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p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer

(DNA repair/tumor-suppressor protein)

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ABSTRACT **The p53 tumor-suppressor protein has previously been shown to bind double-stranded and single-stranded DNA. We report that the p53 protein can bind single-stranded DNA ends and catalyze DNA renaturation and DNA strand transfer. Both a bacterially expressed wild-type p53 protein and a glutathione *S*-transferase–wild-type p53 fusion protein catalyzed renaturation of different short (25- to 76-nt) complementary single-stranded DNA fragments and promoted strand transfer between short (36-bp) duplex DNA and complementary single-stranded DNA. Mutant p53 fusion proteins carrying amino acid substitutions Glu-213, Ile-237, or Tyr-238, derived from mutant p53 genes of Burkitt lymphomas, failed to catalyze these reactions. Wild-type p53 had significantly higher binding affinity for short (36- to 76-nt) than for longer (≥462-nt) single-stranded DNA fragments in an electrophoretic mobility-shift assay. Moreover, electron microscopy showed that p53 preferentially binds single-stranded DNA ends. Binding of DNA ends to p53 oligomers may allow alignment of complementary strands. These findings suggest that p53 may play a direct role in the repair of DNA breaks, including the joining of complementary single-stranded DNA ends.**

Inactivation of the wild-type p53 gene through deletion or point mutation is believed to play a critical role in the development of a large variety of human tumors (1). p53 gene "knockout" mice appear normal at birth but show a dramatically increased incidence of spontaneous tumors during their adult life (2). The p53 protein acts as a cell cycle regulator in the G_1 phase (see ref. 3 for a review). It accumulates by posttranslational stabilization mechanisms following certain types of DNA damage (4–6). These and other findings have led to the "molecular policeman" model. Upon DNA damage, p53 levels increase. This leads to a cell cycle block in G_1, permitting the cell to repair its DNA or, if the damage has been too great, exit by apoptosis (7). The p53 mutations found in many tumors prevent the ability of the protein to cause growth arrest, as a rule. Cells with damaged DNA can therefore replicate, providing a vast breeding ground for mutations and, hence, tumor development.

Several biochemical activities have been ascribed to the p53 protein. It can bind DNA in a sequence-specific manner (8–10). It also has a transactivating domain and is able to activate transcription from a minimal promoter downstream of a specific p53 binding motif (11). Wild-type p53 negatively regulates various genes that lack a p53 binding site, presumably by direct interaction with the TATA-binding protein, TBP (12, 13). p53 mutations found in human tumors compromise sequence-specific DNA binding, transactivation, and TBP binding. In addition to the sequence specific binding, the p53 protein binds nonspecifically to double-stranded (ds) and single-stranded (ss) DNA as well (14, 15). At least the binding to dsDNA is impaired in mutant p53 proteins.

Here we describe the ability of p53 to catalyze the renaturation of short complementary ssDNA fragments and promote DNA strand transfer reactions. We have also characterized the ssDNA-binding properties of the p53 protein as the basis for the reactions and found that the protein preferentially binds to ssDNA ends.

MATERIALS AND METHODS

Plasmids and Proteins. p53, glutathione *S*-transferase (GST), and GST–p53 fusion proteins were produced and purified as outlined (16). GST–mutant p53 plasmids were generated by PCR amplification of the following human mutant p53 cDNAs: Arg-213 to Gln (m2), Met-237 to Ile (m3), and Cys-238 to Tyr (m6) (17). Plasmids pCAT-Promoter (Promega) and pCAT1 (carrying a 0.2-kb human genomic DNA fragment) were prepared by alkaline lysis (18) and linearized with *Bam*HI.

Oligodeoxynucleotides and DNA. The following oligonucleotides were used: the prodynorphin gene exon 4 fragments 5′-GTACAAACGCTATGGGGGCTTCTTGCGGCGCATTCGT-3′ [(+)-strand, nt 513–549, 37 nt; N1], 5′-GACGAATGCGCCGCAAGAAGCCCCCATAGCGTTTGT-3′ [(−)-strand, nt 515–550, 36 nt; N2], and 5′-GTACAAACGCTATGGGGGCTTCTTGCGGCGCATTCGTCCCAAGCTGTCCAGTCAAGTCGCCTAGGACAGC-3′ [(+)-strand, nt 513–582, 70 nt; N3] (19); a mutant fragment of the human immunodeficiency virus enhancer, m-κB, 5′-GGTGATCATTCACTTTCCGCTATTCACTTTCCAGGAT-3′ [(+)-strand, 37 nt; N4] (20); a fragment of oligonucleotide N2 with GG added to the 5′ end, 5′-GGGACGAATGCGCCGCA-3′ (17 nt; N5); oligonucleotide N1 extended at the 5′ end, 5′-CGACAGGATCCGCTGAACTGACCTGACAAACGTATGGGGGCTTCTTGCGGCGCATTCGCT-3′ (60 nt; N6); an oligonucleotide with random sequence, 5′-CAGGTCAGTTCAGCGGATCCTGTCG(N_{26})GAGGCGAATTCAGTGCAACTGCAGC-3′ (76 nt; N7), and the complementary oligonucleotide 5′-GCTGCAGTTGCACTGAATTCGCCTC-3′ (25 nt; N8); the mutant 1 oligonucleotide N1, 5′-GTACAAACGCTATATTCGCTTCTTGCGGCGCATTCGT-3′ (37 nt; N9) and the complementary oligonucleotide (N10); the mutant 2 oligonucleotide N1 5′-GTACAAACGCTATGGCGGCTTCTTGCGGCGCATTCGT-3′ (37 nt; N11) and the complementary oligonucleotide (N12) (mutant nucleotides are underlined). The 462-bp fragment (HD3) of the 5′ end

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Abbreviations: GST, glutathione *S*-transferase; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

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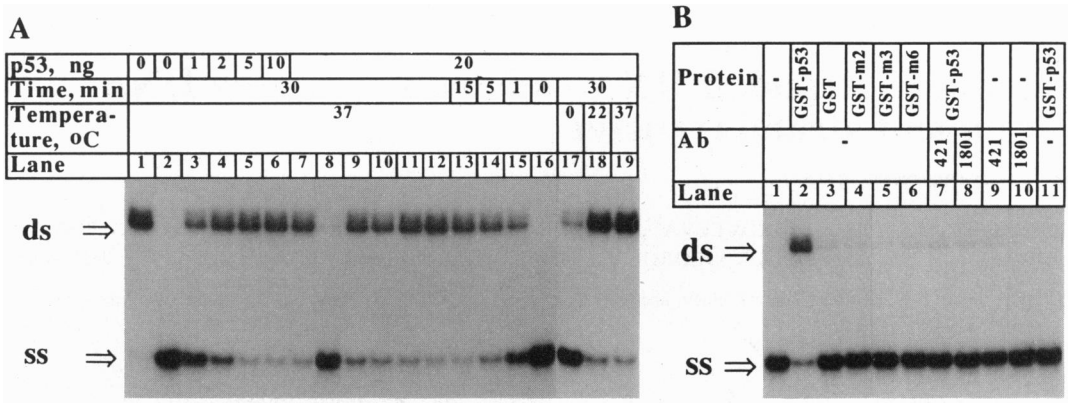

FIG. 1. DNA renaturation by the p53 protein. (*A*) The labeled oligonucleotide N2 (0.05 ng) and the nonlabeled oligonucleotide N3 (0.12 ng), containing a 36-nt complementary region, incubated in the presence or absence of bacterially produced wild-type p53 protein (lanes 2–19), or preformed double-stranded oligonucleotide, containing labeled N2 and nonlabeled N3 oligonucleotides (lane 1), were analyzed in a nondenaturing 10% polyacrylamide gel. Arrows indicate the positions of labeled double-stranded (ds) and single-stranded (ss) DNA. The reaction was inhibited by 5 mM GTP (lane 8), but not by 5 mM GDP, GMP, or nonhydrolyzable analog guanosine 5′-[β,γ-imido]triphosphate (lanes 9–11). (*B*) DNA renaturation activity of wild-type and mutant p53 proteins and effect of anti-p53 antibodies. Labeled N2 and complementary (N3; lanes 1–10) or noncomplementary (N7, 0.12 ng; lane 11) oligonucleotides were incubated with GST–wild-type or mutant p53 fusion proteins (10 ng). Renaturation was inhibited by 0.2 μg of anti-p53-antibodies (Ab) (lanes 7 and 8).

of the dopamine D3 receptor gene was prepared as described (21). *Escherichia coli* DNA (4–15 kb) was obtained from Sigma.

DNA Renaturation, Strand Exchange and Electrophoretic Mobility-Shift Assays. All reactions were performed in 10 μl of 5 mM Tris·HCl, pH 7.5/10 mM KCl/0.5 mM EDTA/3.7% (vol/vol) glycerol/1.5 mM dithiothreitol with 10 μg of bovine serum albumin, and 4000–10,000 cpm of boiled ^{32}P-end-labeled oligonucleotide, in the presence (renaturation and binding) or absence (strand transfer) of 5 mM $MgCl_2$. Reaction mixtures were incubated for 30 min at 37°C, unless otherwise stated. After renaturation and strand transfer reactions, stop solution containing EDTA (pH 8.0) and SDS was added to give 26 mM EDTA and 0.17% SDS. Reaction products were analyzed by electrophoresis in a nondenaturing 5% or 10% polyacrylamide gel in 0.5× TGE buffer (25 mM Tris·HCl, pH 8.5/0.19 M glycine/1 mM EDTA), followed by autoradiography of the dried gel. PAb421 and PAb1801 antibodies (0.1 μg/μl) (Oncogene Science) and control anti-c-Rel antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with the p53 protein for 1 hr at 4°C before reactions.

Electron Microscopy. *Pvu* II-digested pUC18 DNA (Boehringer Mannheim), double-stranded or heat-denatured single-stranded (60 ng), was incubated with 20 ng of bacterially produced p53 or GST–p53 fusion protein in 10 mM Tris·HCl, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM dithiothreitol for 30 min at 37°C. Samples were spread onto 20-μl droplets of redistilled water as hypophase and absorbed on carbon-coated grids as described (22, 23). Micrographs of rotary-shadowed samples were taken in a JEOL TEM-SCAN 100-CX electron microscope. To calculate the proportion of DNA molecules that had bound p53, at least 500 molecules were analyzed for each sample.

RESULTS

p53 Protein Catalyzes Renaturation of Complementary Single-Stranded Oligonucleotides. Complementary single-strand-

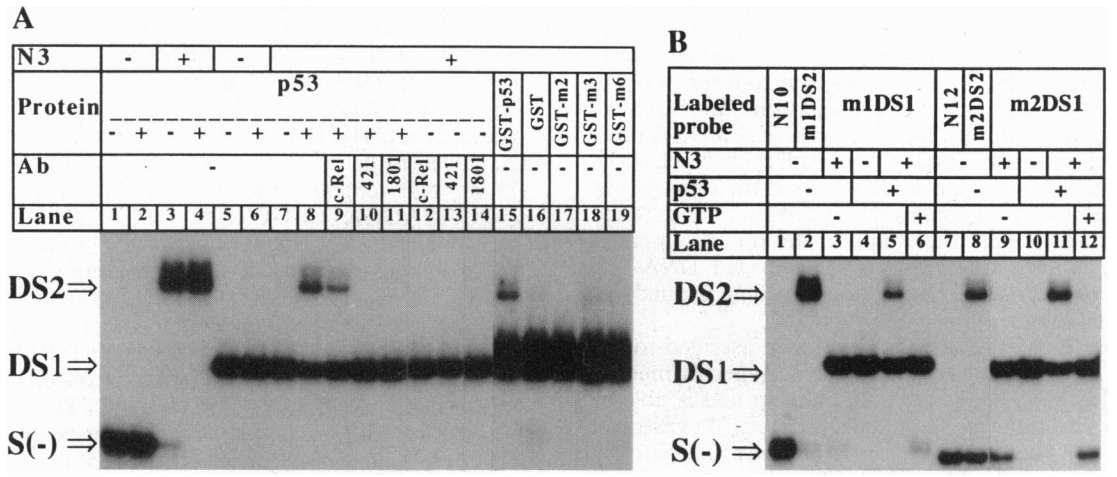

FIG. 2. Strand transfer activity of the p53 protein. (*A*) Both p53 and GST–wild-type p53 catalyzed transfer of the labeled strand (oligonucleotide N2; 36 nt) of a short duplex molecule, DS1 (N2/N1), to the (+)-acceptor complementary oligonucleotide (N3; 70 nt). A novel duplex molecule, DS2 (N2/N3), was the product of the reaction. Arrows indicate the positions of labeled N2 [S(−)], DS1, and DS2. Labeled N2 oligonucleotide (lanes 1 and 2; 0.1 ng), DS2 (lanes 3 and 4; 0.3 ng), and DS1 (lanes 5–19; 0.2 ng) were incubated with or without p53, GST, GST–wild-type p53, or GST–mutant p53 proteins (20 ng) in the presence or absence of acceptor oligonucleotide N3 (1 ng; 5-fold molar excess over N2 oligonucleotide) and then analyzed by nondenaturing 10% polyacrylamide gel electrophoresis. Anti-p53 antibodies (0.2 μg), but not control anti-c-Rel antibodies (0.2 μg), inhibited strand transfer. (*B*) Strand transfer reaction with duplex oligonucleotides that carry one single or four contiguous mismatches relative to the (+)-acceptor strand (N3). Duplex oligonucleotides m1DS1 (N9/N10) and m2DS1 (N11/N12) were used instead of N1/N2 as DS1. GTP was added at 5 mM.

ed oligonucleotides were incubated with the p53 protein and analyzed by nondenaturing polyacrylamide gel electrophoresis after addition of SDS/EDTA stop solution to disrupt DNA–protein complexes. In the presence of bacterially produced, purified human wild-type p53 protein (Fig. 1*A*, lanes 3–7) or GST–human wild-type p53 fusion protein (Fig. 1*B*, lane 2), complementary single-stranded oligonucleotides were quantitatively converted into a slower migrating form with a mobility identical to that of the duplex oligonucleotide. The double-stranded product was formed neither in the absence of protein (Fig. 1*A*, lane 2) nor after incubation with control proteins, including GST and three different GST–mutant p53 fusion proteins, GST–p53 m2, m3, and m6, derived from mutant p53 genes in Burkitt lymphomas (Fig. 1*B*, lanes 3–6). When a noncomplementary oligonucleotide was used as a substrate instead of the complementary oligonucleotide, no dsDNA was formed (Fig. 1*B*, lane 11). The anti-p53 antibodies PAb421 and PAb1801, but not control anti-cRel antibodies (data not shown), completely inhibited duplex formation induced by p53 (Fig. 1*B*, lanes 7 and 8). Duplex formation was dependent on p53 protein concentration and on the duration and temperature of incubation. Most of the reaction product was formed within 5 min of incubation with p53 (Fig. 1*A*, lanes 13–16). The recovery of the reaction product was significantly higher at 37°C than at 22°C, and no product was formed at 0°C (lanes 17–19). Incubation with GTP (lane 8), but not GDP, GMP, or the nonhydrolyzable GTP analog guanosine 5′-[β,γ-imido]triphosphate (lanes 9–11), inhibited duplex formation induced by p53. ATP, CTP, and TTP (ribothymidine 5′-triphosphate) also inhibited the reaction (not shown). Since no NTPase activity is known to be associated with p53, these results indicate that ATP, CTP, GTP, and TTP, but not GDP, GMP, or guanosine 5′-[β,γ-imido]triphosphate, either are allosteric effectors or occupy a DNA binding site in the p53 protein.

Different pairs of oligonucleotides with regions of complementarity located at the 3′ end (N7/N8; 25 complementary nucleotides) or in the middle (N5/N3; 17 complementary nucleotides) of the longer oligonucleotide, and at the ends of two relatively long (60- and 76-nt) oligonucleotides (N6/N7; 25 complementary nucleotides) were quantitatively converted into duplex form by incubation with the GST–p53 protein (data not shown). Hence, the renaturation reaction promoted by the p53 protein is not sequence specific.

Strand Transfer Reaction Catalyzed by the p53 Protein. The ability of wild-type p53 to catalyze strand transfer was tested by incubating the protein with a short duplex oligonucleotide (DS1; N1/N2, 35 bp), containing one labeled strand (N2; 36 nt), and a nonlabeled acceptor strand (N3; 70 nt), complementary to the labeled strand of DS1 (Fig. 2*A*). The transfer of labeled N2 strand from DS1 duplex to the longer N3 acceptor strand would result in a novel labeled duplex molecule, DS2, with slower electrophoretic mobility than that of DS1. Control incubation of p53 with labeled N2, DS2, or DS1 oligonucleotides (Fig. 2*A*, lanes 2, 4, and 6) did not give rise to any changes in the mobility of the labeled DNA fragments. Likewise, labeled DS2 did not appear after incubation of labeled DS1 with N3 acceptor strand (lane 7). Formation of labeled DS2 was observed only when the incubation mixture contained both p53 and the nonlabeled acceptor strand in addition to labeled DS1 (lane 8). PAb421 (lane 10) and PAb1801 (lane 11) antibodies, but not control antibodies (lane 9), completely inhibited the formation of DS2. The weak effect of control antibodies could be due to the presence of ions and carrier protein (gelatin) in the antibody solution. GST–wild-type p53 also catalyzed strand transfer (lane 15). In contrast, GST alone and three GST–mutant p53 fusion proteins failed to catalyze the formation of the DS2 duplex (lanes 16–19).

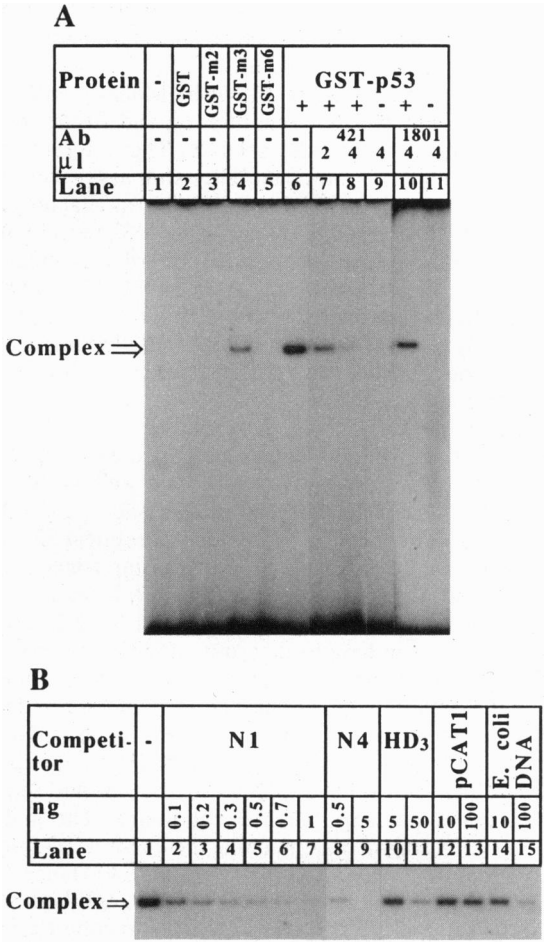

FIG. 3. Electrophoretic mobility-shift assay of ssDNA-binding activity of GST–p53 proteins. (*A*) Binding of GST–wild-type and mutant p53 fusion proteins to a short single-stranded oligonucleotide, N1, and effects of anti-p53 antibodies on DNA–p53 complex formation. The reaction mixture, containing the labeled oligonucleotide N1 (0.34 ng), was incubated with GST proteins (10 ng) and analyzed in a nondenaturing 5% polyacrylamide gel. An arrow indicates the position of the retarded complex. (*B*) Competition by short single-stranded oligonucleotides (N1 and N4) and longer ssDNA fragments (HD3, pCAT1, and *E. coli* DNA) with the labeled oligonucleotide N1 for binding to the GST–wild-type p53 protein.

The recovery of DS2 was dependent on protein concentration, with maximal strand transfer at 20–100 ng. The amount of DS2 increased in the course of incubation for 60 min. The reaction was very slow at 22°C and did not occur at all at 0°C (data not shown). DS2 recovery reached a maximum at a 2-fold excess of (+)-acceptor strand over DS1 concentration. The DS2 product was completely absent when unrelated oligonucleotides were used instead of the complementary acceptor oligonucleotide (data not shown).

Some proteins or compounds can promote spontaneous strand transfer that can be blocked by a single base mismatch in the region of branch migration (24). To determine whether or not the p53-promoted strand transfer was due to an enzymatic activity of p53, the strand transfer reaction was carried out with duplexes that had one single or four contiguous mismatches relative to the (+)-acceptor strand (N11/N12 or N9/N10 duplexes, respectively) instead of the N1/N2 duplex. p53 catalyzed strand transfer of the perfectly matched oligonucleotide (Fig. 2*A*, lane 8) and the oligonucleotides carrying a single mismatch or four contiguous mismatches (Fig. 2*B*, lanes 5 and 11) with a similar efficiency. Thus, strand transfer promoted by p53 is not a result of

spontaneous branch migration. GTP inhibited this reaction (Fig. 2*B*, lanes 6 and 12), as well as strand transfer from the N1/N2 duplex (data not shown).

Analysis of p53 Binding to ssDNA by Mobility-Shift Assay. In order to promote DNA renaturation and strand transfer, the p53 protein has to bind ssDNA. This interaction was examined by electrophoretic mobility-shift assay with a short single-stranded oligonucleotide (N1; 37 nt) as a labeled probe (Fig. 3*A*). Incubation of GST–wild-type p53 protein with the labeled oligonucleotide gave rise to a retarded complex (Fig. 3*A*, lane 6), whereas GST (lane 2) and GST–mutant p53 proteins (lanes 3 and 5) did not complex with ssDNA or showed only weak affinity for ssDNA (GST–p53 m3; lane 4). PAb421 (lanes 7 and 8) and PAb1801 (lane 10) antibodies, but not control antibodies (data not shown), inhibited complex formation between GST–p53 and ssDNA.

The specificity of binding was examined by using a set of short single-stranded oligonucleotides as well as the longer (462-nt) HD3 DNA fragment and pCAT1 and *E. coli* DNA as competitors. The labeled N1 oligonucleotide–p53 protein complex gradually disappeared at increasing concentrations of nonlabeled N1 oligonucleotide (Fig. 3*B*, lanes 2–7). Nonlabeled oligonucleotides N4 (lanes 8 and 9), N2, N7, and others, 36–76 nt in length (data not shown), also competed efficiently with labeled N1 oligonucleotide for p53 binding. Thus, GST–wild-type p53 exhibited high and approximately equal affinity for all short oligonucleotides tested (36–76 nt). Since these oligonucleotides all have different sequences, the binding of GST–wild-type p53 to ssDNA is not sequence specific under our experimental conditions. The ability of longer ssDNA fragments, including the 462-nt HD3 fragment (lanes 10 and 11), denatured plasmids pCAT1 (lanes 12 and 13) and pCAT-Promoter (data not shown), and high molecular weight *E. coli* DNA (lanes 14 and 15) to compete for p53 binding was lower by about 2 orders of magnitude. These data indicate that short ssDNA fragments and/or DNA ends are the prime binding targets for p53.

Electron Microscopy Reveals Preferential Binding of p53 to ssDNA Ends. Electron microscopy showed that p53 bound poorly to dsDNA (Fig. 4*B*). Only 0.5–1% of the dsDNA molecules were found to complex with p53. However, p53 bound to ssDNA (Fig. 4 *C–E*). Around 50% of ssDNA molecules were observed in complexes with the protein. Importantly, p53 bound to the ends of DNA in 90% of p53–ssDNA complexes. In the remaining p53–ssDNA complexes, p53 bound to the internal portions of DNA. No difference was observed between the interaction of wild-type p53 and GST–wild-type p53 with DNA. The GST–mutant p53 m2 protein did not bind to ssDNA (data not shown).

DISCUSSION

We have shown that both bacterially produced wild-type p53 and a GST–wild-type p53 fusion protein can bind ssDNA ends and catalyze DNA renaturation and strand transfer. The GST protein itself failed to do so. Anti-p53 antibodies PAb421 and PAb1801, which recognize epitopes in the carboxyl-terminal and amino-terminal parts of the protein, respectively, can inhibit all three activities of p53. This is consistent with the interpretation that binding to ssDNA ends and promotion of DNA reannealing and strand transfer are intrinsic properties of the p53 protein. The fact that antibodies that react with two different epitopes in p53 were inhibitory may indicate that both domains of p53 are needed for these activities. Another possibility is that the antibodies induce a conformational change in the p53 protein that interferes with ssDNA-end binding and/or DNA renaturation and strand transfer.

The failure of mutant p53 proteins to bind ssDNA ends and promote DNA reannealing and strand transfer shows that these activities are dependent on the wild-type conformation of p53, suggesting that they are relevant to the tumor-

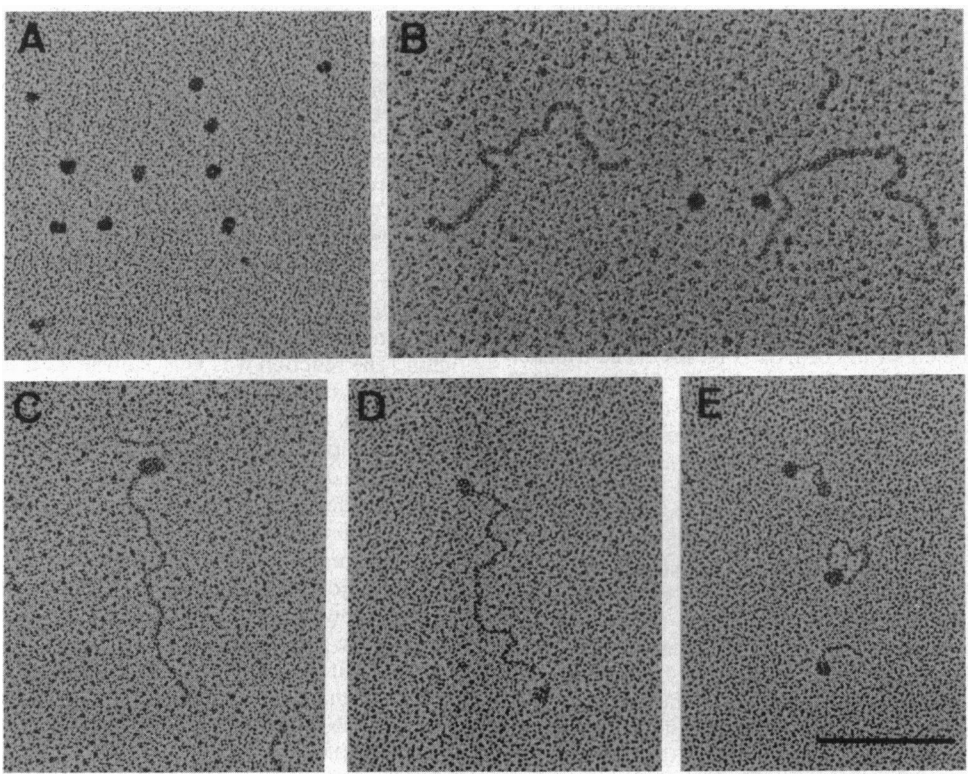

FIG. 4. Electron micrographs of wild-type p53 binding to ss- or dsDNA. (*A*) Bacterially produced p53 without DNA. (*B*) p53 incubated with blunt-ended pUC18 dsDNA fragments (2.4 and 0.32 kb) generated by digestion with *Pvu* II. No p53–dsDNA complexes were observed. (*C–E*) p53 incubated with denatured pUC18 DNA fragments. p53 bound to the ends of ssDNA. (Bar = 250 nm in *A–E*).

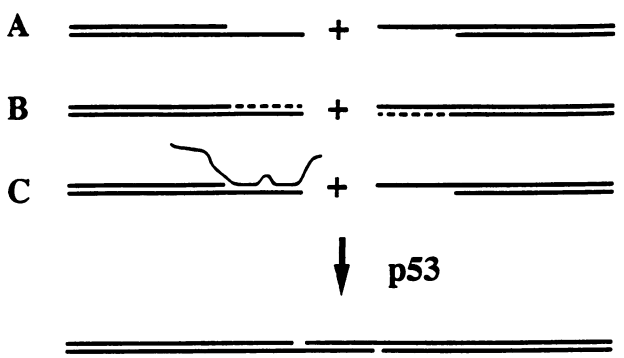

FIG. 5. Model for p53-catalyzed joining of staggered duplex DNA ends. p53 catalyzes the renaturation of protruding complementary ssDNA ends (*A*). p53 may also promote the proper joining of blunt DNA ends created by fill-in DNA synthesis (*B*) or remove incorrectly annealed ssDNA, by catalyzing strand transfer (*C*).

suppressor function of the wild-type protein. However, we cannot rule out that the mutant proteins might have some residual activity *in vivo* that was lost due to denaturation during preparation of the proteins *in vitro*.

The ability to bind ssDNA ends is a novel property of p53. This finding suggests that p53 may serve as an intracellular sensor of DNA strand breaks *in vivo*. The interaction of p53 with ssDNA ends could trigger a conformational change in the protein, leading to activation of a DNA repair pathway. Conceivably, the binding of two DNA ends to a p53 oligomer may align DNA fragments and thereby promote DNA renaturation and strand transfer *in vitro* and, possibly, *in vivo*.

How can the binding to ssDNA ends and the renaturation and strand transfer activities of p53 be linked to the function of p53 as a tumor suppressor? The accumulation of p53 in response to DNA damage is thought to cause G_1 arrest, allowing DNA repair before replication, or apoptosis, if the DNA damage was extensive. In addition, p53 activates GADD45, a gene involved in DNA repair (25). The results described here raise the possibility that p53 also participates in DNA repair in a more direct way. Exposure of cells to DNA-damaging agents may give rise to DNA with protruding single-stranded ends. One function of the p53 protein could be to find such ends and promote their renaturation (Fig. 5*A*). The protruding 5′ ends can also be converted to double-stranded blunt ends through filling in by DNA polymerases (Fig. 5*B*). The p53 protein could promote the appropriate joining of such ends, as well as incorrectly renatured DNA ends (Fig. 5*C*) *in vivo* through its ability to catalyze DNA strand transfer.

While this manuscript was under preparation, Oberosler *et al.* (26) reported that baculovirus-produced wild-type p53 binds RNA and ssDNA, has DNA–DNA and RNA–RNA annealing activity, and promotes RNA strand exchange. Our results concerning DNA renaturation and strand transfer are in agreement with the findings of Oberosler *et al.* and extend the strand exchange results to DNA. On the basis of our own data and the results of Oberosler *et al.*, an additional mechanism for the appropriate joining of DNA ends that involves p53 might be envisaged. In case of severe DNA damage, RNA present in excess in the nucleus could be used as single-stranded complementary linker for joining of DNA ends through RNA–DNA reannealing and/or strand exchange catalyzed by p53.

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