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p53 Inhibits DNA Replication In Vitro in a DNA-Binding-Dependent Manner

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The p53 tumor suppressor gene product is a sequence-specific DNA-binding protein that is necessary for the G_1 arrest of many cell types. Consistent with its role as a cell cycle checkpoint factor, p53 has been shown to be capable of both transcriptional activation and repression. Here we show a new potential role for p53 as a DNA-binding-dependent regulator of DNA replication. Constructs containing multiple copies of the ribosomal gene cluster (RGC) p53 binding site cloned on the late side of the polyomavirus origin were used in in vitro replication assays. In the presence of p53, the replication of these constructs was strongly inhibited, while the replication of constructs containing a mutant version of the RGC site was not affected by p53. Several tumor-derived mutant p53 proteins were unable to inhibit replication of the construct with wild-type RGC sites. Additionally, the transactivator GAL4-VP16 was unable to inhibit replication of a construct containing GAL4 binding sites adjacent to the polyomavirus origin. We also show that the inhibition by p53 can occur from sites cloned as far as 600 bp from the origin. Preincubation experiments suggest that p53 inhibits replication at a step mediated by ATP, possibly by inhibiting the binding of polyomavirus T antigen to the core origin. The presence of an endogenous p53 binding site in the polyomavirus origin suggests potential mechanisms for the observed inhibition.

The cellular protein p53 has been implicated in the control of cell proliferation and tumor progression. In the wild-type form, p53 acts as a tumor suppressor, while several of the mutant forms display oncogenic activities (for reviews, see references 12, 30, and 43). Wild-type p53 is a sequence-specific DNA-binding protein that is able to activate transcription from promoters containing p53 response elements both in vitro and in vivo (for a review, see reference 50). In addition, p53 is able to repress transcription from many promoters that lack p53 binding sites. This is possibly accomplished through its interaction with the TATA-binding protein (33, 37, 38, 45, 48). Thus, p53 could potentially regulate growth in the cell by activating genes that repress growth or by repressing genes that activate growth or both. In sharp contrast to the wild-type protein, many of the mutant forms of p53 found in human cancers are unable to bind DNA specifically and have therefore lost transcriptional activation capabilities (50, 61).

p53 accumulates in the nucleus of cells that have undergone DNA damage and is postulated to act in a damage response pathway that can result in either cell cycle arrest at G_1 (28, 36) or apoptosis (29, 35, 59). Several genes containing p53 response elements have been identified as potential targets for activation by p53 after cellular DNA damage and may be components of the damage response pathway; GADD45 (25), WAF1/p21 (15), and mdm2 (2, 42, 58) can all potentially be activated by p53 following DNA damage.

Although p53 is clearly active in regulating transcription, there is suggestive evidence from several sources that p53 may play a role in DNA replication as well. Simian virus 40 (SV40) T antigen when complexed with p53, is unable to replicate SV40 origin-containing DNA both in vitro (17, 52) and in vivo (5), suggesting that p53 could inactivate some host cellular counterpart of the viral protein, thus inhibiting cellular replication. Further evidence for a direct interaction with the DNA replication machinery is provided by the observation that p53 is localized to foci of replication in the nuclei of herpesvirus-infected cells (55) and that a C-terminally truncated form of p53 which is constitutively active for DNA binding inhibits nuclear DNA replication in transcription-free DNA replication extracts from *Xenopus* eggs (9). Additionally, p53 binds the single-stranded-DNA-binding protein RP-A (13, 21, 31). It is therefore possible that p53 functions directly in both transcription and replication in its role as a checkpoint for cell growth. The fact that p53 binding sites have been identified in the vicinity of viral (3; this study) and putative cellular (26) origins of replication, combined with the striking loss of DNA binding among mutant p53s, suggested the possibility of a DNA-binding-dependent role for wild-type p53 in DNA replication.

It has recently been shown that p53 is able to restore polyomavirus (Py) replication in vivo at origins that lack the requisite enhancers when p53 binding sites are placed adjacent to the origin (24). This restoration is similar to that seen when other transcription factors are allowed to bind in this region (8, 18, 53). However, we believe it is interesting to look at the effect of p53 bound adjacent to a Py origin that is able to replicate in the absence of p53 because this situation may more closely mimic the way that p53 confronts cellular origins.

MATERIALS AND METHODS

Plasmids. The constructs used in this work described as Vw_nPyCAT and Vm_nPyCAT and the constructs with p53 binding sites placed at a distance from the origin described previously as PG_n-CAT, MG_n-CAT, and PG_n-MG_n-CAT (where *n* is the number of repeats of the wild-type or mutant p53 binding site derived from the ribosomal gene cluster [RGC]) (27) were kindly provided by B. Vogelstein. pJSCATori(5×17-mer), the construct containing five GAL4 sites adjacent to the Py origin and referred to as 5×GAL4-PyOri elsewhere in the text, was created by cloning the five GAL4 sites from pG5E4T (32) into the *Bgl*II site of pJSCATori (4) and was kindly provided by J. Hassell. p373.A2 carries Py A2 strain DNA inserted into the *Bam*HI site of pAT153 (34).

Proteins. Wild-type and mutant p53 proteins were immunopurified from Sf21 insect cells infected with recombinant baculoviruses as previously reported (52). Py large T antigens were also immunopurified from baculovirus-infected Sf21 cells by previously published procedures (52). Protein concentrations were esti-

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mated by the Bradford assay and silver staining of protein gels. Purification of GAL4-VP16 protein from *Escherichia coli* was done by previously published procedures (7), and protein was dialyzed into BC100 buffer (20 mM Tris [pH 8.0], 100 mM potassium acetate, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], 20% glycerol). RP-A was purified as previously described (39).

Replication of Py ori DNA in vitro. The reaction conditions and methods used for the preparation of mouse FM3A cell extracts have been described previously (52). Standard reaction mixtures (50 μl) contained 40 mM creatine phosphate (pH 7.7); 7 mM $MgCl_2$; 0.5 mM DTT; 4 mM ATP; 200 mM each CTP, UTP, and GTP; 100 mM each dATP, dGTP, and dCTP; 20 mM [α-^{32}P]dTTP; and 100 mg of creatine kinase per ml. FM3A extract (300 to 400 μg of protein), Py T antigen, and reaction mixtures were incubated at 33°C and subsequently analyzed by either acid precipitation or phosphorimaging analysis of agarose gels following *Dpn*I treatment of purified and linearized replication products (52) with a Molecular Dynamics PhosphorImager. Preincubation experiments were performed as outlined in the figure legends; 10 min of preincubation with Py T antigen was sufficient for binding of the protein to the Py origin in footprint assays under replication conditions (unpublished data), and this preincubation time was used in replication reactions when binding of Py T antigen was a prerequisite for further addition of protein.

DNase I footprint reactions. The DNase I footprint method was performed as previously described (3). A p373.A2 Py origin-containing fragment was produced by digestion with *Dde*I, filling in with ^{32}P-labeled deoxynucleoside triphosphates (dNTPs), digestion with *Hin*fI, and gel purification of the labeled fragment. This produced the end-labeled whole 408-bp origin fragment representing nucleotides 5075 to 187 in the Py genome. Reaction mixtures (50 μl) containing 40 mM creatine phosphate, 4 mM ATP, 7 mM $MgCl_2$, 0.2 mg of bovine serum albumin per ml, 0.5 mM DTT, 10 ng of plasmid pAT153, and 1 ng of 5′-^{32}P-labeled fragment were incubated with various amounts of protein at 33°C for 10 min. The amount of DNase I required to produce an even pattern of partial cleavage products was tested empirically. Following digestion, 50 μl of DNase stop solution (2 M ammonium acetate, 100 mM EDTA, 0.2% sodium dodecyl sulfate [SDS], 100 μg of sheared salmon sperm DNA per ml) was added, and the DNA was extracted with phenol-chloroform and then ethanol precipitated. The DNA was subjected to electrophoresis on denaturing 8% polyacrylamide gels.

RESULTS

p53 inhibits the in vitro replication of constructs containing multiple copies of the RGC site adjacent to the Py ori. To investigate a DNA-binding-dependent role for p53 in DNA replication, the Py in vitro replication system was used as previously described (52). The constructs, containing multiple copies of the RGC site cloned adjacent to or at a distance from the Py ori on the late side, have been used previously to demonstrate transcriptional activation by p53 both in vivo (27) and in vitro (16). DNA containing 16 copies of the wild-type site (Vw_{16}PyCAT) or 15 copies of a mutated site that does not bind p53 (Vm_{15}PyCAT) was incubated in the standard reaction mixture with increasing amounts of immunopurified wild-type human p53. Replication of Vw_{16}PyCAT was strongly inhibited by p53, while replication of Vm_{15}PyCAT was not affected (Fig. 1a, compare lanes 5 to 10 with lanes 15 to 18). Although starting levels of replication vary from experiment to experiment, we found that this inhibition occurred over a wide range of DNA and T antigen concentrations.

To confirm the need for DNA binding in the inhibition of replication by p53, tumor-derived mutant p53 proteins were used in the Py in vitro replication assay. The Trp-248 and His-273 mutant p53 proteins have been shown to have poor DNA-binding abilities and are thus unable to activate transcription from constructs containing p53 binding sites (16). The Vw_{16}PyCAT construct was used in replication mixtures containing increasing amounts of either wild-type or mutant (Trp-248 or His-273) human p53 protein (Fig. 1b). While the wild-type p53 protein strongly inhibited replication, the mutant p53 proteins were not inhibitory even at high concentrations (Fig. 1b, compare lanes 1 and 5 and 6 and 10). The slight stimulation at lower levels (200 to 400 ng) has not yet been elucidated. Taken together (Fig. 1c), our experiments show that the inhibition of replication by p53 is DNA binding dependent.

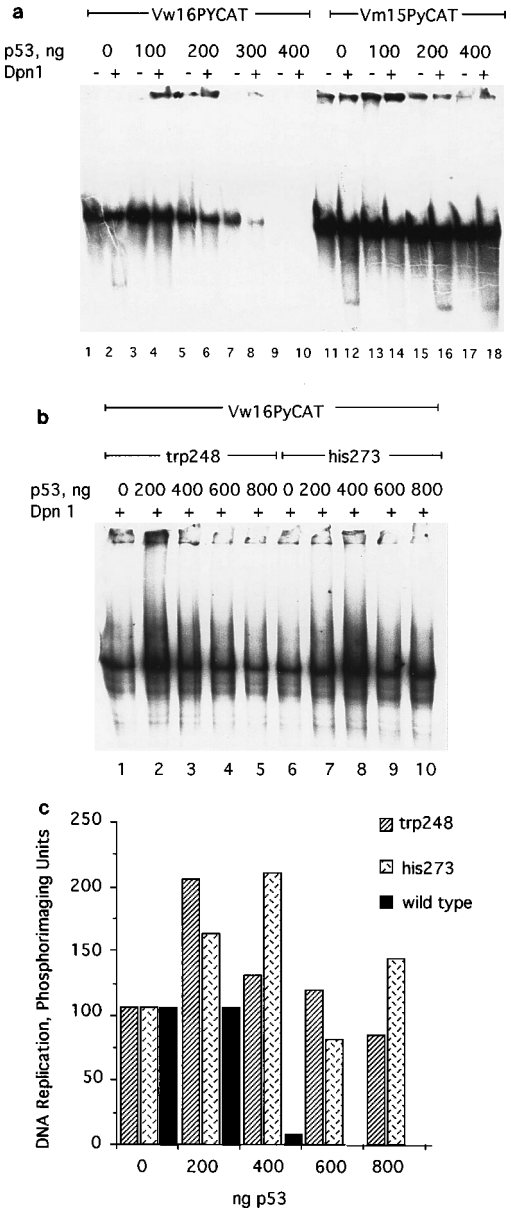

FIG. 1. (a) Wild-type p53 inhibits the in vitro replication of Py ori-containing constructs with wild-type but not mutated p53 binding sites. DNA (Vw_{16}PyCAT or Vm_{15}PyCAT) was added to reaction mixtures containing Py T antigen, murine FM3A cell extracts, and other components as described in the text. Mixtures were incubated in the presence of increasing amounts of wild-type human p53, and replicated products are shown. (b) Mutant p53 does not inhibit the in vitro replication of the Vw_{16}PyCAT construct. DNA was incubated in the presence of increasing amounts of the tumor-derived mutant p53 protein Trp-248 or His-273. *Dpn*I-resistant linearized products are shown. p53 concentrations are indicated. (c) Phosphorimaging analysis of replicated products.

p53 inhibits the replication of a construct containing four RGC sites, but GAL4-VP16 does not inhibit replication of a construct containing five GAL4 sites adjacent to the Py ori. Constructs with four RGC sites or one RGC site cloned adjacent to the Py ori were also tested in reaction mixtures containing increasing amounts of wild-type p53. Replication of the construct with one RGC site was only inhibited by p53 at very high levels of p53, at which the construct without RGC sites was also inhibited (data not shown). Inhibition of replication by p53 of the construct containing four RGC sites (Vw_4

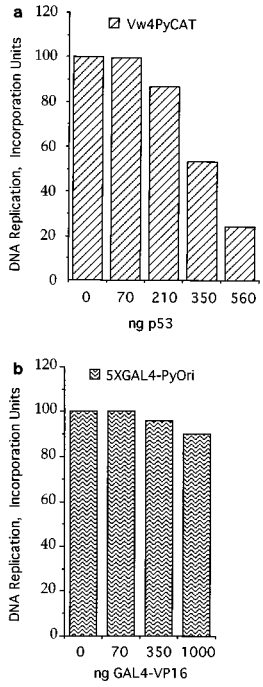

FIG. 2. p53 but not GAL4-VP16 protein inhibits Py replication when bound near the origin. (a) The Vw_4PyCAT construct, containing four copies of the wild-type p53 binding site, was added to reaction mixtures. Replication was measured by acid precipitation of replicated DNA. (b) DNA (5×GAL4-PyOri) was replicated in reaction mixtures containing increasing amounts of GAL4-VP16 protein, and replicated products were analyzed by acid precipitation of replicated DNA.

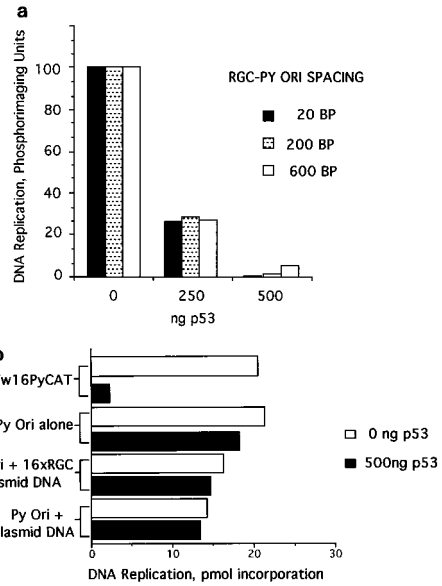

FIG. 3. p53 inhibits in vitro replication of Py ori-containing constructs with p53 binding sites placed at a distance from the origin but does not inhibit in *trans*. (a) Constructs with sites placed adjacent to (20 bp) or 200 or 600 bp from the origin were incubated under replication conditions in the presence of increasing amounts of p53. Replicated products were analyzed by phosphorimaging of the product gel. (b) Py origin-containing DNA was replicated in the presence of 200 ng of plasmid DNA containing 16 copies of the RGC site; 500 ng of p53 protein was added as indicated, and replication was measured by acid precipitation of replicated DNA. $Vw_{16}PyCAT$, Py origin plasmid alone, and Py origin plus plasmid DNA containing mutated RGC sites were present as controls.

PyCAT) was intermediate at levels of p53 that completely abolished replication of the construct containing 16 sites. At higher concentrations of p53, replication of the construct containing four sites was greatly inhibited (Fig. 2a). In this case, replication products were quantitated by acid precipitation. The results with this method are generally in close agreement with the results of autoradiographic analysis of *Dpn*I-resistant products (44). The greater inhibition of replication with more RGC sites is similar to the greater activation of transcription previously seen with more p53 binding sites (27).

It was possible that any protein bound adjacent to the Py ori could disrupt the replication process through physical hindrance of T antigen binding or simply by impeding the progress of the replication fork. To address this, a construct containing five GAL4 sites adjacent to the Py ori was used in the in vitro replication reaction with increasing amounts of GAL4-VP16 protein that had been shown to be transcriptionally active (16a). The GAL4 DNA-binding domain shows very high affinity for its cognate site, much more so in fact than the p53 protein for the RGC site (16a, 23). However, GAL4-VP16 was unable to inhibit Py replication even at very high protein concentrations (Fig. 2b). It is therefore unlikely that the inhibition by p53 is due to simple roadblock effects. The result also establishes that the inhibition by p53 is not a general effect caused by the binding of transcription factors adjacent to the origin during replication. Because we believe that the inhibition of replication by p53 of Vw_4PyCAT and $Vw_{16}PyCAT$ results from similar mechanisms, in subsequent experiments the $Vw_{16}PyCAT$ construct was used to ensure strong inhibition by p53 so that the mechanism of inhibition could be defined.

p53 can inhibit replication when bound to RGC sites at a distance from the origin but cannot inhibit in *trans*. To further examine the inhibition by p53, constructs that contained the RGC sites placed either 200 or 600 bp from the Py ori on the late side were acquired (27). The replication of these constructs was compared with that of the construct with the sites adjacent to the ori when increasing amounts of wild-type p53 were present in the reaction mixture (Fig. 3a). Surprisingly, the dose-dependent inhibition by wild-type p53 of all constructs was very similar. One possibility suggested by this result was that when p53 bound DNA, the protein underwent a conformational change (19) that enabled it to bind or sequester some replication factor, thus inhibiting DNA replication. If this were the case, then p53 should also be able to inhibit in *trans*. To test this, replication-incompetent ori-minus plasmid DNA bearing 16 copies of the RGC site was added to mixtures containing Py ori DNA in the presence of increasing amounts of wild-type p53. The concentration of p53 binding sites was made equal to that in previous experiments in which inhibition of replication by p53 was seen. p53 was not able to inhibit in *trans* (Fig. 3b). Thus, while p53 is able to inhibit from a distance of 600 bp, it must be bound to the plasmid containing the origin.

RP-A does not relieve the inhibition caused by p53. RP-A is an essential component of the machinery required to replicate ori DNA (51, 57). Since p53 binds RP-A, it was an interesting possibility that p53, when bound to a plasmid containing an origin, might be able to sequester this protein from the replication fork, thus inhibiting replication from that origin. To examine this, purified RP-A was added to replication reactions to see whether the protein could overcome the inhibitory effects of p53 (Fig. 4). RP-A greatly stimulated ori DNA replication, indicating that the FM3A murine cell extracts were limiting in this protein (Fig. 4, compare lane 1 with 4 or 7). However, replication of the construct containing p53 binding sites was still strongly inhibited by p53 (Fig. 4, compare lane 3

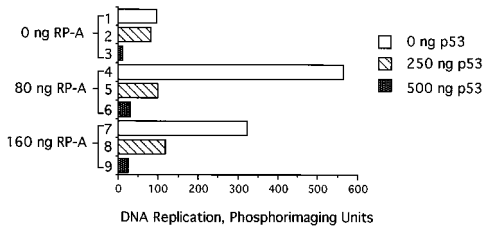

FIG. 4. RP-A does not relieve the inhibition of in vitro DNA replication caused by p53. Plasmid DNA containing copies of the RGC site adjacent to the Py origin (Vw_{16}PyCAT) was replicated in the presence of 0 (lanes 1 to 3), 80 (lanes 4 to 6), or 160 (lanes 7 to 9) ng of RP-A. Increasing amounts of p53 were then added to each set, as indicated.

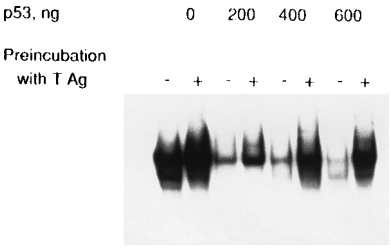

FIG. 5. Preincubation with Py T antigen relieves the inhibition of DNA replication caused by p53. Reaction mixtures which lacked deoxynucleotides were prepared. Vw_{16}PyCAT was preincubated in the reaction mixture for 20 min in the presence (+) or absence (−) of Py T antigen. p53 was then added in increasing concentrations, and Py T antigen was added to reactions initially lacking it. After 10 min of further incubation, deoxynucleotides were added, and the reactions were allowed to proceed. The autoradiogram shows *Dpn*I-resistant products.

with 6 or 9). As a general rule, we found that the higher fold inhibitions by p53 were always seen in extracts with the greatest competency. Similarly, over the course of this work, several different preparations of Py T antigen were used, and it was also true that the greatest fold inhibitions were seen with the more competent T antigen preparations.

Since p53 can bind RP-A, the possibility remained that the inhibition of replication seen by p53 in the RP-A-stimulated extract did not rely solely on sequence-specific DNA binding by p53. We therefore tested the construct containing mutated p53 binding sites in this context and found that, indeed, p53, at the highest concentration tested (500 ng), was able to inhibit replication of this construct by approximately 25% (data not shown). However, because the inhibition by p53 was significantly greater when p53 binding site-containing constructs were used (Fig. 4), it is likely that, in the context of added RP-A, inhibition by p53 has two components; one based on DNA binding, as previously seen, and one based on sequestering or inactivating RP-A in a non-DNA-binding-dependent manner.

Preincubation of DNA with T antigen relieves the inhibition by p53. The fact that RP-A did not stimulate DNA replication in the presence of p53 suggested that p53 might inhibit DNA replication at a step prior to the involvement of RP-A. The nature of the in vitro reactions allowed us to design preincubation experiments in order to predict at what step replication was being inhibited by p53. As demonstrated with SV40 ori DNA replication, when reaction mixtures are deprived of deoxynucleotides and preincubated with T antigen, the presynthesis complex forms (56), but the complex is unable to begin elongation. p53 could then be added, followed by addition of deoxynucleotides, to see if inhibition of DNA replication occurred at the elongation or initiation stage. Preincubation with T antigen was carried out for 10 min, increasing amounts of p53 were added and incubated for a further 10 min, and then deoxynucleotides were added to allow elongation. DNA replication after this treatment was compared with that in replication reactions when p53 was added without preincubation (Fig. 5). While p53 was able to inhibit DNA replication in reactions without T antigen preincubation, when T antigen was allowed to form the presynthesis complex at the origin, p53 was no longer able to inhibit replication (Fig. 5). Full restoration of replication was not seen, most likely because any initiation events occurring after the incubation with T antigen would take place in the presence of p53 and would be equally inhibited; the slight differences among levels of replicated products in the reactions preincubated with T antigen are due to experimental variation.

This preincubation experiment was repeated with the construct containing the RGC sites placed 600 bp from the origin to ensure that the mechanism of inhibition by p53 was the same in both cases, and it was (data not shown). This experimental result supports the idea that p53 inhibits replication at a step prior to stimulation by RP-A and that the inhibition is possibly linked to the prevention of T antigen binding.

p53 inhibits DNA replication at a step mediated by ATP. Further preincubation experiments were performed to more closely pinpoint the step at which p53 inhibits replication. Depriving the reaction mixture of ribonucleotides (other than ATP) allows T antigen to bind and begin unwinding the origin, but replication would cease because primase would be unable to synthesize RNA primers (reference 51 and references therein). Depriving the reaction mixture of all ribonucleotides (including ATP) would stop T antigen from being able to form the double hexamer-DNA complex at the origin (34). After these preincubations, p53 was added, and after incubation to allow binding, the withheld components were added to the reaction mixtures. While deprivation of ATP resulted in similar levels of inhibition compared with those in the situation without preincubation (Fig. 6a, compare lanes 1 and 10, 2 and 11, and 3 and 12), in the two cases when T antigen was capable of binding the origin before p53 addition, the strong inhibition of replication was significantly relieved (Fig. 6a, compare lanes 6 and 9 with lane 3 or 12; also see Fig. 6b). Once again, full restoration of replication levels would not be expected because any initiation events that occurred after the 10-min incubation would be inhibited by p53.

Endogenous p53 binding site present in the Py core origin region may influence p53 binding at adjacent RGC sites. Because the previous experiments suggested that not all 16 RGC sites were necessary and perhaps only facilitated inhibition by increasing local concentrations of p53, we were interested in directly examining in detail the binding of p53 to the constructs used in this study. To do this, we used the DNase I protection method under replication conditions on a fragment containing the origin region alone. We were surprised to discover that the Py origin contained an endogenous p53 binding site (spanning Py nucleotides 66 to 85) that overlaps part of site A (Fig. 7). Site A contains strong binding sites for Py T antigen and is indispensable for normal levels of replication (54). Examination of the region protected by p53 revealed that there is indeed a strong candidate sequence for p53 binding (Fig. 7). The consensus sequence for p53 contains two copies of the 10-mer 5′-RRRC(A/T)(T/A)GYYY-3′, where R is a purine and Y is a pyrimidine (14). The region within the Py ori that was well protected by p53 contains two such adjacent 10-mers, each with an 8 of 10 match to the p53 consensus sequence. Although p53 binding to this region could potentially inhibit

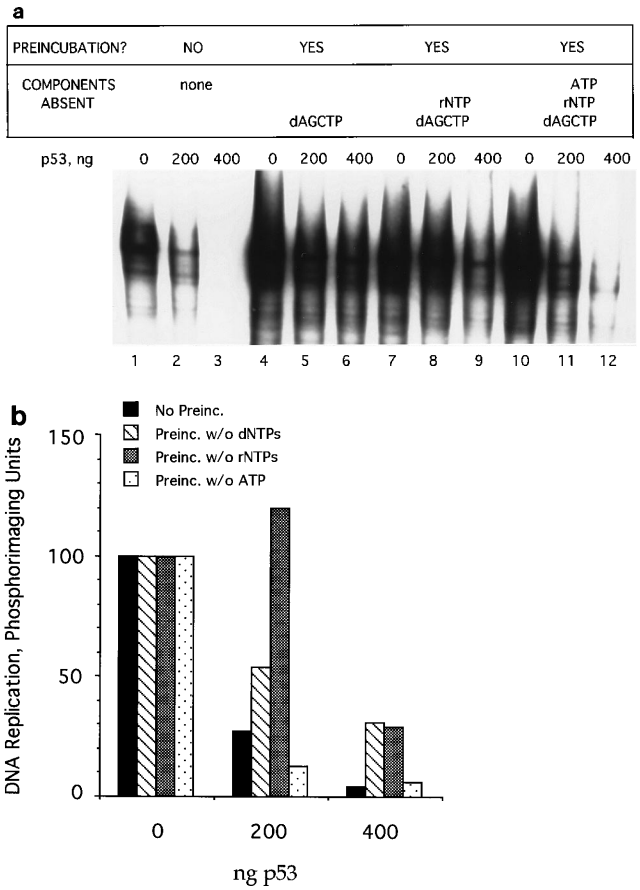

FIG. 6. p53 inhibits DNA replication at a step mediated by ATP. Vw_{16}PyCAT was added to replication mixtures as described previously with the following exception. For the preincubations, Py T antigen was incubated with the DNA and FM3A extract and all components of the reaction mixture except those indicated for 10 min. Wild-type p53 was then added, and the mixture was incubated for a further 10 min. The withheld components were then added, allowing the reaction to proceed. (a) *Dpn*I-resistant replicated products. (b) Phosphorimaging analysis and normalization of replication levels reveal relief of inhibition when Py T antigen is allowed to bind before p53 addition (preincubation without dNTPs and without rNTPs).

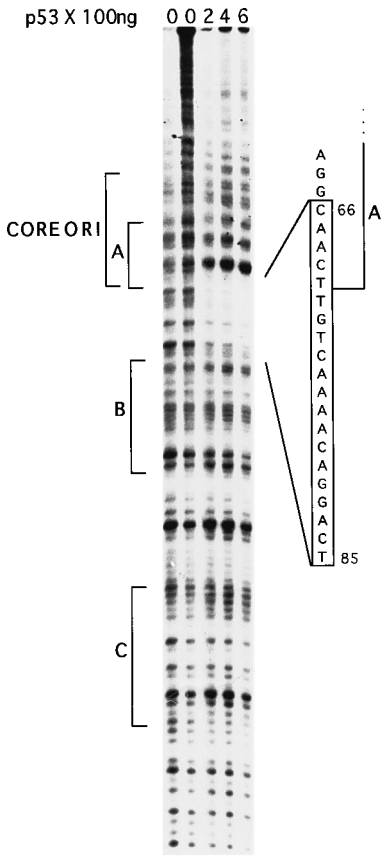

FIG. 7. The Py origin contains an endogenous p53 binding site. The DNase I fragment footprint of the Py origin region after binding by increasing levels of wild-type p53 (as indicated above lanes) is shown. Lanes 0 (no protein) show the DNase I digestion pattern at two different DNase I concentrations. Landmarks of the Py ori region are indicated at the left. The sequence within the protected site is indicated by the expanded region to the right (which also depicts the relationship of the protected site to site A of the Py ori).

binding by Py T antigen to site A, inhibiting DNA replication, we do not see inhibition of replication unless additional p53 binding sites are present in the plasmid.

We have attempted footprint anasysis of the constructs containing both the origin region and adjacent p53 binding sites; however, to date we have been unable to produce definitive results. As discussed further below, p53 molecules could bind both the endogenous site in the core origin and the adjacent p53 binding sites and then, through self-association, loop the DNA (46). This might influence DNase I digestion. We are currently working to extend our results in this area.

DISCUSSION

Transcriptional elements and transcription factors are known to play important roles in the regulation and initiation of DNA replication in many diverse biological systems (10, 22). The Py replicon has been extensively studied in this context (18, 20, 60). In this study, using the Py in vitro replication system, we have demonstrated a potential new role for p53 as a repressor of DNA replication. We have shown, in two ways, that sequence-specific DNA binding by p53 is an absolute requirement for the strong inhibition of Py replication in vitro. First, wild-type p53 is unable to inhibit replication when mutated binding sites are used. Second, tumor-derived mutants of p53 that are unable to bind DNA in a sequence-specific manner do not inhibit Py replication. GAL4-VP16, a potent transactivator that binds specifically to GAL4 binding sites, is unable to inhibit Py replication when GAL4 sites are adjacent to the Py origin, demonstrating that the inhibition by p53 is not caused simply by bound protein impeding the progress of the replication fork. Furthermore, this illustrates that the inhibition of replication that we see with p53, unlike the stimulation seen with previous in vivo studies, is not a general property of all transcription factors.

How does p53 inhibit Py ori DNA replication in vitro? At least three possibilities can be suggested: (i) modulation of DNA structure, (ii) sequestration of DNA replication factors, and (iii) DNA renaturation. Although p53 activities supporting all three possibilities have been documented, we favor the first possibility, that the presentation of the core origin is altered by p53, for the following reasons. p53 inhibits at a very early stage of the replication reaction and is also able to inhibit replication when bound to sites placed as far as 600 bp from the Py origin. This could occur through bending or looping of the DNA bound by p53. Support for the looping idea comes from our observation that there is a single p53 binding site within the Py

core origin. This site alone is clearly not sufficient for strong inhibition by p53, since constructs lacking additional p53 binding sites are not inhibited. However, the existence of this site would provide the basis for possible protein-protein interactions mediated by the DNA site within the origin and sites adjacent to or even at a distance from the origin. The subsequent distortion of the origin region could preclude the binding of replication factors. It has recently been reported that p53 is able to loop DNA containing separated p53 binding sites and that this ability may be linked to transcriptional activation (46). If activation of transcription and inhibition of DNA replication can both result from p53-mediated looping near origin regions, this suggests that p53 could simultaneously upregulate genes and downregulate origins in response to DNA damage. However, it is also possible that, through looping, the RGC sites in fact function to increase the local concentration of p53 at the endogenous site and that p53, when bound continually to this site within site A of the Py origin, is able to block Py T antigen binding and therefore replication. Continuous binding in this case would depend on high local concentrations of the protein; in vivo, it is possible that regulated forms of p53 (9) or activation by single-stranded DNA (23) could significantly enhance DNA binding, making additional sites unnecessary.

The likelihood that p53, when bound to the DNA, may sequester other components of the replication reaction is not supported by our experiments. RP-A, a replication protein shown to bind p53, did not relieve the inhibition of replication caused by p53. The physiological importance of the minor non-DNA-binding-dependent inhibition that we see by p53 in RP-A-stimulated extracts is unclear; mutant forms of p53 that do not bind DNA retain their ability to bind RP-A (13). Finally, the ability of p53 to reanneal complementary single strands (1, 16b, 41) and thus function as an antihelicase is a plausible explanation for its inhibition of DNA replication. However, since p53 reannealing is both DNA sequence independent and a property of mutant as well as wild-type p53, it is unlikely that this function pertains to the inhibition of DNA replication seen in this study. The fact that p53 can inhibit replication when its binding sites are distal to the origin also argues strongly against p53's reannealing function being relevant to the inhibition noted in our experiments.

Our results appear to be at odds with studies in which the effects of transcriptional activators bound to sites in the vicinity of the Py origin were required for Py DNA replication in vivo. The entire Py enhancer (11) or subsets of the enhancer region (6, 40, 49) have been shown to be essential for Py T antigen-mediated Py ori DNA synthesis in permissive mouse cells. Indeed, a number of heterologous transcription factors, including *Saccharomyces cerevisiae* GAL4, mammalian AP1, and hybrid transactivators such as GAL4-Jun and GAL4-VP16, can activate replication from the Py core origin in vivo when binding sites for these proteins replace the Py enhancer (18). In vitro studies with the related virus SV40 indicated that one function of transcriptional activators bound adjacent to the origin is to relieve repression or occlusion brought about by the presence of nucleosomes on templates in cells (8). Given that, in the in vitro system used in our experiments, the origin-containing template is naked DNA, while in cells the origin DNA replicates as chromatin, this may be at least one basis for the differences observed. However, in some cases, the transcriptional activation domains of the proteins were shown to be required for full activation of DNA replication (4). Another notable difference in the roles of transactivators in transcription and replication is the position independence of the former but not of the latter process (20, 47).

p53 itself was shown to be capable of activating the Py core origin when constructs containing a single p53 binding site were used in similar cell transfection studies (24). Although this would appear to directly contradict our results, it must be stressed that there are two basic differences between the effects of p53 in cell transfection and in our in vitro approaches. First, in the in vivo study, only the replication of templates containing single and not multiple p53 binding sites was compared. Second, in cells, the origin functions very poorly without an activator, while in vitro, there is no requirement for the presence of enhancer sequences (44), and in fact, there is no effect of at least some activators bound in the vicinity of the SV40 origin (8) or the Py origin (this study). Thus, in the in vivo case, p53 was tested against a background of extremely weak replication of an enhancerless ori DNA plasmid, while in vitro, the enhancerless template system has strong replication capability that is not increased by the presence of bound transactivators. Indeed, in the in vivo case, the extent to which p53 activated replication from templates bearing p53 binding sites but lacking an enhancer was markedly less than that seen when the enhancer but not p53 binding sites were present (24). Whether this suggests that the relatively lesser stimulatory effect by p53 represents the sum of p53 activation and inhibitory activities is difficult to assess. Experiments to determine whether activation by p53 is greater or less than that seen with GAL4-VP16 or whether a construct containing both the Py enhancer and p53 binding sites replicates less well than a construct with the enhancer alone are in progress.

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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 J. **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. **Bennett-Cook, E. R., and J. A. Hassell.** 1991. Activation of polyomavirus DNA replication by yeast GAL4 is dependent on its transcriptional activation domains. EMBO J. **10:**959–969.
5. **Braithwaite, A. W., H.-W. Sturzbecher, C. Addison, C. Palmer, K. Rudge, and J. R. Jenkins.** 1987. Mouse p53 inhibits SV40 origin-dependent DNA replication. Nature (London) **329:**458–460.
6. **Campbell, B. A., and L. P. Villarreal.** 1988. Functional analysis of the individual enhancer core sequences of polyomavirus: cell-specific uncoupling of DNA replication from transcription. Mol. Cell. Biol. **8:**1993–2004.
7. **Chasman, D. I., J. Leatherwood, M. Carey, M. Ptashne, and R. D. Kornberg.** 1989. Activation of yeast polymerase II transcription by herpesvirus VP16 and Gal4 derivatives in vitro. Mol. Cell. Biol. **9:**4746–4749.
8. **Cheng, L., J. L. Workman, R. E. Kingston, and T. J. Kelly.** 1992. Regulation of DNA replication *in vitro* by the transcriptional activation domain of GAL4-VP16. Proc. Natl. Acad. Sci. USA **89:**589–593.
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. **DePamphilis, M. L.** 1993. Eukaryotic DNA replication: anatomy of an origin. Annu. Rev. Biochem. **62:**29–63.
11. **de Villiers, J., W. Schaffner, C. Tyndall, S. Lupton, and R. Kamen.** 1984. Polyoma virus DNA replication requires an enhancer. Nature (London) **312:**242–246.
12. **Donehower, L. A., and A. Bradley.** 1993. The tumor suppressor p53. Biochim. Biophys. Acta **1155:**181–205.
13. **Dutta, A., S. M. Ruppert, J. C. Aster, and E. Winchester.** 1993. Inhibition of DNA replication factor RPA by p53. Nature (London) **365:**79–82.
14. **el-Deiry, W. S., S. E. Kern, J. A. Pietenpol, K. W. Kinzler, and B. Vogelstein.** 1992. Definition of a consensus binding site for p53. Nat. Genet. **1:**45–49.
15. **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.
16. **Farmer, G., J. Bargonetti, H. Zhu, P. Friedman, R. Prywes, and C. Prives.**

1992. Wild-type p53 activates transcription *in vitro*. Nature (London) **358:** 83–86.
16a. **Farmer, G., and C. Prives.** Unpublished data.
16b. **Ferrari, E., U. Hubscher, and C. Prives.** Unpublished data.
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. **Guo, Z.-S., and M. L. DePamphilis.** 1992. Specific transcription factors stimulate simian virus 40 and polyomavirus origins of DNA replication. Mol. Cell. Biol. **12:**2514–2524.
19. **Halazonetis, T. D., L. J. Davis, and A. N. Kandil.** 1993. Wild-type p53 adopts a "mutant"-like conformation when bound to DNA. EMBO J. **12:**1021–1028.
20. **Hassell, J. A., W. J. Muller, and C. R. Mueller.** 1986. The dual role of the polyomavirus enhancer in transcription and DNA replication. Cancer Cells **4:**561–569.
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. **Heintz, N. H., L. Dailey, P. Held, and N. Heintz.** 1992. Eukaryotic replication origins as promoters of bidirectional DNA synthesis. Trends Genet. **8:**376–381.
23. **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.
24. **Kanda, T., K. Segawa, N. Ohuchi, S. Mori, and Y. Ito.** 1994. Stimulation of polyomavirus DNA replication by wild-type p53 through the DNA-binding site. Mol. Cell. Biol. **14:**2651–2663.
25. **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-atelangiectasia. Cell **71:**587–597.
26. **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.
27. **Kern, S. E., J. A. Pietenpol, S. Thiagalingam, A. Seymour, K. W. Kinzler, and B. Vogelstein.** 1992. Oncogenic forms of p53 inhibit p53-regulated gene expression. Science **256:**827–830.
28. **Kuerbitz, S. J., B. S. Plunkett, W. V. Walsh, and M. B. Kastan.** 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA **89:**7491–7495.
29. **Lane, D. P.** 1993. A death in the life of p53. Nature (London) **362:**786–787.
30. **Levine, A. J.** 1993. The tumor suppressor genes. Annu. Rev. Biochem. **62:**623–651.
31. **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.
32. **Lin, Y.-S., M. F. Carey, M. Ptashne, and M. R. Green.** 1988. GAL4 derivatives function alone and synergistically with mammalian activators in vitro. Cell **54:**659–664.
33. **Liu, X., C. W. Miller, P. H. Koeffler, and A. J. Berk.** 1993. The p53 activation domain binds the TATA box-binding polypeptide in holo-TFIID, and a neighboring p53 domain inhibits transcription. Mol. Cell. Biol. **13:**3291–3300.
34. **Lorimer, H. E., E. H. Wang, and C. Prives.** 1991. The DNA-binding properties of polyomavirus large T antigen are altered by ATP and other nucleotides. J. Virol. **65:**687–699.
35. **Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne, and T. Jacks.** 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature (London) **362:**847–852.
36. **Lu, X., and D. P. Lane.** 1993. Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? Cell **75:**765–778.
37. **Mack, D. H., J. Vartikar, J. M. Pipas, and L. A. Laimins.** 1993. Specific repression of TATA-mediated but not initiator-mediated transcription by wild type p53. Nature (London) **363:**281–283.
38. **Martin, W. W., M. A. Subler, R. M. Munoz, D. R. Brown, S. P. Deb, and S. Deb.** 1993. p53 and SV40 T antigen bind to the same region overlapping the conserved domain of the TATA-binding protein. Biochem. Biophys. Res. Commun. **195:**428–434.
39. **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.
40. **Muller, W. J., D. Dufort, and J. A. Hassell.** 1988. Multiple subelements within the polyomavirus enhancer function synergistically to activate DNA replication. Mol. Cell. Biol. **8:**5000–5015.
41. **Oberosler, P., P. Hloch, U. Ramsperger, and H. Stahl.** 1993. p53-catalyzed annealing of complementary single-stranded nucleic acids. EMBO J. **12:** 2389–2396.
42. **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.
43. **Prives, C., and J. J. Manfredi.** 1993. The p53 tumor suppressor protein: meeting review. Genes Dev. **7:**529–534.
44. **Prives, C., Y. Murakami, F. G. Kern, W. Folk, C. Basilico, and J. Hurwitz.** 1987. DNA sequence requirements for replication of polyomavirus DNA in vivo and in vitro. Mol. Cell. Biol. **7:**3694–3704.
45. **Seto, E., A. Usheva, G. P. Zambetti, J. Momand, N. Horikoshi, R. Weinmann, A. J. Levine, and T. Shenk.** 1992. Wild-type p53 binds to the TATA-binding protein and represses transcription. Proc. Natl. Acad. Sci. USA **89:**12028–12032.
46. **Stenger, J. E., P. Tegtmeyer, G. A. Mayr, M. Reed, Y. Wang, P. Wang, P. V. C. Hough, and I. A. Mastrangelo.** 1994. p53 oligomerization and DNA looping are linked with transcriptional activation. EMBO J. **13:**6011–6020.
47. **Tang, W. J., S. L. Berger, S. J. Triezenberg, and W. R. Folk.** 1987. Nucleotides in the polyomavirus enhancer that control viral transcription and DNA replication. Mol. Cell. Biol. **7:**1681–1690.
48. **Truant, R., H. Xiao, C. J. Ingles, and J. Greenblatt.** 1993. Direct interaction between the transcriptional activation domain of human p53 and the TATA box-binding protein. J. Biol. Chem. **268:**2284–2287.
49. **Veldman, G. M., S. Lupton, and K. R.** 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. **5:**649–658.
50. **Vogelstein, B., and K. W. Kinzler.** 1992. p53 function and dysfunction. Cell **70:**523–526.
51. **Waga, S., and B. Stillman.** 1994. Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. Nature (London) **369:**207–212.
52. **Wang, E. H., P. N. Friedman, and C. Prives.** 1989. The murine p53 protein blocks replication of SV40 DNA in vitro by inhibiting the initiation functions of SV40 large T antigen. Cell **57:**379–392.
53. **Wasylyk, C., J. Schneikert, and B. Wasylyk.** 1990. Oncogene v-jun modulates DNA replication. Oncogene **5:**1055–1058.
54. **Weichselbraun, I., G. Haider, and E. Wintersberger.** 1989. Optimal replication of plasmids carrying polyomavirus origin regions requires two high-affinity binding sites for large T antigen. J. Virol. **63:**961–964.
55. **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 (London) **349:**429–431.
56. **Wobbe, C. R., F. B. Dean, Y. Murakami, L. Weissbach, and J. Hurwitz.** 1986. Simian virus 40 DNA replication *in vitro*: study of events preceding elongation of chains. Proc. Natl. Acad. Sci. USA **83:**4612–4616.
57. **Wold, M. S., D. H. Weinberg, D. M. Virshup, J. J. Li, and T. J. Kelly.** 1989. Identification of cellular proteins required for simian virus 40 DNA replication. J. Biol. Chem. **264:**2801–2809.
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. **Yonish-Rouach, E., D. Grunwald, S. Wilder, A. Kimchi, E. May, J.-J. Lawrence, P. May, and M. Oren.** 1993. p53-mediated cell death: relationship to cell cycle control. Mol. Cell. Biol. **13:**1415–1423.
60. **Yoshimoto, K. K., and L. P. Villarreal.** 1991. Replication dependent and cell specific activation of the polyomavirus early promoter. Nucleic Acids Res. **19:**7067–7072.
61. **Zambetti, G. P., and A. J. Levine.** 1993. A comparison of the biological activities of wild type and mutant p53. FASEB J. **7:**855–865.