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Complex Formation between p53 and Replication Protein A Inhibits the Sequence-Specific DNA Binding of p53 and Is Regulated by Single-Stranded DNA

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Human replication protein A (RP-A) (also known as human single-stranded DNA binding protein, or HSSB) is a multisubunit complex involved in both DNA replication and repair. Potentially important to both these functions, it is also capable of complex formation with the tumor suppressor protein p53. Here we show that although p53 is unable to prevent RP-A from associating with a range of single-stranded DNAs in solution, RP-A is able to strongly inhibit p53 from functioning as a sequence-specific DNA binding protein when the two proteins are complexed. This inhibition, in turn, can be regulated by the presence of various lengths of single-stranded DNAs, as RP-A, when bound to these single-stranded DNAs, is unable to interact with p53. Interestingly, the lengths of single-stranded DNA capable of relieving complex formation between the two proteins represent forms that might be introduced through repair and replicative events. Increasing p53 concentrations can also overcome the inhibition by steady-state levels of RP-A, potentially mimicking cellular points of balance. Finally, it has been shown previously that p53 can itself be stimulated for site-specific DNA binding when complexed through the C terminus with short single strands of DNA, and here we show that p53 stays bound to these short strands even after binding a physiologically relevant site. These results identify a potential dual role for single-stranded DNA in the regulation of DNA binding by p53 and give insights into the p53 response to DNA damage.

As the many cellular pathways that pass through or emanate from the phosphoprotein p53 are identified and defined, it becomes more and more obvious that proper regulation of p53 protein activity is a very important determinant in the normal cellular life cycle (for reviews, see references 11, 19, 29, and 31). When the regulation is in place, growth arrest or apoptotic pathways initiated by p53 can help monitor and eliminate errant growth; when this regulation is perturbed, establishment and maintenance of aberrant cellular growth can result. Wild-type p53 is a sequence-specific DNA binding protein that can activate transcription from promoters containing p53 response elements both in vitro and in vivo (for a review, see reference 53). But when the DNA binding ability of the protein is lost, as it is in many of the mutant forms of p53 found in human cancers, the protein loses the sequence-specific transcriptional activation capabilities otherwise inherent to the protein.

The transcriptional activation function of wild-type p53 can be temporarily or permanently disrupted when the protein associates with any one of several viral oncogenes (3, 46, 47, 60), some cellular proteins (58), or even mutated forms of p53 itself (28); all of these different associations can lead to marked changes in the regulation of cell growth. DNA binding by p53 may be further regulated through conformational shifts within the protein or stabilization of the protein brought about by transient or permanent interactions between p53 and either small peptides (25, for review see references 24 and 35), cellular proteins (33), or single-stranded DNA (ssDNA) (26). In addition, an alternate form of p53 that possesses enhanced DNA binding abilities and performs potentially unique functions has been identified (5, 30, 57). It is likely, therefore, that a potent mechanism of regulating cellular pathways dependent on wild-type p53 activity involves the regulation of the DNA binding ability of p53.

gadd45 (27), *WAF1/CIP1* (13), *mdm2* (2, 44, 58), the cyclin G gene (40), and *bax* (38, 48, 59) are among the growing list of target genes that can be activated by p53 when the protein is bound to specific sequences in the respective promoters of these genes. They are particularly interesting because their gene products have potential relevance in cellular growth arrest and/or apoptotic pathways. GADD45 has been shown to bind proliferating-cell nuclear antigen in vivo (51). WAF1 inhibits the activity of cyclin-dependent kinases necessary for cell cycle progression and interferes with the DNA replicative functions of proliferating-cell nuclear antigen (16, 54), and although the role of p53 in apoptosis is proving much more complicated (20, 45), the *bax* gene product binds bcl-2, demonstrating a potential mechanism for p53 in transcription-dependent regulation of apoptosis.

Beyond the role of p53 in transcription, many lines of evidence suggest that p53 may be directly involved in the regulation of DNA replication: p53 is localized to replication foci in herpesvirus-infected cells (56), p53 is able to inhibit polyomavirus replication in vitro in a DNA binding-dependent manner (37), a C-terminal truncated form of p53 that is constitutively active for DNA binding inhibits nuclear DNA replication in transcription-free DNA replication extracts from *Xenopus* eggs (9), p53 can bind ssDNA ends and catalyze DNA renaturation (1, 7), and p53 is able to complex with the heterotrimeric ssDNA binding protein replication protein A (RP-A) (12, 21, 32). This last association is particularly striking because of the role RP-A plays in both DNA replication and repair. RP-A is a required factor for the initiation and elongation stages of DNA replication both in vitro (36, 55) and in vivo (15), and cells with deletions in any of the three subunits are not viable. It is the major ssDNA binding protein seen in mammalian cell extracts (49), and it is the 70-kDa subunit that is responsible

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for this binding (14); it is also this largest subunit that interacts with p53 (12). Although RP-A levels stay constant throughout the cell cycle, the p34 subunit of RP-A may be responsible for regulating the activities of the heterotrimer through its phosphorylation at the G_1/S transition and dephosphorylation during mitosis (10, 41); however, evidence is mixed on this point (18, 42). ssDNA binding by the heterotrimer may be an important event in this regulation, as in vitro, phosphorylation of p34 occurs only after the association of RP-A with ssDNA (15). The function of the 11-kDa subunit is unknown; however, it is necessary for complex formation between the p34 and p70 subunits (52). RP-A also functions in homologous recombination (39) and nucleotide excision repair (NER) (22). Potentially important to its role in excision repair, RP-A binds specifically to the xeroderma pigmentosum damage-recognition protein (XPA) (34) and the endonuclease XPG (22). Beyond RP-A's role in replication and repair, there is now evidence that RP-A may be directly involved in the transcriptional activation of some genes (50).

Because p53 and RP-A are involved in similar aspects of cellular growth (although with a possibly antagonistic relationship) and because both proteins depend on DNA binding for much of their activities, it was interesting to investigate whether the association between the two affected the DNA binding ability of either or both proteins. It has been previously reported that RP-A is not able to bind ssDNA when the RP-A is bound to an N-terminal fragment of p53 fused to glutathione *S*-transferase (12). However, we felt it was appropriate to reevaluate and extend these observations, using full-length proteins in assays designed to give insight into the potential physiological ramifications of the interaction between p53 and RP-A.

MATERIALS AND METHODS

Purification of proteins. Sf-21 cells were infected with human p53 recombinant baculovirus, harvested 48 h postinfection, and extracted, and full-length p53 protein was immunopurified essentially as described previously (17). Influenza virus peptide-tagged p53Δ30 was constructed by PCR using an oligonucleotide corresponding to fixed 5′ and 3′ endpoints (positions 1 and 363, respectively) of the p53 coding sequence, resulting in the second residue of p53 being affixed to the influenza virus sequence: MGYPYDVPDYA. Human central core p53(96-312) was a gift from N. Pavletich and was prepared as described previously (43). RP-A proteins were purified from exponentially growing 293 cells essentially as described previously (36). 293 cells (5×10^5 cells/ml) were lysed in hypotonic buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dithiothreitol [DTT]) and spun, and supernatant was passed over a phosphocellulose column equilibrated in buffer A (25 mM Tris-HCl [pH 7.5], 1 mM Na_2EDTA, 10% glycerol, 0.01% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM DTT) with 0.2 M NaCl. The flowthrough was passed over a Q Sepharose column equilibrated in buffer A8 (25 mM Tris-HCl [pH 8.0], 1 mM Na_2EDTA, 10% glycerol, 0.01% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM DTT) with 0.1 M NaCl. The column was eluted with a linear gradient of buffer A8 (0.1 to 0.6 M NaCl). RP-A protein peak fractions were then passed over an ssDNA cellulose column equilibrated in buffer A containing 0.5 M NaCl, washed with buffer A containing 0.75 M NaCl, and eluted with buffer A containing 1.5 M NaCl and 50% ethylene glycol. Peak fractions were pooled and dialyzed into buffer A containing 20% glycerol.

EMSA. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (8). The WAF1 p53 binding site-containing fragment was prepared by cloning the synthetic WAF1 DNA binding site (5′-AAT TCT CGA GGA ACA TGT CCC AAC ATG TTG CTC GAG-3′) into the *Eco*RI site of Bluescript vector. This plasmid was digested with *Acc*I and *Sac*I to generate the 115-bp fragment which was then labeled with the Klenow fragment of *Escherichia coli* DNA polymerase. This probe (1 ng) was used in the EMSA. RP-A and p53 proteins were added to the probe-containing reaction mixture simultaneously. The mixture was incubated for 30 min at room temperature, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) was performed at 4°C. The gels were then dried and exposed to autoradiographs. Phosphorimaging was performed with a Molecular Dynamics PhosphorImager. Oligonucleotides of specified sizes (19, 30, 40, and 70 nucleotides; Operon Technologies) were used as ssDNA substrates (5′-CGC CCA CGG ATC TGA AGG G-3′, 5′-CCA GCT GCA GCT CCA CTG GAT GGA GAA TAT-3′, 5′-GAT CGA ATT CTC AGT CAT CGT CAG GCC CTT CTG TCT TGA AC-3′, 5′-GAT CAG ATC TAC CAT GGG CTA CCC ATA CGA TGT TCC AGA TTA CGC GTC GAA CAC CAG CCA GA-3′). These probes were either labeled with T4 polynucleotide kinase, phenol-chloroform extracted, and passed over a G-50 column or used without labeling as described in text. Labeled ssDNA probe (1 ng) was used in the EMSAs. The SCS p53 binding site was prepared by annealing oligonucleotides that represent a consensus binding site for p53 (5′-TCG AGC CGG GCA TGT CCG GGC ATG TCC GGG CAT GTC-3′).

Protein binding assay. Protein A-Sepharose beads were rocked with the p53 monoclonal antibody 1801 supernatant containing approximately 1 μg of antibody for 2.5 h at 4°C. Approximately 1 μg of p53 was incubated separately with 1 μg of RP-A in 30 μl of reaction mixture containing mock extract from Sf-21 cells and ssDNA where noted. After a 30-min incubation, mixtures were added to washed beads and the samples were rocked for 2 h at room temperature. Beads were then washed four times in buffer A, and samples were run on 10% polyacrylamide gels. The gels were transferred to nitrocellulose by Western transfer, and the nitrocellulose was probed with 1801 antibody specific for p53 or 9H8 antibody specific for the p34 subunit of RP-A. Antibody for RP-A was kindly provided by Z.-Q. Pan (Mount Sinai Medical Center, New York, N.Y.).

RESULTS

p53 does not inhibit ssDNA binding by RP-A. In order to address the functional implications of the p53–RP-A interaction, we designed experiments utilizing the EMSA. We first tested the ability of RP-A to bind a ss labeled oligomer of 70 nucleotides over a range of RP-A concentrations, in the presence and absence of p53. The reaction mixtures each contained 25 ng of purified p53 protein, and this amount of p53 was able to bind a labeled fragment containing the p21/WAF1 binding site (Fig. 1A, compare lanes 4, 7, 10, and 13 with lane 14). The ssDNA was in molar excess to the RP-A at the lowest concentration of RP-A, approximately equimolar in the middle concentration range, and less than equimolar at the highest RP-A concentration. p53 was in molar excess to RP-A (ranging between a 25-fold and 1.5-fold excess) and ssDNA at all points. ssDNA binding by RP-A was not inhibited in the presence of p53 at any of the RP-A concentrations tested. Even at the lowest RP-A concentration, when ssDNA binding is at a minimum and the molar ratio of p53 to RP-A is the highest, the ability of RP-A to bind the ss probe was unaffected by p53 (compare lanes 2 and 3). It was, however, of interest to us that there seemed to be a slight inhibition of WAF1 binding by p53 in the presence of RP-A (compare lanes 14 and 13).

RP-A has been reported to bind ssDNAs containing as few as 8 to 12 nucleotides, although the preferred binding length of one heterotrimer is postulated to be approximately 30 nucleotides (49). As the length of the strand increases, RP-A binds with greater affinity and EMSA reveals new forms representing multiple RP-A heterotrimers bound to individual strands (6). It was therefore possible that RP-A binding to different lengths of ssDNA might be selectively inhibited by p53. We examined RP-A binding to oligomers of 19, 40, and 70 nucleotides at very low concentrations of RP-A over a curve of p53 concentrations, reasoning that if an inhibition were to be seen it would be at the highest ratio of p53 to RP-A (Fig. 1B). We found that even the weak binding to the 19-mer was unaffected by high concentrations of p53, even as the molar ratio of p53 to RP-A approached 100 to 1. Although p53 was able to slightly inhibit RP-A binding to the 40-nucleotide DNA, reaching a maximum of approximately 25% inhibition at the highest level of p53 tested, we feel that this is not significant. p53 was also unable to inhibit the ability of RP-A to recognize and bind the 70-nucleotide ssDNA. We also tested the effect of p53 on RP-A binding to ssDNA in the presence of unlabeled p53 binding site DNA on the chance that p53 may have altered or enhanced abilities when bound to its consensus site. However, the presence of a p53 binding site in the reaction mixture had no effect (data not shown).

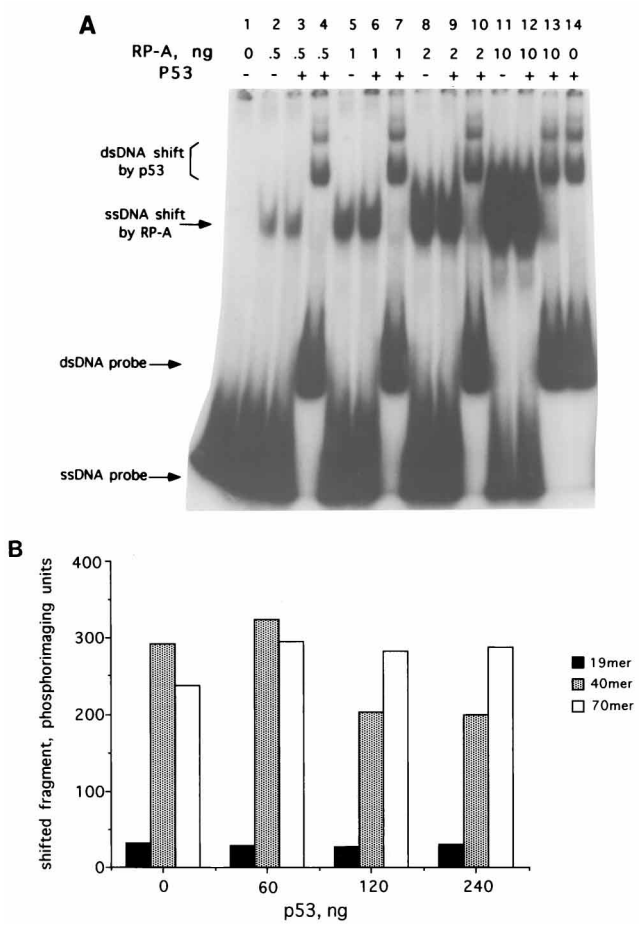

FIG. 1. p53 is not able to inhibit ssDNA binding by RP-A. (A) RP-A, at the indicated concentrations, was incubated with 70-mer ^{32}P-end-labeled ssDNA in the absence (lanes 2, 5, 8, and 11) or presence (lanes 3, 6, 9, and 12) of 25 ng of p53. A ds fragment containing the WAF1 site was used as a control for p53 binding efficacy (lanes 4, 7, 10, 13, and 14). (B) Phosphorimaging analysis of EMSA using ^{32}P-labeled ssDNA probes of 19, 40, and 70 nucleotides. RP-A (1 ng) was used to bind oligonucleotides in the absence of p53 or in the presence of increasing amounts of p53 protein as indicated.

RP-A is unable to complex with p53 in the presence of ssDNAs. One explanation of the result described above is that when RP-A is complexed with ssDNA it is physically unable to interact with p53 and is therefore immune to any inhibitory effect of p53–RP-A complex formation. We explored this possibility based on the published observation that when labeled ssDNA and RP-A are passed over a column with immobilized p53, only RP-A that is not associated with ssDNA complexes with p53 (12). This result suggested either that p53 blocks RP-A from binding ssDNA or that ssDNA blocks RP-A from associating with p53. Based on our previous experiment, the latter prospect warranted investigation. In order to test whether ssDNA does in fact disrupt the interaction between p53 and RP-A, protein binding assays were performed in the absence and presence of ssDNA. The p53 antibody 1801 was linked to protein A-Sepharose beads and incubated with a reaction mixture containing 1 μg of RP-A, 1 μg of p53, and increasing amounts of ssDNAs of various lengths as noted. Equimolar concentrations of ssDNAs of each length were used in the concentration curves, and the highest level of ssDNA represents a point where the DNA and proteins are approximately equimolar. The samples were subjected to SDS-PAGE,

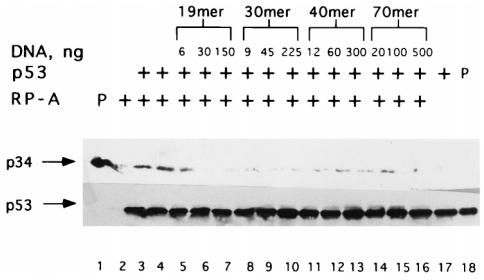

FIG. 2. ssDNAs inhibit complex formation between p53 and RP-A. Shown are the results of Western analysis of the RP-A p34 subunit and p53 protein brought down by p53 antibody bound to protein A-Sepharose beads. Incubation was performed in the presence or absence of ssDNA of either 19, 30, 40, or 70 nucleotides. Equimolar amounts of the 19-, 30-, 40-, and 70-nucleotide single strands were used as shown. P (lanes 1 and 18) indicates purified protein run as a marker.

and RP-A levels were determined by probing with antibody specific for the p34 subunit of RP-A (Fig. 2). The p34 subunit is a good marker, as it is only brought down when p53 associates with the p70 subunit of a fully intact RP-A heterotrimer. The ability of RP-A to complex with p53 was markedly inhibited by the presence of the 19-mer (lanes 5 to 7), 30-mer (lanes 8 to 10), 40-mer (lanes 11 to 13), and 70-mer (lanes 14 to 16). Complex formation between p53 and RP-A was not affected by nonspecific double-stranded DNA (dsDNA) (data not shown). ssDNA does not affect the level of p53 binding to the beads, confirming that only the interaction between p53 and RP-A is altered by the presence of ssDNA (Fig. 2).

RP-A inhibits the ability of p53 to bind the WAF1 p53 binding site. Although p53 was unable to inhibit RP-A's ssDNA binding ability, the possibility still remained that RP-A might have some effect on the DNA binding ability of p53. The p53 binding site identified in the WAF1 promoter has been previously demonstrated to be a strong p53 recognition sequence (13), and p53 binding to (as well as activation of transcription from) this site in vivo appears to be important in damage response and subsequent cell cycle regulation by p53. We therefore chose the WAF1 site as the p53 binding site for our EMSA. As expected, p53 was able to shift fragments containing the WAF1 site very well (Fig. 3A, lanes 2, 8, and 9). However, in the presence of RP-A the ability of p53 to bind its cognate site was markedly inhibited (lanes 3 to 6). With 50 ng of RP-A (representing a twofold molar excess of RP-A to p53), the inhibition of sequence-specific DNA binding by p53 reaches 75%. This did not appear to be a result of competition for the site by RP-A, because (i) RP-A bound dsDNA very poorly (lane 7), (ii) there is nonspecific dsDNA in the reaction, and (iii) the labeled fragment is in obvious excess. RP-A was also able to inhibit p53 binding to 40-bp fragments generated by annealing oligomers representing the WAF1, GADD45, and RGC p53 binding sites, demonstrating that this effect is not specific to the WAF1 site but may represent a more universal regulation of p53 site-specific binding by RP-A (data not shown). However, because the effect of free single strands in these annealed mixtures complicated the analysis, we chose to limit the study to the WAF1 purified fragment.

It has been previously shown that RP-A interacts with the N terminus of p53, although C-terminal contacts were also suggested (12). We therefore chose to examine the effect of RP-A on p53 deletion mutants to further prove that the direct interaction of RP-A with p53 is responsible for the inhibition of p53 site-specific DNA binding. The influenza-tagged p53Δ30 protein lacks the final C-terminal 30 amino acids but retains the

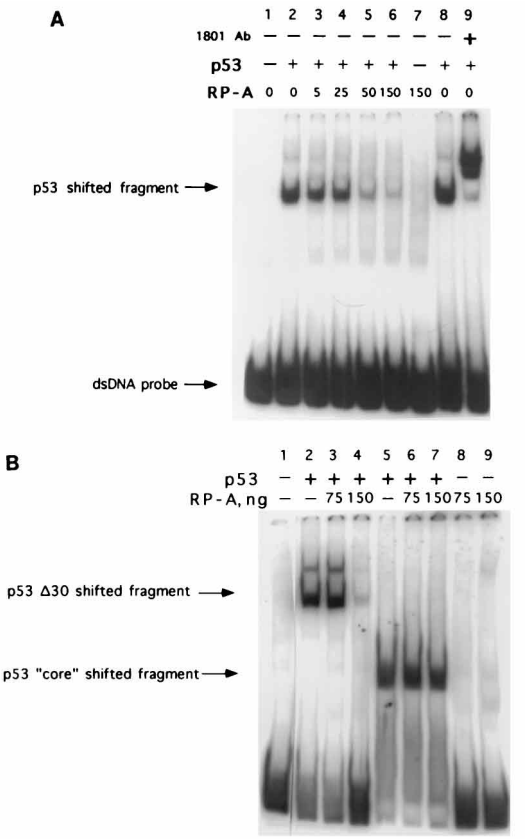

FIG. 3. RP-A inhibits the sequence-specific binding ability of wild-type p53 through interacting with the N terminus. (A) Gel mobility shift analysis of p53 (10 ng) binding to a 115-bp ^{32}P-labeled fragment containing the WAF1 site was carried out in the presence of increasing amounts of RP-A as indicated (measured in nanograms) (lanes 3 to 6). The fragment is shifted as a result of p53 binding as shown by antibody supershift with 1801 (compare lanes 8 and 9). (B) RP-A inhibits the sequence-specific binding of p53Δ30 lacking the C terminus but does not inhibit the p53 core which lacks the N-terminal and C-terminal regions. Gel mobility shift analysis of p53Δ30 (10 ng) binding to a 115-bp ^{32}P-labeled fragment containing the WAF1 site was carried out in the presence of increasing amounts of RP-A as indicated (lanes 2 to 4). This was also done with the p53 core protein containing amino acids 96 to 312 (lanes 5 to 7). RP-A binding to the WAF1 fragment was also tested (lanes 8 and 9).

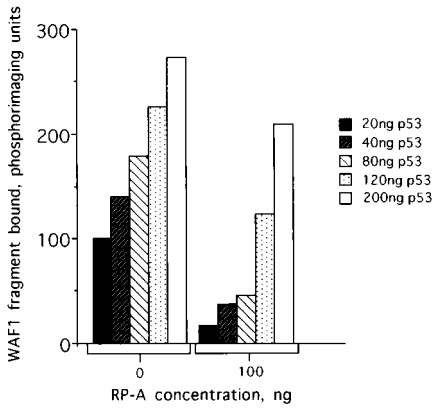

FIG. 4. The concentration curve of p53 in the presence of RP-A shows the restoration of p53 binding to the WAF1 fragment as the ratio of p53 to RP-A increases. Gel mobility shift analysis of p53 binding to a 115-bp ^{32}P-labeled fragment containing the WAF1 site was carried out over a range of p53 concentrations in the absence or presence of 100 ng of RP-A. Phosphorimaging analysis of DNA-protein complexes was performed by a typical EMSA. Inhibition of p53 binding by RP-A was approximately 80% at the lowest p53 level and 20% at the highest p53 level.

full DNA binding region and the activation domain of the N terminus. The protein bound the WAF1 site well (Fig. 3B, lane 2) but was completely inhibited from binding by RP-A (lane 4). The relative lack of inhibition at the intermediate level of RP-A compared with wild-type p53 suggests that, at lower RP-A concentrations, C-terminal contacts may increase the affinity of RP-A for p53; however, at higher concentrations the N-terminal contacts suffice for full binding and inhibition. The "core" deletion contains only amino acids 96 to 312, and thus lacks both the C-terminal and N-terminal portions of the protein. It too was able to bind DNA well (lane 5); however, it was unaffected by increasing concentrations of RP-A (lanes 6 and 7). This was expected, as the core protein lacks the regions that have been implicated in complex formation between p53 and RP-A and should therefore be immune to any effect mediated through protein-protein interaction.

The ability of RP-A to inhibit p53 binding to WAF1 can be overcome as p53 concentration is increased. p53 levels are known to increase in cells that have been subjected to DNA-damaging events, and transcription from genes containing p53 response elements then occurs. We tested whether the inhibition of p53 binding by RP-A could be overcome by increasing p53 concentrations (Fig. 4). We observed that p53 was able to overcome the inhibition by RP-A as the concentration of p53 increased (compare the magnitude of inhibition by RP-A at 20 ng of p53 with that at 200 ng of p53). This result is significant, because while RP-A levels within the cell stay constant, p53 levels fluctuate in response to cellular events. This fluctuation may be more extreme with regard to local concentrations of p53 and RP-A in the vicinity of repair events (see Discussion). The observation that the ability of RP-A to inhibit p53 binding depends on the ratio of RP-A to p53 further demonstrates that the inhibition by RP-A is due to a stable protein-protein interaction with a strict stoichiometry that can be tipped back and forth to favor inhibition by RP-A at low p53 concentrations or binding by p53 at high p53 concentrations. It is also interesting that because the binding of p53 is restored to near normal levels at the highest p53 concentration, the overall effect of RP-A on the p53 binding curve is actually to make it much more responsive to p53 levels by magnifying the fold increase in DNA binding as p53 concentrations are raised.

RP-A is not able to inhibit p53 binding to WAF1 in the presence of ssDNA. Because RP-A functions within cells as a strong ssDNA binding protein and because of our previous finding that ssDNA can disrupt complex formation between p53 and RP-A in protein binding assays, we felt it would be interesting to examine the effect of ssDNA on the ability of RP-A to inhibit p53 binding to the WAF1 site. This prospect was especially intriguing in a physiological sense, because both DNA damage and DNA replication can produce ssDNA that would attract RP-A and it is known that p53 activity (as well as DNA binding) is responsive to both DNA damage and cell cycle events. We examined the affect of RP-A on p53 binding at two concentrations of p53 in the absence of ssDNA (Fig. 5A, lanes 2 to 5 and lanes 6 to 9) and in the presence of ssDNA (lanes 12 to 15 and lanes 16 to 19). The addition of unlabeled ssDNA to our assay completely abolished the ability of RP-A to inhibit p53 binding to the WAF1 site. Or, to look at the same result in a different way, the appearance of ssDNA in a mixture containing p53 and RP-A increases binding of p53 to the WAF1 site by approximately 50-fold (compare lanes 5 and 15). This stimulation appears to be due completely to ssDNA in-

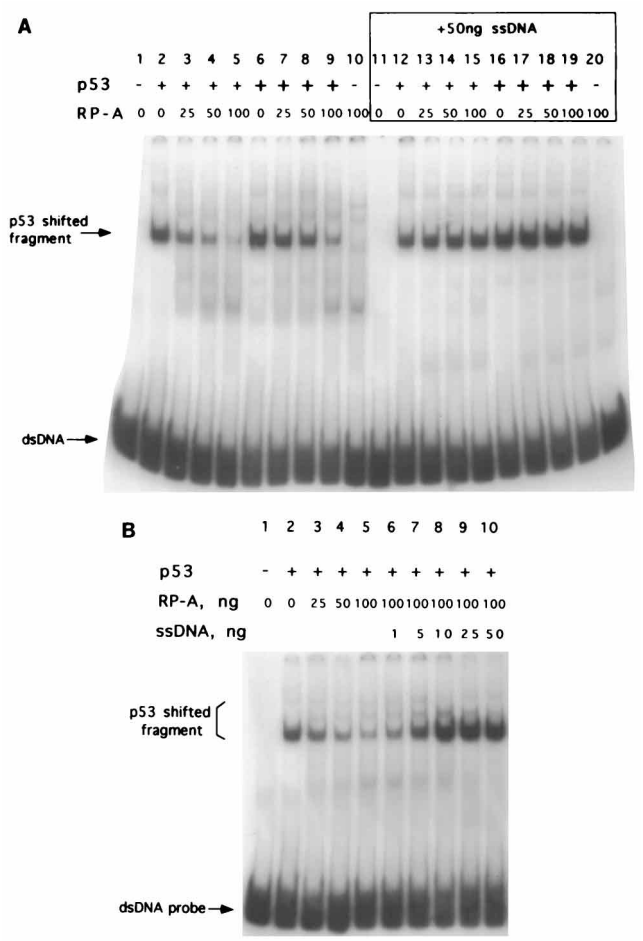

FIG. 5. RP-A is not able to inhibit p53 sequence-specific DNA binding in the presence of ssDNA. (A) Gel mobility shift analysis of p53 binding to a 115-bp ^{32}P-labeled fragment containing the WAF1 site was carried out in the presence of increasing amounts of RP-A as indicated by using either 10 ng of p53 (lanes 2 to 5 and lanes 12 to 15) or 20 ng of p53 (lanes 6 to 9 and lanes 16 to 19). Incubations were done in the absence (lanes 1 to 10) or presence (lanes 11 to 20) of 50 ng of unlabeled ssDNA of 70 nucleotides. (B) Titration of ssDNA into an EMSA reaction mixture containing p53 and RP-A shows restoration of p53 binding. Gel mobility shift analysis of 15 ng of p53 binding to a 115-bp ^{32}P-labeled fragment containing the WAF1 site was carried out in the presence of increasing amounts of RP-A as indicated (lanes 2 to 5). Increasing amounts of unlabeled ssDNA of 70 nucleotides were then added to the mixture (lanes 6 to 10).

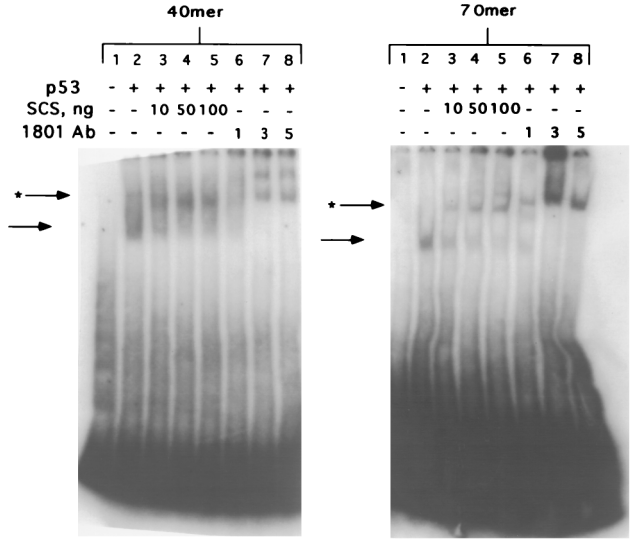

FIG. 6. p53 is able to bind site-specific dsDNA and ssDNA simultaneously. ^{32}P-labeled ssDNA of 40 and 70 nucleotides were used as probes for p53 binding (in lane 2 for each gel, the arrow indicates the p53 shifted single strand). Increasing concentrations of a p53 binding site (SCS) were titrated into the reaction mixture as shown (lanes 3 to 5 for each gel). The supershifted form is indicated by an arrow with an asterisk. The monoclonal pAb1801 was used to confirm that the mobility-shifted ssDNA fragment contained p53 (lanes 6 to 8 for each gel).

teracting with RP-A, as the same concentration of this ssDNA was unable to stimulate p53 binding to WAF1 in the absence of RP-A (compare lanes 2 and 12 or lanes 6 and 16). We next repeated this result over a broad curve of ssDNA concentrations and found that ssDNA can begin to restore p53 binding at the lowest concentration tested and that 10 ng was able to completely restore p53 binding to normal levels (Fig. 5B, compare lanes 2 and 8). This amount of ssDNA is approximately equimolar to the amount of RP-A in the reaction mixture. In this case, we did see a small fold stimulation of p53 binding by ssDNA that went beyond the starting levels (compare lanes 2 and 8 to 10).

p53 is capable of binding ssDNA and DNA containing the WAF1 p53 binding site simultaneously. It has been shown previously that ssDNA can stimulate p53 site-specific binding, possibly by inducing the formation or stabilizing the existence of the DNA binding-competent conformation of p53 (26). Because our assays suggest that p53 and RP-A are unable to interact when RP-A is bound to ssDNA, it seems clear that the relief of inhibition of p53 DNA binding in the presence of ssDNA is due primarily to this lack of complex formation between p53 and RP-A. However, it is possible that ssDNA additionally affects p53 DNA binding in a specific manner, so we felt it would be interesting to examine the DNA binding of p53 in the presence of both ssDNA and p53 binding site-containing dsDNA. When labeled dsDNA containing a p53 binding site was used as the probe, increasing concentrations of ssDNA showed no effect on the retardation of the protein-bound fragment (data not shown) (25). However, when labeled ssDNA was used as the probe, increasing concentrations of cold dsDNA containing a p53 consensus site (SCS) supershifted the fragment represented by p53 bound to ssDNA of either 40 or 70 nucleotides (Fig. 6, lanes 2 to 5 for each gel). This demonstrated that p53 could bind both nonspecific ssDNA and ds p53 binding site DNA simultaneously. It also suggests that while all p53 bound to ssDNA favors the additional binding of dsDNA site specifically, p53 which is already bound site specifically to dsDNA may be resistant to ssDNA binding. The p53-specific antibody, pAb1801, was used to show that the shifted fragment represents p53 binding (lanes 6 to 8 for each gel). While the EMSA did not show a significant overall increase in p53 site-specific DNA binding caused by ssDNA, as was seen in DNase I footprinting assays (26), it is possible that the quality of binding is changed in some way (see Discussion).

DISCUSSION

As the regulatory role of p53 in cell cycle control, apoptosis, damage response, and damage repair has become elucidated, it has also become clear that p53 can itself be regulated in a posttranslational manner through its interaction with cellular or viral proteins, through phosphorylation, or through contact

with ss- or dsDNA. Indeed, induction of p53 function in response to some types of DNA damage appears to have post-translational components that act by stabilizing the protein (extending its normally short half-life) and/or by increasing its sequence-specific DNA binding activity. Once stabilized and activated for site-specific binding, p53 becomes a multipotent protein capable of participating in numerous pathways as a transcriptional activator of damage response genes. However, it is also possible to envision nontranscriptional roles for p53 in the regulation of cell growth that depend on the direct interaction between p53 and other proteins, especially proteins involved in DNA replication or repair. One potential mechanism for growth arrest by p53 is provided by the observation that when p53 is complexed with RP-A, RP-A is unable to bind ssDNA. In this scenario, RP-A would be unable to perform its function in DNA replication when p53 levels become elevated, and growth arrest would result. One troubling aspect of this mechanism is that mutant p53 also complexes with RP-A (12), and it is well documented that DNA replication, and therefore RP-A function, proceeds unimpaired in cells containing high levels of mutant p53. We feel that our current study helps resolve this contradiction and provides new insights into the relationship among p53, RP-A, and ssDNA.

In our ssDNA binding studies using EMSA, we found no evidence that p53 inhibits ssDNA binding by RP-A. This was true over a range of p53 and RP-A concentrations and with several different sizes of ssDNA. While our protein binding studies confirm the interaction between p53 and RP-A, we found that p53 and RP-A are strongly inhibited from interacting by the presence of ss- but not dsDNA. The fact that the p70 subunit of RP-A is responsible for interactions between both ssDNA and p53 suggests that the manners in which it binds these two are mutually exclusive, with RP-A exhibiting a preference for ssDNA over p53.

But our most striking result came when we examined the affect of RP-A on the ability of p53 to bind dsDNA site specifically. RP-A strongly inhibits this, and the inhibition is dependent on the ratio of RP-A to p53 and could therefore be modulated by rising or falling levels of p53. In fact, this observation could be one explanation for the observed latency of p53 in cells. p53 levels would have to rise above a critical threshold before the inhibition by RP-A was overcome. In confirmation of our protein binding studies, RP-A is unable to inhibit the DNA binding of p53 in the presence of ssDNA. This relief of inhibition appears to be due completely to ssDNA binding by RP-A and presumedly blocks any interaction between RP-A and p53. Therefore, in a DNA damage situation, even as p53 levels increase within the cell, the recruitment of RP-A to sites of DNA damage and ssDNA would release previously bound p53, thus magnifying the response by p53 to situations of cellular stress. Although RP-A is more abundant than p53 in cells, we feel that our current study uses molar ratios of the two proteins that are physiologically relevant. Localization of the proteins to sites of repair and replication is likely to produce intracellular inconsistencies in their concentrations that would not be revealed by gross analysis of cell extracts. In this way, we believe that the results of this study represent a potential model for activation of p53 and p53 response genes by processes such as NER (Fig. 7). Further, during NER a fragment of 30 nucleotides is excised, and the potential proximity of p53 to this event (because of its interaction with RP-A) and the affinity RP-A would have for filling the gap left behind would make p53 available to bind this ssDNA fragment (Fig. 7D). Once bound to this fragment, p53 could have an increased site-specific binding affinity, as has been previously reported based on a footprint analysis of p53 binding site-containing

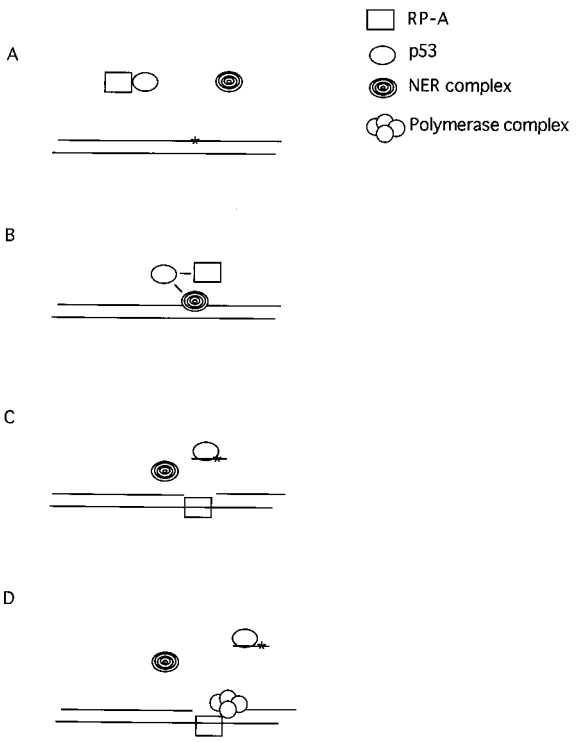

FIG. 7. Model for liberation of p53 from RP-A after DNA damage. (A) p53 is complexed with RP-A and remains unable to bind DNA sequence specifically in normal cells until DNA damage occurs (*). (B) Affinity of p53 and RP-A for elements of the repair machinery localize the p53–RP-A complex to the site of DNA damage. (C) Affinity of RP-A for the ss gap region of DNA liberates p53, freeing it to bind DNA sequence specifically. Binding by p53 to the NER-excised single strand may stimulate the sequence-specific binding of p53 or may inhibit a free RP-A molecule from complexing with p53. The increased DNA binding potential of p53 results in activation of the damage response gene (i.e., WAF1). (D) Repair synthesis begins to fill in the gap.

supercoiled DNA (26). Although we were unable to see much stimulation of DNA binding by p53 with ssDNA in the EMSA we performed, our observation that p53 can bind both ss- and dsDNA simultaneously, resulting in a supershifted fragment, suggests that the presence of ssDNA could change the quality of site-specific p53 binding rather than the quantity of p53 able to bind. p53 has been postulated to have a very high "on/off" rate in site-specific DNA binding (4). This would be visualized in a footprint assay as less binding, because while the protein was "off," DNase I could digest the temporarily unprotected DNA. If ssDNA reduced the off rate for bound p53, this would essentially appear to be a stimulation of DNA binding in a footprint assay. In an EMSA, on the other hand, the gel matrix might stabilize the protein "on" the fragment, artificially reducing the off rate and thus eliminating the potential to observe p53 binding as being affected by ssDNA. In that manner, the appearance of ssDNA in the cell, whether through damage-induced lesions and subsequent NER or possibly during replication, could potentially affect p53 in two ways: first, by liberating p53 from its complex with RP-A, thus enabling p53 to be available for site-specific binding, and second, by interacting with the C terminus of p53 and fixing the conformation of the protein in a state capable of binding DNA site specifically with a reduced off rate (Fig. 7).

It has been recently reported that nuclear injection of linearized plasmid DNA, circular DNA with a large gap, or ss circular phagemid is sufficient to induce a p53-dependent ar-

rest; however, shorter duplex molecules were less effective, and ss molecules did not induce arrest (23). It is possible, however, that in these injection experiments the short single strands were quickly degraded while the larger molecules were resistant to degradation. In that light, these data potentially support some of the predictions of our current study. It has been demonstrated in vivo that p53 and RP-A both segregate to similar sites within the nucleus of some cells (56); however, functional ramifications of complex formation between the two in vivo has yet to be directly shown. It is possible that localization of the two proteins is an important determinant in the regulation of p53 binding by RP-A, and this too may be a reason for inconsistencies between this study and the analysis of DNA substrates in microinjection experiments. It will be interesting to extend our findings into a cellular context; it is possible that p53 response to DNA damage might be slower in cells that overexpress the p70 subunit of RP-A, as would be predicted by our model. It would also be interesting to examine whether p53 response genes are more quickly activated in S-phase cells when RP-A molecules are recruited to replication forks and the ratio of RP-A to p53 is therefore reduced. Further, it would be interesting to employ the techniques in this study with other potential substrates generated by repair and replication; specifically, duplex molecules with short gaps could be used to mimic repair intermediates and the affect of these molecules on the interaction between p53 and RP-A could be assessed.

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REFERENCES

1. **Bakalkin, G., T. Yakovleva, G. Selivanova, K. P. Magnusson, L. Szekely, E. Kiseleva, G. Klein, L. Terenius, and K. G. Wiman.** 1994. p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. Proc. Natl. Acad. Sci. USA **91:**413–417.
2. **Barak, Y., T. Juven, R. Haffner, and M. Oren.** 1993. Mdm-2 expression is induced by wild-type p53 activity. EMBO **12:**461–468.
3. **Bargonetti, J., P. N. Friedman, S. E. Kern, B. Vogelstein, and C. Prives.** 1991. Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. Cell **65:**1083–1091.
4. **Bargonetti, J., I. Reynisdottir, P. N. Friedman, and C. Prives.** 1992. Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53. Genes Dev. **6:**1886–1898.
5. **Bayle, J. H., B. Elenbaas, and A. J. Levine.** 1995. The carboxyl-terminal domain of the p53 protein regulates sequence-specific DNA binding through its nonspecific nucleic acid-binding activity. Proc. Natl. Acad. Sci. USA **92:**5729–5733.
6. **Blackwell, L. J., and J. A. Borowiec.** 1994. Human replication protein A binds single-stranded DNA in two distinct complexes. Mol. Cell. Biol. **14:** 3993–4001.
7. **Brain, R., and J. R. Jenkins.** 1994. Human p53 directs DNA strand reassociation and is photolabelled by 8-azido ATP. Oncogene **9:**1775–1780.
8. **Chen, X., G. Farmer, H. Zhu, R. Prywes, and C. Prives.** 1993. Cooperative DNA binding of p53 with TFIID (TBP): a possible mechanism for transcriptional activation. Genes Dev. **7:**1837–1849.
9. **Cox, L. S., T. Hupp, C. A. Midgley, and D. P. Lane.** 1995. A direct effect of activated human p53 on nuclear DNA replication. EMBO J. **14:**2099–2105.
10. **Din, S.-U., S. J. Brill, M. P. Fairman, and B. Stillman.** 1990. Cell-cycle-regulated phosphorylation of DNA replication factor A from human and yeast cells. Genes Dev. **4:**968–977.
11. **Donehower, L. A., and A. Bradley.** 1993. The tumor suppressor p53. Biochim. Biophys. Acta **1155:**181–205.
12. **Dutta, A., S. M. Ruppert, J. C. Aster, and E. Winchester.** 1993. Inhibition of DNA replication factor RPA by p53. Nature **365:**79–82.
13. **el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
14. **Erdile, L. F., W.-D. Heyer, R. Kolodner, and T. J. Kelly.** 1991. Characterization of a cDNA encoding the 70-kDa single-stranded DNA-binding subunit of human replication protein A and the role of the protein in DNA replication. J. Biol. Chem. **266:**12090–12098.
15. **Fang, F., and J. W. Newport.** 1993. Distinct roles of cdk2 and cdc2 in RP-A phosphorylation during the cell cycle. J. Cell Sci. **106:**983–994.
16. **Flores-Rozas, H., Z. Kelman, F. B. Dean, Z.-Q. Pan, J. W. Harper, S. J. Elledge, M. O'Donnel, and J. Hurwitz.** 1994. CDK-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase δ holoenzyme. Proc. Natl. Acad. Sci. USA **91:**8655–8659.
17. **Friedman, P. N., S. E. Kern, B. Vogelstein, and C. Prives.** 1990. Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. USA **87:**9275–9279.
18. **Georgaki, A., and U. Hubscher.** 1993. DNA unwinding by replication protein A is a property of the 70 kDa subunit and is facilitated by phosphorylation of the 32 kDa subunit. Nucleic Acids Res. **21:**3659–3665.
19. **Gottlieb, M. T., and M. Oren.** 1996. p53 in growth control and neoplasia. Biochim. Biophys. Acta **1287:**77–102.
20. **Haupt, Y., S. Rowan, E. Shaulian, K. H. Vousden, and M. Oren.** 1995. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. Genes Dev. **9:**2170–2183.
21. **He, Z., B. T. Brinton, J. Greenblatt, J. A. Hassell, and C. J. Ingles.** 1993. The transactivator proteins VP16 and GAL4 bind replication factor A. Cell **73:**1223–1232.
22. **He, Z., L. A. Henricksen, M. S. Wold, and C. J. Ingles.** 1995. RPA involvement in the damage-recognition and incision steps of nucleotide excision repair. Nature **374:**566–569.
23. **Huang, L.-C., K. C. Clarkin, and G. M. Wahl.** 1996. Sensitivity and selectivity of the DNA damage sensor responsible for activating p53-dependent G_1 arrest. Proc. Natl. Acad. Sci. USA **93:**4827–4832.
24. **Hupp, T. R., and D. P. Lane.** 1994. Allosteric activation of latent p53 tetramers. Curr. Biol. **4:**865–875.
25. **Hupp, T. R., A. Sparks, and D. P. Lane.** 1995. Small peptides activate the latent sequence-specific DNA binding function of p53. Cell **83:**237–245.
26. **Jayaraman, L., and C. Prives.** 1995. Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. Cell **81:**1021–1029.
27. **Kastan, M. B., Q. Zhan, W. S. El-Deiry, F. Carrier, T. Jacks, W. V. Walsh, B. S. Plunkett, B. Vogelstein, and A. J. Fornace, Jr.** 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and *GADD45* is defective in ataxia-telangiectasia. Cell **71:**587–597.
28. **Kern, S. E., K. W. Kinzler, A. Bruskin, D. Jarosz, P. Friedman, C. Prives, and B. Vogelstein.** 1991. Identification of p53 as a sequence-specific DNA-binding protein. Science **252:**1708–1711.
29. **Ko, L. J., and C. Prives.** 1996. p53: puzzle and paradigm. Genes Dev. **10:**1054–1072.
30. **Kulesz-Martin, M. F., B. Lisafeld, H. Huang, N. D. Kisiel, and L. Lee.** 1994. Endogenous p53 protein generated from wild-type alternatively spliced p53 RNA in mouse epidermal cells. Mol. Cell. Biol. **14:**1698–1708.
31. **Levine, A. J.** 1993. The tumor suppressor genes. Annu. Rev. Biochem. **62:**623–651.
32. **Li, R., and M. R. Botchan.** 1993. The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication. Cell **73:**1207–1221.
33. **Maheswaran, S., C. Englert, P. Bennett, G. Heinrich, and D. A. Haber.** 1995. The WT1 gene product stabilizes p53 and inhibits p53-mediated apoptosis. Genes Dev. **9:**2143–2156.
34. **Matsuda, T., M. Saijo, I. Kuraoka, T. Kobayashi, Y. Nakatsu, A. Nagai, T. Enjoji, C. Masutani, K. Sugasawa, F. Hanaoka, A. Yasui, and K. Tanaka.** 1995. DNA repair protein XPA binds replication protein A (RPA). J. Biol. Chem. **270:**4152–4157.
35. **Maxwell, S. A., and J. A. Roth.** 1994. Posttranslational regulation of p53 tumor suppressor protein function. Crit. Rev. Oncog. **5:**23–57.
36. **Melendy, T., and B. Stillman.** 1993. An interaction between replication protein A and SV40 T antigen appears essential for primosome assembly during SV40 DNA replication. J. Biol. Chem. **268:**3389–3395.
37. **Miller, S. D., G. Farmer, and C. Prives.** 1995. p53 inhibits DNA replication in vitro in a DNA-binding-dependent manner. Mol. Cell. Biol. **15:**6554–6560.
38. **Miyashita, T., S. Krajewski, M. Krajewska, H. G. Wang, H. K. Lin, D. A. Liebermann, B. Hoffman, and J. C. Reed.** 1994. Tumor suppressor p53 is a regulator of *bcl-2* and *bax* gene expression in vitro and in vivo. Oncogene **9:**1799–1805.
39. **Moore, S. P., L. Erdile, T. Kelly, and R. Fishel.** 1991. The human homologous pairing protein HPP-1 is specifically stimulated by the cognate single-stranded binding protein hRP-A. Proc. Natl. Acad. Sci. USA **88:**9067–9071.
40. **Okamoto, K., and D. Beach.** 1994. Cyclin G is a transcriptional target of the p53 tumor suppressor protein. EMBO J. **13:**4816–4822.
41. **Pan, Z.-Q., A. Amin, E. Gibbs, H. Niu, and J. Hurwitz.** 1994. Phosphorylation of the p34 subunit of human single-stranded-DNA-binding protein in cyclin A-activated G1 extracts is catalyzed by cdk-cyclin A complex and DNA-dependent protein kinase. Proc. Natl. Acad. Sci. USA **91:**8343–8347.
42. **Pan, Z.-Q., C.-H. Park, A. A. Amin, J. Hurwitz, and A. Sancar.** 1995. Phosphorylated and unphosphorylated forms of human single-stranded DNA-binding protein are equally active in simian virus 40 DNA replication and in nucleotide excision repair. Proc. Natl. Acad. Sci. USA **92:**4636–4640.

43. **Pavletich, N. P., K. A. Chambers, and C. O. Pabo.** 1993. The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. Genes Dev. **7**(12B)**:**2556–2564.
44. **Perry, M. E., J. Piette, J. A. Zawadzki, D. Harvey, and A. J. Levine.** 1993. The mdm-2 gene is induced in response to UV light in a p53-dependent manner. Proc. Natl. Acad. Sci. USA **90:**11623–11627.
45. **Sabbatini, P., J. Lin, A. J. Levine, and E. White.** 1995. Essential role for p53-mediated transcription in E1A-induced apoptosis. Genes Dev. **9:**2184–2192.
46. **Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine.** 1982. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54kd cellular protein in transformed cells. Cell **28:**387–394.
47. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129–1136.
48. **Selvakumaran, M., H.-K. Lin, T. Miyashita, H. G. Wang, S. Krajewski, J. C. Reed, B. Hoffman, and D. Liebermann.** 1994. Immediate early up-regulation of bax expression by p53 but not TGFB1: a paradigm for distinct apoptotic pathways. Oncogene **9:**1791–1798.
49. **Seroussi, E., and S. Lavi.** 1993. Replication protein A is the major single-stranded DNA binding protein detected in mammalian cell extracts by gel retardation assays and UV cross-linking of long and short single-stranded DNA molecules. J. Biol. Chem. **268:**7147–7154.
50. **Singh, K. K., and L. Samson.** 1995. Replication protein A binds to regulatory elements in yeast DNA repair and DNA metabolism genes. Proc. Natl. Acad. Sci. USA **92:**4907–4911.
51. **Smith, M. L., I.-T. Chen, Q. Zhan, I. Bae, C.-Y. Chen, T. Y. Gilmer, M. B. Kastan, P. M. O'Connor, and A. J. J. Fornace.** 1994. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science **266:**1376–1380.
52. **Stigger, E., F. B. Dean, J. Hurwitz, and S.-H. Lee.** 1994. Reconstitution of functional human single-stranded DNA-binding protein from individual subunits expressed by recombinant baculoviruses. Proc. Natl. Acad. Sci. USA **91:**579–583.
53. **Vogelstein, B., and K. W. Kinzler.** 1992. p53 function and dysfunction. Cell **70:**523–526.
54. **Waga, S., G. J. Hannon, D. Beach, and B. Stillman.** 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature **369:**574–578.
55. **Waga, S., and B. Stillman.** 1994. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro. Nature **369:**207–212.
56. **Wilcock, D., and D. P. Lane.** 1991. Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes infected cells. Nature **349:**429–431.
57. **Wolkowicz, R., A. Peled, N. B. Elkind, and V. Rotter.** 1995. Augmented DNA-binding activity of p53 protein encoded by a carboxyl-terminal alternatively spliced mRNA is blocked by p53 protein encoded by the regularly spliced form. Proc. Natl. Acad. Sci. USA **92:**6842–6846.
58. **Wu, X., J. H. Bayle, D. Olson, and A. J. Levine.** 1993. The p53-mdm-2 autoregulatory feedback loop. Genes Dev. **7:**1126–1132.
59. **Zhan, Q., S. Fan, I. Bae, C. Guillouf, D. A. Liebermann, P. M. O'Connor, and A. J. Fornace.** 1994. Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. Oncogene **9:**3743–3751.
60. **Zhang, Q., D. Gutsch, and S. Kenney.** 1994. Functional and physical interaction between p53 and BZLF1: implications for Epstein-Barr virus latency. Mol. Cell. Biol. **14:**1929–1938.