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The Amino-Terminal Functions of the Simian Virus 40 Large T Antigen Are Required To Overcome Wild-Type p53-Mediated Growth Arrest of Cells

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High levels of the p53 tumor suppressor protein can block progression through the cell cycle. A model system for the study of the mechanism of action of wild-type p53 is a cell line (T64-7B) derived from rat embryo fibroblasts transformed by activated *ras* and a temperature-sensitive murine p53 gene. At 37 to 39°C, the murine p53 protein is in a mutant conformation and the cells actively divide, whereas at 32°C, the protein has a wild-type conformation and the cells arrest in the G_1 phase of the cell cycle. Wild-type simian virus 40 large T antigen and a variety of T-antigen mutants were assayed for the ability to bypass the cell cycle block effected by the wild-type p53 protein to induce colony formation at 32°C. The results indicate that two functions within the amino terminus of T antigen are essential to induce cell growth: (i) the ability to bind to the retinoblastoma protein, Rb, and (ii) the presence of a domain in the first exon that appears to interact with the cellular protein, p300. Thus, the cell cycle arrest triggered by wild-type p53 may be overcome by formation of a T-antigen complex with Rb, p300, or both that could then function to either remove p53-mediated negative growth regulatory signals or promote a positive cell growth signal. Surprisingly, T antigen-p53 complexes are not required to overcome the temperature-sensitive p53 block to the cell cycle in these cells. These data suggest that simian virus 40 T antigen associated with Rb, p300, or both proteins can communicate in a cell with the functions of the wild-type p53 protein.

The p53 protein is encoded by a tumor suppressor gene. About 60% of human cancers contain mutations at the p53 locus (23, 30). Most commonly, one p53 allele contains a missense mutation producing a faulty protein, while the second allele is lost by a reduction to homozygosity, leaving no wild-type protein in the cancer cells (1). This is the expected pattern for a tumor suppressor gene, in which a loss of function results in abnormal growth control. The validity of this interpretation was demonstrated by the creation of mice without normal p53 genes; all of these animals developed cancers in the first 3 to 6 months of life (8). Reintroduction of the wild-type p53 gene into cells which are being transformed by oncogenes (15) or are already transformed (36) blocks the transformation process or reduces the tumorigenic potential of the cells (4). When the wild-type p53 protein is expressed at high levels in these cells, it stops cell proliferation (35, 38). Cells transformed with a temperature-sensitive p53 mutant divide at 37 to 39°C when the p53 protein is in the mutant form but fail to divide at 32°C when the p53 protein is primarily in a wild-type conformation and acts as a tumor suppressor gene product (35, 38). The wild-type p53 protein blocks progression through the cell cycle in G_1 (35, 38). Thus, under certain circumstances, the wild-type protein can regulate the passage of cells through the cell cycle. Several studies have demonstrated that the wild-type p53 protein, but not the mutant p53 protein, can act as a transcription factor or a negative regulator (14, 26, 50, 62), and so it is tempting to suggest that the p53-mediated transcription of a set of genes can block passage through the cell cycle in the G_1 phase of this cycle.

Simian virus 40 (SV40) initiates tumors in animals (11, 17) and transforms cells in culture (56) because of the transforming properties of the virus-encoded oncogene product, large T antigen (3). An extensive genetic analysis of large T antigen (5, 27, 31, 45, 51–53, 55, 60, 65, 66) has localized three distinct domains of this protein that are required for the transformation of a variety of cell types in culture. SV40 large T antigen is composed of 708 amino acid residues, and amino acid sequences within residues 1 to 82, 105 to 114, and 351 to 626 are critical for the transformation process. Mutations within the first exon of T antigen (residues 1 to 82) are complemented for transformation by the adenovirus oncogene E1A (60). E1A proteins bind to a cellular protein termed p300 (10), and E1A mutant proteins that have lost the E1A-p300 interaction fail to complement SV40 T-antigen mutants with mutations in residues 1 to 82 (60). Although not yet demonstrated directly, these results suggest that some of these SV40 large T-antigen residues may bind to the cellular p300 protein and alter p300 functions, resulting in cellular transformation. Residues 105 to 114 of SV40 large T antigen are critical for binding to the retinoblastoma susceptibility protein (Rb) (6), which is a tumor suppressor protein regulating the transcription factor E2F, which in turn is thought to regulate entry into the S phase of the cell cycle (10, 58). SV40 T antigen also binds to the wild-type p53 protein (28, 32). The ability of T antigen to form a complex with p53 correlates with its ability to immortalize primary murine cells in culture (27, 67) and is also required for extending the life span of human cells (31) and for the transformation of certain immortal cell lines (53, 65). While in vitro binding studies showed that T-antigen residues 273 to 517 were required for T-antigen binding to the wild-type p53 protein (41, 48), immortalization assays have demonstrated that both the immortalization and T antigen-p53 binding functions are affected by mutations within residues 351 to 626

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TABLE 1. Characteristics of wild-type and mutant SV40 virus large T-antigen proteins

Phenotype[a]	Construct	Amino acid change[b]	Transformation[c]			Immortalization, primary MEF
			C3H10T1/2	REF52	Rat-1	
Wild type	B3		+	+	+	+
−p300 complementing function	2803	Δ35 + in (5)				
	*dl*1135	Δ17–27	−	−	ND	+
	2831	Δ5–35 + in (5)				
−Rb binding	pVU-1	Δ106-113 + in (1)	+	−	−	+
	K1	E107K				
−p53 binding	*dl*1137	Δ122-708 + in (11)				
	2809	Δ409 + in (5)	+	−	ND	−
	2811	in 424 (4)				
−NLS	pSVcT	K128N	+	+	ND	+
−DNA binding Zn finger	2837/p6-1	Δ168–346 + in (4)	+	+	ND	+

[a] A region in the extreme amino terminus of T antigen, required to transform the immortal murine line, functionally complements a non-p300-binding E1A deletion mutant in a transformation assay (60). NLS, nuclear localization signal.

[b] Δ, amino acid deletion; in, amino acid insertion (number of amino acids inserted). Letters denote amino acid residues; numbers not in brackets denote amino acid positions.

[c] The activities of mutant and wild-type T antigens in transformation and immortalization assays are as reported from the following sources: C3H10T1/2 (44, 53, 68), REF52 (45, 53, 68), Rat-1 (25), mouse embryo fibroblasts (MEF) (5, 66). ND, not determined.

(27, 66) in two noncontiguous regions, residues 351 to 450 and 533 to 626 (27). When T antigen binds to p53, it inactivates its ability to bind to DNA and act as a transcription factor at a p53-responsive element (14, 39, 49), just as mutations in the p53 gene block this same function (14, 26, 62). Thus, T antigen mediates its ability to transform cells by binding to or interacting with three cellular proteins, Rb, p53, and possibly p300, which are thought to play critical roles in cell cycle events.

The study reported here used a cell line transformed by an activated *ras* oncogene and a temperature-sensitive p53 mutant. At 32°C, these cells arrest in the G_1 phase of the cell cycle, and the ability of the SV40 large T antigen to overcome this temperature-dependent block in cell division was examined. The role of the T-antigen N terminus (p300) and the Rb and p53 binding sites in T antigen was assessed by using mutants in each of these regions of the T-antigen gene and determining their ability to overcome the p53-mediated block to cell division. Surprisingly, the T-antigen p53 binding site was not required to overcome the p53-initiated G_1 block in the cell cycle. Instead, both the p300 site and Rb-T antigen complexes were needed to inactivate the p53 temperature-sensitive block to cell division. T-antigen proteins with mutations in either the N-terminal region (p300) or the Rb binding site failed to inactivate the p53 block in the cell cycle, but these individual mutations could complement each other to produce cell lines that grew at 32°C. These results demonstrate that the N terminus of T antigen (p300) and the Rb binding site functionally interact with the p53 protein. Two distinct models that predict different sets of interactions are proposed.

MATERIALS AND METHODS

Plasmids. The following vectors that code for mutant SV40 T antigen contain the entire *Eco*RI-digested SV40 genome cloned into the *Eco*RI site of pUC18: pSVcT (29), 2803, 2809, and 2811 (67), 2831 (68), and 2837/p6-1 (66). T-antigen mutant vectors K1 and pVU-1 contain early-region SV40 DNA cloned into pBR328 (25). Vector B3 (43) (which codes for wild-type SV40 T antigen) and the mutant vectors *dl*1135 and *dl*1137 contain the entire *Bam*HI-digested SV40 genome cloned into the *Bam*HI site of pBR322 (45). Vector pSVgem codes for wild-type T antigen and contains early-region SV40 DNA cloned into pGEM (Promega). The pBluescript vector BSKS− (Stratagene) was used as a negative control. The properties of these mutant vectors are summarized in Table 1 and Fig. 3.

Cell culture and DNA transfections. T64-7B cells (22) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified, 5% CO_2 atmosphere. For the colony formation assay, 2×10^5 T64-7B cells were seeded at 37°C into 10-cm-diameter dishes. Cells were transfected 1 day later by the calcium phosphate procedure (18) with equimolar amounts of plasmid DNA and 10 or 15 μg of salmon sperm DNA as carrier. Eighteen hours later, the transfected cells were rinsed twice with phosphate-buffered saline (PBS), replenished with fresh DMEM, and shifted to 32°C. Culture medium was changed every 5 to 6 days, and colonies were cloned and/or scored by staining with crystal violet 21 to 25 days later.

Immunoprecipitations. Cells were metabolically labeled for 4 h with 100 μCi of [^{35}S]methionine EXPRESS (Dupont-NEN) in 2 ml of methionine-free DMEM plus 2% PBS-dialyzed fetal bovine serum. Labeled cells were rinsed with cold PBS, scraped from the plates, pelleted, and stored at 80°C prior to lysis. Cellular protein lysates were prepared in lysis buffer (50 mM Tris-Cl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.28 trypsin-inhibiting units of aprotinin [Sigma] per ml) and precleared with protein A-Sepharose (Sigma). Immunoprecipitations were carried out with 10^6 to 10^7 trichloroacetic acid-precipitable cpm of lysates with appropriate antibodies and protein A-Sepharose, rotating at 4°C for at least 2 h. Immune complexes were washed twice in SNNTE buffer (50 mM Tris [pH 7.4], 5 mM EDTA, 5% sucrose, 1% Nonidet P-40, 0.5 M NaCl) and once in radioimmunoprecipitation assay buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.5% sodium dodecyl sulfate, 1% [wt/vol] sodium deoxycholate), boiled, and separated on polyacrylamide gels. Gels were fixed (30% methanol, 10% acetic acid), washed in H_2O, and prepared for fluorography in 1 M sodium salicylate–5% glycerol. The gels were dried under vacuum and exposed to Kodak XAR-5 film at −80°C. The following monoclonal antibodies were used: M73 (21), 2A6 (47), 246 (61), 240 (16), 416, 419, and 421 (20), 412 (19), and 901 (27). Ascites fluid for 901 was kindly provided by S. Tevethia (Pennsylvania State University).

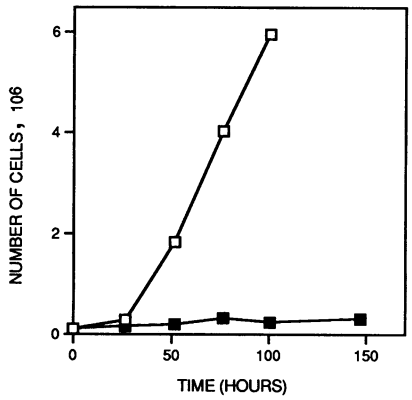

FIG. 1. Growth curves for T64-7B cells at 39°C (open squares) or 32°C (filled squares). A total of 10^5 cells in 10-cm-diameter plates were shifted to 39 or 32°C and counted over a 150-h period.

RESULTS

The p53 temperature-sensitive cell line. A temperature-sensitive p53 protein is encoded by a murine mutant p53 allele in which valine replaces alanine at codon 135 (38). This mutant p53 allele ($p53^{Val-135}$), plus the activated *ras* oncogene, will transform primary rat embryo fibroblasts at 37 to 39°C but not at 32°C (22, 35, 38). Cells transformed by these oncogenes grow at 37°C, but at 32°C, the cells are blocked in the G_1 phase of the cell cycle. The $p53^{Val-135}$ protein is in a mutant conformation at 37 to 39°C and a wild-type conformation at 32°C (35). In some of these cell lines, such A1-5 (22, 35), the p53 levels are very high, and transfecting wild-type SV40 T antigen into these cells does not relieve the block to cell division at 32°C, presumably because wild-type p53 is in excess to SV40 T antigen in these cells (46). However, other temperature-sensitive p53-plus-*ras*-transformed rat embryo fibroblast cell lines, such as T64-7B (22), have been generated which express lower levels of wild-type p53 and still are blocked in their progression through the cell cycle at 32°C (Fig. 1) predominantly in the G_1 phase of the cycle (a fluorescence-activated cell sorting analysis of the DNA content of these cells shows that 82% of the cells are in G_1 and 15.7% of the cells are in G_2 or M, while the remainder are in S phase; by contrast, at 37°C, 20 to 25% of the growing cells are in S phase). This cell line will grow at 32°C when the SV40 large T antigen gene is introduced and expressed in these cells.

SV40 T antigen overcomes the temperature-sensitive p53-mediated block in cell division. When T64-7B cells are plated at 32°C in a culture dish, they fail to form colonies. By contrast, when T64-7B cells are transfected with DNA containing the entire SV40 genome (B3 DNA) or the SV40 large T-antigen gene alone (pSVgem DNA) (transfected at 37°C for 18 h and then shifted to 32°C to test for the formation of colonies on the surface of a culture dish), then colonies of these cells are readily detected (Fig. 2). When the vector BSKS alone is used for these DNA transfection experiments, no colonies are formed (Fig. 2). The colonies that arise in this assay are readily cloned and expanded and grow continuously at 32°C. The cells from these colonies all express SV40 T antigen, which is found in cell extracts to be bound to the p53 protein (28, 32).

SV40 T-antigen mutant analysis of the rescue of p53 temperature-sensitive cell lines. To determine which functional domains of SV40 large T antigen are required for this T-antigen-mediated rescue of the p53 temperature-sensitive block in cell division, a variety of mutant T-antigen plasmids (described in Table 1) were used. These mutants fall into five

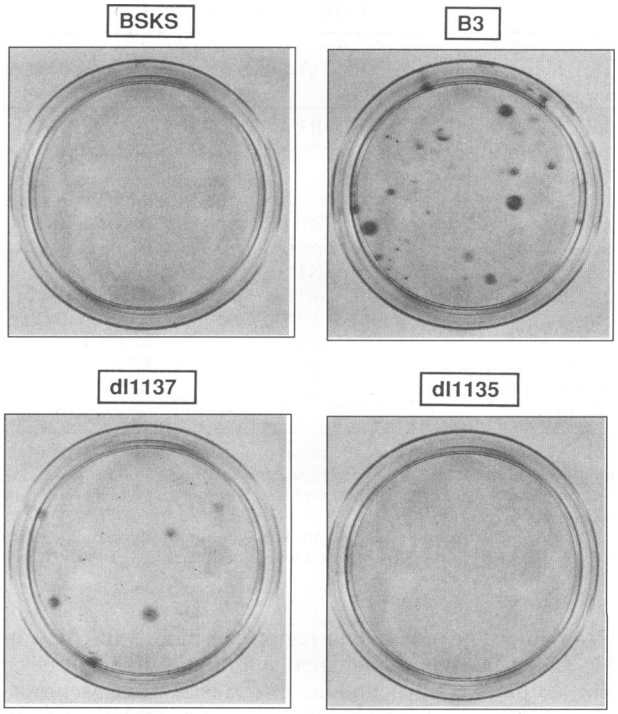

FIG. 2. Morphology of T64-7B colonies that form at 32°C. Cells were transfected as described in Materials and Methods with 5 μg of wild-type (B3) or mutant (*dl*1137 or *dl*1135) T-antigen vectors or with the molar equivalent of a control vector (BSKS) and stained with crystal violet 3 weeks following the temperature shift to 32°C.

classes: mutants that fail to provide the T-antigen p300-complementing function (2803, *dl*1135, and 2831) (class 1), mutants that fail to bind the Rb protein (pVU-1 and K1) (class 2), mutants that fail to bind the p53 protein (*dl*1137, 2809, and 2811) (class 3), a mutant lacking the nuclear localization signal (pSVcT) (class 4), and a mutant that is defective in the T-antigen zinc finger and does not bind to DNA (2837/p6-1) (class 5). The N-terminal binding site (p300), Rb, and p53 mutants are defective in various assays for transformation of cells in culture, while the other two classes of mutants (classes 4 and 5) are not defective in these assays (Table 1).

Each of these mutant T-antigen genes and the wild-type gene (B3) were tested by transfecting these DNAs into T64-7B cells and shifting them to 32°C. When 2×10^5 cells were analyzed in this fashion, wild-type T antigen yielded about 30 colonies (26 to 38 in three separate experiments) at 32°C, 14 days after the temperature shift (Fig. 2 and 3). Mutants that were defective in the p300-complementing function failed to form colonies (Fig. 3), giving only 0 to 5% of the wild-type values, depending on the mutant allele tested. Mutants defective in Rb binding similarly failed to overcome the p53 temperature-sensitive block in cell division, yielding 2 to 3% of the wild-type values (Fig. 3). By contrast, mutants defective in p53 protein binding were 39 to 63% as efficient as wild-type T antigen in rescuing the cells from the p53 block. Similarly, the nuclear localization mutant and zinc finger mutant were quite efficient (76 to 87%) in overcoming the p53 block to cell division. Examples of the colonies produced at 32°C with *dl*1137 (a T-antigen mutant that does not bind p53) or the failure to form colonies with *dl*1135 (a mutant for p300-complementing function) are shown in Fig. 2, and all of the results are summarized in Fig. 3.

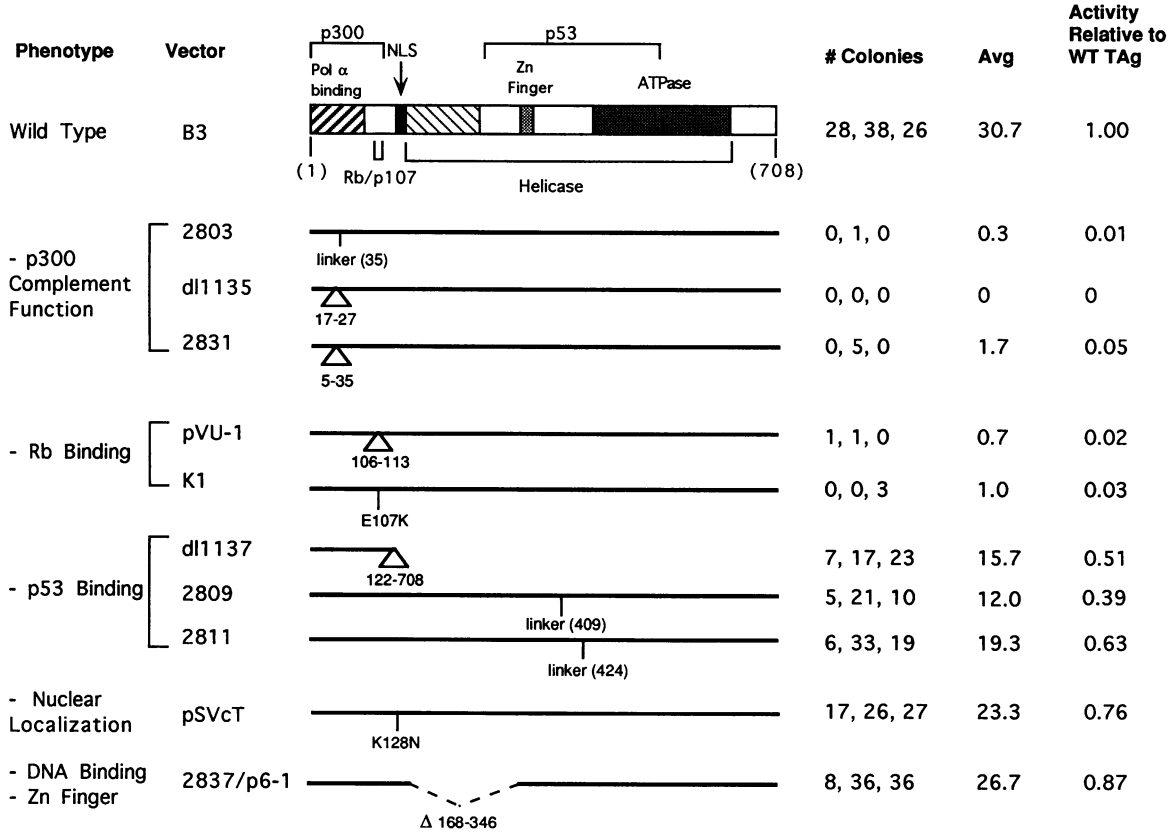

FIG. 3. Compiled results of three complete assays testing the ability of wild-type and mutant T-antigen proteins to induce T64-7B cells to form colonies at 32°C. The mutational lesions affecting particular amino acid residues in each mutant T-antigen protein are indicated schematically below a diagram of the wild-type SV40 T-antigen protein, where various functional domains have been delineated (pol α, polymerase α; NLS, nuclear localization signal). The three values reported for each T antigen tested reflect the number of colonies formed on a single plate of cells transfected in each of three separate experiments. Avg, average number of colonies for the three experiments; WT TAg, wild-type T antigen. Quantities of plasmid used for the transfections were in molar equivalents to 5 μg of wild-type vector B3. No colonies formed at 32°C following transfection with control vector BSKS.

These results indicate that the large T-antigen functions clustered in the first 121 amino acids of T-antigen (*dl*1137 has only those residues) are sufficient to overcome the p53 temperature-sensitive block to cell division. Cell lines expanded at 32°C with either the wild-type T antigen (Fig. 4A) or the *dl*1137 T-antigen mutant (Fig. 4B) expressed T-antigen proteins. These cells were labeled with [^{35}S]methionine for 4 h, and soluble protein extracts were prepared. These extracts were subjected to immunoprecipitation analyses using a variety of monoclonal antibodies. M73 (recognizes adenovirus E1A proteins) and 2A6 (detects the adenovirus E1B 55-kDa protein) were used as negative controls in these experiments; 419 and 412 detect an N-terminal set of residues of SV40 T antigen; 901 detects residues 684 to 698 of SV40 T antigen, while 416 interacts within residues 82 to 271 of T antigen; 421 detects all forms of the p53 protein; 240 detects only mutant forms of the p53 protein; and 246 detects only the wild-type form of the p53 protein. The wild-type T antigen was observed in a T antigen-p53 complex in extracts from these cells (Fig. 4A), while the *dl*1137 T antigen which was present in the T64-7B cells transfected with this plasmid was not complexed to wild-type p53 which was also present in these cells (Fig. 4B). In these experiments, it is common that PAb240 detects a small percentage of wild-type p53 protein that is presumably denatured during preparation of the antigen (Fig. 4B). The levels of p53 in the wild-type T antigen-p53 complex are much higher than in the parental T64-7B cells (data not shown), probably because of the enhanced stability of p53 in a T antigen-p53 complex (33). p53 levels (100% is in the wild-type conformation at 32°C) were much lower in the *dl*1137-rescued T64-7B cells, in which T antigen is not complexed with p53. There was no suggestion in these experiments that *dl*1137 mutant T antigen stabilized p53 levels in these cells.

Colonies grown at 32°C expressing a variety of different T-antigen mutants (Table 1 and Fig. 3) were cloned, expanded, and grown into cell lines. These lines were assayed for the presence of the SV40 T antigen in the same manner as described above. Nine cell lines derived from either of two wild-type constructs and 17 cell lines rescued by the different SV40 T-antigen mutants capable of inducing colonies at 32°C all expressed the transfected SV40 T antigen and retained this expression after extensive growth at 32°C (Table 2).

The amino-terminal T-antigen functions complement to overcome the p53-induced growth arrest. These experiments demonstrate that the T-antigen p300-complementing and Rb-binding functions are both required to overcome the p53 temperature-sensitive block to cell division. If both are required as independent genetic units or functions, then mutations affecting each function may complement each other in a cell. Alternatively, the T antigen-p300 and T antigen-Rb

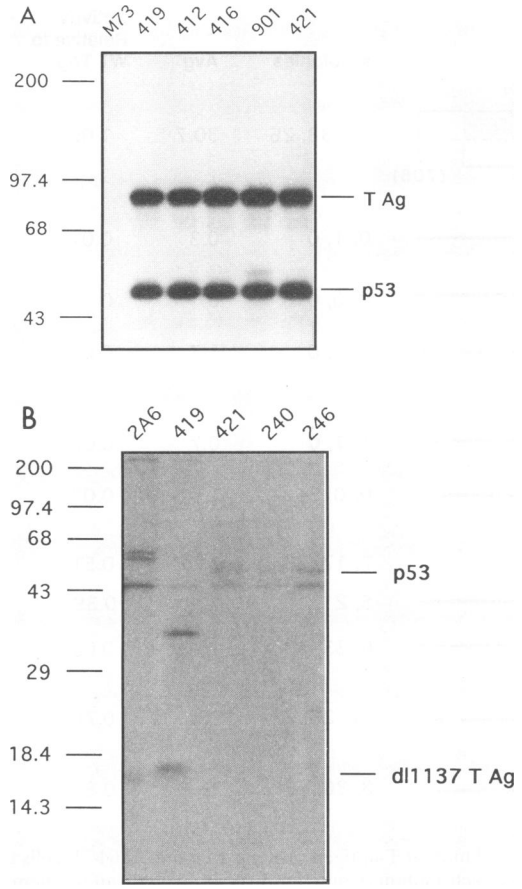

FIG. 4. Expression of T antigen and p53 in T64-7B-derived cell lines that grow at 32°C. (A) A cell line that was rescued for growth at 32°C by wild-type T antigen (T Ag) was metabolically labeled with [^{35}S]methionine, and equal amounts of trichloroacetic acid-precipitable protein (8.8×10^6 cpm) were subjected to immunoprecipitation and gel electrophoresis as described in Materials and Methods. This autoradiograph is an 18-h exposure. Monoclonal antibody M73, which is specific for adenovirus E1A protein, was used as a negative control. Monoclonal antibodies 419, 412, 416, and 901 recognize different epitopes on the T-antigen protein, and antibody 421 recognizes p53. (B) Immunoprecipitation analysis of a cell line rescued for growth at 32°C by the truncated T-antigen mutant *dl*1137. A total of 1.4×10^6 precipitable counts was used for each immunoprecipitation. The adenovirus 55-kDa E1B protein-specific antibody 2A6 served as a negative control and detects cross-reacting proteins in the cell; antibody 240 recognizes only mutant p53, and antibody 246 recognizes only wild-type murine p53. Sizes are given in kilodaltons.

functions might need to act on the same molecule in *cis* to each other, in which case no complementation would be observed. To test this hypothesis, 2831 (p300 defective) and K1 (Rb defective) were transfected separately or were cotransfected into T64-7B cells, which were then shifted to 32°C. The SV40 wild-type T antigen (B3) yielded 21 colonies at 32°C, while 2831 yielded no colonies and K1 produced one colony at 32°C (Table 3). The 2831-plus-K1 complementation test produced about 25% of the wild-type level of colonies, corresponding to a fivefold increase above the background of the K1 mutant clone alone, indicating complementation between these defective gene functions.

When the pVU-1 (Rb-defective) mutant was used in complementation tests with an N-terminal T-antigen-defective mutant (*dl*1135 or 2831), no complementation was ever observed (eliminating recombination as an explanation for the previous results). It has been suggested, however (34), that this deletion mutation also alters the binding of p300 protein to T antigen. All of the cell lines grown at 32°C containing K1 plus 2831 were found to express T-antigen proteins that form a complex with the p53 protein at 32°C (data not shown). This experiment also demonstrates that mutants K1 and 2831 produce stable T-antigen protein levels comparable to those of the wild-type protein. Therefore, the inability of these mutants to overcome the p53 block to cell division, when expressed individually in T64-7B cells, is very likely due to the loss of growth-promoting functions in these proteins.

TABLE 2. Summary of immunoprecipitation analyses of cell lines cloned from colonies that were selected at 32°C

Input T-antigen construct	No. of cell lines expressing T antigen/no. examined
Wild type	
pSVgem	7/7
B3	2/2
Mutant	
*dl*1137	5/5
2809	4/4
2811	3/3
pSVcT	2/2
2837/p6-1	3/3

DISCUSSION

The SV40 large T-antigen gene encodes three distinct gene functions that are required to transform a variety of cells in culture: (i) a p300 protein-complementing activity that maps to within residues 1 to 82, (ii) an Rb-inactivating activity that maps to residues 105 to 114, and (iii) a p53-binding and inactivating region that maps to sequences within residues 351 to 626. It should be pointed out that the Rb binding site of T antigen also binds to two other proteins, p107 and p130. Thus, the properties ascribed to Rb-binding mutants of T antigen in this report could also be mediated by p107 and/or p130. Similarly, other functions of the T-antigen domain from residues 1 to 82 (not only the putative p300 binding site) could mediate the release from a p53 G_1 arrest. Each of these three regions of T antigen and their functions can contribute to the transformation of some cell types (Table 1). In this study, different T-antigen mutants deficient in each of these functions were tested to determine which domains are required to overcome a p53-mediated block in cell division. This is an assay which is clearly distinct from transformation, but it is expected

TABLE 3. Complementation analysis

Protein	Growth[a]				Activity relative to wild-type SV40 large T antigen
	Expt 1	Expt 2	Expt 3	Avg	
Negative control[b]	0	0	0	0	0
B3	27.5	16.5	20.5	21.5	1.0
2831	0	0	0	0	0
K1	2	0	0	0.5	0.02
K1 + 2831	6.5	7	2.5	5.33	0.25

[a] Average number of colonies per plate that grew at 32°C (one to three plates per experiment).
[b] BSKS plus carrier DNA was assayed.

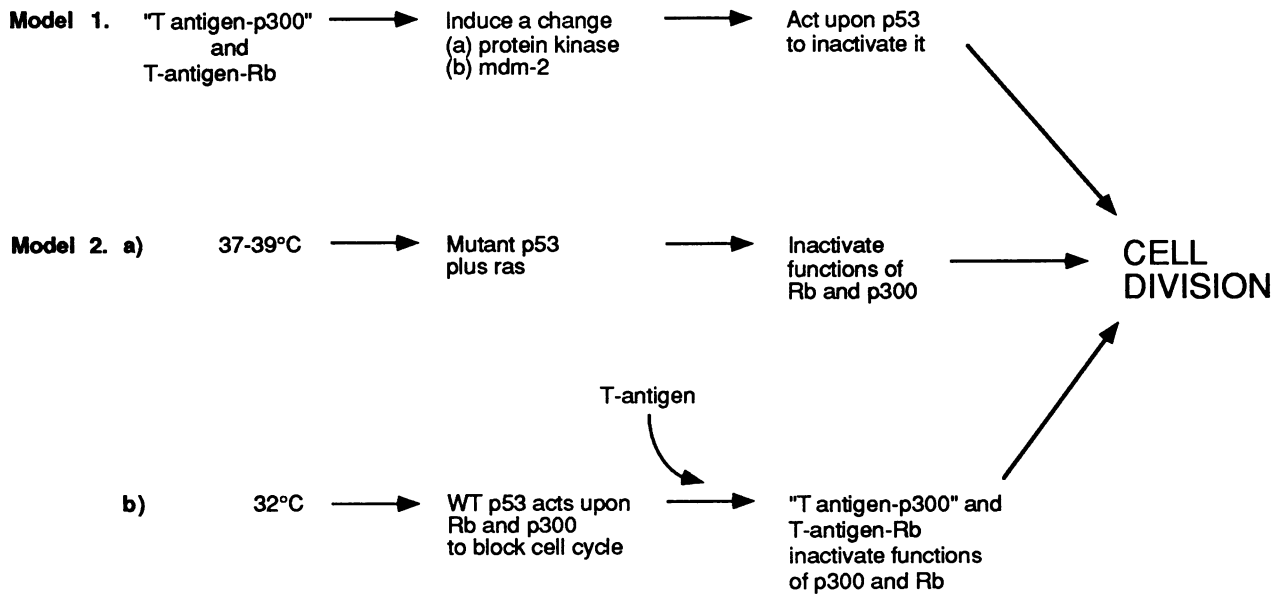

FIG. 5. Alternative models that interpret the results and predict relationships between the functions of p300, Rb, and p53.

to be related in some fashion. Surprisingly, the results demonstrate that p53 binding by T antigen was not required to overcome the p53 block to cell division. In fact, a mutant (*dl*1137) containing only T-antigen amino acid residues 1 to 121 was 50% as efficient as the wild-type T antigen in overcoming this block. Because all of the experiments in this study were carried out in a single cell line, T64-7B, one might be concerned that an additional temperature-sensitive event (in addition to the p53 protein) could affect these results. This is unlikely, however, because Michael-Michalovitz et al. (37) showed, with completely different cells, that an SV40 T-antigen mutant containing residues 1 to 272 was sufficient to overcome the ability of wild-type p53 to suppress transformation and to induce growth arrest. Thus, these results appear to be of general applicability to T antigen-p53 interactions.

While the T antigen-p53 binding site is not required for inactivation of p53-mediated growth arrest, the T-antigen N terminus (p300) and T antigen-Rb interactions are required to overcome the wild-type p53 cell cycle block. The complementation of T-antigen N-terminal mutants (p300) and T antigen-Rb-defective mutants indicates that each can act in *trans* and provides an independent function, without requirement for a single protein with both T antigen-p300 and T antigen-Rb interactive activities. These complementation studies also demonstrate that these mutants fail to relieve p53-mediated growth suppression despite the fact that the levels of mutant T antigen in a cell should be sufficient to do so.

These experimental results demonstrate that the T-antigen N-terminus (p300) and T antigen-Rb functions can communicate with the wild-type p53 and reverse its G_1 block in the cell cycle. Two distinct models can explain these results (Fig. 5). First, complexes between T antigen and cellular proteins (p300, Rb, or both) could induce a set of enzyme activities in these cells, such as protein kinases or protein-modifying enzyme activities, which may act on p53 and alter its activity in the cell cycle. For example, it has been suggested that phosphorylation modification at the casein kinase II site (human p53 residue 392) may alter p53 DNA-binding activity (24) and may be important to its growth suppressor activity (40). p53 has a serine residue that appears to be phosphorylated by a cyclin-dependent kinase activity (2), and p107, which binds to the T antigen-Rb binding site (9, 12), is associated with cyclin A-p34 protein kinase activities (10). It is not clear what effect phosphorylation of the cyclin-dependent kinase site has on p53 activity, but the results presented here suggest that T-antigen functions that may affect phosphorylation pathways are required to overcome p53-mediated growth arrest. A cellular oncogene product, Mdm-2 (13), has been shown to bind to p53 and inactivate its function as a transcription factor (42). A T antigen-p300-Rb complex might affect the level or activity of Mdm-2 in these cells, which could then play a role in the inactivation of wild-type p53 by T antigen. In either case, this model (Fig. 5, model 1) suggests that the putative T antigen-p300 complex and the T antigen-Rb complex induce a set of functions which bypass or inactivate the p53 block to G_1 cell cycle progression. The experimental data do not eliminate the possibility that repression is also involved in these activities.

In contrast to that interpretation (Fig. 5, model 2), it remains possible that the T64-7B cells rely on the expression of mutant p53 protein at 37 to 39°C to continue to divide and progress through the cell cycle. Mutant p53 proteins have recently been shown to have a gain of a new function in transformed cells (7). A mutant p53 protein expressed in a cell with no endogenous p53 protein does indeed provide the ability to divide (form tumors in animals) under circumstances in which the parental cells fail to grow. In addition, it has been clearly shown that in mutant p53-plus-*ras*-transformed rat embryo fibroblasts (such as T64-7B cells), the expression of the mutant p53 protein is continuously required for cells to replicate at an optimal growth rate (63). The presence of mutant p53 protein enhanced the growth rate of these cells by about 3.7-fold. In the present study, the mutant p53 gain of function would be lost when cells are shifted to 32°C, and the cells would then fail to divide because the p300 and Rb functions halt progression through the cell cycle, resulting in a G_1 block. The presence of SV40 T antigen with p300-complementing and Rb-binding properties would alter p300 and Rb functions and therefore permit cell division at 32°C. If this interpretation, which is

consistent with all of the results presented here, is correct, then the mutant p53 gain of function at 37 to 39°C would act on p300 and Rb and, like T antigen, inactivate their functions.

Thus, model 1 (Fig. 5) suggests that the putative T antigen-p300 complex and the T antigen-Rb complex communicate with p53 via the induction or repression of activities that can inactivate p53 function in the cell cycle. Model 2 suggests that mutant p53 can act on p300 and Rb to alter their activities and promote cell division much as T antigen-p300-Rb complexes function to bypass or alter p300 and Rb functions. Both models link p53 to p300 and Rb functionally. The two alternative models either place T antigen-p300-Rb epistatic to wild-type p53 or place mutant p53 epistatic to p300 and Rb in the G_1 phase of the cell cycle. The virtue of these models is that they make a set of predictions which can be tested. For example, model 2 suggests that a mutant p53 protein that is not temperature sensitive should overcome the p53 temperature-sensitive block at 32°C when DNA clones for this protein are transfected into such cells. For the p53 mutant KH215 (54), this indeed appears to be the case, although at a reduced efficiency compared with the SV40 T antigen (46). Moreover, the adenovirus E1A protein is known to bind to both p300 and Rb proteins (58, 64), and the adenovirus p300 binding site complements the SV40 T-antigen p300-defective mutants for transformation (60). This finding suggests that the adenovirus E1A proteins (10) might overcome the temperature-sensitive p53 block in the T64-7B cells. Preliminary experiments demonstrate that the E1A oncogene can rescue these cells for growth and colony formation at 32°C (46). Vousden and coworkers (57) have recently shown that both E1A and the human papillomavirus oncoprotein E7 can overcome wild-type p53-mediated growth arrest. Thus, it is clear that the models presented in Fig. 5 make testable predictions that should lead to new insights into the relationships between p53, Rb, and p300 and their roles in the regulation of cell division.

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