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The Ability of Simian Virus 40 Large T Antigen To Immortalize Primary Mouse Embryo Fibroblasts Cosegregates with Its Ability To Bind to p53

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The large T antigen encoded by simian virus 40 (SV40) plays essential roles in the infection of permissive cells, leading to production of progeny virions, and in the infection of nonpermissive cells, leading to malignant transformation. Primary mouse embryo fibroblasts (MEFs) are nonpermissive for SV40, and infection by wild-type SV40 leads to immortalization and transformation of a small percentage of infected cells. We examined the ability of an extensive set of mutants whose lesions affect SV40 large T antigen to immortalize MEFs. We found that immortalization activity was retained by all mutants whose lesions are located upstream of codon 346. This includes a mutant lacking amino acids 168 to 346. We previously showed (M. J. Tevethia, J. M. Pipas, T. Kierstead, and C. Cole, Virology 162:76–89, 1988) that sequences downstream of amino acid 626 are not required for immortalization of primary MEFs. Studies by Thompson et al. (D. L. Thompson, D. Kalderon, A. Smith, and M. Tevethia, Virology 178:15–34, 1990) indicate that all sequences upstream of residue 250, including the domain for binding of tumor suppressor protein Rb, are not required for transformation of MEFs. Together, these studies demonstrate that the immortalization activity of large T antigen for MEFs maps to sequences between 347 and 626. Several mutants with lesions between 347 and 626 retained the ability to immortalize at nearly the wild-type frequency, while others, with small insertions at amino acid 409 or 424 or a deletion of residues 587 to 589, failed to immortalize. The abilities of mutant T antigens to form a complex with tumor suppressor protein p53 were examined. We found that all mutants able to immortalize retained the ability to complex with p53, while all mutants which lost the ability to immortalize were no longer able to bind p53. This suggests that inactivation of the growth-suppressive properties of p53 is essential for immortalization of MEFs.

Simian virus 40 (SV40) encodes two tumor antigens, large T antigen and small t antigen. Large T antigen is a multifunctional phosphoprotein. It is localized primarily in the nucleus and subjected to a number of posttranslational modifications, including phosphorylation, adenylylation, glycosylation, and ADP-ribosylation. During lytic infection by SV40, large T antigen initially activates and subsequently represses transcription from its own promoter and activates the late promoter (6, 28, 55, 79). It also has the potential to activate a number of cellular and other viral promoters, but the biological importance of these activations remains unclear (1, 56).

T antigen plays an essential role in initiation of viral DNA replication, and its helicase activity appears to be involved in elongation during viral DNA replication (for reviews, see references 5 and 54). In addition, large T antigen immortalizes primary cells and transforms established rodent cell lines (for a review, see reference 58). Large T antigen is sufficient to cause full transformation of primary rodent cells in most cases. Small t antigen may help large T antigen to transform cells in certain circumstances (4).

Large T antigen possesses both specific and nonspecific DNA-binding activities, ATPase activity, and ATP-dependent helicase activity. These biochemical activities are directly involved in viral DNA replication (5), but none cosegregates with the immortalizing or transforming capability of T antigen (10, 43, 44, 71).

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Large T antigen encodes a number of activities which could potentially be involved in the mechanisms of immortalization and transformation. Like some nuclear oncogenes, large T antigen is a transcriptional activator (6, 27), and the spectrum of genes whose expression is altered following infection could play a fundamental role in immortalization and transformation. T antigen is able to induce host cell DNA synthesis (8) and activate rRNA synthesis from silent rRNA genes (65). Most importantly, T antigen physically associates with a number of cellular proteins. Large T antigen forms a specific complex with the underphosphorylated species of $p105^{Rb}$ through amino acid (aa) residues 105 to 114 of large T antigen, a sequence homologous to conserved region 2 of the adenovirus E1A proteins (11, 38). A similar region, also interacting with $p105^{Rb}$, is found in the E7 proteins of oncogenic strains of human papillomaviruses (49). T antigen also binds another cellular protein, 107K/120K protein, through the same region through which it binds Rb; the role of either $p105^{Rb}$ or the 107K/120K protein in growth control is not clear (14, 17). Reintroduction of an Rb gene into tumor cells which have lost both Rb alleles restores normal growth properties (20). Mutants with lesions in the Rb-binding domain of T antigen have reduced transforming ability (25, 71), indicating that binding to $p105^{Rb}$, 107K/120K, or another related protein may be involved in the mechanisms by which large T antigen causes transformation.

T antigen also associates with the p53 antioncogene protein and stabilizes it. Loss or inactivation of both alleles of the p53 gene is found in a substantial fraction of several

different human tumors (2, 22, 23, 39, 50, 69, 75). Introduction of wild-type copies of p53 into transformed cells suppresses their growth (3, 12, 45–47); the wild-type gene is also able to reduce the frequency of transformation of cultured cells by several oncogenes (16, 18). In contrast, some mutant forms of p53 are able to immortalize cells in culture and cooperate with the *ras* oncogene to transform primary rat embryo fibroblasts (REFs) (24). The presence of some mutated alleles in a heterozygous state with a wild-type copy of p53 may confer a growth advantage on these cells (32). Thus, wild-type p53 appears to be a tumor suppressor gene while some mutant alleles behave as dominantly acting oncogenes.

The regions of T antigen essential for this interaction have not been well mapped. A few mutants unable to bind p53 have been described (35, 52). These mutants have small deletions in the vicinity of aa 580 to 590 of large T which render the mutant proteins unstable and may also bring about major changes in their conformation. Manfredi and Prives (41) showed that hybrid SV40-polyomavirus T antigens containing residues 337 to 708 of SV40 large T bound p53 while a hybrid containing residues 411 to 708 did not. p53 also forms complexes with other viral proteins which play roles in transformation. The adenovirus E1B 55-kDa polypeptide interacts with p53, as does the E6 protein of oncogenic strains of human papillomaviruses (59, 61, 76). Association of p53 with these tumor virus proteins could play a role in their transforming or immortalizing activities or both.

T antigen also associates with DNA polymerase α (through aa residues 1 to 83) (13) and a 73-kDa hsp-like protein (through aa residues 1 to 178) (60). These proteins are also possible cellular targets through which T antigen interactions could lead to immortalization or transformation.

The transforming proteins of DNA tumor viruses have long been model systems for studying oncogenesis. Adenovirus E1A proteins resemble SV40 large T antigen in that both are transcriptional regulatory proteins and are able to alter cellular growth control. E1A proteins can immortalize primary cells and cooperate with adenovirus E1B proteins and other oncogene proteins, such as activated *ras* gene products, to bring about full transformation of primary rat cells (57). E1A proteins can also activate and repress viral and cellular gene expression (19, 33, 34, 36, 63). Two regions of E1A, aa residues 30 to 60 and 121 to 127, respectively, were found to be responsible for the immortalization and *ras* cooperation functions. These regions are conserved among different adenovirus serotypes (78) and have homology to portions of SV40 large T antigen and human papillomavirus E7 protein (15). This region of homology has been shown to be essential for binding to $p105^{Rb}$ (11, 15, 49, 77).

Immortality of cells is a recessive phenotype, and immortal human cell lines have been assigned to at least four complementation groups (53). All SV40-immortalized cell lines except one belong to one complementation group, suggesting that SV40 disrupts one of several distinct cellular functions whose alteration can lead to immortalization of primary cells. Assays for the immortalization function of T antigen generally have used primary mouse or rat cells. Primary C57/BL6 mouse embryo fibroblast (MEF) cells can be immortalized by transfection of the gene encoding large T antigen (70), and the amino-terminal 626 aa of T are sufficient for immortalization (71). Deletion mutants which encode T antigens missing aa 1 to 127 or 121 to 250 were also able to immortalize primary MEF cells (73). Therefore, specific and nonspecific DNA-binding activities; DNA helicase activity; binding to $p105^{Rb}$, 107K/120K, and hsp73; and the adenovirus helper-host range function are not essential for immortalization of primary MEF cells. In contrast, the amino-terminal 135 aa of large T appear to be sufficient for immortalization of primary REFs (31, 51, 64).

To explore the mechanisms of immortalization by SV40 large T antigen further, a large set of point, linker insertion, and deletion mutants were assayed for immortalization by using primary C57/BL6 MEFs. In agreement with earlier studies, we found that the N-terminal portion of large T, including the $p105^{Rb}$-binding site, was dispensable for immortalization of primary mouse cells. Analysis of immortalization by mutants with large deletions allowed us to narrow the domain important for immortalization further to residues 347 to 626. We examined several mutants with small insertions or deletions within the 347 to 626 region and found perfect cosegregation between the abilities of these mutant large T antigens to immortalize primary mouse cells and their abilities to bind p53.

MATERIALS AND METHODS

Plasmids, cells, and tissue culture. REF52 cells are an established REF cell line (37) and were a gift from Jim Pipas (University of Pittsburgh, Pittsburgh, Pa.). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal bovine serum (FCS). This medium was supplemented with 500 μg of G418 per ml when appropriate for drug selection following transfection with pSV2neo. pSV2neo contains a bacterial neomycin phosphotransferase gene (66). Wild-type and all mutant SV40 DNAs were cloned into the *Eco*RI site of pUC18 in an orientation in which the two *Bam*HI sites were located close together. Standard techniques were used for bacterial growth and transfection (40), and large-scale plasmid purification was done by CsCl-ethidium bromide buoyant density centrifugation (42).

Mutants and mutant preparation. Many of the mutants used in these studies have been described previously. Mutants in the 2400 series that carry deletions at various *Dde*I sites within the early region of SV40 were described previously (74) and produce nearly full-size T antigens lacking 1 to 4 amino acids. Mutants with a 12-bp linker (5′-TCGCGATC GCGA-3′) inserted at various sites within the SV40 early region belong to the 2800 series and have also been described previously (83). This linker contains two sites for *Nru*I and one for *Pvu*I. Also included in the 2800 series are some deletion mutants. Mutant *dl*A2834 has a deletion of codon 85 (84). Mutants *dl*A2837, *dl*A2838, and *dl*A2839 were prepared from linker insertion mutants by digesting appropriate mutants with *Nru*I, which cuts within the linker, and *Xba*I, which cuts each plasmid once within the polylinker region. By combining appropriate fragments from two linker insertion mutants, sequences between the sites of the linkers were deleted. Mutant *dl*A2837 was prepared by using mutants *in*A2815 and *in*A2819; it lacks sequences encoding aa 168 to 346 and contains the 12-bp linker at the site of the deletion. Mutants *dl*A2838 and *dl*A2839 were prepared by using mutants *in*A2809 and *in*A2827 and lack sequences encoding aa 409 to 520. Mutant *dl*A2838 contains the 12-bp linker at the site of the deletion, while mutant *dl*A2839 contains only half of the linker (a single *Nru*I site).

Mutant designations. Within the text, mutants are designated by their numbers. For insertion mutants, the amino acid codon at the site of the insertion is given in parentheses following the mutant number. For deletion mutants, the amino acids missing from the T antigen encoded by the

mutant genome are listed in parentheses. Two point mutants prepared in other laboratories [K1 (Q107K) (25) and SVcT (K128Q) (30)] and one deletion mutant [Met-128 (Δaa 1–127) (73)] were also included in these studies. For point mutants, the amino acid change created by the mutation is given in parentheses following the mutant number.

Preparation of primary MEF cells. Primary MEF cells were prepared from 11 to 14-day-old C57/BL6 mouse embryos as previously described (70). The heads and limbs were removed, and the torsos were cut into 1- to 2-mm chunks and minced. The tissue was then trypsinized to produce a single-cell suspension. The cells were cultured in DMEM containing 10% FCS, 7.5% $NaHCO_3$, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.3), and 3% glutamine or frozen in liquid nitrogen for subsequent use. Immortalization assays were performed on cells prior to their fourth 1:3 subculturing. The primary MEFs used in some experiments were a gift from M. J. Tevethia (Hershey, Pa.).

Immortalization assays. MEF cells (1.5×10^5) were seeded into 100-mm-diameter plates on the day before transfection. Cells were cotransfected with 5 μg of SV40 wild-type or mutant plasmid DNA, 1 μg of pSV2neo, and 10 μg of high-molecular-weight salmon sperm DNA as the carrier. The standard calcium phosphate transfection procedure described elsewhere (83) was used. The cells were exposed to the calcium phosphate precipitate for 6 to 8 h. The medium was removed, and the cells were washed twice with Tris-buffered saline (TS; 25 mM Tris-Cl [pH 7.5], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.05 mM $MgCl_2$, 0.7 mM $CaCl_2$) and fed with fresh medium containing 10% FCS. The medium was changed every 3 to 4 days, and 400 μg of G418 per ml was added to the medium 6 days after transfection. The cells were stained with 0.5% methylene blue 2 to 3 weeks after transfection, and the number of colonies was scored.

Immunoprecipitations and analyses of p53 binding. The stabilities of the T antigens encoded by *dl*A2837, *dl*A2838, and *dl*A2839 were analyzed in pools of REF52 cells expressing mutant T antigens. The abilities of wild-type and mutant T antigens to bind p53 were examined in BALB/c 3T3 cell lines or pools expressing mutant T antigens, except that C57/BL6 MEF cell lines expressing mutant T antigens were used for mutants in the 2400 series. The abilities of the T antigens encoded by mutants *dl*A2837 and *dl*A2838 to complex with p53 were analyzed in transiently transfected BALB/c 3T3 cells. Cells (2×10^5) were seeded onto 60-mm-diameter plates on the day before labelling. These exponentially growing cultures were washed twice with TS and incubated for 1 to 1.5 h at 37°C in 2 ml of DMEM lacking methionine and containing 2% dialyzed FCS. The medium was removed, and the cells were labelled for 2 to 3 h with 100 μCi of [^{35}S]methionine (Dupont, Boston, Mass.) in 0.7 ml of DMEM lacking methionine and containing 2% dialyzed FCS. Cultures were harvested by washing twice with TS, followed by lysis of cells with 0.5 ml of EBC buffer (50 mM Tris-Cl [pH 8.0], 100 mM NaCl, 0.5% Nonidet P-40, 0.1 U of aprotinin per ml, 1 nM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml [11]) for 20 m at 4°C. The cellular debris were removed by centrifugation for 15 min at 4°C in a microcentrifuge. REF52 cell extracts were precleared by addition of 20 μl of Sepharose A beads for 30 min at 4°C. Extracts from identical numbers of cells were mixed with 5 to 10 μl of a mixture of mouse ascites fluids containing monoclonal antibodies pAB901 and pAB902 (a gift from S. and M. J. Tevethia) or tissue culture supernatant containing monoclonal antibody pAB421. pAB901 and pAB902 antibodies recognize denaturation-resistant determinants located at the carboxy and amino termini of large T antigen, respectively (72). pAB421 antibody recognizes p53. Mixtures were rocked for 1 h at 4°C. A 20-μl volume of protein A-Sepharose beads (1:1 mixture in TS-bovine serum albumin [11]) (Sigma) was added to the mixture, and rocking was continued for 30 min at 4°C. The Sepharose beads were pelleted by centrifugation and washed three times with 1 ml of EBC buffer at 4°C. Immunoprecipitated proteins were released by heating in sample buffer for 15 min at 70°C and analyzed by electrophoresis in sodium dodecyl sulfate (SDS)–7.5% or 10% polyacrylamide gels, followed by fluorography. When stabilities of mutant proteins were being examined, cultures were labelled as described above for 3 h, washed twice with TS, and incubated in DMEM containing 10% FCS. Cultures were harvested after chase periods of 0, 5, and 17 h and processed as described above.

RESULTS

The experiments reported here were conducted to determine more precisely which regions of SV40 large T antigen are required for immortalization of primary MEFs and to search for correlations between this activity of T and its other properties. We used primary MEFs to study immortalization because the assay is quantitative and because cells which do not become immortal through expression of wild-type or mutant T antigen undergo senescence during the course of the experiment (70).

New mutants prepared for these studies. While most of the mutants used in these studies have been described in previous work from this laboratory or others, three mutants not previously described were also used. Each of these new mutants was prepared from a pair of linker insertion mutants by combining appropriate DNA fragments to generate new mutants deleted for the information between the sites of the inserted linker in each parent. By using pairs of mutants with the linker in the same reading frame, these new mutants were expected to encode large T antigen shortened by the removal of internal amino acids encoded between the sites at which the linkers were inserted in the parental viral genomes. Two of the mutants have the same deletion and produce T antigens lacking aa 409 to 520. One of these two mutants (*dl*A2839) has an insertion of two aa and the other (*dl*A2838) has an insertion of four aa at the site of the deletion.

Pools of REF52 cells resistant to G418 and expressing each mutant T antigen were obtained as described in Materials and Methods. To confirm that the T antigens encoded by the new mutants were of the expected sizes and to examine their stabilities, cell pools were radiolabelled with [^{35}S]methionine for 3 h and chased in medium containing cold methionine for 0, 5, or 17 h. Extracts of these cells were immunoprecipitated with monoclonal antibodies recognizing the N and C termini of large T. The autoradiogram resulting from this experiment is shown in Fig. 1. These T antigens were readily detected and were of the expected sizes. The T antigen produced by *dl*A2837 was as stable as wild-type T antigen (compare lanes 1 to 3 with lanes 10 to 12), and those produced by *dl*A2838 and *dl*A2839 were quite unstable, with half-lives of less than 1 h (lanes 4 to 6 and 7 to 9). Mutant *in*A2807(aa 302) was previously shown to encode a very unstable T antigen (83) and is included here for comparison.

Immortalization of primary C57/BL6 MEF cells. Primary MEF cells were seeded on 100-mm-diameter plates at a low

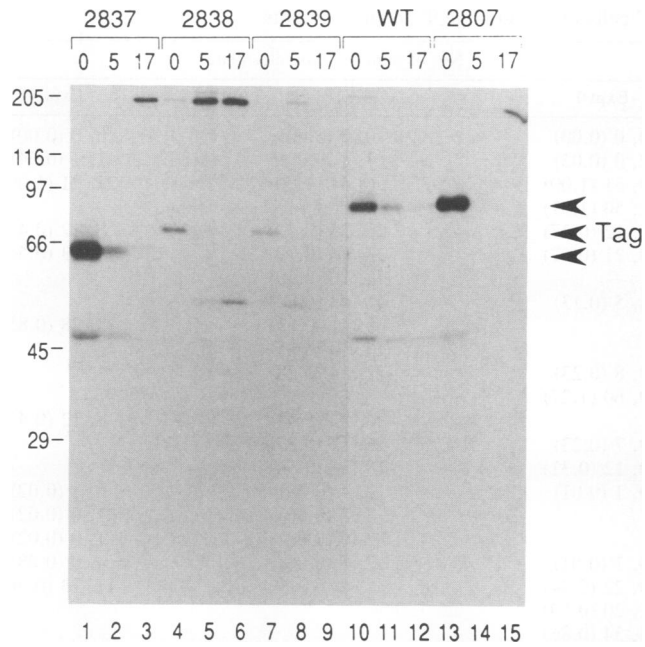

FIG. 1. Stabilities of mutant SV40 large T antigens (Tag). Immunoprecipitates of labelled cell extracts were electrophoresed on SDS–10% polyacrylamide gels and autoradiographed. The molecular sizes in kilodaltons are indicated at the left. The positions of the T antigens are indicated by the arrowheads to the right. Hours of chase time are indicated above the lanes. WT, wild type.

density so that the cells would become senescent before a monolayer of cells was formed. Only immortalized cells form colonies under these conditions. In each experiment, duplicate plates were transfected with each viral DNA. A set of linker insertion mutant and a smaller number of deletion and point mutants were examined in this study. Most mutants were examined in two independent experiments. The results are presented in Table 1 and summarized in Fig. 2.

All mutants with lesions in the first exon [*in*2801(aa 5), *in*A2803(aa 35), and *dl*A2831(aa 5 to 35)] were able to immortalize primary MEF cells, as was mutant Met-128(Δaa 1 to 127), which lacks the first 127 aa of large T antigen. These results are consistent with those of Thompson et al., who showed that the first 250 aa of large T antigen were not required for immortalization (73). Furthermore, mutant *dl*A2837 (aa 168 to 346) was also able to immortalize primary MEF cells. These results set the proximal boundary of the minimum sequences needed to immortalize primary MEFs at residue 347 and indicate that the zinc finger motif of large T antigen is not required for immortalization. Previously, we showed (71) that a mutant missing aa residues 627 to 708 immortalized as well as the wild type. Thus, only the sequences between 347 and 626 are required for immortalization.

Mutants *in*A2809(aa 409) and *in*A2811(aa 424) were unable to immortalize MEF cells. These two mutants encode stable mutant T antigens and retain the host range function. In addition, *in*A2809 retains the abilities to bind specifically to viral DNA (84) and form oligomers (81). These results suggest that the mutations are in a specific region essential for immortalization of MEFs. Furthermore, deletion mutants *dl*A2838(aa 409 to 520) and *dl*A2839(aa 409 to 520) also failed to immortalize. The only other mutant completely defective for immortalization in these assays was *dl*A2433(aa 587 to 589), which we previously reported to be defective for immortalization (71). The inability of a mutant T antigen to immortalize could be due to instability, and several of the T antigens defective for immortalization encode T antigens with reduced stability. We note, however, that mutant *in*A2807(aa 302) was able to immortalize, although only about one-quarter to one-half as well as wild-type T. The T antigen of this mutant was no more stable than those of immortalization-defective mutants such as *dl*A2838(aa 409 to 520) and *dl*A2839(aa 409 to 520) (cf. Table 1, Fig. 1, and reference 83). All of the other linker insertion mutants tested were able to immortalize primary MEFs. Two mutants affecting the Rb-binding site [K1 (Q107K) and *dl*2441(aa 106)] immortalized well. All four mutants affecting small t antigen and one which affects only small t antigen (*in*A2829) also immortalized MEF cells, indicating that small t was dispensable for this function.

p53 binding by mutant T antigens. Formation of complexes between T antigens and mouse p53 was examined in extracts of mouse cell pools, cloned mouse cell lines, or cloned rat cell lines expressing wild-type or mutant T antigens. The ability of p53 to associate with various T antigens was analyzed by immunoprecipitation. Exponentially growing cells were labelled with [^{35}S]methionine. Labelled-cell extracts were immunoprecipitated with anti-T or anti-p53 monoclonal antibodies and analyzed on SDS-polyacrylamide gels. The results are summarized in Fig. 2; representative autoradiographs of the gels are shown in Fig. 3.

In Fig. 3a, coimmunoprecipitation of p53 and large T antigen by anti-T antigen (pAb902) or anti-p53 (pAb421) antibodies was examined for the two insertion mutants *in*A2809(aa 409) and *in*A2811(aa 424) and compared with that for wild-type T antigen and unstable mutant *in*A2807(aa 302). Although the amounts of p53 precipitated by an anti-p53 antibody were within two- to fourfold of each other (lanes 2 to 5), only wild-type and *in*A2807(aa 302) T antigens were coprecipitated (lanes 2 and 5). Examination of the immune complexes retrieved from the anti-T antigen precipitate revealed the presence of p53 from the extracts of *in*A2807- and wild-type-expressing cells (lanes 7 and 10) but no increase over the neomycin-resistant control from either *in*A2809- or *in*A2811-expressing cells (lanes 6, 8, and 9).

Figure 3b shows immunoprecipitates from extracts of transfected cells expressing mutant T antigens *dl*A2837(aa 168 to 346) and *dl*A2838(aa 409 to 520) compared with those expressing the wild type and *in*A2807(aa 302). Note the different mobilities of the deletion mutant T antigens indicated by the arrowheads at the right of the panel. Anti-p53 antibodies coprecipitated T antigen from cells producing *dl*A2837(aa 168 to 346), *in*A2807(aa 302), and wild-type T antigens (lanes 1, 3, and 4) but not from cells producing *dl*A2838(aa 409 to 520) T antigen (lane 2). When portions of the same extracts were immunoprecipitated with pAb901 plus pAb902 (anti-T antigen), p53 coprecipitated with all but *dl*A2838(aa 409 to 520) (compare lanes 6, 8, and 9 with lane 7). Although the data shown in Fig. 3b represent immunochemical analyses of REF52 cells expressing various T antigens, the same results were obtained with transfected murine BALB/c 3T3 cells (data not shown).

Thus, the T antigens of five mutants with lesions in the 347 to 626 region, *in*A2809(aa 409), *in*A2811(aa 424), *dl*A2838(aa 409 to 520), *dl*A2839(aa 409 to 520), and *dl*A2433(aa 587 to 589), all failed to bind to p53. *dl*A2433(aa 587 to 589), *dl*A2838(aa 409 to 520), and *dl*A2839(aa 409 to 520) encode unstable large T antigens, while *in*A2809(aa 409) and *in*A2811(aa 424) T antigens are stable (83). These were the

TABLE 1. Immortalization of primary MEF cells by SV40 large T antigen mutants

DNA	Amino acid lesion	No. of immortal colonies/plate[a]		
		Expt 1	Expt 2	Expt 3
None (mock infection)		0, 0 (0.00)	0, 0 (0.00)	0, 0 (0.00)
pSV2neo		2, 0 (0.03)	3, 4 (0.07)	1, 1 (0.04)
Wild type		39, 31 (1.00)	56, 44 (1.00)	27, 21 (1.00)
2801	*in*5	42, 50 (1.31)		
2831	*dl*5–35 + *in*	14, 12 (0.37)	22, 28 (0.50)	8, 12 (0.42)
2803	*in*35	19, 21 (0.57)	23, 16 (0.39)	14, 13 (0.56)
Met-128	*dl*1–127		14, 16 (0.30)	
2441	*dl*106	4, 5 (0.13)	41, 43 (0.84)	
K1	Q107K		48, 41 (0.89)	13, 28 (0.85)
SVcT	K128Q		15, 30 (0.45)	
2815	*in*168	8, 8 (0.23)	12, 14 (0.28)	
2817	*in*219	29, 60 (1.27)		
2837	*dl*168–346 + *in*		26, 22 (0.48)	8, 12 (0.42)
2807	*in*303	9, 7 (0.23)	16, 11 (0.27)	
2819	*in*346	10, 12 (0.31)	18, 36 (0.54)	
2809	*in*409	0, 1 (0.01)	2, 4 (0.06)	0, 1 (0.02)
2838	*dl*409–520 + *in*		4, 2 (0.06)	1, 0 (0.02)
2839	*dl*409–520 + *in*		2, 0 (0.02)	1, 0 (0.02)
2811	*in*424	0, 1 (0.01)	2, 6 (0.08)	4, 0 (0.08)
2821	*in*460	9, 22 (0.44)		11, 13 (0.50)
2823	*in*464	21, 20 (0.59)		
2827	*in*520	26, 34 (0.86)		
2828	*in*520	26, 28 (0.77)		
2433	*dl*587–589		1, 3 (0.04)	
2829	*in*173 (small t)	40, 47 (1.24)		

[a] The first two numbers are the numbers of colonies from two parallel plates in each experiment. The number in parentheses is the level relative to that of wild-type SV40 DNA in each experiment.

only mutants with deletions or insertions that failed to immortalize primary MEF cells. Four other mutants with lesions in this region, *in*A2821(aa 460), *in*A2823(aa 464), *in*A2827(aa 520), and *in*A2828(aa 520), were able to bind p53 (data not shown) and immortalize primary MEFs. We reported previously (71) that mutants *dl*A2432(aa 507 to 509) and *dl*A2462(aa 509) were able to bind p53 and immortalize primary MEFs as well as wild-type large T. Thus, all mutants able to bind p53 immortalized primary MEFs, while none of the mutants defective for p53 binding were able to immortalize these cells.

All the mutant large T antigens examined with lesions

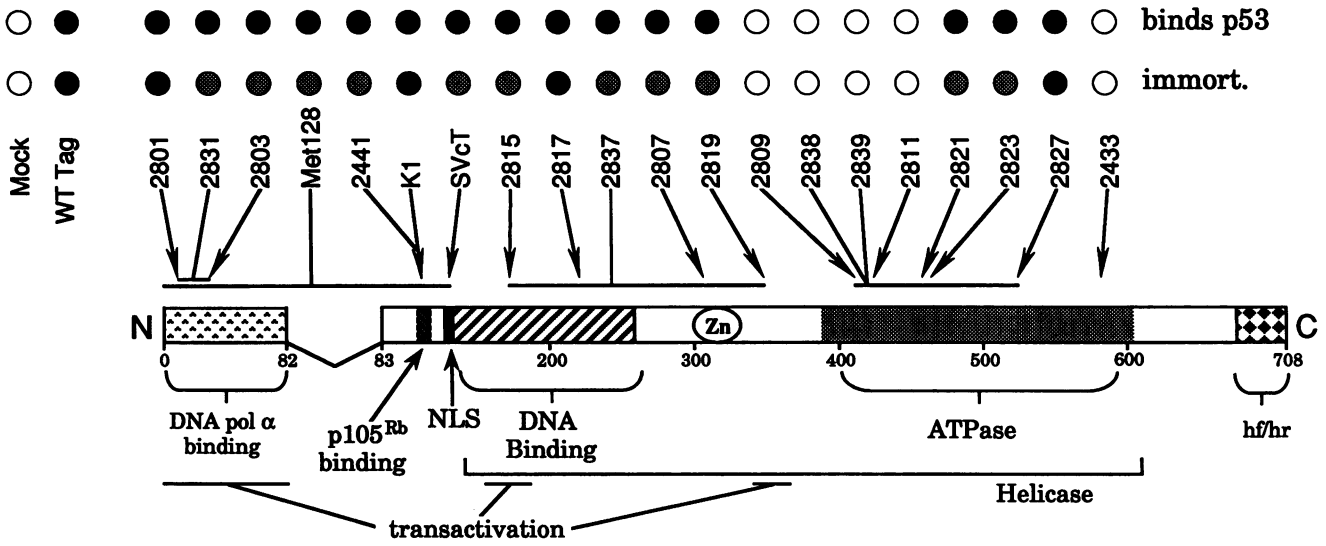

FIG. 2. Immortalization ability (immort.) and p53-binding data of mutants used in this study and regions of T antigen involved in its different functions. The data from Table 1 for MEF immortalization are summarized as follows. Less than 10% of the level of immortalization by wild-type T antigen (WT Tag) is indicated by open symbols, 10 to 70% is indicated by grey symbols, and greater than 70% is indicated by black symbols. p53 binding is indicated by closed symbols, and failure to bind is indicated by open symbols. The locations of mutations are indicated over the map of T antigen. Arrows indicate linker insertion or point mutations, and horizontal bars indicate deleted regions. NLS, nuclear localization signal; pol, polymerase.

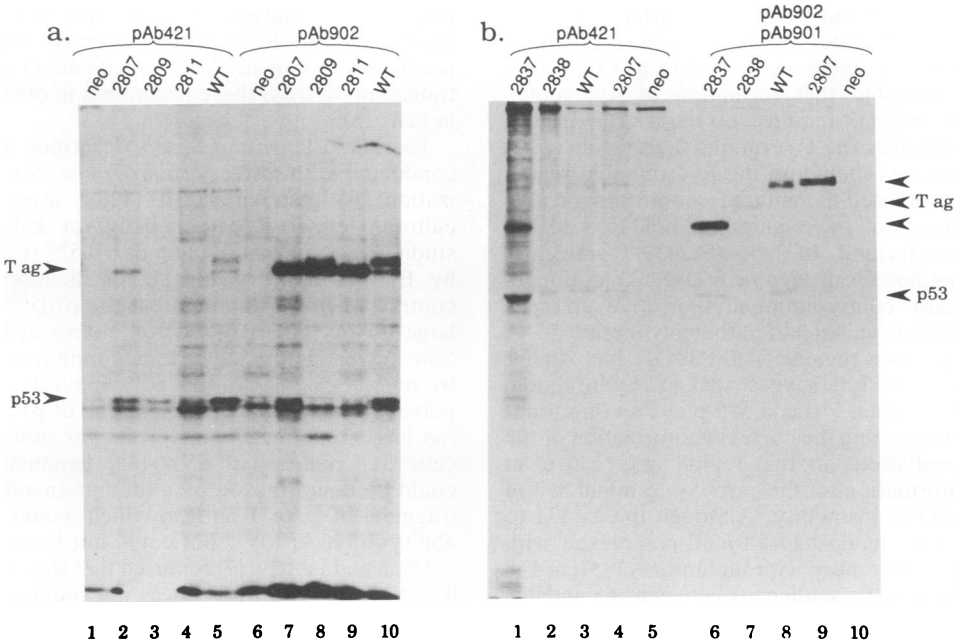

FIG. 3. p53 binding by mutant T antigens. The positions of T antigens and p53 are indicated by arrowheads. The antibody used to immunoprecipitate the labelled cell extract is indicated over the sample names above the lanes. (a) SDS–75% polyacrylamide gel of immunoprecipitates from labelled MEF extracts. Lanes 1 to 5, pAB421 (anti-p53); 6 to 10, pAB902 (anti-T antigen). (b) SDS–10% polyacrylamide gel of immunoprecipitates from labelled extracts of REF52 cells. Lanes 1 to 5, pAB421 (anti-p53); 6 to 10, pAB902 and pAB901 (anti-T antigen).

within the first 400 aa residues bound to p53. These include *dl*A2837(aa 168 to 346), indicating that T antigen sequences upstream of residue 346 are not required for binding of p53 (Fig. 3b, cf. lanes 1 and 6). This finding, coupled with the earlier finding that mutant *dl*A2465(aa 627 to 708) bound p53 (71), indicates that the p53-binding domain is contained within residues 347 to 626 of large T.

DISCUSSION

Tumorigenesis is a multistep process. Tumors may result from alteration of multiple proto-oncogenes, loss of multiple tumor suppressor genes, or a combination of proto-oncogene mutations and tumor suppressor gene loss. Many studies have shown that the combined action of multiple altered proto-oncogenes is sufficient to convert primary cells into immortalized, fully transformed cells (29, 57; for a review, see reference 21). SV40 large T antigen is a multifunctional protein and, acting alone, is able to cause immortalization and transformation of primary rodent cells. The recent finding that T antigen associates with at least two tumor suppressor proteins, $p105^{Rb}$ and p53, has shed light on the mechanisms whereby a single protein can drive primary cells all the way to full transformation.

In the studies described here, primary MEF cells were used to measure the immortalization activities of mutant large T antigens. Immortalization of primary cells is a critical step along the path to full transformation.

Immortalization of primary MEFs by large T antigen. Immortalization of primary MEF cells has now been localized to the carboxy-terminal half of the T antigen. Deletion mutants missing the first 127 aa residues or residues 125 to 250 were able to immortalize primary MEF cells at the wild-type frequency (73). The results shown here (Table 1), together with our previous results obtained with the 2400 series of deletion mutants (71), further narrow down the minimum sequences sufficient for immortalization of primary MEFs to sequences between aa residues 347 and 626 of large T antigen. $p105^{Rb}$ and p107/p120 binding cannot be essential for the immortalization function of SV40 large T antigen, since mutants missing the $p105^{Rb}$-binding site were still capable of immortalizing MEF cells [reference 73 and Met-128(Δaa 1 to 127) in Table 1].

Mutants *dl*A2838(aa 409 to 420), *dl*A2839(aa 409 to 520), and *dl*A2433(aa 587 to 589) were unable to immortalize primary MEF cells, suggesting that the region between aa 400 and 600 encodes an activity essential for immortalization and transformation of large T antigen. However, the instability of the large T antigens encoded by these mutants makes it difficult to draw firm conclusions based on these mutants. However, mutants *in*A2809(aa 409) and *in*A2811(aa 424) also failed to immortalize primary MEF cells, and these mutants encode stable T antigens (83). These mutant T antigens failed to bind p53, suggesting that p53 binding plays an essential role in the immortalization of primary MEFs. In contrast, mutants *in*A2821(aa 460), *in*A2823(aa 464), *in*A2827(aa 520), and *dl*A2432(aa 507 to 509) (71) retain the abilities to immortalize primary MEFs and bind p53. Thus, T antigen between residues 347 and 626 contains some portions where mutations do not affect p53 binding or immortalization and other portions where mutations knock out both p53 binding and immortalization. It remains to be tested whether the p53 binding is sufficient for a mutant large T antigen to immortalize primary MEF cells.

The mutation of *dl*A2433(aa 587 to 589) may not be within the p53-binding site, because a 46-kDa proteolytic fragment spanning residues 131 to 517 was able to bind p53 (62). However, those studies used proteolysis under conditions in

which both free T antigen and T bound to various proteins, including p53, were substrates for proteolysis. Therefore, they reveal the nature of fragments bound to p53 following proteolysis. It is possible that formation of a complex between T antigen and p53 requires a region of T antigen extending further towards the C terminus than residue 517. For example, sequences including the hydrophobic region (570 to 590) may be required to maintain a conformation able to bind p53 but may not be required to hold p53 in the complex once it has formed. In fact, *dl*A2433 T antigen is very unstable, does not form oligomers larger than dimers (81), and lacks many conformationally sensitive epitopes located in the carboxy-terminal half of the polypeptide (9). A point mutant at the same region, 5080(P584L), has similar properties (68). These results suggest that the hydrophobic region between aa residues 570 and 590 plays an important structural role in maintaining the correct conformation of the protein and that mutations in this region may cause an altered overall conformation for the carboxy-terminal half of the protein, resulting in instability. Although the aa 131 to 517 fragment was the smallest one found complexed with p53, the studies presented here with mutant *dl*A2837(aa 168 to 346) demonstrate that the sequences between 168 and 346 are not required for p53 binding. Thus, the upstream boundary for p53 binding must lie between residues 346 and 409 (the most proximal site at which an inserted linker led to loss of immortalization activity and p53 binding). Similarly, the distal boundary for these functions must lie between residues 587 and 626.

We have examined the abilities of several of these mutants to bind rat and monkey p53; in all cases, mutants which were able to bind mouse p53 also bound p53 from the other species examined. Similarly, mutants defective for binding to mouse p53 were defective for binding to p53 of other species (82).

How is immortalization related to other properties of large T antigen? One of the overall goals of our investigations is to gain an insight into how the various activities of large T antigen are related to one another. Figure 2 shows a schematic illustration of the portions of T antigen required for its various activities. Amino acid residues responsible for transformation of 10T1/2 cells are located within the first 121 aa residues of large T antigen (67), where some are shared with small t antigen. Clearly, the sequences required for transformation of 10T1/2 cells are completely nonoverlapping with those required for immortalization of primary MEFs. This part of the large T antigen is involved in DNA polymerase α binding (13), transcriptional activation of viral and cellular genes (84), and binding to $p105^{Rb}$ and p107/p120 (14, 17).

The finding that mutants able to immortalize as well as wild-type T antigen include some [*dl*A2411(aa 143 to 146) and *in*A2815(aa 168)] that were completely defective for transactivation of the SV40 late and Rous sarcoma virus long terminal repeat promoters (84) is interesting and raises the possibility that the activities of large T antigen responsible for transactivation of the SV40 late and Rous sarcoma virus long terminal repeat promoters are not involved in immortalization. This is reminiscent of results obtained in studies on the adenovirus E1A protein, in which the transcriptional activation activity of E1A maps to the unique portion of the 289-aa 13S mRNA product of the E1A gene, a region known to be completely dispensable for E1A immortalization (48). Nevertheless, it remains possible that transactivation of some cellular genes is important for immortalization and that mutants which failed to immortalize primary mouse cells might not be able to transactivate these genes. It is also possible that mutants defective for transactivation of the SV40 late and Rous sarcoma virus long terminal repeat promoters in monkey kidney cells might be able to activate transcription from these promoters in other types of cells or in cells from other species.

The immortalization assays described in this report were conducted with primary mouse cells. Studies on immortalization by adenovirus E1A have used primary rat cell cultures (either REFs or baby rat kidney cells). Those studies indicate that binding of $p105^{Rb}$ (or a related protein) by E1A is required for immortalization, and this result contrasts with our finding that the $p105^{Rb}$-binding region of large T was not required for mouse cell immortalization. Some investigators have studied immortalization of rat cells by mutants of SV40 and polyomavirus. The studies with polyomavirus indicate that binding of $p105^{Rb}$ to polyomavirus large T antigen is important for immortalization of rat cells (31); studies with SV40 (64) demonstrated that rat cells could be immortalized by a mutant encoding an N-terminal fragment of large T antigen which would have retained the ability to bind $p105^{Rb}$ but could not have bound p53.

Chen and Paucha (7) reported that the ability of SV40 large T antigen to bind $p105^{Rb}$ was not required for immortalization of either MEFs or rat REFs. Their findings obtained with MEFs agree with the results presented here and with those obtained by Thompson et al. (73). Chen and Paucha's finding that immortalization of rat cells did not require $p105^{Rb}$ binding is in contrast to the findings of other investigators who have examined rat cell immortalization by polyomavirus and human adenoviruses. However, the protocol they used for immortalization involved subculturing of colonies which arose on plates of cells transfected by wild-type or mutant DNAs. They estimated that expansion of colonies in this manner represented approximately 17 cell doublings. While primary MEFs undergo senescence within 17 doublings, this may not be true for REFs. Consequently, it is uncertain that colonies of REFs expressing mutant T antigens were truly immortal. We are extending our studies to primary rat cells to understand and, we hope, resolve the disagreements among studies conducted previously by others. These studies are required to determine whether these earlier results reflect fundamentally different requirements for and mechanisms of immortalization of REFs and MEFs by SV40 and other DNA tumor viruses.

While only a small portion of large T is required to transform mouse 10T1/2 cells, Srinivasan et al. (67) showed that much more of the protein is required for transformation of REF52 cells and similar results have been obtained with other established rodent cell lines. The set of mutants used in those studies did not permit mapping with precision the sequences required and those dispensable for transformation. We are currently examining the abilities of the mutants used in the studies described here to transform 10T1/2 and REF52 cells. It is clear, however, that the immortalization function of large T requires some of the sequences required for REF52 transformation. Whether REF52 transformation also requires p53 binding is not known.

In this light, it is worth noting that E1A and E1B can cooperate to bring about full transformation of primary REFs and primary baby rat kidney cells. While E1B is also a p53-binding protein, it binds sequences of p53 different from those bound by SV40 large T and the ability to form a complex with p53 is not essential to the transformation activity of E1B (26, 80). The studies currently under way should provide insight into whether p53 binding plays a role in transformation by SV40 large T antigen.

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