streptavidin-alkaline phosphatase.

tivation or suppression of transformation is not, therefore, correlated with the ability of the mutant p53 proteins to oligomerize.

DISCUSSION

We have investigated the roles of individual cysteine residues in the suppression of transformation by p53. Other investigators have used biochemical approaches to implicate redox regulation (21, 28) and zinc coordination (20) in the site-specific DNA binding function of wild-type p53. Recently, crystallographic studies have identified, in the core DNA-binding domain of p53, three cysteines and one histidine that interact with a single zinc molecule (6). Cho et al. (6) have proposed that these interactions are crucial for establishing the correct DNA-binding conformation of p53. In the present study, we show that redox regulation and zinc coordination of p53 are related but distinct processes. We have substituted serines for each cysteine in p53 in order to study the contribution of individual residues to the suppression of transformation by p53 and to molecular activities thought to be crucial for suppression.

Relationship of redox regulation and metal interaction. Reduction of p53 appears to play a role in sequence-specific DNA binding that is distinct from its role in zinc binding (Fig. 5). Presumably, intracellular mechanisms for the reduction of p53 generate free sulfhydryl groups for the coordination of zinc ions in cells. Since we made no effort to remove zinc already bound to p53 before extraction and purification, some of these ions would remain associated with p53 during purification and would contribute to the correct p53 conformation for site-specific DNA binding. The fact that exogenous trace metals enhance the DNA binding function of p53 (Fig. 5) suggests that metal interactions may protect p53 from the effects of oxidation. We have found that DTT enhances the DNA binding activity of p53 even in the absence of exogenous trace metals. Redox regulation and metal binding are, therefore, related but distinct processes.

Redox regulation of p53. p53 appears to belong to a growing number of transcriptional regulatory proteins subject to redox modulation. Our data suggest that reduction of p53 produces an active DNA-binding conformation and that oxidation of p53 produces a non-DNA-binding conformation of the protein (Fig. 1). The activity of transcriptional regulatory proteins, such as NF-κB and AP-1, can be affected by treatment of intact cells with thiol-oxidizing or -reducing agents (9, 36, 47). Alterations in the redox state of the cells may modulate the transactivation function of wild-type p53, but evidence of such an intracellular effect has not yet been obtained. Whether changes occur in the redox state of the cell during the cell cycle under normal circumstances is not known. If, however, reducing conditions are cell cycle related, then wild-type p53 might be stimulated to bind regulatory DNA and induce expression of relevant target genes at the appropriate stage of the cell cycle.

The intracellular redox state of wild-type p53 might also be regulated by its intracellular localization. In serum-stimulated BALB/c 3T3 cells, p53 accumulates in the cytoplasm during the G_1 phase but then moves into the nucleus at the beginning of S phase, where it remains for several hours (49). In addition, p53 rapidly accumulates in the nuclei of cells in which DNA damage has occurred (29). The concentrations of the redox regulators glutathione and Ref-1 are greater in the nucleus than in the cytoplasm (5, 58). Entry of p53 into the nucleus, therefore, may enhance its reduction.

The molecular mechanism by which reduction of p53 stimulates site-specific DNA binding is not known. Considerable evidence indicates that p53 has active and inactive DNA-binding conformations (21, 26, 27, 44). p53 reduction may be one requirement for the active conformation. We have made the striking observation that DTT visibly affects the electrophoretic properties of native p53 tetramers bound to DNA (Fig. 4A) and also changes the electrophoretic migration of cross-linked p53 oligomeric forms in SDS gels (Fig. 4B). The altered gel mobility probably reflects changes in p53 conformation. The crystallographic studies of Cho et al. (6) were done under anaerobic reducing conditions which would maintain the active DNA-binding conformation of p53. Because the effects of oxidation can be rapidly reversed by DTT, it would be interesting to know the structure of oxidized p53. It is unclear which cysteines in p53 are required for the altered electrophoretic properties of p53. We have noted a sequence in p53 that is homologous to a sequence downstream of a critical cysteine residue in the DNA-binding domain of fos. Since DNA binding by fos is dependent on reduction of this cysteine (1), we wondered if the corresponding cysteine in p53, Cys-239, affected its structure and function. We substituted serine for this cysteine and for all other cysteines in murine p53 to determine which residues are important for redox regulation. No single cysteine-to-serine substitution, however, significantly affected the ability of DTT to change the electrophoretic mobility of p53 or to enhance DNA binding at room temperature (Fig. 8 and data not shown). We conclude that more than one Cys-to-Ser substitution is probably required to change this property of p53.

Zinc coordination of p53. Treatment of wild-type p53 with OP, a chelator of zinc ions, abolishes DNA binding (21) (Fig. 4). This finding argues that zinc binding is essential for the binding of p53 to target DNA. Crystallography studies with the central DNA-binding domain of human p53 (6) show that each monomer of human p53 does in fact coordinate one zinc ion through amino acids Cys-176, His-179, Cys-238, and Cys-242.

We show here that the corresponding cysteine residues (Cys-173, Cys-235, and Cys-239) in murine p53 are essential for the suppression of transformation by E1A and ras. Alteration of these residues actually leads to enhancement of transformation (Table 1). The same residues are required for the transactivation of a p53-responsive model promoter (Fig. 7). The natural

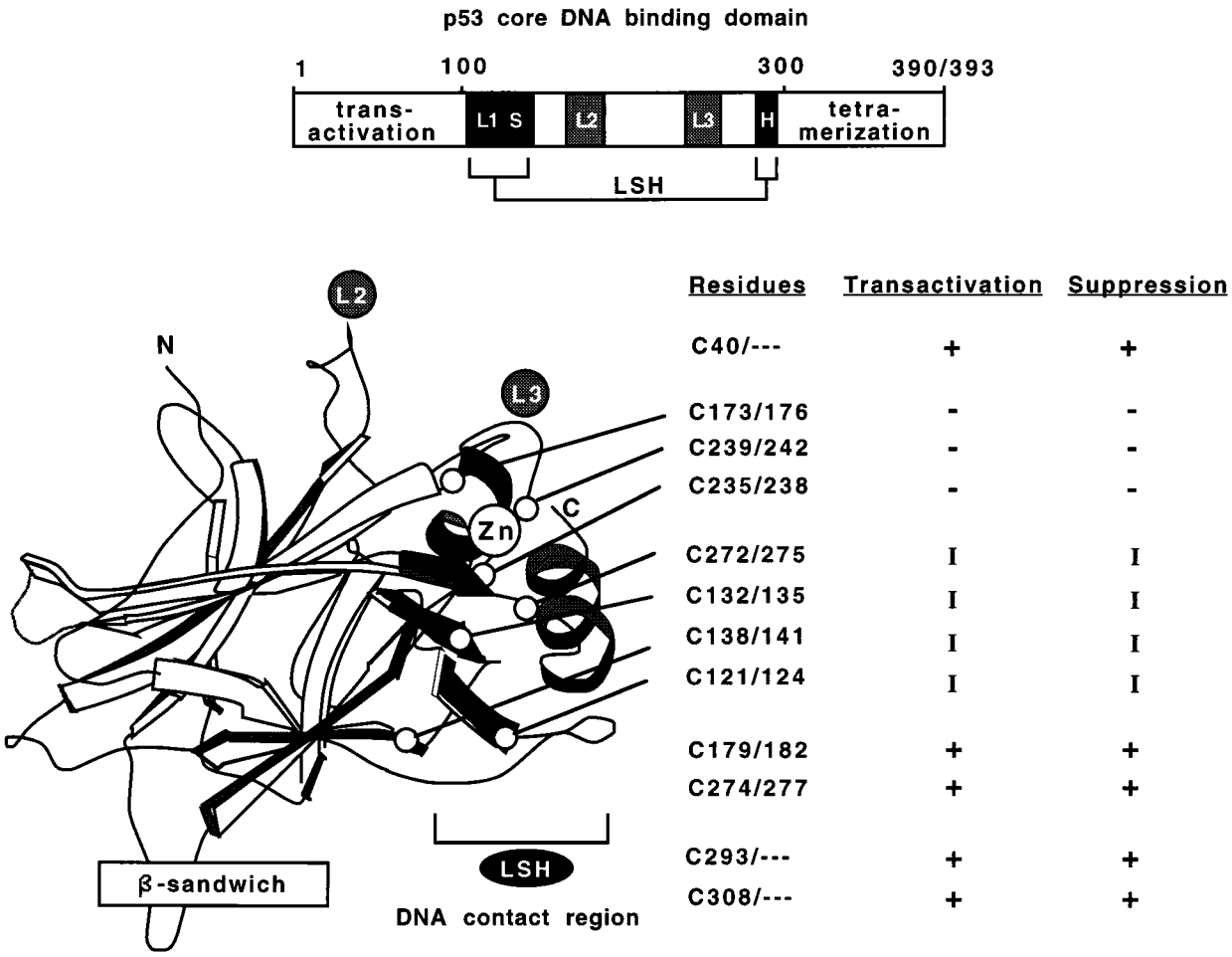

FIG. 10. Comparison of locations and functions of cysteines in p53. The cysteines important to the transactivation and suppression functions of murine p53 are conserved in human p53, whose crystal structure has been solved. The primary structure of p53 is shown at the top. The tertiary structure of the site-specific DNA-binding domain of p53 is shown below. L1, L2, and L3 indicate loops, and LSH indicates a loop-sheet-helix structure demonstrated by Cho et al. (6). Positions of the cysteine residues in murine/human p53s are indicated by open circles, and the transactivation and suppression activities of p53s with serine substitutions at each cysteine position are summarized. +, function similar to that of wild-type p53; −, nearly complete loss of function; I, intermediate loss of function.

promoters activated by wild-type p53, but not by the three cysteine-mutant p53 proteins, in REF cells have not been investigated. The gene known as *WAF1* (11) or *CIP1* (23) is one likely target, since it plays a role in the progression of cells through the cell cycle (10) and since it has a regulatory sequence recognized by wild-type p53 but not by transforming mutant p53 (11, 23). The 21-kDa protein encoded by the *WAF1/CIP1* gene binds to and inhibits the cyclin-dependent protein kinases (23) and thereby prevents phosphorylation events presumably required for cell cycle progression. In vitro DNA-binding studies (Fig. 8A) confirm that the mutant p53C173S, p53C235S, and p53C239S proteins have lost the ability to bind effectively to DNA containing the consensus sequence described by Funk et al. (18).

Zinc binding by p53, therefore, is essential for the normal functioning of the wild-type protein. One purpose of the zinc ion bound by each p53 monomer may be to prevent oxidation, under nonreducing conditions, of the three key cysteine residues located in the central domain of p53 and thereby to stabilize the DNA-binding conformation of the p53 protein. Under reducing conditions, however, chelation of zinc by OP does not shift the tetramer-DNA complex to the high-mobility electrophoretic form associated with oxidation (Fig. 4A). Hainaut and Milner (20) have shown that the chelation of zinc by OP shifts the conformation of the wild-type p53 protein to a mutant conformation characteristic of transforming p53 proteins which do not bind well to target DNA (31). Our results show that the chelation of zinc by OP does not affect the ability of wild-type p53 to form tetramers or higher-order oligomeric structures (Fig. 4B). This finding, in conjunction with our finding that alteration of single cysteine residues does not significantly affect p53 oligomerization (Fig. 9), suggests that failure of the mutant p53s to bind to consensus DNA is not related to tetramer assembly.

Structure and function relationships in p53. Comparison of our functional results with the crystallographic structure of p53 (6) reveals two clusters of cysteines in which serine substitutions give rise to distinctive phenotypes (Fig. 10). Of the 12 cysteines of murine p53, 9 are in the core DNA-binding domain. All of these cysteines, in contrast to those in the N and C termini, are conserved in murine and human p53s. One striking feature of these cysteines is that they are all located in one area of p53 between the β-sandwich and the specific DNA-binding loops and helix. Murine cysteines at positions 173, 235, and 239 in one cluster directly interact with a zinc ion and thereby organize the structure of the L2 and L3 loops on the

DNA-binding surface. Serine substitutions for any of these cysteines severely compromise the transactivation and suppression functions of p53 (Table 1; Fig. 7). This phenotype is consistent with the nearly complete loss of DNA binding by p53 after zinc chelation by OP. A second cluster of cysteines at positions 121, 132, 138, and 272 is adjacent to the zinc-binding region and is located in or near the loop-sheet-helix region that is involved in DNA contacts. These cysteines are in a position to modulate the presentation of the loop-sheet-helix region to the p53 DNA recognition site. Mutation of any of the cysteines in this cluster reduces but does not eliminate the transactivation and suppression functions of p53. This phenotype could reflect a partial loss of DNA-binding potential such as that associated with protein oxidation in the presence of zinc. Because cysteines at positions 121, 132, 138, and 272 have a common location and phenotype, we propose that they may cooperate to modulate function of the DNA-binding domain. We propose that this cluster of cysteines may account for the redox regulation of p53 that is apparently not associated with zinc interaction. The presence of two distinct phenotypes among Cys-mutant p53s suggests that p53 is subject to more than one level of redox control at or near the p53-DNA interface.

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