

## Alternatively Spliced Forms in the Carboxy-Terminal Domain of the p53 Protein Regulate Its Ability to Promote Annealing of Complementary Single Strands of Nucleic Acids

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**The carboxy-terminal domain of the p53 protein comprising amino acid residues 311 to 393 is able to promote the reassociation of single-stranded RNA or DNA into duplex hybrids. This domain is as efficient as the intact p53 protein in both the rate and the extent of the double-stranded product produced in this reaction. Both wild-type and mutant p53 proteins from cancerous cells carry out this reaction. The monoclonal antibody PAb421, which detects an epitope between residues 370 and 378, blocks the ability of p53 to reassociate single strands of RNA or DNA. Similarly, the alternative splice form of the murine p53 protein, which removes amino acid residues 364 to 390 and replaces them with 17 new amino acids, does not carry out the reassociation reaction with RNA or DNA. This is the first indication of functionally distinct properties of the alternative splice forms of p53. These results suggest that this splice alternative can regulate a p53-mediated reaction that may be related to the functions of this protein.**

The tumor suppressor gene product p53 functions as a transcription factor (9, 17, 44) that can positively or negatively regulate a set of genes (11, 32). These activities increase in response to DNA damage (16, 22) and result in a program that can negatively regulate progression through the cell cycle (23, 24) or the initiation of apoptosis or cell death (35, 43). Increase in p53 levels in response to DNA damage stimulates the transcription of a gene, p21-pic-1 (also termed Cip-1 [15], Sdi-1 [28], or WAF-1 [8]), which encodes a protein that binds to and inhibits the cyclin-dependent kinase 2 (Cdk-2) (15). p21-pic-1 has been found associated with cyclin A, D1, and E forming complexes with Cdk-2. p53-mediated apoptosis has been demonstrated in thymocytes in response to DNA damage (6, 21) and in fibroblasts that simultaneously overexpress the transcription factor E2F-1 and wild-type p53 (40). The p53 protein has been structurally and functionally divided into three domains (4, 30, 38). The N-terminal 42 amino acids can act to transactivate the transcription of a test gene (19), when it is linked to a DNA binding domain whose responsive element is localized near the test gene (10, 31). The middle portion of the protein, amino acid residues 120 to 290 (out of 393), is the sequence-specific DNA binding domain (4, 30, 38). The carboxy-terminal domain, composed of residues 311 to 393, contains the nuclear localization signals for the protein (34), the p53 protein oligomerization (tetrameric) sequences (30, 36), and several sites for phosphorylation by cellular protein kinases (5, 37). This domain is sufficient for the transformation of cells in culture, presumably utilizing the protein oligomerization signals to form heterodimeric or heterotetrameric (wild-type and mutant) p53 protein complexes in cells (33). Thus, the C terminus can mediate the *trans*-dominant phenotype of p53 mutant proteins.

Two distinct p53 mRNAs which are derived from alternative use of two exons at the 3' end of the p53 mouse gene have been detected (39). Different levels of the normal splice (NS) and alternative splice (AS) mRNAs of p53 have been found in

different tissues (12), and the two different p53 proteins made by these mRNAs have been detected in mouse epidermal cells (18). The translation of the AS p53 mRNA produces a protein with 17 new amino acids substituting for the 26 carboxy-terminal residues of the NS p53 protein. It even appears that the NS and AS forms of p53 protein are expressed at different levels at different times in the cell cycle (18).

In addition to its transcription factor activity, the p53 protein has recently been shown to be able to promote the annealing of complementary single-stranded RNA and DNA (29). This property of the p53 protein could have physiological significance in one of several ways. First, cells that have no p53 gene or protein are able to amplify DNA copies about one million times better than cells with a functional wild-type p53 protein (20, 42). Such gene amplifications, common in cancers, are thought to be mediated by unequal crossing over or recombination events which are promoted by aggressive single-stranded intermediates initiating recombination. The p53 protein might directly antagonize such events. In addition, the p53 protein might directly recognize DNA damage by binding to the ends of DNA or the single-stranded intermediates that result from the repair processes. It is in response to such signals that p53 levels increase in a cell (22) by increasing the half-life of the p53 protein. Thus, the p53 protein could become stabilized because of its recognition of DNA damage by the protein directly. Alternatively, this p53 anti-helicase-like activity could be involved in regulating DNA replication, transcription, ribosome biogenesis, or even translation of mRNAs with extensive secondary structure (the p53 protein is in the cytoplasm under some circumstances [26]). All of these processes require denaturation of the secondary structure of RNA or DNA.

The experiments reported here set out to determine which domain of the p53 protein harbored the activity that catalyzed single-strand nucleic acid hybridization. The results demonstrate that all of this activity resides in the carboxy-terminal domain of p53 delineated by residues 311 to 393 (C<sub>311-393</sub>). Furthermore, the NS form of the p53 protein, but not the AS form, contains this activity. Thus, the C-terminal 27 amino

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acids of p53, removed by the AS, participates in promoting the reassociation of single-stranded nucleic acids into a double-stranded form.

## MATERIALS AND METHODS

**Baculovirus-produced p53 proteins.** Recombinant baculoviruses for expression of human wild-type and mouse AS p53 protein were generated by cloning each cDNA into the pVL1393 plasmid and cotransfection with Baculogold viral DNA into SF9 cells (Pharmingen). Viruses were then subjected to one round of plaque purification. Recombinant baculoviruses for expression of the mouse wild-type p53 protein and the human mutants His-175 and His-273 have been previously described (3). SF9 cells were infected with baculoviruses and harvested 48 h postinfection.

**RNA and DNA substrates.** The single-stranded RNA substrates were prepared by runoff transcription of the linearized plasmid pGEM-7Z (Promega) in the opposite orientations either from the SP6 promoter to the *Eco*RI site or from the T7 promoter to the *Hind*III site. These two transcripts were 80 and 76 bases in length and had a 30-bp complementary region at the 3' ends. [ $\alpha$ - $^{32}$ P]UTP was used for the SP6-*Eco*RI transcript.

The single-stranded DNA substrates were obtained by heat denaturing an 82-bp fragment from the plasmid pBluescript SK. Briefly, the plasmid was linearized with *Sal*I and the ends were filled in with the Klenow fragment of DNA polymerase I, [ $\alpha$ - $^{32}$ P]dCTP, and the other nucleotide triphosphates. This radiolabeled plasmid was further digested with *Sac*I, and the 82-bp *Sal*I-*Sac*I fragment was isolated from an agarose gel and subjected to heat denaturation.

**Anti-p53 monoclonal antibodies and anti-p53 immunoaffinity columns.** Anti-p53 monoclonal antibodies PAb421 (13); PAb242, PAb246, and PAb248 (41); PAb1801 (2); and PAb1620 (25) were purified by passing the hybridoma supernatants through protein A-Sepharose and eluted with weak acids as described previously (14).

The PAb421 immunoaffinity column was prepared by passing the hybridoma supernatant over protein A-Sepharose, followed by covalent linking with 20 mM dimethyl pimelidate as described by Harlow and Lane (14). The PAb242 immunoaffinity column was prepared similarly, except the antibodies were purified from ascites fluid from the 242 hybridoma.

**Purification of p53 proteins and the C<sub>311-393</sub> carboxy-terminal fragment.** Baculovirus-expressed p53 proteins were purified by immunoaffinity chromatography. Infected SF9 cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1  $\mu$ M pepstatin A, 1  $\mu$ M E-64) by sonication and incubation on ice for 30 min. For the human p53 proteins (wild type, mutant His-175, or mutant His-273) or the mouse wild-type p53 protein, the lysate was centrifuged at 40,000  $\times$  g for 20 min at 4°C. The supernatant was precipitated in 50% ammonium sulfate, followed by centrifugation at 40,000  $\times$  g for 20 min at 4°C. The pellet was resuspended in lysis buffer and passed over a PAb421 immunoaffinity column, and the column was washed extensively with buffer containing 500 mM NaCl and 1% Nonidet P-40. The p53 protein was eluted from the column by incubation at 4°C for 2 to 4 h with rocking in lysis buffer with 0.25 mg of a 14-amino-acid peptide corresponding to the PAb421 epitope per ml (7). A Centricon-30 microconcentrator (Amicon, Inc.) was used to reduce the amount of peptide remaining in the purified p53 protein and to concentrate the purified p53 protein.

Murine p53 proteins (both the wild-type NS and AS forms) were purified from infected insect cells by lysis in TEN (100 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) at 4°C with 10 s of sonication at 50% efficiency with a Branson Sonicator. Debris was removed by centrifugation in a microcentrifuge, and the supernatant was passed in a batch over a column of monoclonal antibody PAb242 cross-linked to protein A-Sepharose for 2 h at 4°C. The column resin was washed in a batch two times with vortexing with 5 column volumes of (i) TEN, (ii) TEN plus 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate}, (iii) TEN plus 350 mM NaCl, (iv) 10% sucrose-500 mM NaCl-1% Nonidet P-40-5 mM EDTA, and (v) TEN. p53 protein was eluted by mixing with 2 column volumes of 100 mM glycine-100 mM NaCl, removing the supernatant, and neutralizing immediately with 250 mM Tris-HCl (pH 7.7)-100 mM NaCl. Protein was dialyzed against 50 mM Tris (pH 8.0)-150 mM NaCl-0.5 mM EDTA-1 mM dithiothreitol-20% glycerol and stored at -80°C.

The C<sub>311-393</sub> fragment was purified as described by Pavletich et al. (30). Briefly, the peptide was expressed in *Escherichia coli* BL21 (D3) cells, extracted from the insoluble fraction of the *E. coli* lysate with 6.4 M guanidine-HCl, and purified by reverse-phase high-performance liquid chromatography on a C<sub>4</sub> column. (The mobile phase contained 0.1% trifluoroacetic acid, and the peptide was eluted with an acetonitrile gradient.)

**Annealing assays.** The RNA-RNA annealing assays were performed as described by Oberosler et al. (29). Briefly, the two single-stranded RNAs (~0.1 ng of the radiolabeled RNA strand and ~0.2 ng of the unlabeled strand) were incubated at 37°C in the reaction buffer containing 40 mM Tris-HCl (pH 7.5), 1.5 mM dithiothreitol, 100 mM NaCl, 1 mM EDTA, 0.1 mg of bovine serum albumin per ml, and 500 U of RNasin per ml in the absence or presence of the indicated amounts of p53 protein. The reactions were stopped at the appropriate time by 0.1 volume of 150 mM EDTA and 3% sodium dodecyl sulfate (SDS). The

double-stranded and single-stranded RNA products were separated in an SDS-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide) gel, and the amounts of each product were quantitated on a Molecular Dynamics Phosphorimager. Reaction conditions for the DNA-DNA annealing assays were the same as those used for the RNA-RNA annealing, except that the final products were separated on an SDS-PAGE (15% polyacrylamide) gel.

The effects of anti-p53 antibodies on the RNA-RNA or DNA-DNA annealing activities of p53 protein were tested by preincubating p53 protein with the indicated antibodies in the annealing buffer for 10 min at room temperature and then adding the RNA or DNA substrates for incubation at 37°C for 1 h, followed by gel electrophoresis and autoradiography.

## RESULTS

**Purity of the p53 proteins and the carboxy-terminal fragment of p53.** The p53 wild-type and mutant proteins were produced by baculoviruses and purified as described in Materials and Methods. The purity of these proteins was determined by using SDS-PAGE, and the stained proteins in these gels are shown in Fig. 1A. The p53 proteins purified by antibody affinity chromatography were judged to be between 80 and 90% pure in these preparations and subsequent studies. The C<sub>311-393</sub> fragment was synthesized in *E. coli* and purified as described in Materials and Methods. The purity of this protein was greater than 95% as determined by SDS-PAGE followed by Coomassie blue staining (Fig. 1B).

**RNA-RNA and DNA-DNA annealing of the human wild-type p53 protein and the C<sub>311-393</sub> fragment of p53 protein.** To test the ability of the full-length and the C<sub>311-393</sub> carboxy-terminal fragment of human p53 protein to catalyze the reassociation of RNA or DNA, complementary single-stranded nucleic acids were prepared. RNA substrates were prepared by runoff transcription from oppositely oriented promoters of the pGEM-7Z plasmid, which produced an 80-base radiolabeled transcript. A 76-base unlabeled transcript with a 30-bp complementary region at the 3' end was also generated. The two RNA substrates were incubated at 37°C for 60 min in the absence and presence of the wild-type p53 protein or the C<sub>311-393</sub> fragment of human p53 protein (Fig. 2A). Double-stranded RNA was produced only when the unlabeled complementary RNA strand and the wild-type p53 protein (Fig. 2A, lanes 6 and 7) or the C<sub>311-393</sub> fragment (Fig. 2A, lanes 10 and 11) were present in the reaction mixture. No double-stranded RNA products were observed in the absence of either the (unlabeled) complementary strand (Fig. 2A, lanes 4 and 8), the wild-type p53 protein (Fig. 2A, lane 5), or the C<sub>311-393</sub> fragment (Fig. 2A, lane 9). The radiolabeled RNA substrate alone without a complementary strand (Fig. 2A, lane 1) and both the labeled and the unlabeled complementary RNA substrates (Figure 2A lane 2) were hybridized in the formamide buffer as a positive control to demonstrate the annealing of the reactants only in the presence of the complementary strand (Fig. 2A, lane 2). Single-stranded DNA substrates, prepared by heat denaturing an 82-bp fragment of plasmid pBluescript SK, were also incubated at 37°C for 60 min (Fig. 2B). Similarly, large amounts of double-stranded DNA were produced only in the presence of the wild-type p53 protein (Fig. 2B, lane 4) or the C<sub>311-393</sub> fragment (Fig. 2B, lane 5). These results demonstrate that the carboxy-terminal amino acids 311 to 393 are sufficient to carry out the reassociation of nucleic acids in vitro.

To further test whether the RNA-RNA or DNA-DNA annealing activity of p53 protein is localized at the carboxy terminus, the effects of different monoclonal anti-p53 antibodies on the annealing reaction were examined (Fig. 3). Several different anti-p53 monoclonal antibodies were incubated with the C<sub>311-393</sub> fragment before the RNA or DNA substrates were added to the reaction mixture. PAb421, whose epitope is mapped to amino acids 370 to 378 (13), completely blocked the

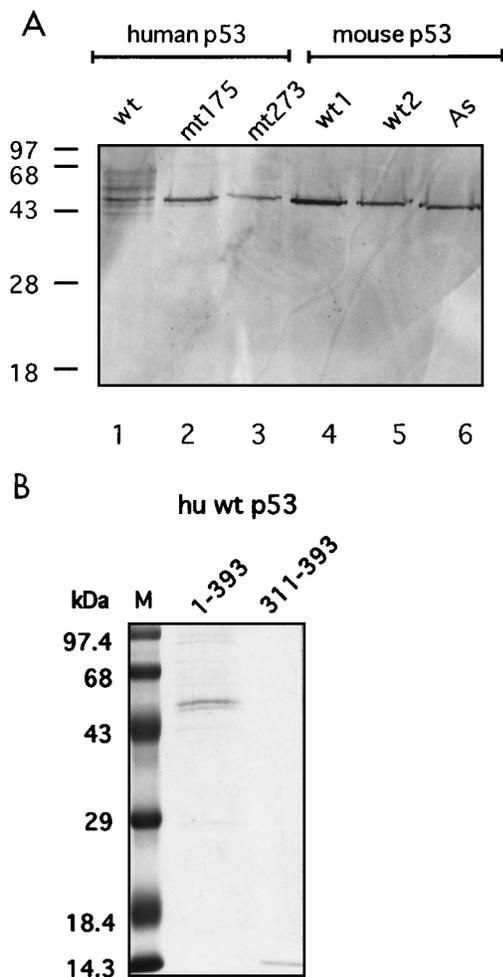


FIG. 1. The baculovirus-produced p53 proteins. The procedures of purification of human and mouse p53 proteins are described in Materials and Methods. The human (hu) wild-type (wt) p53 (A, lane 1), His-175 mutant (mt175) p53 (lane 2), His-273 mutant (mt273) p53 (lane 3), and mouse wild-type p53 (lane 4) proteins were purified by a PAb421-immunoaffinity column and eluted from the column with a solution containing the PAb421-epitope peptide. The mouse wild-type p53 (lane 5) and the AS p53 (lane 6) were purified through a PAb242-immunoaffinity column and eluted from the column with glycine buffer at pH 2.5. The  $C_{311-393}$  fragment (B) was purified as described by Pavletich et al. (30). The purified p53 proteins were loaded on the SDS-PAGE (13.5% polyacrylamide) gel and visualized by silver staining.

$C_{311-393}$  fragment's activity to promote the RNA-RNA annealing (Fig. 3A, lane 2) or DNA-DNA annealing (Fig. 3B, lane 3). However, PAb1801, PAb1620, PAb242, PAb246, and PAb248 did not significantly interfere with the  $C_{311-393}$  fragment in both annealings (Fig. 3A, lanes 3 to 5; Fig. 3B, lanes 4 to 6). All of these antibodies recognize an epitope mapped to the N terminus or middle domain of the p53 protein (2, 25, 41). The murine wild-type p53 protein (as well as the human p53 protein) also promotes DNA-DNA annealing (Fig. 3C, lane 2). Consistently, this activity was completely blocked by the binding of PAb421 (detects residues 370 to 378) (Fig. 3C, lane 3) but not by the binding of PAb242 and PAb246 (which detect residues outside of the region of amino acids 311 to 390) (Fig. 3C, lanes 4 and 5).

**Comparison of annealing activity between the wild-type p53 protein and the  $C_{311-393}$  fragment.** To determine if the carboxy-terminal fragment of the p53 protein could account for all

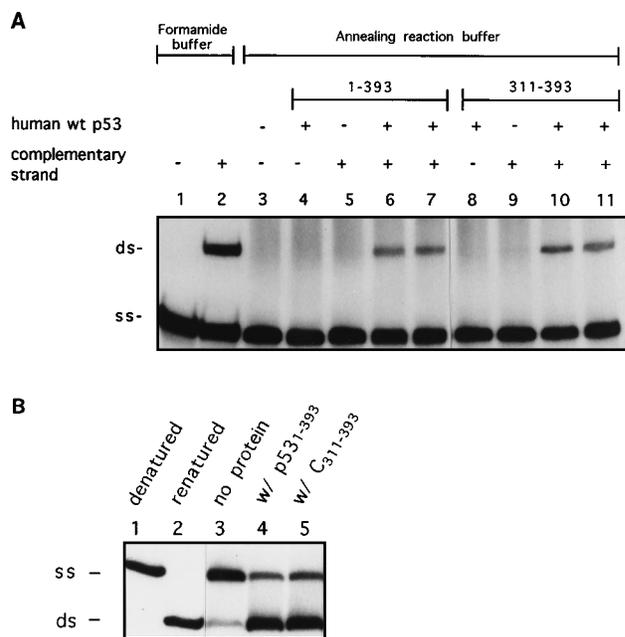


FIG. 2. Annealing of complementary single-stranded nucleic acids by the  $C_{311-393}$  fragment of human p53 protein. The annealing assays were performed as described in Materials and Methods. ds and ss indicate the positions of double-stranded and single-stranded nucleic acids, respectively. (A) RNA-RNA annealing. Lanes 1 and 2 are controls which contain substrate RNAs and hybridization of substrate RNAs in the presence of 80% formamide–0.4 M NaCl–40 mM PIPES [piperazine- $N,N'$ -bis(2-ethanesulfonic acid) (pH 6.4)–1 mM EDTA. Lanes 3 to 7 are annealing reactions promoted by the human wild-type (wt) p53 protein (10 ng per reaction mixture) at the indicated conditions. Lanes 8 to 11 are the annealing reactions catalyzed by the  $C_{311-393}$  fragment (3 ng per reaction mixture) of human p53 protein at the indicated conditions. (B) DNA-DNA annealing. Ten nanograms of the wild-type p53 protein (w/p53 $_{1-393}$ ) or 3 ng of the  $C_{311-393}$  fragment (w/ $C_{311-393}$ ) was present in the indicated reaction mixture.

of the p53 annealing activity, the initial rate of the reaction as a function of time and the extent of the reaction as a function of protein concentration were measured. Figure 4 shows a time course for the annealing of the complementary single-stranded nucleic acids by the human wild-type p53 protein and the  $C_{311-393}$  fragment at a p53 protein concentration of 10 nM. In the DNA-DNA annealing reaction (Fig. 4A), the initial rate for the wild-type p53 protein is 5.7% annealed per min, while the initial rate for the  $C_{311-393}$  fragment is 6.2% annealed per min. The wild-type p53 protein and the  $C_{311-393}$  fragment also show similar initial rates in the RNA-RNA annealing reactions (Fig. 4B). At the concentration of 500 nM (a 50-fold difference), lysozyme and cytochrome *c* do not increase the rate of formation of double-stranded DNA (Fig. 4A) and double-stranded RNA (Fig. 4B). It is well known that many different basic proteins such as lysozyme and cytochrome *c* will enhance the rate of DNA-DNA or RNA-RNA annealing by neutralizing the charges of the phosphate backbone of the nucleic acids. The fact that the carboxy-terminal domain of the p53 protein, which is quite basic, does this at least 50-fold more efficiently than other basic proteins suggests that this property might be functionally significant for the p53 protein in a cell.

The amounts of reassociated double-stranded RNA and double-stranded DNA were also measured as a function of increasing the protein concentration in the annealing assays (Fig. 5). In the DNA-DNA annealing assay (Fig. 5A), the human wild-type p53 protein and the  $C_{311-393}$  fragment, as well

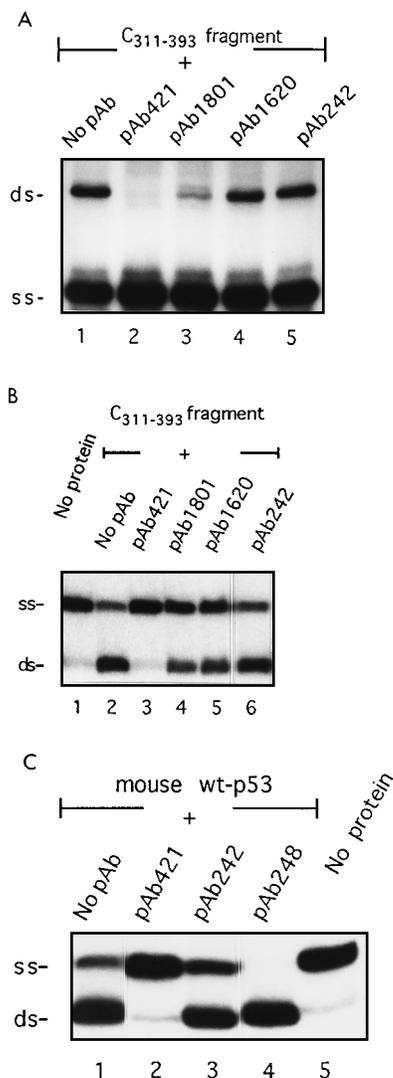


FIG. 3. The effects of anti-p53 monoclonal antibodies on the annealing activity of p53 protein. ds and ss indicate the positions of double-stranded and single-stranded nucleic acids, respectively. Various anti-p53 monoclonal antibodies (1  $\mu$ g) were added to the annealing reaction mixture containing 3 ng of the C<sub>311-393</sub> fragment or 20 ng of the mouse wild-type (wt) p53 protein. (A) RNA-RNA annealing by the C<sub>311-393</sub> fragment. Lane 1 contains no antibodies; lanes 2 to 5 contain PAb421, PAb1801, PAb1620, and PAb242 as indicated. (B) DNA-DNA annealing by the C<sub>311-393</sub> fragment. Lane 1 contains no protein, lane 2 contains the C<sub>311-393</sub> fragment only, and lanes 3 to 6 contain PAb421, PAb1801, PAb1620, and PAb246 as indicated. (C) DNA-DNA annealing by the mouse wild-type p53. The mouse wild-type p53 protein was purified through a PAb242 column and eluted from the column with glycine buffer as described in Materials and Methods. Twenty nanograms of this protein was incubated with 1  $\mu$ g of no pAb, PAb421, PAb242, or PAb248 as indicated (lanes 1 to 4) before the single-stranded DNA was added.

as the human mutant p53 proteins (His-175 and His-273), show similar activities to promote about 75% reassociation of single-stranded DNA. In the RNA-RNA annealing assay (Fig. 5B), the C<sub>311-393</sub> fragment also has activity similar to that of the wild-type p53 protein. However, neither the lysozyme nor the cytochrome *c* has the annealing activity at the range between 0 and 50 nM (Fig. 5). When the protein concentrations of lysozyme and cytochrome *c* were increased to 1.2  $\mu$ M, they started to anneal single-stranded DNA (Fig. 5A). Lysozyme also promotes RNA annealing at the micromolar range, while

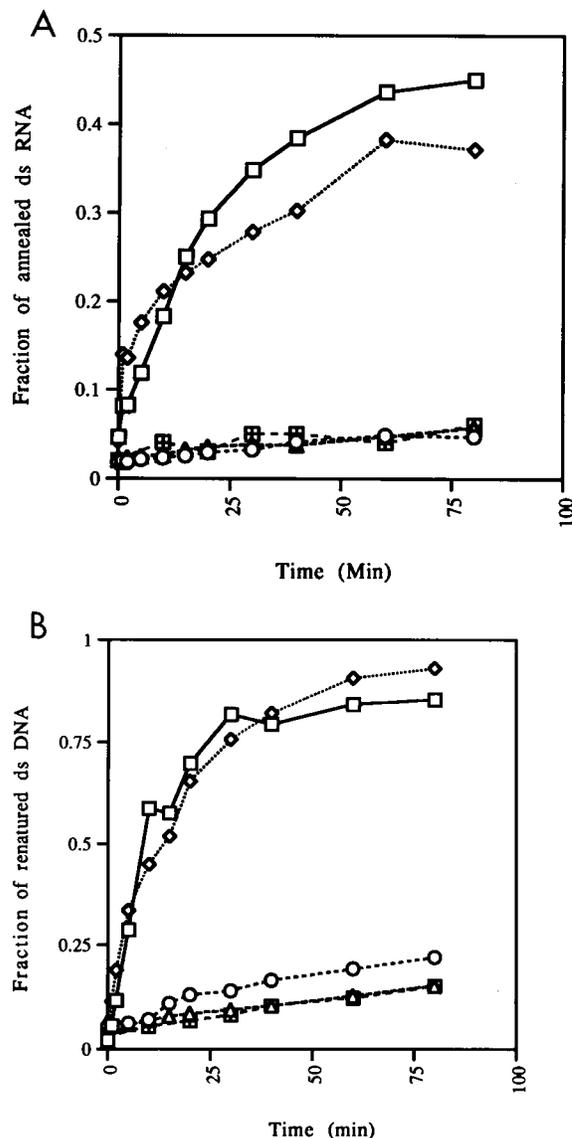


FIG. 4. Comparison of the rate of the annealing reaction promoted by the C<sub>311-393</sub> fragment with that promoted by the full-length p53 proteins. The final concentrations used in the annealing reaction mixtures are indicated as follows: wild-type p53 protein ( $\square$ ), 10 nM; C<sub>311-393</sub> fragment ( $\diamond$ ), 10 nM; cytochrome *c* ( $\circ$ ), 500 nM; lysozyme ( $\triangle$ ), 500 nM. ( $\square$ ), no protein. (A) RNA-RNA annealing. (B) DNA-DNA annealing. ds, double stranded.

cytochrome *c* promotes the association of RNA less well (Fig. 5B). These results indicate that the C<sub>311-393</sub> fragment contains all of the annealing activity of the full-length p53 protein for both the RNA-RNA and DNA-DNA reactions. In addition, the p53 protein is at least 100-fold more efficient than lysozyme in promoting RNA annealing.

**The NS and AS p53 proteins differ in their abilities to anneal RNA and DNA.** The previous experiments demonstrated that the PAb421 monoclonal antibody, which detects an epitope between amino acid residues 370 and 378, blocks the ability of the carboxy-terminal domain fragment (C<sub>311-393</sub>) from reannealing DNA or RNA. The AS form of the p53 protein deletes amino acids 364 to 390 in the NS protein and substitutes 17 new amino acids at the carboxy-terminal end of the protein. Thus, it appeared possible that the NS and AS

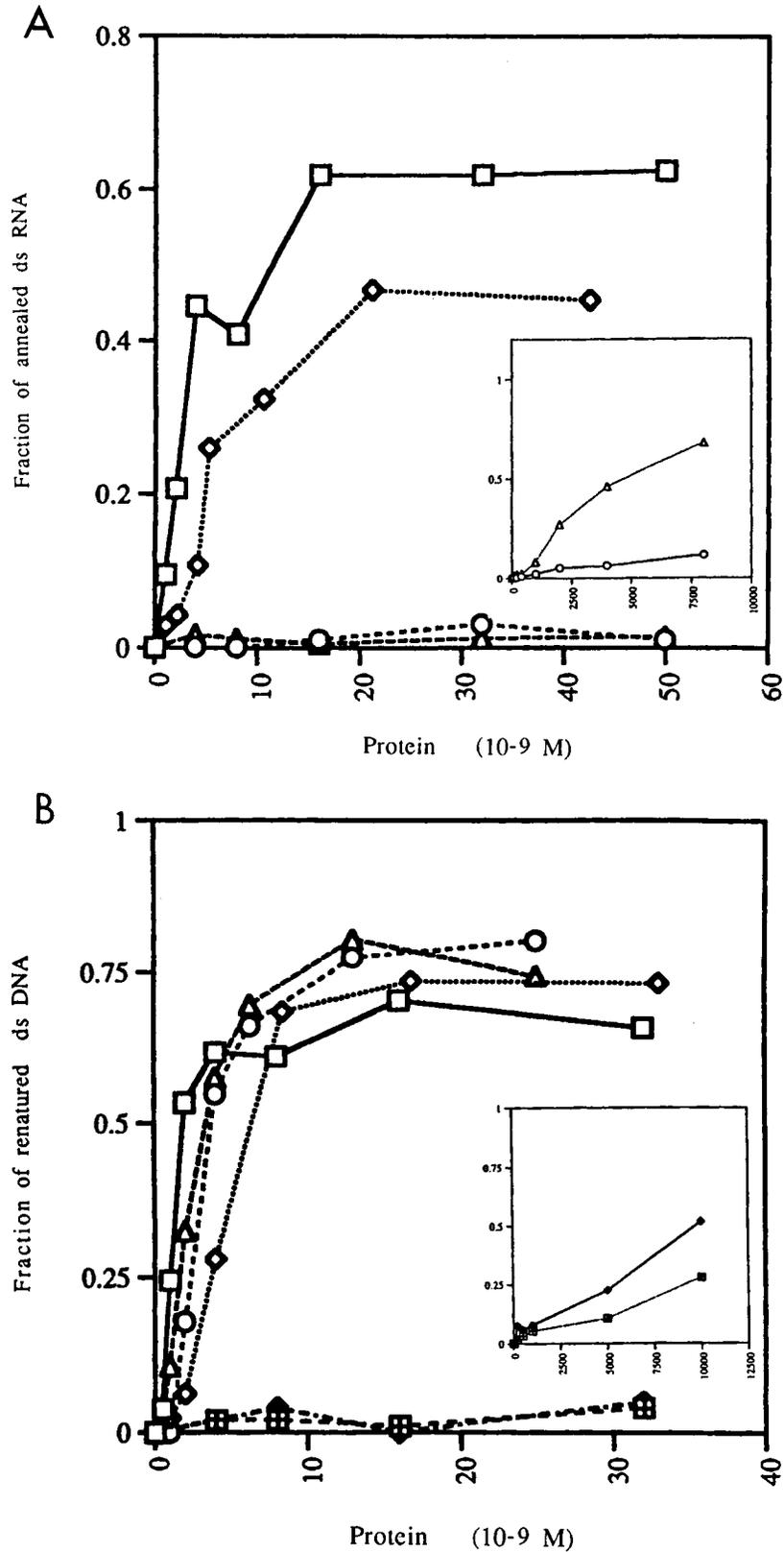


FIG. 5. Comparison of the effects of different concentrations of the C<sub>311-393</sub> fragment and full-length p53 proteins on promoting the annealing reaction. Increasing amounts of protein were used in the annealing reaction mixtures, and the types of protein used are as follows: panel A, □, wild-type p53; ◇, C<sub>311-393</sub> fragment; ○, cytochrome c; △, lysozyme; panel B, □, wild-type p53; ◇, C<sub>311-393</sub>; ○, p53-mt175; △, p53-mt273; ▣, cytochrome c; ◆, lysozyme. Each plot is the average of at least two duplicate experiments with standard deviation less than 15%. (A) RNA-RNA annealing. (B) DNA-DNA annealing. mt175 and mt273 are the His-175 and His-273 mutants, respectively. Insets show results with lysozyme and cytochrome c only. ds, double stranded.

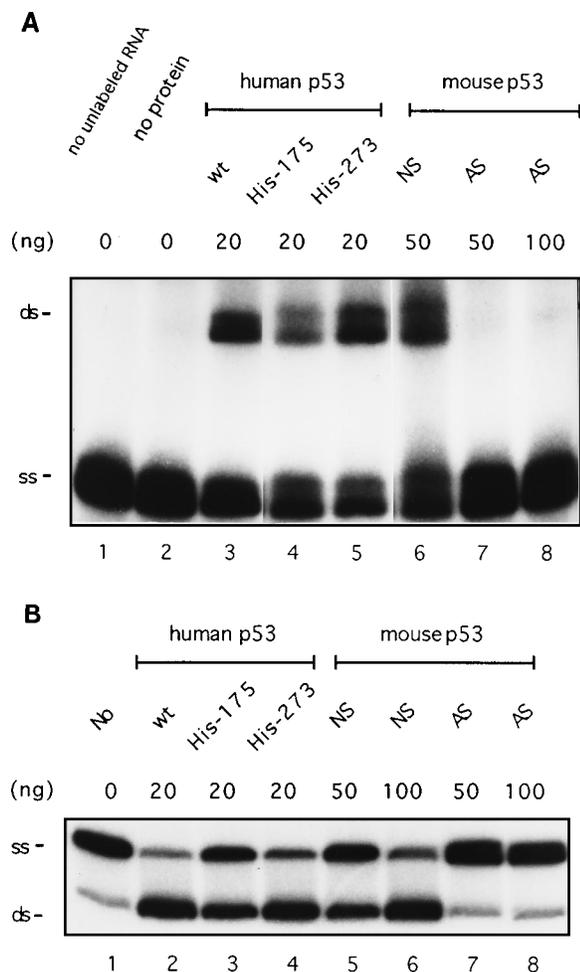


FIG. 6. Annealing of complementary single-stranded nucleic acids by the mutant p53 proteins and by the murine NS and AS forms of these proteins. The amount of each p53 protein added to each reaction mixture is indicated above each lane. ds and ss indicate the positions of double-stranded and single-stranded nucleic acids, respectively. (A) RNA-RNA annealing; (B) DNA-DNA annealing. wt, wild type.

forms of the p53 protein could differ in their abilities to promote the association of RNA or DNA. Both the AS and NS forms of the murine p53 protein were synthesized in baculovirus expression vectors and purified as described in Materials and Methods. For comparison, these murine p53 proteins were assayed with the human p53 wild-type protein and two different p53 mutant proteins (codon 175 and codon 273 mutants) commonly found in cancerous cells. The human wild-type and mutant p53 proteins were efficient in promoting the formation of double-stranded RNA (Fig. 6A, lanes wt, His-175, and His-273) or DNA (Fig. 6B, lanes wt, His-175, and His-273). Similarly, the murine NS wild-type protein efficiently promoted the formation of double-stranded RNA (Fig. 6A, mouse NS) or DNA (Fig. 6B, mouse NS). By contrast, the AS form of p53 completely failed to promote the reassociation of RNA or DNA (Fig. 6, mouse AS). Thus, the carboxy-terminal 27 amino acids of p53, found in the NS but not the AS form of the protein, are critical for the reassociation of RNA and DNA mediated by the p53 protein. The fact that different splice forms of p53 have these distinct properties suggests that this activity is functionally meaningful and regulated.

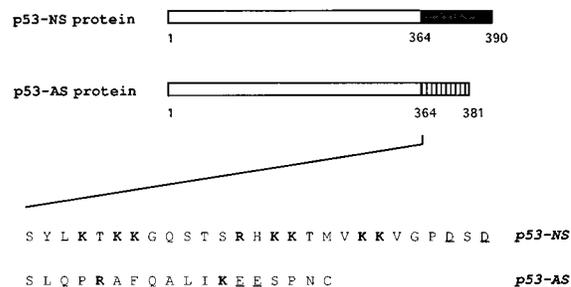


FIG. 7. Amino acid sequences of the NS and AS forms of the p53 protein. The sequences are presented in the one-letter code for amino acids.

## DISCUSSION

Two previous publications have demonstrated that the p53 protein binds to single-stranded DNA and the ends of DNA and can promote the reassociation of single-stranded RNA or DNA to a double-stranded form (1, 29). The significance of these observations, however, remained in doubt because the carboxy terminus of the p53 protein is quite basic and many basic proteins have these activities in common. Basic proteins neutralize the phosphate backbone charges of RNA or DNA and enhance the rate or extent of reannealing. In addition, it remained unclear just what such a function could contribute to the p53 protein, which is a sequence-specific transcription factor (9, 17, 44) that responds to DNA damage (16, 22).

The results presented in this communication begin to answer some of these questions. First, the ability of the p53 protein to promote the reassociation of complementary strands of RNA or DNA can be localized to the carboxy-terminal domain of the protein between residues 311 and 393. This domain is functionally equivalent to the entire protein in this property, both in its rate and extent of DNA or RNA annealing. Two lines of evidence further localize a critical portion of the reannealing activity to the last 27 amino acids of the p53 protein. First, the monoclonal antibody PAb421, which recognizes amino acid residues 370 to 378, blocks the ability of the C<sub>311-393</sub> C-terminal domain fragment of p53 to promote either DNA or RNA reannealing. This could result from blocking critical amino acids in that epitope from functioning or could be due to steric blocking of amino acids outside of the epitope. As such, this is not definitive evidence. Second, the p53 protein containing the AS, which substitutes 17 new amino acids for the last 27 residues in the p53 NS protein (Fig. 7), fails to promote the reassociation of double-stranded DNA or RNA in vitro. While this experiment also localizes the last 27 amino acids of p53 NS as critical in the reassociation reaction, it does not eliminate the possibility that the 17 new amino acids block other p53 residues (residues 311 to 367) from promoting the reassociation of DNA or RNA. These experiments do, however, demonstrate a critical role for the carboxy-terminal 27 residues of the NS p53 protein in this function.

Two lines of evidence suggest that the ability of p53 NS to bind to single-stranded DNA or RNA without any sequence preference is indeed a physiologically meaningful observation. First, other basic proteins such as lysozyme or cytochrome *c* require micromolar concentrations of protein to enhance the rate or extent of nucleic acid hybridization (Fig. 5A or B). The p53 protein acts optimally at 10 to 20 nM. This suggests a real distinction between p53 and proteins that just neutralize the phosphate backbone of RNA or DNA. In addition, the PAb421 epitope peptide (27) composed of 14 amino acids, 7 of which are basic (residues 370 to 383), fails to promote the

reassociation of complementary RNA or DNA in the 20 nM concentration (results not presented). Clearly then, the carboxy-terminal domain of p53 and in particular the last 27 residues of the NS protein contain specificity for this reaction. The second reason to favor this idea is that only one (the NS form) of the p53 splice forms acts to enhance the reassociations of RNA or DNA. The fact that these two splice forms of p53 appeared to be regulated distinctly in a cell (18) suggests that functional differences between the AS forms of p53 could well be meaningful.

What could this function of the carboxy-terminal domain of p53 accomplish in the cell? p53 plays a role in DNA damage control; recognition of DNA ends or single-stranded DNA generated as a product of repair could promote reannealing and prevent abnormal recombination events. In the absence of p53, protein gene amplifications, which likely arise from unequal crossover events, increase dramatically (20, 42). In response to DNA damage, p53 levels increase (22), and so the ability to bind to DNA ends or single-stranded intermediates could result in a stabilization of the p53 protein, which normally has a very short (20-min) half-life. It should be pointed out, however, that mutant forms of the p53 protein detected in cancerous cells (His-175 and His-273 mutants) are not in the least defective for enhancing the reassociation of DNA or RNA. This function may not be related to the cancerous phenotype of p53. This analysis also fails to explain why the efficiencies of annealing of p53 to two single strands of RNA or DNA were similar (Fig. 4 and 5). When p53 levels increase after DNA damage, it is possible that p53 or p53-mdm-2 protein complexes (27) may act upon RNA templates in transcription, ribosome biogenesis, or even the translation of mRNA. An RNA anti-helicase activity could regulate these events, and under some circumstances, p53 proteins have been readily detected in the cytoplasm (26).

This communication localizes a new function to the carboxy-terminal domain of the p53 protein; the ability to enhance the rate of reassociation of RNA and DNA. This function can be regulated by one of the AS forms (p53-AS) of the protein, which fails to carry out this activity. The fact that these two AS forms of the protein have distinct functional activities does indeed suggest that the ability of p53 to enhance the rate of RNA and DNA reannealing is a functionally significant property of this protein.

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