[CANCER RESEARCH 53, 4469–4473, October 1, 1993]

*Advances in Brief*

# Redox Modulation of p53 Conformation and Sequence-specific DNA Binding *in Vitro*[1]

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## Abstract

**The p53 protein is a transcription factor, the function of which is abrogated by oncogenic mutations which affect a flexible domain in the central portion of p53, altering its reactivity with conformation-specific antibodies. Here we show that both conformation and sequence-specific DNA binding of p53 translated *in vitro* can be modulated by metal chelators and oxidizing agents. Oxidation disrupted wild-type p53 conformation and inhibited DNA binding. Conversely, reduction favored folding of p53 into the wild-type form and restored DNA binding. Redox regulation of p53 protein conformation could represent an important mechanism for the control of p53 function.**

## Introduction

The tumor suppressor protein p53 plays a role in the control of important processes such as cell proliferation, differentiation, and cell survival (see review in Ref. 1). Sequence-specific DNA binding is an essential biochemical property of wild-type p53 and the ability to transactivate gene expression from a specific promoter sequence is lost in p53 mutants associated with cell transformation and oncogenesis (2). The p53 protein is also capable of forming specific protein-protein complexes and, as in the case of the products of the *MDM-2* and *WT1* genes, such interactions can in turn regulate the ability of p53 to *trans*-activate genes adjacent to p53 binding sites (3, 4).

Overexpression of the wild-type form of p53 arrests cell cycle progression in $G_1$ and suppresses cell proliferation (see review in Ref. 1). Studies using conformation-specific monoclonal antibodies indicate that single point mutations in p53 can affect the conformation of the entire polypeptide. The suppressor form of wild-type p53 is characteristically reactive with PAb246 (murine specific) and PAb1620, but not with PAb240, whereas the reverse is true for many mutants of p53 associated with cancer (5, 6). PAb246 and PAb1620 recognize topologically related epitopes which are destroyed by protein denaturation. In contrast, PAb240 recognizes a conserved amino acid motif (RHSVV, residues 213–217 in human p53) which is cryptic in wild-type p53 but commonly exposed in many oncogenic mutants (7). The function of p53 appears crucially dependent upon a conformationally flexible domain encompassing about 150 residues in the central portion of the molecule and in which mutations found in human tumors predominate (see review in Refs. 1 and 8).

We have recently presented evidence that cysteine residues located in conserved regions 2, 3, 4, and 5 of p53 could be involved in binding metal ions, thus stabilizing the wild-type conformation of the polypeptide (9). A hypothetical model of wild-type p53 incorporating these putative metal-binding domains proposes the existence of two "metal loops" flanking the cryptic epitope recognized by PAb240. This model provides a structural basis to explain how mutations scattered over more than 150 residues may have a common effect on the tertiary structure of p53 and suggests that factors affecting the metal-dependent folding of wild-type p53 could also regulate its biochemical activity.

In this report, we have investigated whether agents that affect the conformation of the p53 polypeptide could also affect its capacity to bind to a specific DNA sequence *in vitro*. Using p53 translated *in vitro*, we show that agents that reversibly perturb the wild-type phenotype of p53 also modulate its DNA-binding capacity. These agents include metal chelators and oxidizing agents. These studies indicate that redox changes could influence the biological activity of p53 by modulating DNA binding through effects on protein conformation.

## Materials and Methods

**Transcription and Translation of p53.** RNA for translation of wild-type murine p53 was produced by *in vitro* transcription of the plasmid pSP6p53$^{Ala135}$, linearized with *Hind*III (10). The plasmid pSP6p53$^{Val135}$, linearized by *Hind*III, was used to generate RNA for translation of p53$^{Val135}$. Rabbit reticulocyte lysates were from Promega. Translations were carried out for 1 h at 37°C, in the presence of 0.75 μM added [$^{35}$S]methionine (40.5 TBq/mmol; Amersham). After 1 h, translations were stopped by addition of anisomycin (2 μg/μl).

**Exposure to Defined Reagents and Immunoprecipitations.** OP[3] and diamide (Sigma) were kept, respectively, as 10 and 25 mM stock solutions in 10 mM Tris-HCl, pH 7.6. Unless otherwise stated, aliquots of translated lysate were incubated for 20 min at 37°C in the presence of either OP, diamide, DTT, or metal ions dissolved in 10 mM Tris-HCl, pH 7.6. After incubation, lysates were chilled on ice and p53 was incubated with DNA and also analyzed by immunoprecipitation using monoclonal antibodies PAb240, PAb1620, PAb246, PAb248, PAb421, and PAb416 as described previously (6) .

**DNA-binding Assay.** Aliquots of translated lysate (4 μl) were diluted in 100 μl of DNA-binding buffer (20 mM Tris-HCl, pH 7.5-100 mM NaCl-0.1% Nonidet P-40-10% glycerol), containing 5 ng of end-labeled double stranded oligonucleotide 5′-GGGCATGTCCGGGCATGTCC-3′, 1 μg of salmon sperm DNA as a nonspecific competitor, DTT (5 mM), and PAb421 (30 μl of hybridoma supernatant). Although binding was detectable in the absence of added antibodies,[4] we found that addition of PAb421 both supershifted and stabilized p53-DNA complexes, as reported by others (11, 12). Samples were incubated for 30 min at 37°C (30°C for p53$^{Val135}$). Reaction products were analyzed by electrophoresis at 120 V onto 4% polyacrylamide gels in Tris-borate (100 mM) containing 1 mM EDTA, under cooling by water circulation (8–10°C).

## Results and Discussion

The growth suppressor function of wild-type p53 *in vivo* is believed to require sequence-specific binding to DNA. Accordingly, several groups have observed that wild-type, but not mutant p53 binds *in vitro* to consensus sequences matching the consensus 5′-(Pu)$_3$C(A/T)(A/

Received 7/12/93; accepted 8/17/93.

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[1] This work was supported by a Yorkshire Cancer Research Campaign program grant to J. M.

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[3] The abbreviations used are: OP, 1,10-phenanthroline; DTT, 1,4-dithio-L-threitol; diamide, diazene dicarboxylic acid bis(*N,N*-dimethylamide).

[4] Unpublished results.

T)G(Py)$_3$-3′ (2, 11–13).[5] Since DNA binding activity appears to correlate with wild-type p53 genotype, we reasoned that agents that disrupt the specific tertiary folding of wild-type p53 would also abrogate its DNA-binding activity. Exposure to the metal chelator OP induces wild-type p53 to adopt a phenotype indistinguishable from that of an oncogenic mutant of p53 [reactive with PAb240, nonreactive with PAb246 and PAb1620, and forming complexes with lysate $M_r$ 70,000 heat shock protein (9)]. Using an electromobility shift assay to investigate specific DNA binding, we now show that exposure of wild-type p53 to OP prior to incubation with DNA also abrogated its ability to complex with DNA (Fig. 1*A*). Inhibition of complex formation was dependent upon the concentration of OP, with 100% inhibition observed at 1.25 mM. At this concentration, the p53 polypeptide adopted an essentially mutant immunological phenotype (Fig. 1*B*). Interestingly, preformed p53-DNA complexes survived exposure to concentrations of OP which are sufficient to disrupt the conformation of uncomplexed wild-type p53 (Fig. 1*A*; compare *Lanes 2–3* to *Lanes 4–5*).

The inhibition of DNA binding by 0.625 mM OP was partially reversed by addition of Zn(II) at concentrations as low as 125 μM, consistent with a requirement for zinc for optimal DNA-binding (Fig. 1*A*; compare *Lane 6* to *Lane 2*). This further substantiates previously published evidence that zinc may play a role in the folding of wild-type p53 (9).

In several classes of transcription factors, zinc stabilizes polypeptidic "fingers" involved in contacting DNA (14). However, p53 does not contain a classical "zinc finger" motif. Zinc also binds to many non-"zinc finger" proteins involved in nucleic acid replication (15). Recently, NF-kB, a transcription factor of the rel family which does not contain a "zinc finger" consensus, was also found to require zinc in order to bind specifically to DNA (16). Our results suggest that p53 is another example of a non-"zinc finger" protein where metal binding is essential for maintaining the protein in a form which is competent for DNA binding.

In the course of our initial studies involving chelating agents, we found that removal of metal ions with concentrations of OP equal or superior to 2.5 mM was associated with oxidation of thiols in p53 (9). This result suggests that cysteine sulfhydryl groups in p53 which are involved in metal liganding may also be affected by redox modifications. To investigate the effect of redox conditions on p53 conformation and DNA-binding activity, wild-type p53 was first translated in reticulocyte lysate, followed by filtration on Sephadex G-25 to remove small redox regulatory factors such as DTT present in the reticulocyte lysate. Wild-type p53 was then exposed to the sulfhydryl reducing agent DTT or to diamide, a specific sulfhydryl oxidizing reagent.

Although wild-type p53 filtered on Sephadex G-25 retained the characteristic wild-type immunological phenotype, addition of DTT increased its reactivity with PAb246 and PAb1620 (Fig. 2), suggesting that reduction of thiols favors the tertiary folding of p53 in the wild-type conformation. In contrast, exposure to diamide at concentrations of 0.5–1 mM induced p53 to lose the wild-type immunological phenotype, as shown by decreased reactivity with PAb246 (Fig. 2) and PAb1620 (data not shown). Moreover, diamide at concentration equal or superior to 2 mM induced cysteine sulhydryl groups in p53 to form both inter- and intramolecular dissulfide bridges (data not shown). Similar effects were observed after oxidation of p53 by $H_2O_2$ alone (2 mM) or by the combined action of $H_2O_2$ (25 μM) and Fe(II) (10 μM),

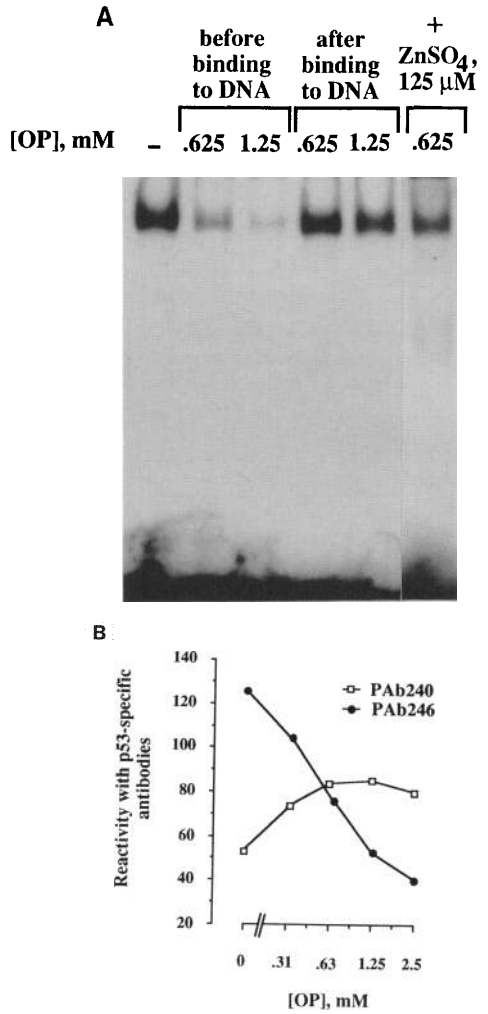


Fig. 1. Effect of the metal chelator OP on conformation and DNA binding activity of wild-type p53. In *A*, wild-type p53 was translated at 37°C, exposed to varying concentrations of OP for 20 min at 37°C, and analyzed for binding to $^{32}$P-labeled double-stranded oligonucleotide 5′-GGGCATGTCCGGGCATGTCC-3′ (before binding to DNA). In parallel, identical aliquots were first incubated with DNA and then exposed to OP for 20 min at 37°C (after binding to DNA). All DNA binding assays were carried out in the presence of PAb421, which supershifts and stabilizes p53-DNA complexes (11, 12). Note that aliquots of lysate were diluted at least 10-fold before incubation with DNA in order to keep the concentration of OP during the binding reaction to less than 0.125 mM, a concentration which did not affect binding activity by itself. In *Lane 6*, wild-type p53 translated at 37°C was exposed to OP in the presence of added $ZnSO_4$, before being assayed for DNA-binding activity. Electromobility shift assays were performed by electrophoresis on 4% polyyacrylamide gel. In *B*, aliquots of p53 used in *A* were immunoprecipitated with anti-p53 monoclonal antibodies as indicated. Immunoprecipitates were quantified by scintillation counting and reactivity with the conformation-specific monoclonal antibodies PAb240 and PAb246 was expressed as a percentage of the amount of p53 immunoprecipitated with PAb421. The amount of p53 immunoprecipitated with PAb421 was unaffected by the concentration of OP.

but not by Fe(II) alone at 100 μM (data not shown). These results indicate that redox conditions influence the conformational folding of wild-type p53.

The ability of wild-type p53 to bind specifically to DNA was also found to depend upon redox conditions. Significantly, wild-type p53 filtered on Sephadex G-25 was unable to complex with DNA in the absence of DTT (Fig. 3*A*), although the protein still expressed an essentially wild-type immunological phenotype (see Fig. 2). That addition of DTT at concentrations of up to 5 mM dramatically restored DNA binding suggests that a reducing environment is essential for the formation of p53-DNA complexes. Conversely, oxidation of the protein with diamide prevented DNA binding even if the binding reaction was performed in the presence of 5 mM DTT (Fig. 3*B*). Preformed

[5] A. R. Hall and J. Milner, unpublished data.

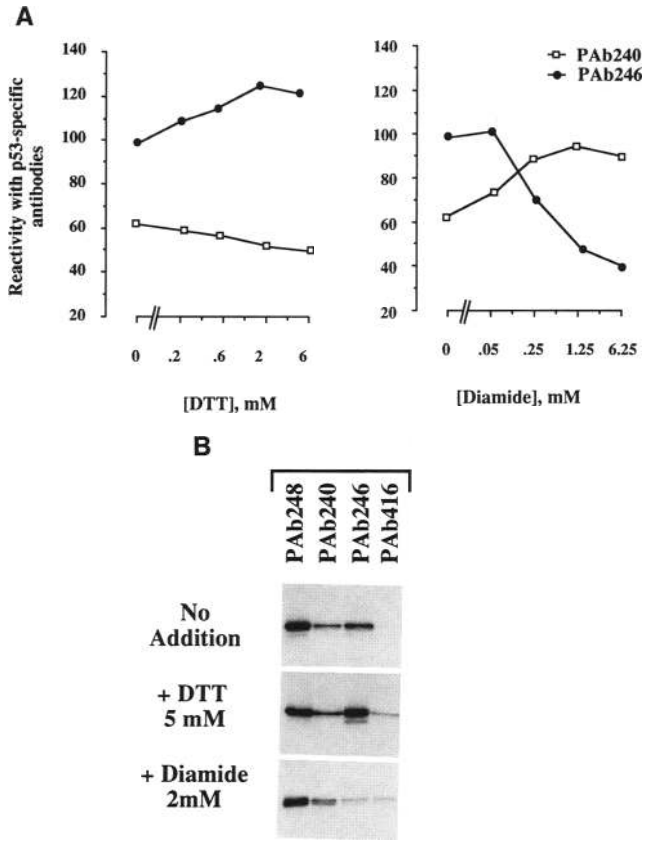


Fig. 2. Effect of DTT and diamide on the reactivity of wild-type p53 with conformation-specific monoclonal antibodies. Wild-type p53 translated *in vitro* in the presence of [$^{35}$S]methionine was filtered on Sephadex G-25 and exposed to varying concentrations of DTT or diamide for 20 min at 37°C, followed by immunoprecipitation with anti-p53 monoclonal antibodies as indicated. In *A*, immunoprecipitates were quantitated by scintillation counting and reactivity with PAb240 and PAb246 was expressed as a percentage of the amount of p53 immunoprecipitated with PAb421, which was unaffected by DTT or diamide. Results similar to those shown for PAb246 were obtained with PAb1620 (not shown). In *B*, immunoprecipitates of p53 filtered on Sephadex G-25 (no addition) or exposed to DTT (5 mM) or to diamide (2 mM) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% gels under reducing conditions. PAb416 was used as a negative control. Note that in addition to full length p53 PAb246 also precipitated a smaller, p53-related product at $M_r$ 42,000 which may represent a truncated version of wild-type p53 (28).

p53-DNA complexes were at least partially resistant to concentrations of diamide which are sufficient to oxidize the uncomplexed protein and abolish its DNA-binding activity (in Fig. 3*B* compare *Lanes 2–3* to *Lanes 4–5*).

The redox requirements in p53 folding and DNA binding were further investigated using a mutant allele of murine p53 ($p53^{Val135}$) which is temperature sensitive for function and conformation. This allele adopts the wild-type phenotype when translated at 30°C and the mutant phenotype at 37°C. Moreover, the conformation of $p53^{Val135}$ can be interconverted between wild-type and mutant forms simply by shifting temperature (17). We have recently found that refolding into the wild-type conformation was facilitated by the addition of ATP and fresh reticulocyte lysate during the temperature shift from 37°C to 30°C (18). In the present study, we found that the requirement for fresh lysate could be replaced by the reducing agent DTT (5 mM, Fig. 4*A*). After refolding in the presence of DTT, $p53^{Val135}$ was again capable of specific binding to DNA (Fig. 4*B*). However, folding from mutant into wild-type phenotype still required ATP hydrolysis, since adenylylimidodiphosphate, a nonhydrolyzable analogue of ATP, inhibited the conversion to wild-type phenotype even in the presence of DTT. Since DTT can overcome the need for addition of fresh lysate, we propose that rabbit reticulocyte lysate may contain redox factors which favor refolding of p53 into the wild-type form. In this context, it is interesting to note that activation of DNA binding by AP1 requires reduction by the cellular redox factor Ref-1 and that the need for Ref-1 can be overcome by DTT (19). Although lysate factors involved in the control of p53 conformation remain to be identified, these results underline the importance of redox conditions in regulating the folding of p53 into the conformation which is capable of binding specifically to DNA.

Overall, the above results indicate that agents which affect p53 conformation also affect DNA binding, thus reinforcing the correlation between p53 "suppressor" conformation and suppressor function via sequence-specific DNA binding. We propose that the redox state of specific cysteine residues in p53 is critical for stabilizing a conformationally flexible domain in p53 in a form permissive for DNA binding. By destabilizing the conformation of this domain, oncogenic mutations would abolish the sequence-specific DNA-binding capacity of p53.

Redox modification is an important posttranslational mechanism regulating the function of many transcription factors, including AP1 (19), NF-kB (20), and Ets (21). In these transcription factors, oxidation of cysteine residues within the DNA binding domain of the molecule inhibits DNA-binding potential. In the case of the bacterial oxidative stress response protein OxyR, it has been proposed that oxidation could convert sulfhydryl groups to a reversible sulfenic acid derivative (22). This hypothesis is based in part upon the observation that under certain conditions metal chelators did not affect the activity of OxyR, thus arguing against the involvement of a metal cofactor (22). With p53, however, metal ions were presumably still bound to

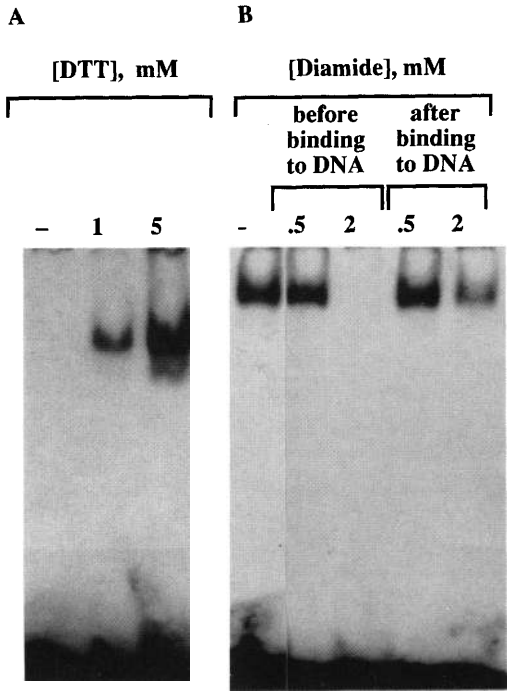


Fig. 3. Effect of redox conditions on the specific DNA binding activity of wild-type p53. In *A*, wild-type p53 was translated at 37°C and filtered on Sephadex G-25. Aliquots were then assayed for DNA binding as described in legend to Fig. 1, in the absence or the presence of varying concentrations of DTT as indicated. In *B*, wild-type p53 was translated at 37°C and aliquots were exposed to varying concentrations of diamide or 20 min at 37°C, before being assayed for DNA-binding activity in the presence of 5 mM DTT (before binding to DNA). In parallel, identical aliquots were first incubated with DNA, then exposed to diamide for 20 min at 37°C (after binding to DNA). Note that *A* and *B* correspond to different gels, thus explaining the apparent difference in the mobility of PAb421-p53-DNA complexes.

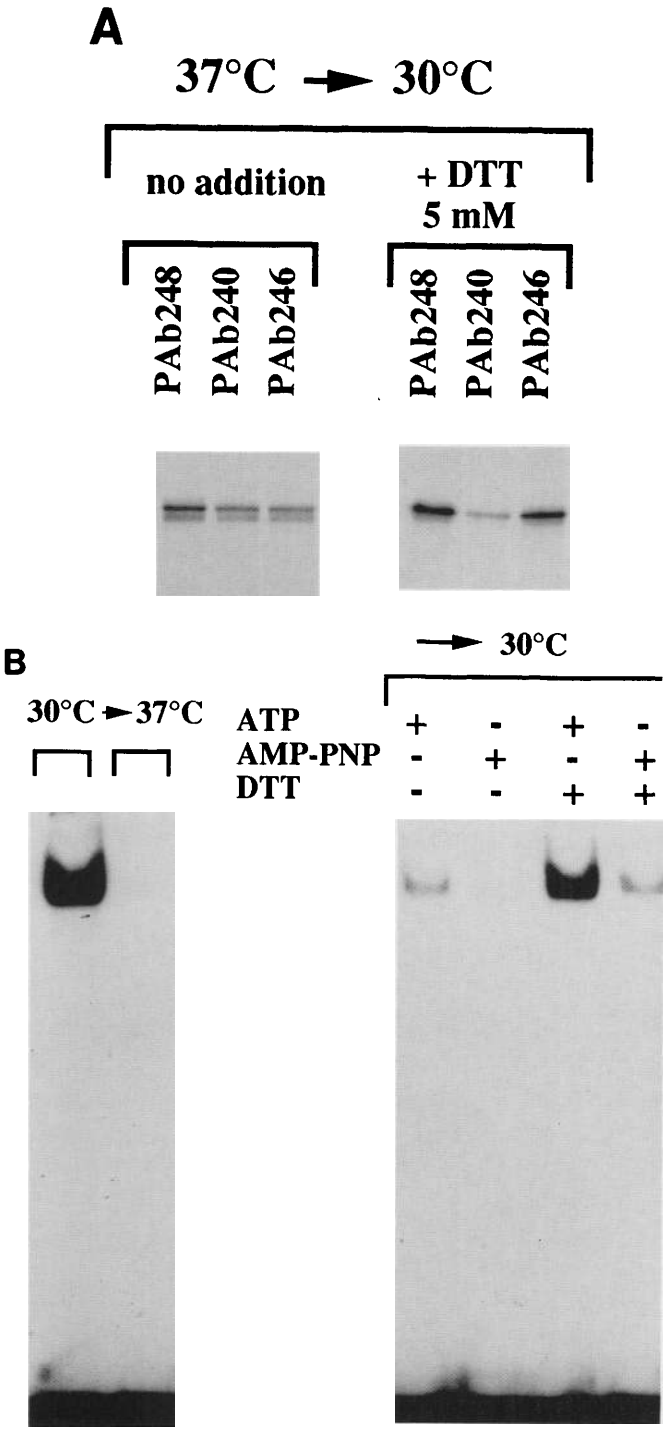


Fig. 4. *A*, effect of DTT on the temperature-dependent folding of $p53^{Val135}$ from mutant to wild-type phenotype and on its DNA binding activity. $p53^{Val135}$ was first translated at 30°C in the presence of [$^{35}$S]methionine and the temperature was shifted to 37°C for 5 min. The lysate was then divided into four aliquots, and each of them was supplemented with ATP (4 mM) or with the nonhydrolyzable analogue adenylylimidodiphosphate (*AMP-PNP*, 4 mM) in the absence or in the presence of 5 mM DTT. Aliquots of lysate were further incubated at 30°C for 30 min. High concentrations of ATP analogue were used to overcome the effects of ATP and ATP-regenerating system present in the lysate (18). *A*, sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis on reducing 15% gels of immunoprecipitates of p53 incubated without (no addition) or with DTT (+DTT 5 mM) in the presence of ATP 4 mM. Under these conditions, p53 resolved as two closely spaced species at $M_r$ 52,000 and 53,000, which may represent distinct charge isoform of full-length p53. *B*, effect of DTT on the specific DNA-binding activity of $p53^{Val135}$. An aliquot of each experimental conditions was assayed for specific DNA binding as described in Fig. 1.

the protein after removal of reducing factors by filtration, since exposure to OP disrupts the conformation of wild-type p53 filtered on Sephadex G-25 (9).

All cells produce oxygen radicals as a consequence of physiological processes and it has been proposed that reactive oxygen species act as second messengers for a variety of agents, including cytokines (23). Moreover, oxygen radicals are also produced in a variety of stress conditions such as acute or chronic inflammation and in response to ionizing radiations (24). Sensitivity of p53 to redox conditions suggests that the function of wild-type p53 as a sequence-specific transcription factor could be inhibited by reactive oxygen intermediates and stimulated by the counteracting cellular reducing response.

Evidence that wild-type p53 is sensitive to redox conditions is particularly interesting since a conformation hypothesis proposes that normal p53 may function both to suppress and promote cell proliferation, a given function depending upon the conformation of the polypeptide. The suppressor form of p53 is equivalent to wild-type phenotype and may be inactivated by growth stimulation which induces a conformational change in the protein (25). The promoter form resembles the mutant phenotype. Redox control of p53 conformation represents an elegant biochemical mechanism to explain how p53 activity could be regulated by physiological processes associated with growth signaling and also with stress-induced responses, such as the response to DNA damage induced by irradiation or by chemotherapeutic agents (26, 27). Further experiments will determine whether p53 conformation and DNA binding activity are also sensitive to redox conditions in intact cells.

## Acknowledgments

We thank S. Butcher for technical assistance and Meg Stark for photographic work.

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