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The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation

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ABSTRACT **p53 accumulates after DNA damage and arrests cellular growth. These findings suggest a possible role for p53 in the cellular response to DNA damage. We have previously shown that the C terminus of p53 binds DNA nonspecifically and assembles stable tetramers. In this study, we have utilized purified segments of human and murine p53s to determine which p53 domains may participate in a DNA damage response pathway. We find that the C-terminal 75 amino acids of human or murine p53 are necessary and sufficient for the DNA annealing and strand-transfer activities of p53. In addition, both full-length wild-type p53 and the C-terminal 75 amino acids display an increased binding affinity for DNA damaged by restriction digestion, DNase I treatment, or ionizing radiation. In contrast, the central site-specific DNA-binding domain together with the tetramerization domain does not have these activities. We propose that interactions of the C terminus of p53 with damaged DNA may play a role in the activation of p53 in response to DNA damage.**

The maintenance of genomic integrity is crucial to cellular survival. Eukaryotic cells delay cell cycle progression after DNA damage, presumably to allow time for repair of DNA lesions (1). p53, a transcriptional activator that accumulates in response to ionizing radiation and other DNA-damaging agents (2, 3), has been implicated in growth arrest in part by induction of the $p21^{Waf1/Cip1/Sdi1}$ gene (4). The p21 protein inhibits activity of cyclin-dependent protein kinases necessary for cell cycle progression (5, 6). Moreover, p53 stimulates the activity of the *GADD45* (*DDTI1*) gene, which is thought to be involved in the response to DNA damage (7–9). Recently, the *GADD45* gene product was found to bind proliferating-cell nuclear antigen *in vivo* and to stimulate DNA excision repair *in vitro* (10). These findings argue that p53 participates in the cellular response to DNA damage through activation of genes involved in cell cycle regulation and DNA repair.

Extensive biochemical characterization of p53 has identified DNA-binding properties unrelated to its recognition of transcription response elements. Several groups have reported that p53 is capable of binding single-stranded DNA ends and of catalyzing DNA renaturation and DNA strand transfer in the absence of ATP (11–14). Although these activities are characteristic of proteins involved in DNA repair and recombination, their significance in p53 function remains unclear. Interestingly, p53 interacts with components of the transcription factor TFIIH transcription complex that participate in nucleotide excision repair (15, 16). These data suggest that, in addition to transactivating genes involved in regulation of cell cycle progression and DNA repair, p53 may interact directly with factors required for repair of DNA lesions.

Studies of human and murine p53 have identified a number of autonomous functional domains. The N-terminal acidic region confers transcriptional activity on the GAL4 DNA-binding domain (17). The large central conserved region of p53 binds DNA specifically (18–21). The C-terminal basic domain assembles stable tetramers (21–23) and binds DNA nonspecifically (14, 20, 21, 24). Together, the N-terminal and central regions of p53 are necessary and sufficient for p53 transactivation and suppression of oncogenic transformation (21, 25). The C-terminal region appears to be dispensable for these activities. Nevertheless, Plummer *et al.* (26) have identified a Li–Fraumeni syndrome family expressing p53 with a deletion of the C-terminal 62 amino acids. Individuals inheriting this mutation have an increased susceptibility to a broad spectrum of cancers. Others have implicated the C terminus of p53 in allosteric regulation of p53 function *in vivo* and *in vitro* (19, 27). Interestingly, the C-terminal region of human p53 catalyzes renaturation of complementary oligonucleotides (14, 28). These data suggest that the C-terminal region has distinct biochemical properties.

In this study, we show that the C-terminal 75 amino acids of human and murine wild-type (wt) p53s are capable of DNA renaturation and DNA strand-transfer activities. Furthermore, we demonstrate that the C terminus of p53 exhibits an increased binding affinity for DNA damaged enzymatically or by ionizing radiation.

MATERIALS AND METHODS

Plasmids. Previously, we described the pIT plasmid for recombination of p53 or segments of p53 with baculovirus (29) and the bacterial expression plasmid pBT (21). Both pIT and pBT have a DNA cassette for insertion of PCR-generated DNA segments of p53, and both encode p53s with a small N-terminal tag containing six histidine residues for purification by metal affinity chromatography. The sequences of wt p53 and p53 segments cloned in these expression vectors were verified by DNA sequencing.

Purification of p53. Murine wt p53, segments of murine p53, and human wt p53 were expressed by infecting insect cells with recombinant baculovirus as described (21). Segments of human p53 were expressed in *Escherichia coli* (HMS174 or BL21) as described (21). The p53s were purified either by metal or by immunoaffinity chromatography (21, 30) and were stored in 20 mM Tris·HCl, pH 8.0/100 mM NaCl/50% (vol/vol) glycerol at −70°C. Purified proteins were analyzed by PAGE in SDS as described by Laemmli (31) and were stained with Coomassie blue. All p53s were >90% pure.

DNA Annealing Assays. Annealing assays were done using one of two methods. In method A, a 150-bp DNA fragment corresponding to pBluescript KS+ (Stratagene) nucleotides 634–784 was labeled on one 5′ end and was purified by agarose gel electrophoresis. The DNA (1 ng) was heat denatured in 30 μl of 30 mM Tris·HCl, pH 7.5/50 mM NaCl/1 mM EDTA/1.5 mM dithiothreitol/5% (vol/vol) glycerol (buffer A). Either wt p53 or segments of p53 (8 ng) were added for 30 min on ice. In method B, 100 pg of a 5′-end-labeled 35-base synthetic

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Abbreviations: wt, wild type; CCC, covalently closed circular.
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oligonucleotide (5′-GCTGGGTACCGGGCCCCCCCTC-GAGGTCGACGGTA-3′) and 100 pg of a 35-base synthetic complementary oligonucleotide were incubated for 20 min at 25°C in the presence of wt p53 or segments of p53 (12 ng) in 20 μl of 5 mM Tris·HCl, pH 7.5/10 mM KCl/0.5 mM EDTA, 3.5% (vol/vol) glycerol/1.5 mM dithiothreitol containing bovine serum albumin at 1 mg/ml (buffer B). In some cases, monoclonal antibody (200 mg/ml) PAb421 or PAb240 was incubated with p53 for 30 min on ice prior to mixing with DNA. The reactions were terminated by the addition of EDTA, SDS, and bromphenol blue. Reaction products were analyzed by SDS/PAGE. Gels were dried and autoradiographed.

DNA Strand-Transfer Assays. Strand-transfer assays were done by using one of two methods. In method A, 1 ng of the 150-bp DNA used for the DNA annealing assay was incubated with 20 ng of the single-stranded, circular, positive strands of pBSKS+ and wt p53 or segments of p53 (30 ng) in 20 μl of buffer A. Reaction mixtures were incubated at 37°C for 30 min. Proteinase K was added to a final concentration of 1 mg/ml, and the reaction mixtures were incubated at 37°C for 15 min. SDS and bromphenol blue were then added to a final concentration of 0.1% and 0.01%, respectively. Samples were electrophoresed in a 1% agarose gel for 2 hr at 100 V. In method B, the DNA substrates were a heteroduplex donor molecule consisting of labeled (5′-GCTGGGTACCGGGC-CCCCCCTCGAGGTCGACGGTA-3′) and unlabeled (5′-T-ACCGTCGACCTCGAGG-3′) strands. The acceptor oligonucleotide (5′-TACCGTCGACCTCGGGGGGGCCCGG-TACCCAGC-3′) was unlabeled. The DNAs were mixed with wt p53 or segments of p53, and the reactions were carried out and analyzed as described in method B for DNA annealing.

Competition Assay for Binding of DNA by p53. A gel shift assay was carried out as described by Wang *et al.* (21) in the presence or absence of competitor DNA. The 35-bp double-stranded DNA probe and the assay conditions were the same as those used in method B for the DNA annealing assay. Two nanograms of the 5′-end-labeled probe was incubated with 100 ng of wt p53 or segments of p53. pSV01ΔEP (32) was used as competitor, double-stranded DNA; the plasmid DNA was >95% supercoiled. The same preparation of DNA was treated in one of several ways. The plasmid (575 ng) was incubated with 60 units of *Alu* I (New England Biolabs) for 60 min at 37°C in 100 μl of 10 mM Tris·HCl, pH 7.0/10 mM $MgCl_2$/1 mM dithiothreitol, after which *Alu* I was heat inactivated at 60°C for 20 min. pSV01ΔEP was also treated with 0.05 unit of DNase I for 5 min on ice. The reaction was terminated by the addition of EDTA to a final concentration of 5 mM. pSV01ΔEP DNA was γ-irradiated (800 rads; 1 rad = 0.01 Gy) in water at a final concentration of 5.75 ng/μl. DNA binding was determined by gel electrophoresis, autoradiography, and densitometry.

RESULTS

To investigate the role of p53 in the cellular response to DNA damage, we analyzed the biochemical properties of p53 segments previously used to characterize the DNA-binding and oligomerization functions of p53. We purified wt p53 and segments of p53 by using either metal affinity or immunoaffinity chromatography. Murine (M) p53s are identified by their amino acid components as numbered by Pennica *et al.* (33), and human (H) p53s are identified by their amino acid components according to Harlow *et al.* (34). The functional domains of human and murine p53s are shown in Fig. 1. wt p53 forms stable tetramers, binds to DNA both specifically and nonspecifically, and strongly induces transcription through binding to DNA recognition sites (21, 23). p53 segments H83–363 and M80–360 form tetramers and bind DNA specifically but have no C-terminal nonspecific DNA-binding domain (21, 23). p53 segments H318–393 and M315–390 form

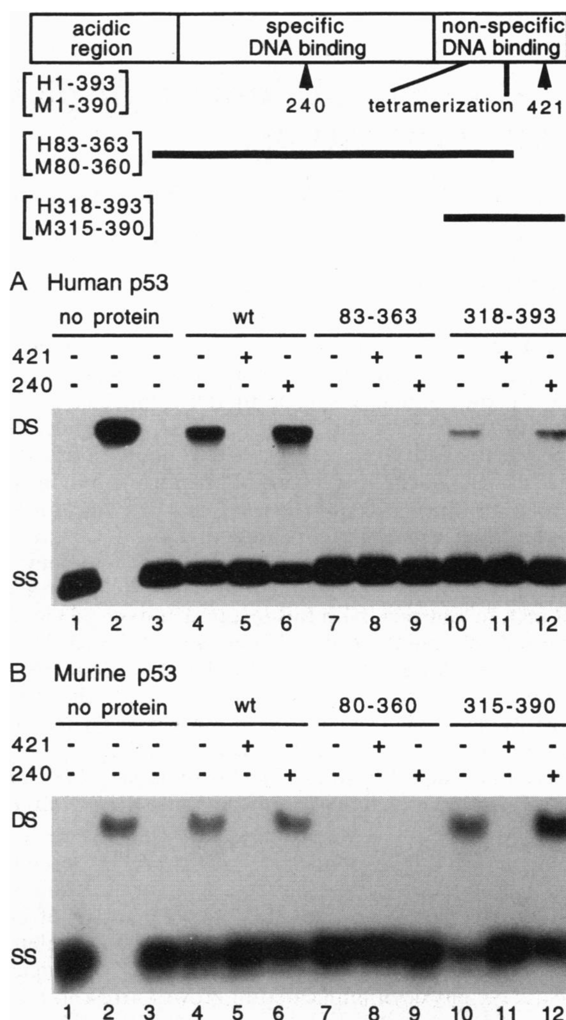

FIG. 1. DNA annealing by wt p53 and segments of p53. The domain organization of human (H) and murine (M) p53s is shown. Locations of epitopes for PAb240 and 421 are indicated. The solid bars show the locations of p53 segments. (*A*) Annealing of 150-bp heat-denatured DNA by human p53 and segments of p53 as described in *Materials and Methods* (method A). DS and SS indicate double- and single-stranded DNAs. (*B*) Annealing of complementary 35-base synthetic oligonucleotides by murine p53 and segments of p53 as described in *Materials and Methods* (method B). p53s and monoclonal antibodies were added to lanes 4–12 as indicated. All samples were treated with proteinase K and SDS before being analyzed by gel electrophoresis.

tetramers and bind DNA but without apparent sequence specificity (21, 23).

DNA Annealing by p53. We examined DNA annealing by p53 using different DNA substrates. First, we used human p53 and denatured 150-bp DNA (Fig. 1*A*). The denatured and duplex DNAs are shown in lanes 1 and 2, respectively. In the absence of p53, DNA annealing was undetectable after 30 min under these assay conditions (lane 3). wt p53 induced the annealing of double-stranded DNA (lane 4). PAb421, a monoclonal antibody whose epitope maps to amino acids H373–386 (35), blocked annealing by wt p53 (lane 5). In contrast, preincubation with PAb240, a monoclonal antibody against p53 amino acids H212–217 (36), did not alter annealing of DNA (lane 6) by p53. Segment H83–363, containing the site-specific DNA-binding and tetramerization domains of p53, failed to catalyze DNA annealing (lanes 7–9). Segments H1–323, H333–393, and H318–363 also failed to anneal DNA at similar protein concentrations (data not shown). p53 segment H318–393, however, had renaturation activity (lane 10),

and preincubation with PAb421 but not with PAb240 abolished this activity (lanes 11 and 12).

We also used murine p53 and complementary 35-bp synthetic oligonucleotides in an independent assay for DNA renaturation (Fig. 1*B*). Single-stranded oligonucleotides (lane 1) annealed under optimal renaturation conditions (lane 2) in the absence of p53. Under our assay conditions, however, the oligonucleotides remained single-stranded in the absence of p53 (lane 3). wt p53 promoted renaturation of the short synthetic oligonucleotides (lane 4). This activity was inhibited by PAb421 but not by PAb240 (lanes 5 and 6). Similar results were obtained using murine segment M315–390 (lanes 10–12). Segment M80–360 did not promote DNA annealing (lanes 7–9).

We conclude that the C-terminal 75 amino acids of both human and murine p53s promote the annealing of complementary DNA strands containing 35–150 nucleotides.

DNA Strand Transfer by p53. We investigated the ability of p53 to catalyze DNA strand transfer. The diagram in Fig. 2*A* shows one assay used to define strand-transfer characteristics of human p53 segments. The donor molecule was a 150-bp duplex DNA radiolabeled on one strand (lane 1) that is fully complementary to a section of a single-stranded, circular DNA. Transfer of the labeled strand to the circular, acceptor DNA would result in a partially double-stranded molecule with reduced electrophoretic mobility compared to that of the donor substrate. To make a control marker (lane 2), we mixed donor DNA with an excess of acceptor DNA, and the mixture was heat-denatured and reannealed. In experimental samples that were not denatured, p53 promoted the transfer of the radiolabeled strand of duplex DNA to the acceptor DNA to create a product (lane 3) that electrophoresed at the same position as the marker DNA. Preincubation of wt p53 with PAb421 prevented formation of product DNA (lane 4). p53 segment H83–363 did not mediate strand transfer (lanes 5 and 6). In contrast, segment H318–393 induced strand transfer in the absence (lane 7) but not in the presence (lane 8) of PAb421.

Similar results were obtained using murine p53 segments in a different assay (Fig. 2*B*). A partially duplex donor DNA was mixed with a linear acceptor strand under assay conditions with no resulting strand transfer (lane 1). Control product DNA was produced by mixing the donor with an excess of acceptor DNA and by heating and annealing the mixture (lane 2). wt p53 (lane 3) and segment M315–390 (lane 7) promoted the transfer of labeled oligonucleotide to the unlabeled single-stranded acceptor molecule but not in the presence of PAb421 (lanes 4 and 8). Segment M80–360 failed to promote strand transfer (lanes 5 and 6).

We conclude that the C-terminal 75 amino acids of p53 promote transfer of complementary strands from duplex DNA to single-stranded linear or circular DNAs. These data suggest that strand transfer by p53 is not dependent on the presence of two complementary ends.

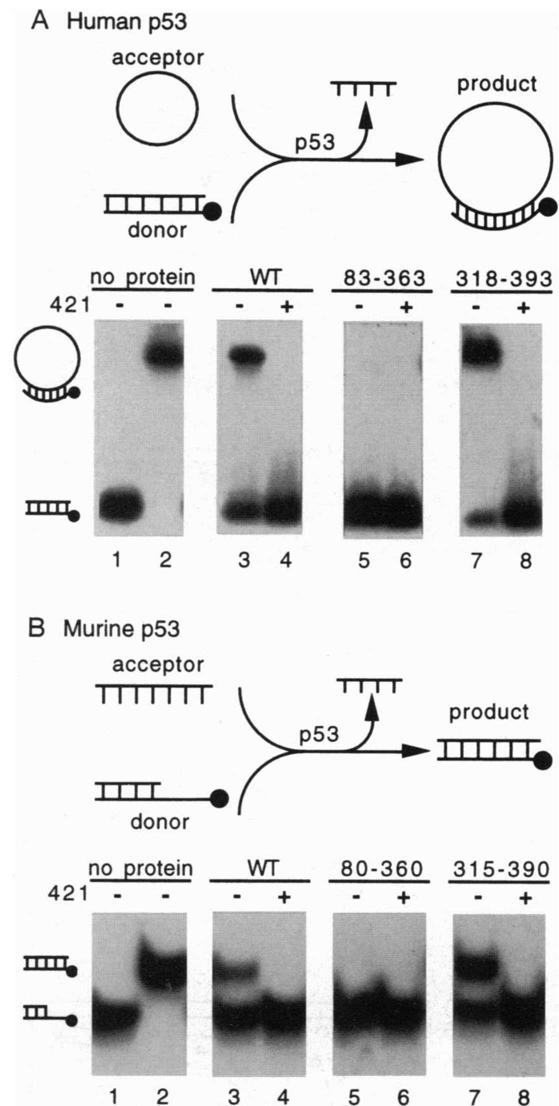

FIG. 2. DNA strand transfer by purified p53 and segments of p53. (*A*) Double-stranded donor DNA labeled on one strand was incubated with human p53 and single-stranded circular acceptor DNA as described in *Materials and Methods* (method A) and as outlined in the figure. (*B*) Partially double-stranded donor DNA labeled on one strand was incubated with murine p53 and linear, single-stranded acceptor DNA as described in *Materials and Methods* (method B) and as outlined in the figure. p53s and monoclonal antibody PAb421 were added to lanes 3–8 as indicated. All samples were treated with proteinase K and SDS, and product DNAs were identified by decreased mobility in an acrylamide gel.

Recognition of Damaged DNA by p53. We used an electrophoretic mobility shift assay to investigate the DNA-binding characteristics of murine p53 in the presence of undamaged and damaged competitor DNAs (Fig. 3). As a probe, we used a small (35 bp) double-stranded DNA substrate with no p53 consensus sequence. Aliquots of covalently closed circular (CCC) supercoiled plasmid DNA were treated with *Alu* I, DNase I, or ionizing radiation and were used as competitors in the DNA-binding experiments. We chose these DNA-damaging agents to introduce a variety of DNA lesions in the competitor DNAs. *Alu* I digestion of CCC DNA generates 15 blunt-ended, double-stranded DNA segments. DNase I produces mostly single-stranded nicks. Ionizing radiation damages bases and sugars and causes a variety of strand breaks.

Fig. 3*A* illustrates the effects of the various competitor DNAs on nonspecific DNA binding by wt p53. Lane 1 shows the position of unbound substrate. In the absence of competitor, wt protein retarded the migration of double-stranded probe (lane 2), and the addition of increasing amounts of CCC DNA did not compete effectively for p53 binding (lanes 3–5). In contrast, *Alu* I-treated CCC DNA (lanes 6–8) at identical concentrations as those used with the untreated CCC DNA competed well for p53 binding. Similar results were obtained with DNase I-treated (lanes 9–11) and γ-irradiated (lanes 12–14) competitor DNAs. Competitor DNAs were added in mass excesses of 1-, 6-, and 12-fold. Although we could not quantitate the precise extent of DNA damage by DNase I or γ-irradiation, we calculate that competitor DNA cut with *Alu* I had 0.2-, 1.2-, and 2.4-fold molar excesses of DNA ends relative to the radiolabeled probe in lanes 6, 7, and 8, respectively.

The gel in Fig. 3*A* was analyzed by scanning densitometry, and the results of that analysis are presented in Fig. 3*B*. Under our assay conditions, CCC DNA did not begin to compete for

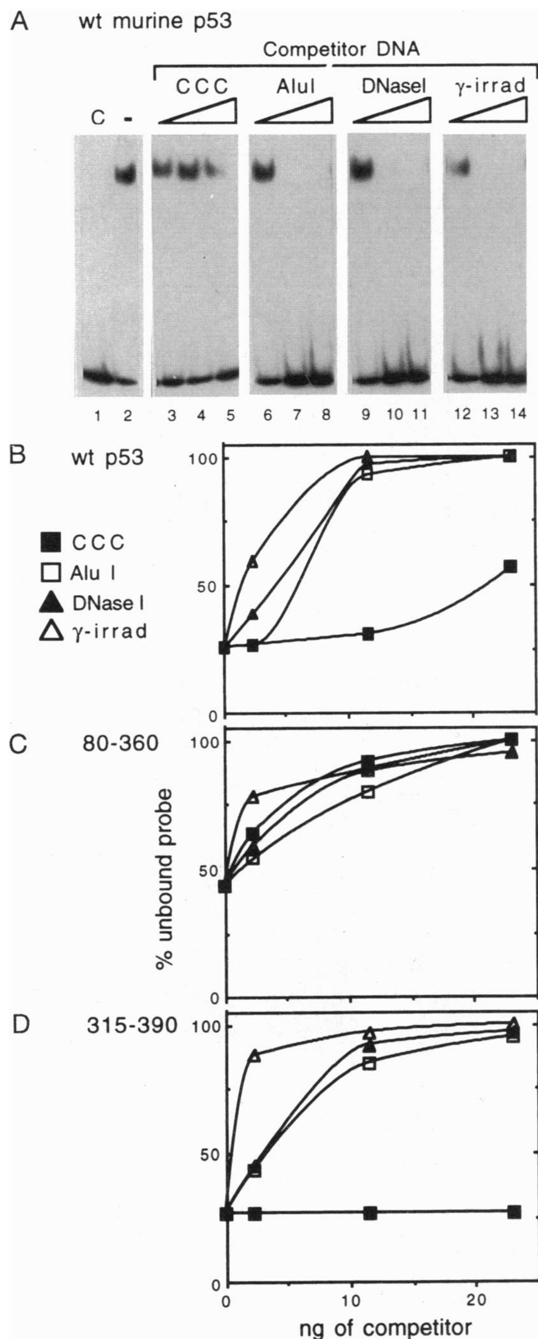

FIG. 3. Competition analysis of p53 DNA binding. Protein–DNA complexes were identified by the mobility shift assay described in *Materials and Methods*. (*A*) Murine p53 (100 ng) was added to a 35-bp synthetic oligonucleotide (2 ng) with no p53 consensus DNA binding site. Lanes 1 (C) and 2 (−) show DNA in the absence and presence of p53 without addition of competitor DNA. Lanes 3–14 show DNA binding in the presence of increasing amounts of competitor DNAs as indicated. Lanes 3, 6, 9, and 12 contain 2 ng of competitor DNA; lanes 4, 7, 10, and 13 contain 12 ng of competitor DNA; lanes 5, 8, 11, and 14 contain 24 ng of competitor DNA. (*B–D*) Competition curves for wt p53, segment M80–360, and segment M315–390, respectively.

wt p53 binding until a 12-fold mass excess of competitor DNA to probe DNA had been reached (lane 5). Damaged DNAs competed well at 1- to 6-fold mass excesses. Similar competitor analyses using p53 segments M80–360 and M315–390 are shown in Fig. 3 *C* and *D*, respectively. All DNAs competed effectively and at similar DNA concentrations for binding to segment M80–360. In contrast, damaged DNAs competed for binding to segment M315–390 much more efficiently than did undamaged DNA (Fig. 3*D*). A 1-fold mass excess of damaged DNAs competed significantly with the labeled probe, whereas a 12-fold mass excess of undamaged competitor DNA failed to inhibit binding. Segment 315–390, therefore, has at least a 12-fold higher affinity for damaged DNA than for undamaged DNA. We conclude that wt p53 recognizes damaged DNA and that this activity resides in the C-terminal 75 amino acids of p53.

DISCUSSION

The tumor suppressor p53 responds to DNA damage (2, 3). Depending on cell type, levels of endogenous p53 may rise rapidly after DNA damage. This accumulation of p53 coincides with induction of p21 (4), *GADD45* (7, 8, 37), and perhaps other genes that are involved in cell cycle arrest at the G_1/S boundary after DNA damage. Cellular arrest would provide time for DNA repair and would prevent the incorrect replication of DNA. Evidently, p53 plays a central role in one or more pathways activated by DNA damage. It will be important to trace these pathways from the induction of genes involved in cell cycle regulation to the repair of lesions.

Our finding that p53 recognizes and interacts strongly with damaged DNA argues that p53 participates in repair pathways as more than a key transcriptional factor. p53 might well act at the earliest stages of the response to DNA damage as a sensor of damage. Perhaps the association of p53 with damaged DNA triggers the accumulation of p53. In normal cells, p53 is rapidly degraded (2); in at least some circumstances, degradation occurs via the ubiquitin-dependent proteolytic pathway (38). Because inhibitors of cellular transcription and translation do not block p53 accumulation after DNA damage (2, 39, 40), p53 accumulation probably reflects a reduction in the rate of p53 degradation. A tight association with DNA could lead to reduced degradation of p53 through changes in p53 conformation, modification, association with other proteins, or compartmentalization within the nucleus. Subsequent DNA repair could lead to release of p53 from damaged DNA and restoration of normal degradation processes.

It is not clear how p53 tightly bound to damaged DNA would facilitate DNA repair. Hupp *et al.* (27) have presented evidence that structural changes in the C terminus of p53 activate site-specific DNA binding by the central domain of p53. Perhaps tight binding of the C terminus of p53 to damaged DNA might also activate site-specific DNA binding and transactivation. If so, the bound p53 would either have to be released in an activated form or act from a distance at promoters involved in DNA repair. Stenger *et al.* (41) have shown that p53 bound to DNA several thousand base pairs from promoters can enhance transcription via DNA looping. Activation over larger distances, however, has not been demonstrated. Given these uncertainties, it would be reasonable to consider other possible functions of p53 bound to damaged DNA. Perhaps p53 acts directly in the repair process itself. p53 apparently interacts with components of the transcription factor TFIIH transcription complex, which participate directly in nucleotide excision repair (15, 16). Furthermore, damage recognition and protein-mediated strand transfer are steps that have been implicated in the DNA repair process (42).

Our results indicate that the p53 central domain and the p53 C-terminal domain bind DNA by quite different mechanisms. p53 segments encompassing the central domain and the tetramerization domain bind to DNA either specifically or nonspecifically but do not promote DNA annealing or strand transfer and have no preference for damaged DNA. In contrast, C-terminal segments that also include the tetramerization domain catalyze DNA annealing and strand transfer and demonstrate a strong preference for damaged DNA. These results argue that the C terminus of p53 has specialized functions.

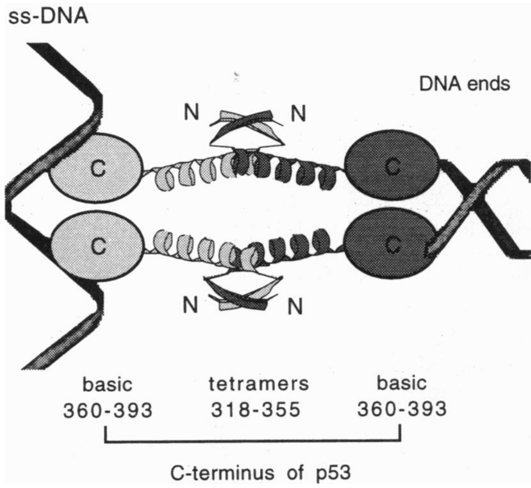

FIG. 4. Model for the interaction of the C-terminal region of p53 with DNA ends and single-stranded DNA (ss-DNA). The tetramer domain is drawn according to Jeffrey *et al.* (43). Human p53 amino acids are indicated at the bottom. N and C refer to N and C termini.

Fig. 4 presents a model to demonstrate possible interactions of the C terminus of p53 with DNA. Previous studies showed that the tetramerization region is needed for DNA binding by the C-terminal region (21). Competition studies indicate that C-terminal tetramers bind ends of DNA and possibly DNA with additional lesions in preference to double-stranded DNA. Furthermore, the strand-transfer experiment shown in Fig. 2*A* argues strongly that the C terminus can also bind single-stranded circular DNA. Perhaps the common denominator underlying each of these interactions is a preference for single-stranded regions of DNA. Fig. 4, therefore, shows the basic region in the C-terminal domain of p53 interacting with single-stranded regions of DNA. The annealing and strand-transfer reactions indicate that p53 can link two separate DNA molecules. Linking would be a natural consequence of the simultaneous binding of subunits of tetramers to separate DNAs. These p53–DNA interactions together with interactions of p53 with repair proteins implicate p53 in the repair process itself.

In conclusion, we have investigated the interaction of p53 with a variety of DNA substrates associated with DNA damage. Consistent with previous reports (13, 14, 28), we find that the C-terminal region of p53 catalyzes annealing of complementary oligonucleotides. We have extended these findings by showing that the C-terminal 75 amino acids of human and murine p53s are necessary and sufficient for p53-promoted strand transfer between complementary DNA molecules and for p53 recognition of DNA damage. Our findings argue that p53 may guard against genetic instability by sensing DNA damage and initiating a cascade of events culminating in cell cycle arrest and repair of DNA lesions.

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