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Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen

(tumor suppressor/protein–protein interaction/DNA tumor virus/eurkaryotic DNA synthesis)

PAULA N. FRIEDMAN*, SCOTT E. KERN†, BERT VOGELSTEIN†, AND CAROL PRIVES*

*Department of Biological Sciences, Columbia University, New York, NY 10027; and †Oncology Center, Johns Hopkins School of Medicine, Baltimore, MD 21231

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ABSTRACT **Murine p53 blocks many of the replication activities of simian virus 40 (SV40) large tumor antigen (T antigen) *in vitro*. As murine cells do not replicate SV40 DNA, it was of interest to determine how p53 from permissive human cells functions. Recombinant baculoviruses encoding either the wild-type form of human p53 or a mutant p53 cloned from a human tumor cell line were constructed, and p53 proteins were purified from infected insect cells. Surprisingly, we found that wild-type human p53 was as inhibitory to the ability of T antigen to mediate replication of an SV40 origin-containing (*ori* DNA) plasmid *in vitro* as was murine p53. Wild-type human p53 also blocked the DNA unwinding activity of T antigen, as did its murine counterpart. In contrast to murine and wild-type human p53, the mutant human p53 did not block *ori* DNA replication or DNA unwinding. Murine p53 formed a complex with mutant human p53 *in vivo*. Furthermore, mutant human p53 reduced the inhibition of SV40 *ori* DNA replication by murine p53 *in vitro*. These results provide a model for the way in which mutant p53 proteins can affect normal functions of p53.**

Simian virus 40 (SV40) replicates in primate but not rodent cells (reviewed in ref. 1). In both primate and nonprimate cells that express the SV40 early region, a fraction of the large tumor antigen (T antigen) is found in a complex with the cellular p53 protein (reviewed in refs. 2 and 3). SV40-transformed cells generally contain considerably higher levels of p53 than do untransformed cells, and a significant proportion of their T antigen is bound to the cellular protein. In lytically infected cells a p53–T-antigen complex also forms, but a smaller proportion of the T antigen is recruited into the complex (4). Although the reasons for the different levels of p53 in these different situations are not entirely clear, it has been proposed that in transformed cells T antigen stabilizes the p53 protein, through formation of the p53–T-antigen complex (5).

p53 is a nuclear DNA-binding phosphoprotein that associates with the 55-kDa product of the adenovirus E1B gene (reviewed in refs. 2 and 3) and also with the E6 protein encoded by human papillomavirus (6). Some forms of mutant p53 bind to the heat shock cognate protein (hsc 70, reviewed in ref. 7). Little is known about the biological activities of p53, but several lines of evidence point to the possibility that wild-type p53 is a tumor suppressor. In a high proportion of human tumors (8–12), as well as in some murine tumors (13, 14), the p53 genes have sustained deletion and/or missense mutations within regions of p53 shown to be highly conserved among all vertebrate forms examined (15). Although mutant forms of p53 can cooperate with the *ras* oncogene to transform primary diploid cells in culture (16, 17), transformation is inhibited when the wild-type p53 is also present in large quantities (18, 19). Moreover, wild-type p53 can inhibit the growth of human tumors containing p53 gene mutations (48). It has been proposed that mutant p53 genes alter cellular growth properties by sequestering and inactivating the resident normal p53 (16, 19, 20). This is supported by evidence of p53 hetero-oligomers immunoprecipitated from transfected cells (16, 19, 20). Thus the propensity for p53 to form oligomers may be highly relevant to its role in oncogenesis.

In the absence of a defined biochemical function for p53, it is difficult to assess how binding to T antigen affects p53 properties. Because the biochemical activities of T antigen are well characterized (reviewed in ref. 21), it is more straightforward to question how p53 binding affects T-antigen function. Accordingly, it has been established that murine p53 inhibits the replication function of T antigen *in vivo* (22, 23) and *in vitro* (24, 25). Murine p53 inhibits the initial stages of the replication reaction by blocking the DNA helicase and unwinding activities of T antigen (24, 25). p53 also competes with DNA polymerase α for binding to T antigen (26). Human cells, unlike murine cells, support SV40 DNA replication, and it was hypothesized that this could be attributed to functional differences in the p53 protein of the two species (23–25). In the present report, we show that wild-type human p53 does not differ from murine p53 in its ability to inhibit SV40 DNA replication. However, a mutant form of p53 derived from a human tumor lacked this inhibitory activity. Moreover, we demonstrate that complexes form efficiently between p53 proteins and that one p53 can modulate the function of another *in vitro*.

MATERIALS AND METHODS

Cells and Viruses. *Spodoptera frugiperda* insect cells (Sf27 cells) and recombinant baculoviruses vEV55SVT (SV40 T antigen) and vEV55p53 (murine p53) were kindly provided by D. O'Reilly and L. Miller (University of Georgia, Athens, GA). To generate recombinant baculoviruses expressing mutant (27) or wild-type human p53, the cDNAs of these genes were cloned into the transplacement vector pEV55, and occlusion body-negative viral isolates were obtained as described (28).

Purification of SV40 T Antigen and p53 Proteins. Sf27 cells (2.5×10^7 per 150-mm dish) were infected with either T-antigen- or p53-expressing recombinant baculoviruses at a multiplicity of infection of 1 plaque-forming unit per cell. Where coinfections were performed, viruses were used at similar multiplicities of infection. Extracts of infected cells were prepared as described (25). T antigen and p53 proteins were purified from cell lysates by immunoaffinity procedures (25) using monoclonal antibodies cross-linked to protein A-Sepharose (monoclonal antibody PAb 419 for SV40 T antigen and monoclonal antibody PAb 421 for p53 proteins). p53–T-antigen complexes were purified using PAb 419 cross-linked

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen.

to protein A-Sepharose to ensure that all the p53 present was originally bound to T antigen.

***In Vitro* SV40 *ori* DNA Replication.** HeLa extracts and replication reactions were prepared according to published procedures (29–31). Reactions were incubated at 37°C for 3 hr, and the incorporation of ^{32}P-labeled dTMP was determined by acid precipitation and scintillation counting. To characterize the reaction products, DNA was purified from replication reactions and then digested to linear form with either *Bst*XI or *Pvu* I, in the presence or absence of *Dpn* I, followed by analysis on 1% agarose gels and subsequent autoradiography.

DNA Unwinding Assay. To measure unwinding of double-stranded DNA by T antigen, published procedures were used (32, 33). Reactions (20 μl) contained 40 mM creatine phosphate (pH 7.7, di-Tris salt), 7 mM $MgCl_2$, 0.5 M dithiothreitol, 4 mM ATP, creatine kinase (100 μg/ml), 300 ng of a 346-base-pair labeled fragment that spans the SV40 origin, 300 ng of *Escherichia coli* single-stranded binding protein (Pharmacia), and 300 ng of SV40 T antigen in the presence or absence of the indicated p53 protein. Reaction mixtures were incubated at 37°C for 30 min and terminated by the addition of 10 μl of a solution containing 3% SDS, 3 mg of proteinase K per ml, and 75 mM EDTA for 30 min at 37°C, followed by electrophoresis on 8% polyacrylamide gels and autoradiography.

RESULTS

Effects of Human p53 on SV40 T-Antigen Function. Purified SV40 T antigen is capable of mediating replication of DNA containing the viral replication origin (*ori* DNA) in the presence of extracts prepared from human cells (29–31). To study the effects of human p53 on SV40 T-antigen replication functions, recombinant baculoviruses were constructed containing either wild-type p53 cDNA or mutant p53 cDNA derived from the human tumor cell line A431. The A431 p53 was the first reported human p53 sequence (27) and was thought to represent the wild-type form. Recent experiments, however, demonstrated that the histidine at codon 273 of the A431 p53 is often found as a somatic mutation in human tumor cells and that Arg-273 is the wild-type form (8). The baculovirus-produced human p53 proteins were recognized by monoclonal antibodies PAb 421 (34) and PAb 1801 (ref. 35; specific for human p53) but not by PAb 242 and PAb 248 (ref. 36; specific for murine p53) (data not shown). Equal quantities of immunoaffinity-purified wild-type or mutant human p53 or, for comparison, murine p53, were added to replication reactions, and the reaction products were analyzed by *Dpn* I digestion of linearized ^{32}P-labeled DNA isolated from reaction mixtures (Fig. 1*A*) or by acid precipitation (Fig. 1*B*). Both the murine and wild-type human p53 proteins were strongly inhibitory to the replication reaction. When either murine or wild-type human p53 was added at a molar ratio of p53 to T antigen of 2:1, SV40 DNA synthesis was inhibited ≈80%, and at a ratio of 4:1, there was no detectable replication over background. By contrast, the mutant p53 protein had virtually no effect on the accumulation of replicated SV40 *ori* DNA at all concentrations tested, even twice those of the highest levels of wild-type human p53 tested.

The initiation functions of T antigen have been well studied (for review, see ref. 21). We previously showed that murine p53 blocks these activities by inhibiting both the unwinding and the DNA helicase activity of T antigen (25). To analyze the effect of human p53 on DNA unwinding, T antigen was incubated with a α-^{32}P-end-labeled double-stranded *ori* DNA fragment in the presence of *E. coli* single-stranded binding protein and other components of the replication reaction. Under these conditions, ≈15% of the labeled double-stranded DNA was converted to single strands, consistent with previously published reports (ref. 32; Fig. 2, lane 2). In the absence of T antigen, no single-stranded fragments were

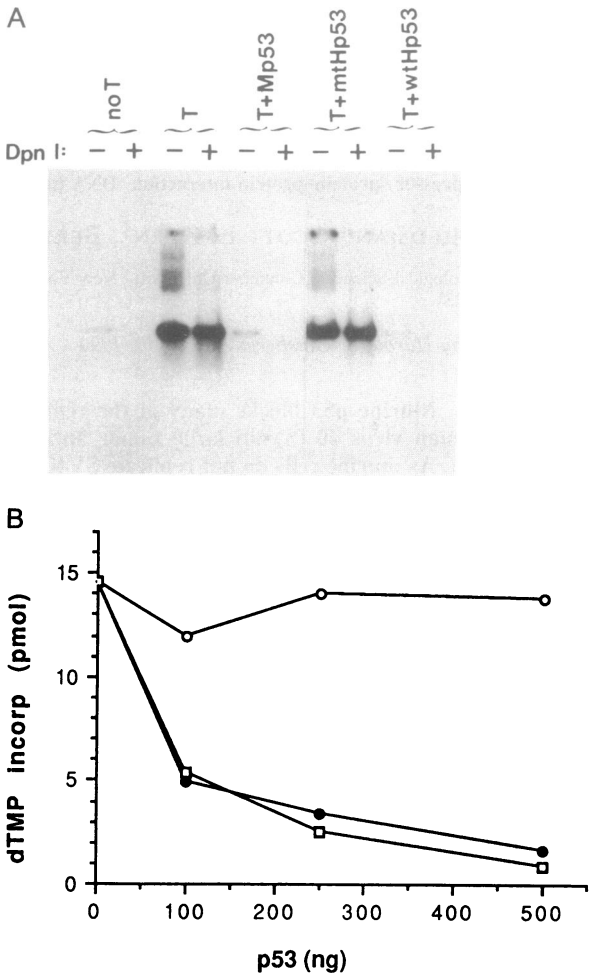

FIG. 1. Wild-type but not mutant human p53 inhibits SV40 *ori* DNA replication *in vitro*. (*A*) Replication reaction mixtures lacking T antigen (no T) or containing 300 ng of SV40 T antigen (T) and other components as described in the text were incubated alone or with 500 ng of the indicated p53 protein. At the end of the reactions, DNA was purified, linearized with *Bst*XI, and incubated with (+) or without (−) *Dpn* I followed by electrophoresis and autoradiography. Mp53, murine p53; mtHp53, mutant human p53; wtHp53, wild-type human p53. (*B*) Reaction mixtures containing 300 ng of SV40 T antigen and the indicated amounts of murine p53 (□), mutant human p53 (○), or wild-type human p53 (●). After 3 hr, 5-μl samples of each reaction were acid precipitated and analyzed by liquid scintillation spectroscopy.

detected under these conditions (Fig. 2, lane 1). Both murine and wild-type human p53 proteins strongly inhibited the T-antigen unwinding reaction such that only 5% and 2% of single-stranded products, respectively, were detected at the highest concentrations of p53 added (Fig. 2, lanes 4 and 8). As seen with the replication of *ori* DNA, mutant human p53 did not inhibit the unwinding reaction (Fig. 2, lanes 5 and 6). Similarly, using a DNA helicase assay in which T antigen displaced a 31-nucleotide oligomer annealed to M13 DNA, murine and wild-type human p53 proteins inhibited the reaction, whereas mutant p53 did not (data not shown).

Mutant Human p53 Reduces the Inhibition by Murine p53 of SV40 *ori* DNA Replication *in Vitro*. In insect cells coinfected with recombinant baculoviruses expressing T antigen and p53, the two proteins form sufficiently tight complexes that they can be coimmunoprecipitated from extracts of infected cells (25, 37). It was of interest to determine whether two different p53 proteins associate with one another in baculovirus-infected insect cells, as was reported in experiments with cultured mammalian cells (16, 19, 20). Human and

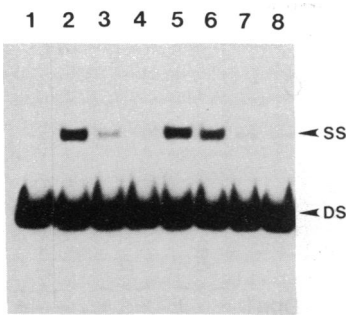

FIG. 2. Wild-type, but not mutant, human p53 inhibits the unwinding activity of SV40 T antigen. Unwinding reaction mixtures containing 0 ng (lane 1) or 300 ng (lanes 2–8) of immunopurified SV40 T antigen and either 250 ng (lanes 3, 5, and 7) or 500 ng (lanes 4, 6, and 8) of immunopurified murine p53 (lanes 3 and 4), mutant human p53 (lanes 5 and 6), or wild-type human p53 (lanes 7 and 8) were incubated with a ^{32}P-labeled SV40 origin-containing double-stranded fragment (DS). Labeled single-stranded DNA (SS) is indicated.

murine p53 proteins can be easily distinguished on the basis of their different electrophoretic mobilities (38). Insect cells were coinfected with the mutant human p53-expressing and murine p53-expressing viruses and labeled with [^{35}S]methionine prior to extraction. Labeled cell extracts were then immunoprecipitated with human- and murine-specific antibodies (Fig. 3). In all cases both human p53 and murine p53 were coimmunoprecipitated. As PAb 248 (murine p53 specific) immunoprecipitated approximately equal quantities of human and murine p53 proteins, this strongly indicated that the majority of the proteins had formed p53 hetero-oligomers. PAb 1801 (human p53 specific) immunoprecipitated both human and murine p53 proteins as well, but this was a relatively weaker reaction than with the other antibodies.

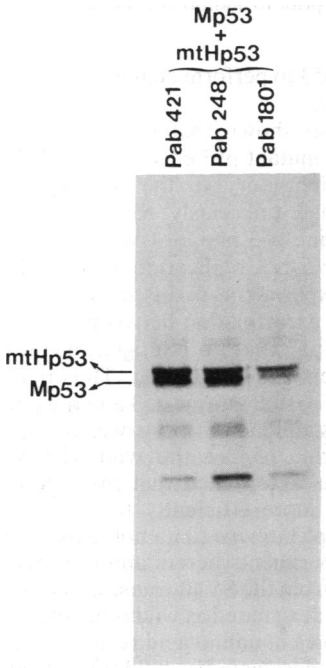

FIG. 3. Wild-type and mutant p53 proteins complex with one another in insect cells. Extracts prepared from ^{35}S-labeled insect cells that were coinfected with recombinant baculoviruses expressing murine p53 and mutant human p53 were immunoprecipitated with p53-specific monoclonal antibodies PAb 421, which recognizes both murine and human p53 proteins, PAb 248, which is specific for murine p53, and PAb 1801, which is specific for human p53, cross-linked to protein A-Sepharose. Immunoprecipitates were washed in RIPA buffer (25) and analyzed on 10% SDS/polyacrylamide gels followed by autoradiography. The relative position of mutant human (mtHp53) and murine (Mp53) p53 proteins are indicated.

That murine and human p53 proteins are capable of complex formation in insect cells suggested that one p53 might be able to modulate the function of the other. We had previously determined that a murine p53–T-antigen complex purified from insect cells coinfected with T antigen and murine p53 recombinant baculoviruses exhibited no replication activity (39). To determine whether the presence of the noninhibitory mutant human p53 could alleviate this inhibition, we infected insect cells with three recombinant baculoviruses so that SV40 T antigen, murine p53, and the mutant human p53 were coexpressed. When extracts of such triply infected insect cells were then passed over a T-antigen-specific PAb 419 column, all three proteins were eluted (Fig. 4*A*, lane 1). Analysis of the purified complex revealed that all three proteins could be immunoprecipitated with either PAb 419 (T antigen specific), PAb 248 (murine p53 specific) or PAb 1801 (human p53 specific), indicating that after purification the three proteins were still associated with each other (data not shown). It is difficult to assess which proteins were bound to each other in this preparation because of the demonstrated ability of each component to bind to either of the other two proteins *in vivo*. However, because we had determined in doubly infected insect cells that murine p53 bound considerably more efficiently to T antigen than did mutant human p53 (data not shown), it is likely that in triply infected cells a significant proportion of the mutant human p53 was not itself directly associated with T antigen but rather was bound to murine p53 (see *Discussion*). Regardless of the mode by which these three proteins were associated, it was now reasonable to compare the ability of these complexes to mediate the replication of SV40 *ori* DNA. Accordingly, similar quantities of T antigen, either alone, in complex with murine p53, or in complex with murine p53 and mutant human p53, were added to the replication reactions. As seen in Fig. 4*B*, T antigen alone generated substantial quantities of *Dpn* I-resistant full-length products. The murine p53–T-antigen complex (Fig. 4*A*, lane 2) yielded no detectable products, consistent with our previous studies. However, when mutant human p53 was also present in the complex, a dramatic difference was noted, in that replication products were readily detected. By determining the radioactivity in the *Dpn* I-resistant DNA products, we estimated that free T antigen was only 3- to 5-fold more efficient than T antigen in the "triple complex."

Additional evidence that the mutant human p53 can influence the ability of murine p53 to inhibit T antigen was obtained when both p53 proteins, individually purified, were added to replication reactions (Fig. 4*C*). On the basis of the assumption that human p53 may mitigate inhibition by murine p53 because a complex can be formed between the two p53 molecules, we first preincubated human and murine p53 proteins at 37°C in the presence of the replication components prior to initiating the replication reaction by adding T antigen. As expected, murine p53 alone strongly inhibited the synthesis of *ori* DNA replication products. The presence of added mutant human p53 led to a substantial reduction in the degree to which murine p53 inhibited replication. Note that smaller quantities of murine p53 were used in this experiment than were in that shown in Fig.1, due to the volume and buffer constraints of the replication reactions. The products of the replication reactions were also analyzed by acid precipitation (data not shown). T antigen alone synthesized 17 pmol of replicated *ori* DNA; T antigen plus murine p53 resulted in 7 pmol of DNA products; and reactions containing T antigen, murine p53, and mutant human p53 yielded 15 pmol of DNA. Thus the mutant human p53 affects the ability of the murine p53 protein to inhibit the replication function of T antigen.

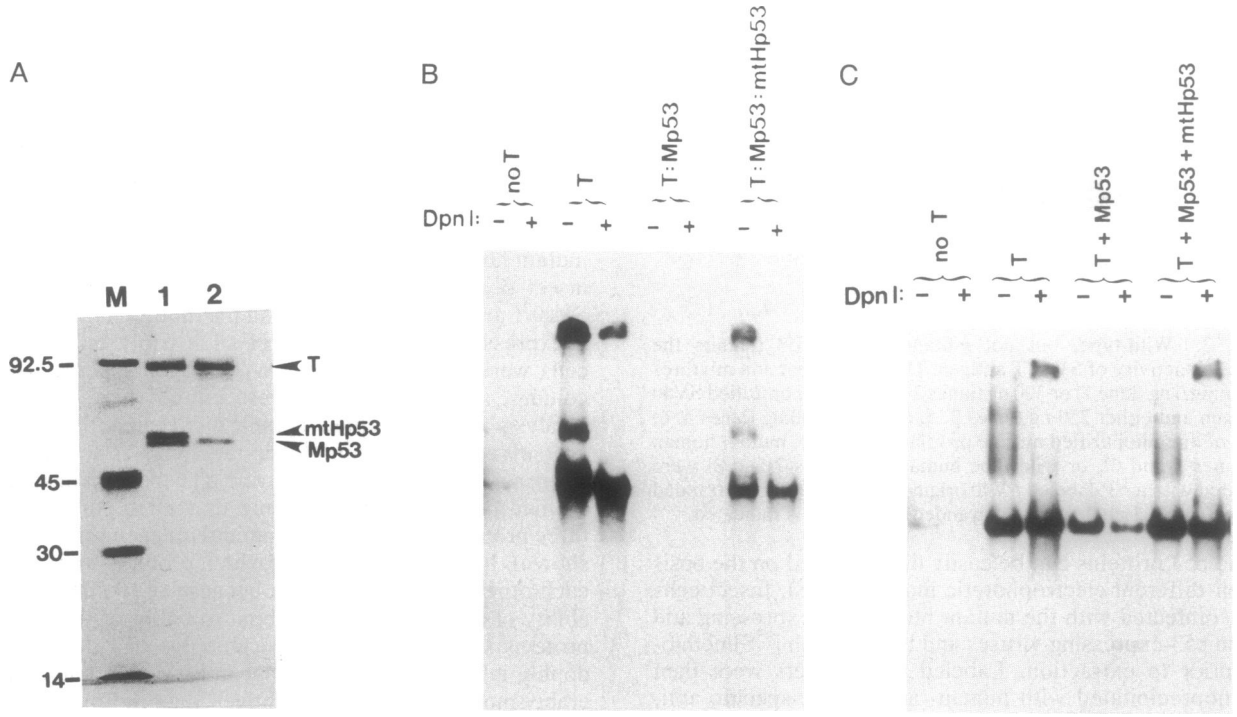

FIG. 4. Mutant human p53 relieves the inhibition by murine p53 of SV40 *ori* DNA replication *in vitro*. (*A*) Insect cells were coinfected with recombinant baculoviruses encoding either SV40 T antigen and murine p53 (lane 2) or SV40 T antigen and murine and mutant human p53 (lane 1). The protein complexes were immunoaffinity purified with the SV40 T-specific monoclonal antibody PAb 419 and analyzed by SDS/PAGE and silver staining. T, SV40 T antigen; Mp53, murine p53; mtHp53, mutant human p53. Lane M contains standard proteins with molecular masses in kilodaltons as indicated. (*B*) Replication reaction mixtures lacked (no T) or contained 300 ng of either free T antigen (T), or T antigen in the preparations shown in *A* containing the indicated p53 proteins. Reaction products were analyzed as in Fig. 1*A*. (*C*) Replication reaction mixtures were incubated either with or without 250 ng of the specified p53 proteins for 30 min at 37°C prior to addition of 300 ng of SV40 T antigen where indicated, and reactions were continued for 3 hr. Products were analyzed as in Fig. 1*A*.

DISCUSSION

Our studies have shown that wild-type human p53 inhibits the replication-associated functions of SV40 T antigen *in vitro*. Because human cells are permissive for SV40 DNA replication, this was a somewhat unexpected observation. It could be argued that our data do not reflect the potential of p53 to affect replication in intact cells. However, the ability of murine p53 to inhibit the replication of SV40 *ori* DNA *in vitro* mimics the situation in transfected cells, where it was demonstrated that murine p53 blocked the replication of SV40 *ori* DNA (22, 23). It was concluded from one such study (22) that, in contrast to murine p53, human p53 does not inhibit SV40 T antigen-mediated *ori* DNA replication *in vivo*. However, that study had utilized a plasmid encoding the mutant human p53 from A431 cells, which was at the time considered the wild-type form, so their data does not contradict ours. If the correspondence of the *in vivo* and *in vitro* results for murine p53 can be extrapolated to those we have obtained with human p53, then how does viral DNA synthesis occur in a human cell? In lytically infected cells only a small fraction of T antigen is bound to p53 (4). Moreover, wild-type p53 is itself normally present in very small quantities (40–42). Our data indicate that both murine and human p53 proteins inhibit T-antigen function only at fairly high concentrations (ref. 25, this study, and P.N.F., E. Wang, and C.P., unpublished results) and that a molar ratio of p53 to T antigen of at least 1:1 is required before significant inhibition can be observed. The ratio of p53 to T antigen in permissive human cells may well fall below the necessary value. It should be noted that Tack *et al.* (43) showed that monkey p53 stimulates the ATPase and helicase activities of SV40 T antigen in p53–T-antigen immune complexes isolated from lytically infected cells. How monkey p53 affects the replication of SV40 *ori* DNA *in vitro* awaits the production of sufficient quantities of this source of p53 to perform analogous experiments to those described above.

We have also shown that in contrast to the wild-type human p53 the mutant p53 encoded by the human A431 cell line was not inhibitory to the replication function of T antigen. We had previously reported (25) that a highly oncogenic murine p53 mutant (Ala-135 → Val) (44) did not inhibit SV40 *ori* DNA replication *in vitro*. By combining the results of our experiments in this and our previous study (25), we have noted a correlation between the extent to which a given source of p53 binds to T antigen and the degree to which it inhibits its replication function. Thus both the oncogenic mutant Ala-135 → Val murine p53 and the mutant human p53 proteins bind significantly more weakly to T antigen than either the murine p53 or the wild-type human p53. For example, it was determined that the wild-type human p53 bound ≈5-fold more efficiently to T antigen than did the mutant human p53 *in vivo* (data not shown). The murine p53 used in the experiments herein and in previous studies (24, 25) was cloned from the SV40-transformed cell line SVA31E7 (45). Originally designated as wild type, the SVA31E7 murine p53 bears changes at amino acid residues 48, 79, and 81 that differ from that designated as wild type by Finlay *et al.* (46). However, these changes do not lie within the highly conserved regions of p53 (15). Furthermore, it is clear that the SVA31E7 murine p53 binds extremely well to T antigen *in vivo* (25, 37).

The current assessment of the role of p53 in cells is that it functions as a negative regulator of cell growth. The suggestion that high levels of mutant p53, when introduced into cells, can bind to and sequester the resident normal p53 within the cells and thus prevent it from carrying out its normal function in suppressing growth is derived from *in vitro* p53 transformation experiments. Our data support this in the

following ways. First, we have shown that different p53 proteins can form complexes with one another efficiently in insect cells. Because of the potential relevance to oncogenesis of the interactions between p53 molecules, establishing the parameters of oligomerization between either different or identical p53 molecules should prove to be very informative. Second, we have shown that the presence of one p53 protein can modulate the effects of another. In the case that we have studied, the noninhibitory mutant human p53 reduced the inhibitory effects of the murine p53. Our experiments did not discriminate between different modes by which human p53 functions in this regard. Although it could act by virtue of a simple competition with murine p53 for binding to T antigen, we consider this unlikely because the mutant human p53 bound far more weakly to T antigen *in vitro* than did murine p53 (data not shown). This suggests that at least some of the mutant p53 in the triple complex was probably associated with the murine p53 rather than directly with the T antigen. This is also supported by the fact that, unlike its weak interactions with T antigen, the mutant p53 bound very well to the murine p53 in insect cells.

How do these data relate to the functions of normal and mutant p53 proteins in cells? The mounting evidence that wild-type p53 is a tumor suppressor raises the possibility that the normal role of p53 may be to maintain cells in or shift them to the resting rather than the dividing mode. This is supported by the fact that wild-type p53 inhibits cell cycle progression in human tumor cells carrying endogenous p53 gene mutations (48). On the basis of its interaction with T antigen, it is possible to speculate on an analogous role for p53 in uninfected cells. A major function of T antigen is to initiate DNA synthesis from the viral replication origin. Analysis of its roles in this process (reviewed in ref. 21) has revealed a remarkable similarity to those provided by other well-studied DNA initiation factors from *E. coli* (reviewed in ref. 47). This suggests a commonality to the function of DNA initiation proteins from widely divergent sources and the possibility that vertebrate DNA synthesis may initiate by mechanisms analogous to those defined for the *E. coli* and SV40 origins. Should this be the case, cellular T-antigen-like initiation proteins might exist that may also exhibit similar interactions with and regulation by normal, but not mutant, p53 proteins. Whatever the function of p53 might normally be, identification of cellular proteins that interact differentially with normal and oncogenically related p53 proteins should provide further insight into this question.

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