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Transformation of a Continuous Rat Embryo Fibroblast Cell Line Requires Three Separate Domains of Simian Virus 40 Large T Antigen

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Mouse C3H 10T1/2 cells and the established rat embryo fibroblast cell line REF-52 are two cell lines widely used in studies of viral transformation. Studies have shown that transformation of 10T1/2 cells requires only the amino-terminal 121 amino acids of simian virus 40 (SV40) large T antigen, while transformation of REF-52 cells requires considerably more of large T antigen, extending from near the N terminus to beyond residue 600. The ability of a large set of linker insertion, small deletion, and point mutants of SV40 T antigen to transform these two cell lines and to bind $p105^{Rb}$ was determined. Transformation of 10T1/2 cells was greatly reduced by mutations within the first exon of the gene for large T antigen but was only modestly affected by mutations affecting the $p105^{Rb}$ binding site or the p53 binding region. All mutants defective for transformation of 10T1/2 cells were also defective for transformation of REF-52 cells. In addition, mutants whose T antigens had alterations in the Rb binding site showed a substantial reduction in transformation of REF-52 cells, and the degree of this reduction could be correlated with the ability of the mutant T antigens to bind $p105^{Rb}$. There was a tight correlation between the ability of mutants to transform REF-52 cells and the ability of their T antigens to bind p53. These results demonstrate that multiple regions of large T antigen are required for full transformation by SV40.

The 5,243-bp genome of simian virus 40 (SV40) encodes two proteins produced early after infection, the 708-amino-acid large T antigen and the 174-amino-acid small t antigen, as well as three capsid proteins and an additional late protein, the agnoprotein, involved in capsid assembly. The large T antigen is a multifunctional nuclear phosphoprotein which has been shown to play central roles both in the lytic infection of monkey cells and in the abortive and transforming infections of rodent cells.

Enzymatically, T antigen possesses ATPase activity, DNA and RNA helicase activities, and both specific and nonspecific DNA-binding activities (for reviews, see references 4 and 45). T antigen is also a transcriptional activator (5, 24). One of the targets for this activation is the SV40 late promoter. T antigen is also able to activate a variety of other viral and cellular promoters, but the biological significance of these activations is unclear (1, 47).

Infection of primary rodent embryo fibroblasts with SV40 leads to their immortalization and transformation (for a review, see reference 50). Large T antigen acting alone is sufficient for both immortalization and transformation, though small t antigen may assist large T antigen in transforming under conditions in which levels of large T antigen are limiting, and under other conditions as well (3). Neither the DNA-binding, ATPase, nor helicase activities of large T antigen appear to be required for immortalization and transformation (9, 38, 39, 56). Furthermore, the ability of large T antigen to transactivate the SV40 late promoter can be separated genetically from its ability to immortalize and transform (68). It remains possible, however, that mutants defective for transactivation of the SV40 late promoter retain the ability to activate various cellular promoters and that these activations are of fundamental importance for immortalization, transformation, or both.

Of potentially greater importance for transformation is the ability of T antigen to form complexes with at least two cellular tumor suppressor gene products, $p105^{Rb}$ (10, 35) and p53. The $p105^{Rb}$ protein is the product of the retinoblastoma susceptibility gene, and loss or inactivation of both alleles of this gene is associated with retinoblastomas, osteosarcomas, and additional malignancies. The p53 gene appears to be altered or absent in a large fraction of human tumors (for a review, see reference 18). Unlike the Rb gene, both copies of which must be mutated or deleted for alterations in cellular growth to occur, mutation of a single allele of the p53 gene is sufficient to cause substantial alterations in cellular growth control, possibly due to the sequestration of wild-type p53 in complexes containing both wild-type and mutant p53 polypeptides. It is also possible that some mutant alleles of the p53 gene acquire additional properties that further potentiate its ability to alter cellular growth control. T antigen is known to associate with other cellular proteins, including DNA polymerase α as well as the cellular heat shock cognate proteins hsc72/73, but there is no evidence suggesting that these latter interactions are relevant to growth control.

Formation of a complex between T antigen and $p105^{Rb}$ requires sequences between amino acids 105 and 114 of T antigen. The region of T antigen involved in forming complexes with p53 extends over a much larger portion of the protein and appears to involve multiple regions between amino acids 347 and 626 (68). T antigen also binds to a 107K/120K cellular protein through the same sequences as it binds $p105^{Rb}$. There may be still additional cellular proteins which form complexes with large T antigen.

The transforming proteins of DNA tumor viruses have been studied for many years as model systems for understanding oncogenesis. The E1A region of human adenoviruses encode two proteins, of 289 and 243 amino acids.

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These proteins share with large T antigen the ability to alter cellular growth control. E1A proteins can immortalize primary rodent cells, and by cooperating with E1B proteins or with other oncogene proteins, such as activated *ras* gene products, they can bring about the full transformation of primary cells (48). E1A proteins can also activate and repress viral and cellular gene expression (16, 29, 30, 32, 51). The regions of E1A responsible for the immortalization and *ras* cooperation functions have been mapped, are contained in both the 289- and 243-amino-acid E1A gene products, and are conserved among different adenovirus serotypes (60). These regions share sequence homologies with SV40 large T antigen as well as with the E7 protein, a transforming protein of human papillomaviruses (13). E1A, E7, and large T antigen all bind to $p105^{Rb}$ (10, 13, 43, 59). Adenovirus E1A proteins also bind another cellular protein, p300, through sequences located near the amino terminus of the E1A proteins (61).

The 55K E1B proteins of some serotypes of human adenoviruses (serotypes 2 and 5 but not serotype 12) form complexes with p53, but the region of p53 required for complex formation with E1B proteins is different from the region important for forming complexes with SV40 large T antigen. The ability of E1A to cooperate with *ras* or E1B to transform primary rodent fibroblasts requires that E1A be able to bind both p300 and $p105^{Rb}$ (61, 63). It is not known whether either SV40 large T antigen or the papillomavirus E7 protein binds to p300.

Mouse 10T1/2 and rat REF-52 cells are two established rodent cell lines. REF-52 cells grow relatively slowly and morphologically resemble primary cells. 10T1/2 cells appear more transformed and may be closer genetically to a fully transformed state. The amino-terminal 121 residues of T antigen are sufficient to transform 10T1/2 cells (54), while transformation of REF-52 cells requires a much larger portion of T antigen and was affected by mutations located throughout the coding region, including within the first 121 amino acids and as close to the carboxy terminus as residue 600 (44). Neither *ras* nor E1A acting alone transforms REF-52 cells. Full transformation of REF-52 cells requires collaboration of *ras* and E1A (15), suggesting that multiple events must happen for REF-52 cells to become transformed.

To further explore the mechanisms of transformation by SV40 large T antigen, a large set of point, linker insertion, and deletion mutants was assayed for transformation using REF-52 and 10T1/2 cells. Transformation of 10T1/2 cells was greatly reduced by mutation within the first exon of the gene for large T antigen but was only modestly affected by mutations affecting the $p105^{Rb}$ binding site. All mutants which were defective for 10T1/2 cell transformation were defective for transformation of REF-52 cells. Mutants defective for binding to $p105^{Rb}$ showed only a low ability to transform REF-52 cells. In addition, there was a tight correlation between the ability of mutants to transform REF-52 cells and the ability of their T antigens to bind p53. These results demonstrate that multiple regions of large T antigen are required for full transformation of REF-52 cells. These include sequences within the first exon of large T antigen, the $p105^{Rb}$ binding region, and the p53 binding domain.

MATERIALS AND METHODS

Plasmids, cells, and tissue culture. REF-52 cells are an established rat embryo fibroblast cell line (34). 10T1/2 cells are an established mouse fibroblast cell line (46). Both were gifts from Jim Pipas (University of Pittsburgh, Pittsburgh, Pa.) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% fetal bovine serum. This medium was supplemented with 400 μg of G418 per ml when appropriate. CV-1 cells are a line of African green monkey kidney cells (21); these cells were cultured in DMEM supplemented with 10% newborn calf serum (GIBCO/BRL). 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. pSV2neo contains a bacterial neomycin phosphotransferase gene (53). Bacterial growth and transfection (36) and large-scale plasmid purification by CsCl-ethidium bromide buoyant density centrifugation (37) were done by standard techniques.

SV40 mutants. The mutants used in these studies have been described previously. Mutants in the 2400 series were constructed by Tornow and Cole (58) and have deletions at various *Dde*I sites within the early region of SV40. Some of these encode nearly full-size T antigens lacking one to four amino acids; due to a reading frame shift, others produce T antigens truncated at sites near and downstream of the deletion. Linker insertion mutants with a 12-bp linker (5′-TCGCGATCGCGA-3′) inserted at various sites within the SV40 early region belong to the 2800 series (67). This linker contains two sites for *Nru*I and one for *Pvu*I. Also included in the 2800 series are some deletion mutants. Mutants *dl*A2837, *dl*A2838, and *dl*A2839 were each prepared from pairs of linker insertion mutants and carry deletions of the SV40 DNA between the linker sites of the two parents (68). Mutant *dl*A2837 lacks sequences encoding amino acids 168 to 346 and contains the 12-bp linker at the site of the deletion. Mutants *dl*A2838 and *dl*A2839 lack sequences encoding amino acids 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).

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 [23] and SVcT [27]) and one deletion mutant (MET128 [57]) 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.

Transformation assays. (i) Monolayer overgrowth assays. REF-52 cells and 10T1/2 cells were seeded into 100-mm plates at a density of 10^5 cells per plate the day before transfection. Each plate was transfected with 5 μg of wild-type or mutant SV40 plasmid DNA and 10 μg of salmon sperm carrier DNA, using the calcium phosphate method as described by Wigler et al. (62). At 12 h posttransfection, the cells were washed twice with TS (0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM Tris-Cl [pH 7.45], 130 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4) and allowed to recover for 24 h in DMEM containing 10% fetal calf serum (FCS). The medium was changed twice a week for 3 weeks. The cells were stained with 0.5% methylene blue, and the formation of dense foci on top of the monolayer of untransformed cells was scored with a MasterScan interpretive densitometer. During the scanning, a threshold was set just above the staining of the control plate for each experiment. The densitometer scored all of the foci above this threshold. Many plates were also

scored by directly counting foci, and in all cases, virtually identical results were obtained.

(ii) G418 selection. The G418 selection assays were similar to the monolayer overgrowth assays except that each plate of cells was also cotransfected with 0.5 μg of pSV2neo DNA together with wild-type or mutant SV40 DNA and carrier DNA. In some experiments, cells were split 1:4 48 h after transfection. Cells were cultured in medium containing 400 μg of G418 per ml, starting 3 days after transfection. The cells were stained with methylene blue 2 to 3 weeks after transfection, and dense colonies were scored with a MasterScan interpretive densitometer. In each assay, the DNA of each mutant to be analyzed was transfected into two identical plates. In several assays using this ratio of T-antigen plasmid to pSV2neo, multiple colonies were examined by immunofluorescence, and all were found to express T antigen.

Interaction of T antigens with $p105^{Rb}$. Rb cRNA was transcribed in vitro from a Stratagene pBluescript SK+ cloning vector containing the human Rb cDNA sequence downstream from a T7 bacteriophage promoter by transcription with T7 RNA polymerase. This plasmid was a kind gift from Ed Harlow. $p105^{Rb}$ protein, labeled with [^{35}S]methionine, was synthesized in vitro by using a Promega (Madison, Wis.) rabbit reticulocyte lysate system programmed with the Rb cRNA. Rabbit reticulocyte lysate containing labeled Rb protein was frozen at −20°C for 1 or 2 days before being used in immunoprecipitation experiments.

Cultures of BALB/c 3T3 cells were cotransfected with plasmids encoding wild-type or various mutant T antigens and pSV2neo. Transfections were performed by the calcium phosphate method as described above. Following recovery, the cells were grown in DMEM–10% FCS–400 μg of Geneticin (Sigma) per ml for 2 weeks to produce stably transformed cell pools. CV-1 monkey kidney cells were calcium phosphate transfected with plasmid encoding mutant or wild-type T antigen. At 16 h posttransfection, the cells were washed twice with TS and allowed to recover for 2 days in DMEM containing 10% newborn calf serum. Stably transformed BALB/c 3T3 cells and transiently transfected CV-1 cells expressing wild-type or mutant T antigens were harvested with lysis buffer containing 250 mM NaCl, 0.1% Nonidet P-40, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES pH 7.0), 1 mM phenylmethylsulfonyl fluoride, 0.1 U of aprotinin (Sigma) per ml, and 10 μg of leupeptin (Sigma) per ml. This and subsequent steps were performed at 4°C. The cellular debris was removed by centrifugation for 15 min in a microcentrifuge.

The ability of wild-type and mutant T antigens to form complexes with $p105^{Rb}$ was determined by slight modification of the method described by Dyson et al. (13). Fifteen microliters of the rabbit reticulocyte lysate containing labeled Rb protein was added to 1 ml of each mouse or monkey cell lysate. Lysate mixtures were rocked gently for 1.5 h and then precleared for 30 min with 20 μl of protein A-Sepharose beads (Pharmacia) which had been washed three times with and resuspended in a volume of TBS-BSA (25 mM Tris-Cl [pH 8.0], 120 mM NaCl, 10% bovine serum albumin) equal to that of the packed Sepharose beads. Following removal of the protein A-Sepharose beads, 10 μl of a mixture of mouse ascites fluids containing monoclonal antibodies pAb901 and pAb902 was added and the lysate mixtures were rocked gently for 1.5 h. These antibodies recognize denaturation-resistant determinants located at the carboxy and amino termini of T antigen, respectively (56). Then 35 μl of protein A-Sepharose beads in TBS-BSA was added and rocking was continued for an additional hour, after which the protein A-Sepharose beads and bound proteins were pelleted, washed three times with cold lysis buffer (without the protease inhibitors), and resuspended in sample loading buffer (10% glycerol, 2% sodium dodecyl sulfate [SDS], 0.2 mg of bromophenol blue per ml, 60 mM Tris-Cl [pH 6.8], 0.05% [vol/vol] β-mercaptoethanol). Samples were heated at 70°C for 15 m, centrifuged in a microcentrifuge for 2 min, and placed on ice. Supernatants were loaded onto a 10% SDS-polyacrylamide gel. Labeled proteins were detected by fluorography.

Comparison of the stabilities of wild-type and mutant T antigens. Pools of REF-52 cells expressing wild-type or mutant T antigens were selected by cotransfection of cells with 0.5 μg of pSV2neo and 5.0 μg of a plasmid expressing wild-type or mutant T antigen by the calcium phosphate method. G418 (400 μg/ml) was added 48 h after transfection. Colonies surviving selection were pooled approximately 2 to 3 weeks after transfection and used in subsequent experiments. Cells in 60-mm dishes were incubated for 1 h in DMEM lacking methionine and containing 2% dialyzed FCS and were subsequently labeled with 100 μCi of [^{35}S]methionine (Translabel; Amersham) in a volume of 0.7 ml of the same medium. After 1 h of labeling, the labeling medium was removed, and cells were washed twice with TS and incubated in 5 ml of DMEM containing 10% FCS. Cultures were harvested 0, 2, 4, and 6 h later and processed for immunoprecipitation as described previously (68).

RESULTS

The experiments described here were conducted to map more precisely the regions of SV40 large T antigen required for growth transformation of established cell lines and to examine the correlations between these activities and the other activities of large T antigen. We also report on the ability of these mutant T antigens to interact with $p105^{Rb}$. To study transformation, we used two established cell lines, mouse 10T1/2 cells and rat REF-52 cells, because these lines are widely used for studies of transformation by DNA tumor viruses and because it has been shown that transformation of 10T1/2 cells requires considerably less of T antigen (as little as the N-terminal 121 amino acids [54]) than does transformation of REF-52 cells (which requires most of T antigen). With both lines, it is easy to distinguish transformed foci from normal cells.

Transformation of 10T1/2 cells. Two different types of assays were conducted. In the monolayer overgrowth assay, cells were transfected with wild-type or various SV40 mutant DNAs and then incubated with periodic changes of media until foci formed. This assay tested for the ability of transformed cells to overgrow a monolayer of normal cells. In the other assay, cell cultures were cotransfected with wild-type or various mutant DNAs and pSV2neo, which confers resistance to the antibiotic G418. This procedure permitted killing of those cells which did not take up DNA. By using a high ratio of SV40 DNA to pSV2neo, most of the cells which become resistant to G418 will also have taken up SV40 DNA and expressed T antigen. The density of the resultant clones of cells is an index of the level to which the particular T antigen has transformed them. This second assay is less stringent, and consequently, some mutants showed a greater transformation potential by using this assay. The data are shown in Table 1 and summarized in Fig. 1. Figure 2 shows representative plates of cells generated by using each of these assays.

TABLE 1. Transformation of 10T1/2 cells by SV40 large-T-antigen mutants

Mutant	Location of lesion[a]	Scores[b]			
		Dense focus assay		G418 assay	
		Expt 1	Expt 2	Expt 3	Expt 4
Mock		1, 1 (0.02)	1, 0 (0.01)	0, 0 (0.00)	0, 0 (0.00)
pSV2neo	No T antigen			1, 0 (0.02)	1, 0 (0.01)
1209	No T antigen		4, 2 (0.03)	5, 3 (0.19)	1, 0 (0.01)
Wild type		50, 63 (1.00)	93, 100 (1.00)	26, 16 (1.00)	30, 46 (1.00)
2801	*in*5	5, 5 (0.09)	5, 2 (0.04)	15, 14 (0.69)	20, 10 (0.39)
2831	*dl*5-35+*in*		0, 3 (0.02)	1, 3 (0.10)	0, 1 (0.01)
2803	*in*35	5, 1 (0.05)	1, 2 (0.02)	2, 0 (0.05)	3, 0 (0.04)
2441	*dl*106		79, 83 (0.84)	20, 15 (0.83)	25, 17 (0.55)
K1	Q107K		62, 69 (0.68)		
SVcT	K128Q		77, 66 (0.74)	14, 11 (0.60)	
2420	1-128+6		60, 66 (0.65)	3, 5 (0.19)	20, 16 (0.47)
2411	*dl*143-146		98, 88 (0.96)	4, 6 (0.24)	31, 25 (0.74)
2815	*in*168	60, 65 (1.11)	118, 105 (1.16)	18, 20 (0.90)	19, 15 (0.45)
2817	*in*219	68, 105 (1.53)	132, 159 (1.51)	11, 14 (0.60)	40, 25 (0.86)
2807	*in*302	52, 51 (0.91)	89, 74 (0.84)	14, 21 (0.83)	13, 12 (0.33)
2819	*in*346	60, 68 (1.13)	75, 72 (0.76)	14, 23 (0.88)	18, 11 (0.38)
2809	*in*409	36, 27 (0.56)	63, 39 (0.53)	19, 14 (0.79)	14, 10 (0.32)
2811	*in*424	35, 39 (0.65)	51, 67 (0.61)	10, 15 (0.60)	9, 11 (0.26)
2821	*in*460	65, 77 (1.26)	76, 70 (0.76)	13, 14 (0.64)	12, 11 (0.30)
2823	*in*464	65, 73 (1.22)	117, 102 (1.13)	22, 7 (0.69)	15, 17 (0.42)
2827	*in*520	76, 116 (1.70)	122, 132 (1.32)	18, 17 (0.83)	22, 39 (0.80)
2828	*in*520	72, 122 (1.72)	138, 135 (1.41)	15, 14 (0.69)	15, 26 (0.54)
2432	*dl*507-510		114, 111 (1.17)	12, 21 (0.79)	36, 24 (0.79)
2416	1-508+3		37, 28 (0.34)	9, 7 (0.38)	3, 4 (0.09)
2433	*dl*587-589		46, 76 (0.63)	12, 5 (0.40)	11, 10 (0.28)
2829	*in*173 (small t)		2, 4 (0.03)	10, 11 (0.50)	18, 26 (0.58)

[a] The prefix "*in*" represents an insertion of a 12-bp linker, except for 2828, which has 6 bp inserted at codon 520. The suffix "+*in*" represents a linker inserted at the site of the deletion. The prefix "*dl*" represents a deletion of the indicated amino acids. Point mutants are indicated by the wild-type single-letter amino acid code, followed by its location and the mutant amino acid which replaces it. Frameshift deletion mutants are represented by the numbers indicating the amino acid residues encoded by the mutants and followed by "+3", "+6", or "+26", indicating the numbers of amino acids from shifted reading frames as a result of reading frame shift.

[b] Two numbers are the scores from two parallel plates in each experiment. Numbers in parentheses are the levels relative to that of wild-type SV40 DNA in each experiment.

Results obtained in the monolayer overgrowth assay agreed with the findings of others that only the amino-terminal part of T antigen is required for transformation of 10T1/2 cells (54). Mutant *dl*A2420(Δaa139-708) was able to transform 10T1/2 cells, though the efficiency of transformation was not as high as that of wild-type large T antigen. This mutant encodes a T antigen consisting of the first 138 amino acids of large T antigen plus 6 amino acids from the shifted reading frame. The ability of T antigen to transform 10T1/2 cells generally was not affected significantly by mutations distal to amino acid 140 of large T antigen. However, transformation efficiency was reduced with some mutants [*dl*A2416(aa509-708) and *dl*A2433(aa587-589)] which produce very unstable fragments of T antigen (58), even though their T antigens contain much more of large T antigen than the N-terminal 121 amino acids.

Some mutations within the first 35 amino acid residues abolished the ability of T antigen to transform 10T1/2 cells. These mutants included *in*A2803(aa35) and *dl*A2831(aa5-35). Mutations at the $p105^{Rb}$ binding site [*dl*2441(aa106) and K1(Q107K)] did not affect the transformation of 10T1/2 cells significantly. K1 has a point mutation converting Glu-107 to Lys, and the mutant protein fails to bind $p105^{Rb}$ (10). *dl*2441(aa106) has a small in-frame deletion at the beginning of the region involved in $p105^{Rb}$ binding and showed very little binding to $p105^{Rb}$ (see Fig. 3). In addition, SVcT (K127Q) (mutation at the nuclear localization signal) transformed 10T1/2 cells at wild-type efficiency.

Interestingly, 10T1/2 cells transfected with *in*2801(aa5) and *in*2829(small t-173) were able to form dense colonies in G418 assays but failed to form dense foci efficiently in monolayer overgrowth assays (Table 1 and Fig. 2). The fact that 10T1/2 cells transfected by either mutant which affected small t antigen failed to form dense foci in the absence of G418 selection suggests that small t antigen might play a role in overcoming the contact growth inhibition by nontransformed cells. While wild-type small t antigen has a half-life of greater than 3 h, the half-life of the small t antigen encoded by *in*2829(small t-173) was less than 30 min (49). We have not examined the stability of the small t antigen encoded by *in*2801(aa5).

We also note that some mutants whose lesions are very close together [*in*A2827(aa520), *in*A2828(aa520), and *dl*A2432 (aa509)] routinely transformed more efficiently than did wild-type SV40 in the monolayer overgrowth assay. The foci produced in cultures transfected with these mutants were detectable earlier than with wild-type SV40 and were always larger and denser than wild-type foci (data not shown).

Transformation of REF-52 cells. The same two types of assays used with 10T1/2 cells were used to study the ability of these mutants to transform REF-52 cells (Table 2 and Fig. 1 and 2). Several mutants were defective for REF-52 transformation in both the monolayer overgrowth and G418 assays. These include the two mutants with lesions in the first exon that were completely defective for transformation of 10T1/2 cells [*in*A2803(aa35) and *dl*A2831(aa5-35)] and

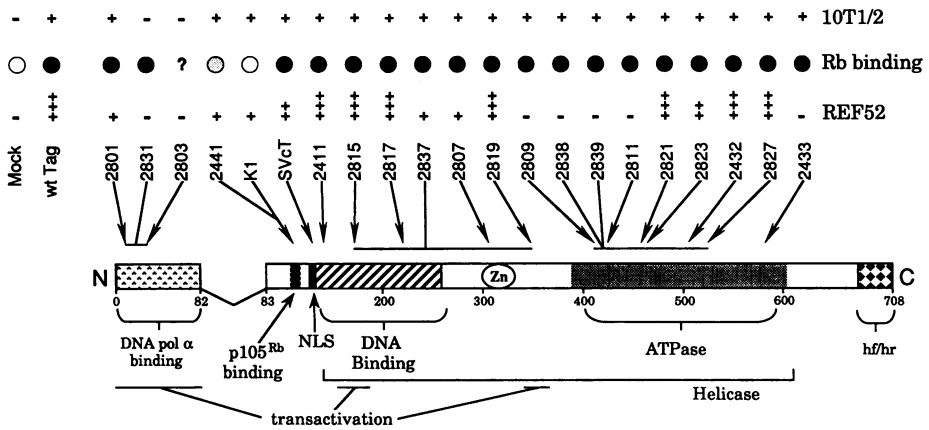

FIG. 1. Comparison of abilities of mutants of T antigen to transform REF-52 and 10T1/2 cell lines and to bind to Rb. Locations of mutations noted in the text are indicated above the cartoon of the protein. Arrows indicate linker insertion mutations and deletions of one to five amino acids. The extent of larger deletions is indicated by a horizontal bar. Other functions of T antigen are indicated below the cartoon of the protein. The abilities of mutants to transform C3H 10T1/2 and REF-52 cells and to bind to Rb are indicated in the first three lines and are summarized from Tables 1 and 2, respectively. Symbols for transformation by C3H 10T1/2 cells: +, greater than 30% of the wild-type control (wt Tag) level; −, when less than 10% of the wild-type control level. Symbols for transformation by REF-52 cells: +++, greater than 50% of the wild-type control level; ++, less than 50% but greater than 20% of the wild-type control level; +, less than 20% and greater than 1% of the wild-type control level; −, less than 1% of the wild-type control level. For Rb binding, an open circle represents no binding, a gray circle represents weak but detectable binding, and a filled circle represents easily detectable binding. pol, polymerase; NLS, nuclear localization signal; hf/hr, helper function/host range.

other mutants which encode T antigens truncated at amino acid 138 (*dl*A2420), 508 (*dl*A2416), or 584 (*dl*A2410). Mutant *dl*A2465, whose T antigen is truncated at amino acid 626, transformed REF-52 cells efficiently, indicating that the distal boundary between sequences required and dispensable for transformation of REF-52 cells is located between 589 and 626. This mutant was examined only in the more stringent monolayer overgrowth assay. Mutant *dl*A2837 (aa168-346) also transformed REF-52 cells. These results suggest that there are multiple discontinuous regions of large T antigen involved in transformation; one or more regions located within the first 138 residues are required along with portions of the sequences between residues 347 and 626.

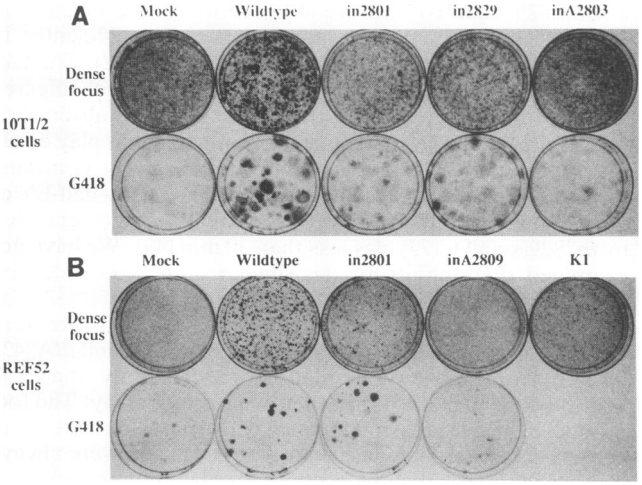

FIG. 2. Transformation of 10T1/2 and REF-52 cells by mutant large T antigens. Cells transfected with SV40 DNA alone (dense focus assay) or SV40 DNA plus pSV2neo (G418 assay) were cultured in the absence or presence, respectively, of G418 (400 μg/ml) for 2 to 3 weeks and stained with methylene blue. (A) Representative plates from transformation of 10T1/2 cells; (B) REF-52 cell transformation assays. The SV40 mutants used are indicated above the plates. SV40 DNA was replaced with an identical amount of pUC18 DNA for all plates labeled "Mock."

Since the T antigens encoded by many mutants with lesions in the first exon of large T antigen produce very unstable T antigens, the stabilities of the T antigens encoded by *in*A2803(aa35) and *dl*A2831(aa5-35) were determined by pulse-chase labeling of REF-52 cell pools expressing these T antigens. The data are shown in Fig. 3. In this assay, wild-type T antigen had a half-life of more than 10 h. It can be seen that the T antigen encoded by *in*A2803(aa35) was quite unstable, with a half-life of considerably less than 2 h. The T antigen of *in*A2831(aa5-35), while not as stable as wild-type T antigen, had a half-life of almost 4 h.

*dl*A2433(aa587-589) did not transform REF-52 cells, confirming that the hydrophobic region between amino acid residues 570 and 590 is important for transformation of REF-52 cells (44). Mutant *dl*A2433(aa587-589) encodes an unstable protein. The instability of this mutant T antigen is not sufficient to explain its total failure to transform REF-52 cells, however, since *in*A2807(aa302) T antigen, with an insertion at the zinc finger region, is as unstable as *dl*A2433(aa587-589) T antigen, and it transformed REF-52 cells to a substantial level although not as well as did wild-type T antigen. Mutants in another region with the carboxyl half of large T antigen, *in*A2809(aa409) and *in*A2811(aa424), were also unable to transform REF-52 cells. Neither *dl*A2433 (aa587-589), *in*A2809(aa409), nor *in*A2811(aa424) T antigen is able to bind p53 (68). The T antigen of *in*A2811(aa424) is relatively stable (67).

Mutations at the $p105^{Rb}$ binding site affected the transformation of REF-52 cells significantly. K1 protein, which does not bind $p105^{Rb}$ (10), transformed REF-52 cells at about 2 to 5% of the wild-type level. The level of transformation by K1(Q107K) was significantly above the levels of the mock

TABLE 2. Transformation of REF-52 cells by SV40 large-T-antigen mutants[a]

Mutant	Location of lesion	Scores			
		Dense focus assay		G418 assay	
		Expt 1	Expt 2	Expt 3	Expt 4
Mock		0, 0 (0.00)	0, 0 (0.00)	0, 0 (0.00)	0, 0 (0.00)
pSV2neo	No T antigen			2, 0 (0.06)	0, 0 (0.00)
1209	No T antigen	0, 0 (0.00)	0, 0 (0.00)	2, 1 (0.09)	
Wild type		396, 387 (1.00)	343, 387 (1.00)	14, 21 (1.00)	85, 104 (1.00)
2801	*in*5	73, 69 (0.18)	25, 31 (0.08)	16, 15 (0.89)	58, 88 (0.77)
2831	*dl*5−35+in	0, 0 (0.00)	1, 0 (0.00)	1, 0 (0.03)	4, 3 (0.04)
2803	*in*35	0, 0 (0.00)	0, 0 (0.00)	4, 3 (0.20)	0, 1 (0.01)
2441	*dl*106	147, 96 (0.31)	62, 45 (0.15)	17, 11 (0.80)	63, 44 (0.57)
K1	Q107K	26, 10 (0.05)	6, 7 (0.02)		
SVcT	K128Q	283, 246 (0.68)	101, 7 (0.15)	10, 12 (0.63)	
MET128	*dl*1-127		0, 1 (0.00)		
2420	1-138+6	0, 0 (0.00)	0, 0 (0.00)	0, 3 (0.09)	
2411	*dl*143-146	304, 327 (0.81)	246, 215 (0.63)	8, 6 (0.40)	
2815	*in*168	364, 351 (0.91)	137, 136 (0.37)	12, 7 (0.54)	24, 19 (0.23)
2817	*in*219	721, 757 (1.89)	366, 380 (1.02)	27, 25 (1.49)	117, 113 (1.22)
2807	*in*302	28, 25 (0.07)	9, 7 (0.02)	8, 11 (0.54)	25, 9 (0.18)
2819	*in*346	235, 254 (0.62)	105, 114 (0.30)	14, 12 (0.74)	12, 29 (0.22)
2809	*in*409	0, 0 (0.00)	0, 0 (0.00)	1, 0 (0.03)	3, 3 (0.03)
2811	*in*424	0, 0 (0.00)	0, 0 (0.00)	0, 1 (0.03)	0, 3 (0.02)
2821	*in*460	213, 195 (0.52)	149, 134 (0.39)	16, 14 (0.86)	68, 51 (0.63)
2823	*in*464	151, 158 (0.39)	33, 49 (0.11)	13, 11 (0.69)	34, 33 (0.35)
2432	*dl*507-510	440, 441 (1.13)	376, 412 (1.08)	19, 17 (1.03)	
2416	1-508+3	2, 1 (0.00)	0, 0 (0.00)	0, 0 (0.00)	
2827	*in*520	607, 622 (1.57)	448, 468 (1.25)	24, 19 (1.23)	168, 166 (1.77)
2828	*in*520	682, 756 (1,84)		20, 17 (1.06)	103, 84 (0.99)
2433	*dl*587-589	0, 0 (0.00)	0, 0 (0.00)	1, 2 (0.09)	
*dl*A2465	1-626+1		246, 190 (0.60)		
2829	*in*173 (small t)	85, 141 (0.29)	42, 77 (0.16)	9, 7 (0.46)	14, 27 (0.22)
Mock		0, 0 (0.00)	0, 0 (0.00)		
Wild type		79 (1.00)	80, 76 (1.00)		
2837	*dl*168-346	9, 17 (0.16)	17, 30 (0.30)		
2838	*dl*408-519+*in*	0, 0 (0.00)	0, 0 (0.00)		
2839	*dl*408-519+6	0, 0 (0.00)	0, 0 (0.00)		

[a] See footnotes to Table 1.

control and a number of transformation-negative mutants, such as *in*A2803(aa35), *dl*A2831(aa5-35), *in*A2809(aa409), and *in*A2811(aa424) (Fig. 1). Another mutant at the p105^{Rb} binding site, *dl*2441(aa106), transformed REF-52 cells 15 to 30% as well as did wild-type SV40 in the monolayer overgrowth assay and about 50 to 80% as well in the G418 assay. SVcT(K127Q) transformed REF-52 cells at near wild-type efficiency, indicating that the nuclear localization signal was not required for transformation of REF-52 cells.

*in*2829(small t-173) transformed REF52 cells at a reduced level in both monolayer overgrowth and G418 assays. Therefore, small t antigen appeared to play a role in the transformation of REF-52 cells. Mutant *in*A2801(aa5) was very defective for transformation of REF-52 cells in the monolayer overgrowth assay but transformed well in the G418 assay. This dependence on the assay used for transformation of REF-52 cells by *in*A2801 was the same as that seen for transformation of 10T1/2 cells by this mutant. Whether this result reflects the activity of the mutated large T, mutated small t, or both antigens is not known. All mutants in regions other than those discussed above transformed REF-52 cells at or near the wild-type level.

p105^{Rb} binding by mutant large T antigens. To examine the importance of binding to p105^{Rb} for transformation, we determined whether these mutant T antigens were able to bind to p105^{Rb} (Fig. 3). Cell extracts containing T antigens were mixed with labeled p105^{Rb}, and the ability of an anti-T-antigen monoclonal antibody (pAb901, pAb902, pAb101, or pAb416) to coprecipitate labeled Rb was examined. This approach has also been used by Dyson et al. (13) to examine Rb interactions with T antigen, adenovirus E1A proteins, and papillomavirus E7 proteins.

For these studies, we used stably transformed pools of rodent cells expressing either wild-type or various mutant T antigens (some from REF-52 cells and some from BALB/c 3T3 cells) as sources for wild-type or mutant T antigens. In other experiments, we used transiently transfected monkey kidney cells (CV-1). The wild type and some mutants were analyzed by using T-antigen-containing extracts from multiple sources, and no dependence of complex formation on the source of T antigens was seen (data not shown).

In this assay, the p105^{Rb} produced by in vitro translation contained polypeptides of many sizes (Fig. 4A, lane 1) which result from protein synthesis initiation at internal AUGs within the p105^{Rb}-coding region (13). Since binding of T antigen to p105^{Rb} requires p105^{Rb} sequences between amino acids 393 and 772 (19), most of the smaller p105^{Rb} polypeptides synthesized in vitro were not able to bind T antigen (Fig. 4A; compare lanes 1 and 4). The data showed that p105^{Rb} was bound efficiently by large T antigen present in an

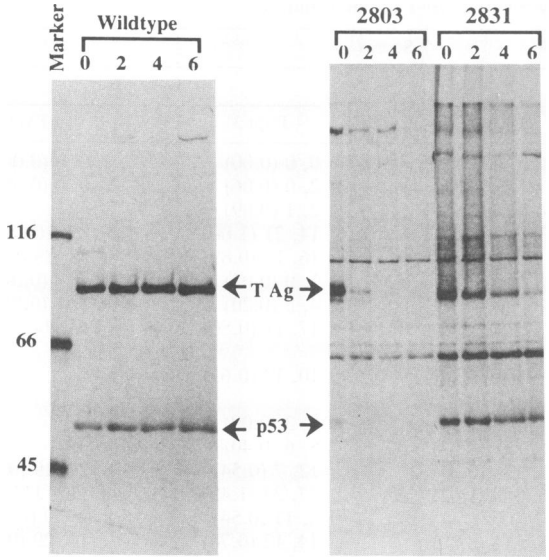

FIG. 3. Analysis of the stabilities of the T antigens encoded by *in*A2803(aa35) and *dl*A2831(aa5-35). Cultures of REF-52 cells expressing mutant or wild-type T antigens (T Ag) were labeled with [^{35}S]methionine for 1 h and chased for 0, 2, 4, or 6 h as described in Materials and Methods. T antigens were immunoprecipitated with monoclonal antibodies pAb901 and pAb902 and separated by electrophoresis. The gel was dried and autoradiographed.

extract of COS-1 cells, but none was bound when an extract of uninfected CV-1 cells was used (Fig. 4A; compare lanes 4 and 3).

Most of the mutant T antigens were able to bind p105^{Rb}. Some of the data are shown in Fig. 4, and all of the data are summarized in Fig. 1. Two of the mutants examined, K1(Q107K) and *dl*2441(aa106), have lesions affecting the binding site for p105^{Rb}. The only T antigen which showed no detectable binding to p105^{Rb} in this assay was K1(Q107K) (Fig. 4A, lane 7; Fig. 4B, lane 5). With *dl*2441(aa106), very weak binding only slightly above background was detected on long exposure of the gel (Fig. 4B, lane 6). Since this mutant encodes a stable T antigen and the concentration of *dl*2441 T antigen was comparable to that of wild-type T antigen (Fig. 4C; compare lanes 2 and 6), we consider this mutant to be defective for normal interactions with p105^{Rb}. Mutant *dl*A2831(aa5-35), which was defective for transformation of both 10T1/2 and REF-52 cells, nevertheless was able to bind p105^{Rb} (Fig. 4B, lane 4). Since *dl*A2831(aa5-35) T antigen was relatively unstable, extracts contained only a low level of this T antigen (Fig. 4C, lane 4), and only a small amount of p105^{Rb} was bound. We were unable to detect p105^{Rb} binding to *in*A2803(aa35) T antigen (Fig. 4B, lane 3), since levels of this mutant T antigen were extremely low (Fig. 4C, lane 3). However, we think it highly likely that this T antigen binds p105^{Rb}, since its T antigen contains all of the sequences deleted in *dl*A2831(aa5-35) T antigen, which bound p105^{Rb}. Thus, the mutants defective for normal binding to p105^{Rb} [(K1(Q107K) and *dl*2441(aa106)] were somewhat to substantially defective for transformation of REF-52 cells (Table 2) but transformed 10T1/2 cells efficiently, though not as well as did wild-type SV40 (Table 1). Together, these data suggest that the amino-terminal portion of large T encodes two functions relevant for transformation: the site for p105^{Rb} binding and a function mapping to the first exon.

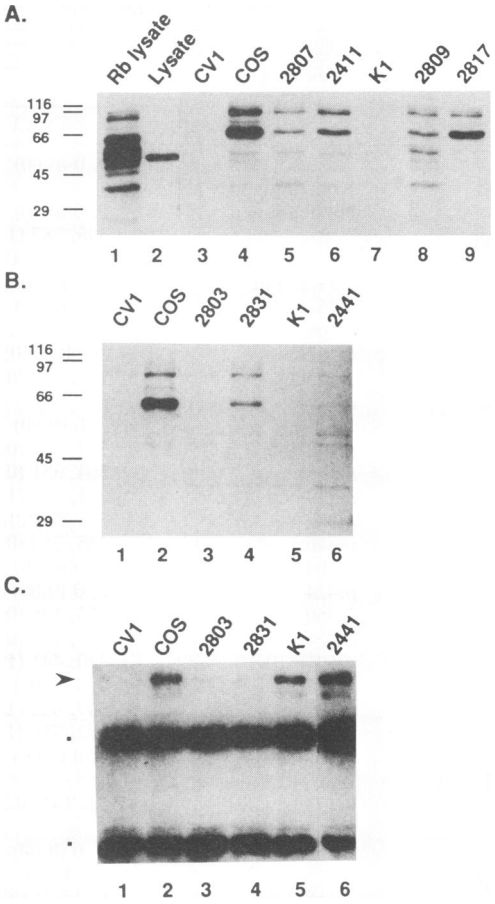

FIG. 4. T antigen-Rb binding. Shown are fluorographs of labeled Rb protein immunoprecipitated with anti-T-antigen antibodies in the absence or presence of wild-type and various mutant T antigens (A) and (B) and immunoblot of T-antigen protein immunoprecipitated from the other half of samples used in panel B (C). Lanes Rb lysate and Lysate contain 1 μl of rabbit reticulocyte lysate from in vitro translation reactions performed with and without, respectively, human Rb cRNA. Immunoprecipitates from mixtures of Rb protein and CV-1 cell extracts containing no T antigen were run in lanes CV1. Immunoprecipitates from mixtures of Rb protein and COS cell extracts containing wild-type T antigen were run in lanes COS. All other lanes are marked according to the mutant T antigen expressed by the CV-1 cells used to make each cellular extract. Molecular weight markers are indicated in kilodaltons at the left of panels A and B. In panel C, T antigen is marked by an arrow, and heavy- and light-chain immunoglobulin proteins are marked by squares.

DISCUSSION

In the studies described here, established REF-52 cells and 10T1/2 cell lines were used to define more precisely the domains of the SV40 early proteins involved in cellular transformation. Both lines are already immortal, and immortalization of primary rodent cells is a critical step along the path to their full transformation. We have recently described the results of studies with SV40 mutants which showed that the ability of mutants to immortalize primary mouse embryo fibroblasts (MEFs) was tightly linked to the ability of mutant T antigens to bind p53 (68).

These cell lines have different genetic requirements for transformation and likely reflect different stages along the pathway to complete malignant transformation. Fragments

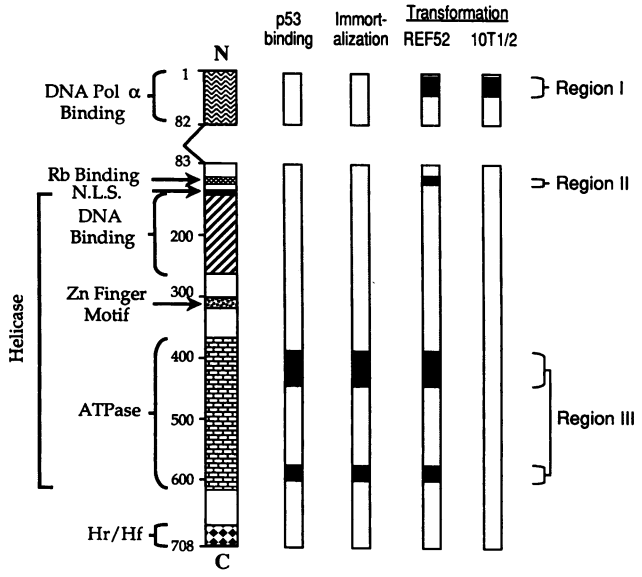

FIG. 5. Summary of the functional domains required for transformation by T antigen. Regions referred to in the text are marked with brackets and are shaded. Pol, polymerase; N.L.S., nuclear localization signal; Hr/Hf, host range/helper function.

from the N terminus of T antigen as short as 121 amino acids are sufficient to transform 10T1/2 cells, while transformation of REF-52 cells requires sequences extending from the N terminus of T antigen to beyond residue 600 (54). The results of the studies presented here suggest that multiple regions of large T antigen are important for transformation of REF-52 cells (Fig. 5). Region I is within the common region of large T and small t antigens and has weak homology to conserved region 1 of the adenovirus E1A proteins. Defects in both large T and small t antigens could be involved in the transformation phenotypes of mutants with lesions in region I. Region II is the $p105^{Rb}$ (and p107/120) binding site, which extends from amino acids 105 to 114 (12, 14). The ability of mutants affecting this site to transform have been studied in several laboratories (6, 7, 23) with variable results. With a few exceptions, mutants with single amino acid changes in the $p105^{Rb}$ binding site were only partially defective for transformation.

Region III is defined by the mutations of *in*A2809(aa409) and *in*A2811(aa424). These mutant T antigens are stable and failed to bind p53. They were defective in transformation of REF-52 cells and immortalization of primary MEF cells (68). Several mutants with lesions between amino acids 460 and 520 retained the ability to transform REF-52 cells (Table 2), immortalize primary MEFs, and bind p53 (68). Distal to this area is a hydrophobic domain (amino acids 570 to 590). Mutants with lesions in this region encode unstable T antigens which failed to bind p53, to form oligomers larger than dimers (65), to transform REF-52 cells, and to immortalize primary MEF cells. Mutants with these properties include *dl*A2433(aa587-589) and 5080(P584L) (44). We suspect that mutations affecting this hydrophobic domain alter the conformation of much of the carboxyl half of large T antigen and prevent it from interacting with p53. In a sense, then, region III is bipartite since mutations at amino acids 409 and 424 as well as mutations within the hydrophobic domain (amino acids 570 to 590) are defective for transformation, immortalization, and p53 binding, while mutations between these two areas retain these properties. We hypothesize that regions I, II, and III each likely specify interactions between large T antigen and various cellular gene products important for growth regulation.

Transformation by SV40 large T antigen. (i) 10T1/2 cells. Data about large T antigen presented here agree with previous observations that only the amino-terminal 121 amino acids of large T antigen are sufficient for transformation of 10T1/2 cells (54). *dl*A2420(aa139-708), which encodes 138 amino acids from the N terminus of T antigen, plus 6 amino acids encoded by a shifted reading frame, was able to transform 10T1/2 cells. In fact, among the mutant SV40 DNAs examined, no mutations in the second exon of the large-T-antigen-coding region abolished or significantly impaired the ability of T antigen to transform 10T1/2 cells (Table 1).

Mutants *in*A2803(aa35) and *dl*A2831(aa5-35) were completely defective for transformation of 10T1/2 cells. This region includes a sequence homologous to E1A conserved region 1 (amino acids 9 to 20) (13). We have shown that *dl*A2831(aa5-35) T antigen binds $p105^{Rb}$ (Fig. 4); because of the very low levels of *in*A2803(aa35) T antigen, we could not tell whether this mutant T antigen also binds Rb, but we think it likely, since binding to $p105^{Rb}$ was retained by *dl*A2831(aa5-35), which has a much larger alteration within the N-terminal portion of large T antigen. *dl*A1135 T antigen, missing residues 17 to 27, also binds $p105^{Rb}$ and a related 107K/120K cellular protein (14). Therefore, some sequences between 5 and 35 likely encode an activity essential for transformation.

It is known that two separate activities of the adenovirus E1A gene are required for cooperation with an activated *ras* gene in the immortalization and transformation of primary baby rat kidney cells (BRK) (64). Two mutants of adenovirus E1A, one defective for $p105^{Rb}$ binding and one defective for p300 binding, can complement one another and cooperate with an activated *ras* gene to transform BRK cells. Recently, Yaciuk et al. (63) showed that mutants of SV40 defective for Rb binding could complement mutants of E1A defective for p300 binding for transformation of BRK cells. Similarly, mutant *dl*A1135(aa17-27) was able to complement an E1A mutant defective for $p105^{Rb}$ binding. This finding suggests that one possible role for T-antigen first-exon sequences is interaction with p300. Given the functional and structural relatedness of the transforming proteins from several papovaviruses, adenoviruses, and papillomaviruses, it would not be surprising if large T antigen interacts with the p300 protein or a related protein. Alternatively, one could also argue that although this region (amino acids 5 to 35) is not required for the binding of $p105^{Rb}$ and p107/120, it might be essential for inactivation of the normal functions of these cellular proteins.

For *in*2801(aa5) and *in*2829(small t-173), two viable mutants affecting small t antigen, different results were obtained when two different protocols of transformation were used. The two mutants failed to transform 10T1/2 cells in the monolayer overgrowth assay but transformed 10T1/2 cells at about 50% of the wild-type level in the G418 assay, suggesting that small t antigen is playing some role in overcoming contact inhibition by neighboring nontransformed cells. Srinivasan et al. reported that *dl*1137t, which encoded no small t antigen, was able to transform 10T1/2 cells (54). This conclusion was based on the results of a G418 resistance assay. Therefore, the role of small t antigen in transformation requires further testing using different types of assays

and a combination of plasmids encoding various wild-type and mutant large and small T antigens.

The transformation of 10T1/2 cells by two mutants in the $p105^{Rb}$ binding domain suggests that the intact $p105^{Rb}$ binding site is not required for large T antigen to transform this cell line. Mutant K1(Q107K) was unable to bind $p105^{Rb}$ (Fig. 4) (10) and is also unable to bind the p107/120 protein (14). The data presented in Fig. 4 indicate that *dl*2441(aa106) T antigen may bind weakly to $p105^{Rb}$, but the amount of binding detected was only slightly above background. This could mean that $p105^{Rb}$ and p107/120 binding are not essential for the transformation of 10T1/2 cells. We think it more likely that the mutations in K1(Q107K) and *dl*2441(106) affect but do not totally prevent an interaction between T antigen and $p105^{Rb}$ or p107/120. This suggests caution in the interpretation of results from $p105^{Rb}$ binding experiments. Although complexes between $p105^{Rb}$ and various mutant T antigens may not be detected by immunoprecipitation, interactions may occur in vivo and be sufficiently stable to suppress the activities of $p105^{Rb}$.

(ii) REF-52 cells. Transformation of REF-52 cells requires the action of multiple oncogenes, e.g., cooperation of activated *myc* and *ras* oncogenes (17, 25). Those first-exon mutants which were unable to transform 10T1/2 cells [*in*A2803(aa35) and *dl*A2831(aa5-35)] also lacked the ability to transform REF-52 cells.

K1(Q107K) and *dl*2441(aa106) transformed REF-52 cells at a substantially reduced level. This finding suggests that binding of T antigen to $p105^{Rb}$, to p107, or to both plays a role in transformation of REF-52 cells. The discussion above about the relationship between the transformation of 10T1/2 cells and binding of cellular proteins also applies to the transformation of REF-52 cells.

Mutants with lesions in other portions of large T antigen, *in*A2809(aa409), *in*A2811(aa424), and *dl*A2433(aa587-589), lost the ability to transform REF-52 cells and retained the ability to transform 10T1/2 cells. All three mutations are located within the sequences required for ATP-binding/ATPase activity. None of the T antigens encoded by these mutants could bind p53 (68). Other mutants with lesions in this region [*in*A2821(aa460), *in*A2823(aa464), *dl*A2432(aa507-510), *in*A2827(aa520), and *in*A2828(aa520)] were able to transform REF-52 cells and retained the ability to bind p53. Thus, there was a tight correlation between the ability of mutants to bind p53 and their ability to transform REF-52 cells.

Two types of mutations might be expected to alter p53 interactions with large T antigen. One class are those which alter residues directly involved in contact between T antigen and p53. The other class are those whose mutations may lie quite distant to residues contacted by p53 but which disrupt the overall structure of the ATP-binding/ATPase domain. We hypothesize that mutants *in*A2809(aa409) and *in*A2811 (aa424) fall into the first class and that mutant *dl*A2433 (587-589) falls into the second. Mutant *dl*A2433(aa587-589) contains a deletion within a very hydrophobic region of large T whose presence appears to correlate with the stability of the protein and its overall structure. Analysis of the structure of *dl*A2433 T antigen by using a panel of monoclonal antibodies indicates that *dl*A2433 T antigen does not react with a substantial number of antibodies which recognize conformationally sensitive sites within the C-terminal half of the molecule (8). The importance of the hydrophobic region (amino acids 570 to 590) for transformation was seen with another mutant affecting this region, 5080(P584L) (55).

Lin and Simmons (31) have described mutants with point mutations close to the position of the mutations of *in*A2809(aa409) and *in*A2811(aa424), at residue 402. These point mutants retained activity for SV40 DNA replication, suggesting that the conformation of these amino acid 402 mutant T antigens is probably disrupted minimally. The mutant T antigens, however, showed substantially reduced interactions with rodent p53. Their ability to transform rodent cells was not examined, but we predict that they will show partial reduction in this activity.

Since the abilities of these mutant T antigens to immortalize primary MEFs, to transform REF-52 cells, and to bind p53 cosegregate, we suggest that all three properties reflect the overall structure of the ATP-binding/ATPase domain of large T. We note that those mutant T antigens which lose the ability to immortalize, transform, or bind p53 in general also lose the ability to react with several monoclonal antibodies which recognize conformationally sensitive epitopes located within the carboxyl half of large T; conversely, those which retained these properties tend to react with most of these antibodies. This finding suggests that the mechanism by which the carboxyl half of large T antigen affects growth regulation is through binding to p53 or related proteins. We have not yet examined the ATP-binding or ATPase activity of the mutant T antigens, but we showed previously that ATPase activity could be uncoupled from transformation; *dl*A2462(aa509) and *dl*A2432(aa507-510) both lack ATPase activity (9) but retain full activity for immortalization (56) and transformation (Table 2) and are able to bind p53.

*in*A2807(aa302) T antigen, with a linker insertion at the zinc finger region (amino acid 302), is unstable (67) and transformed REF-52 cells at a reduced but significant level. This mutant also appeared to transform more efficiently in the G418 assay than in the monolayer overgrowth assay, though the differences were not always large. The zinc finger motif has been shown to play a structural role. Mutants with lesions in this domain encode unstable proteins which have reduced DNA-binding and transforming capability (2, 33). Although *in*A2807(aa302) T antigen retained the ability to bind p53 (68) and $p105^{Rb}$, its levels may be too low to permit the mutant T antigen to sequester all of the p53 or $p105^{Rb}$ in the cell.

One possible concern in interpretation of these data is the instability of some of the mutant T antigens which showed reduced transformation activity. The critical point here is to understand whether failure to transform reflected reduced levels of mutant T antigens with normal transformation activities, altered activities of the mutant T antigens, or both. For several reasons, we believe that the failure of relatively unstable mutant T antigens to transform at wild-type efficiency reflects qualitative defects of the mutant T antigens and implicates the amino-terminal region of T antigen in performing a specific function necessary for transformation. First, mutant *in*A2807(aa302) retained considerable transformation activity in 10T1/2 cells and transformed REF-52 cells at a modest efficiency in the G418 assay. The T antigen of this mutant is very unstable, indicating that relatively low levels of T antigen are sufficient for transformation in some cell lines and with some assays. Second, the ability of *dl*A1135(aa17-27) to complement an E1A mutant defective for binding to $p105^{Rb}$ but not an E1A mutant defective for binding to p300 (63) suggests that the levels of *dl*A1135 T antigen in transfected rat cells are sufficient for performing some of the transformation-related activities of large T antigen; Marsilio et al. (40) showed that *dl*A1135 T antigen was also very unstable. Third, Marsilio et al. investigated the transformation activities of a number of

mutants which each lack six amino acids in the large T/small t common region (40). All of these mutants were defective for transformation, but this could reflect the fact that all were very unstable. Stabilizing one of the mutant T antigens by insertion of six different amino acids at the site of the deletion stabilized the mutant protein but failed to restore transformation to normal or near normal levels, suggesting that the large T/small t common region plays an active role in transformation. However, it is not possible to determine what role the substituted amino acids might by playing. Additional evidence that the amino terminus of large T antigen plays an essential role in transformation of REF-52 cells is the finding that mutant *in*A2831(aa5-35), which is defective for transformation, encodes T antigen which is considerably more stable than that of any other mutant with a lesion in this region. Furthermore, REF-52 cells could be transformed by complementation between mutant *in*A2831(aa5-35) and an adenovirus E1A mutant defective for binding to $p105^{Rb}$ (66).

The results obtained in these studies are in general agreement with results obtained by Srinivasan et al. (54) and by others who used more limited sets of mutants than we did in the studies reported here but are in disagreement with results obtained by Sompayrac and Danna (52), who showed that a fragment of T antigen comprising the N-terminal 147 amino acids was sufficient to transform the rat F111 cell line and to immortalize and transform rat embryo fibroblasts. They suggest that when sufficient quantities of transformation-active T antigen are found in an appropriate cellular compartment within the nucleus, transformation and immortalization can occur, and that their N-terminal fragment of T antigen is such a transformation-active form of T antigen. Our studies do not address the question of whether or how small fragments of T antigen could transform rat cells. Possibly, such fragments are more effective than full-length T antigens in inactivating some tumor suppressor proteins or acquire new activities. Rather, our studies are directed at understanding the requirements for transformation by wild-type T antigen and derivatives of it which are of approximately wild-type size. However, we (Tables 1 and 2) and others (22, 28, 41) have found that mutants of SV40 whose T antigens are defective for nuclear localization were able to transform established rat cell lines even though T antigens were detected exclusively in the cytoplasm. The model advanced by Sompayrac and Danna (52) does not account for the behavior of these cytoplasmic T-antigen mutants, since they do not accumulate to detectable levels within the nuclear compartment.

How is transformation related to other properties of large T antigen? A central goal of the studies described here and other studies ongoing in our laboratory is to understand how the various activities of SV40 large T antigen are related and to gain insight into which of its properties are reflections of the same biochemical activity. Figure 5 shows a diagram illustrating the portions of large T antigen associated with its various activities. Clearly, sequences critical for transformation of both 10T1/2 and REF-52 cell lines are located within the N-terminal portion of the protein. This part of the large T antigen is involved in DNA polymerase α binding (11), transcriptional activation of viral and cellular genes (69), and binding to $p105^{Rb}$ and p107/120 (12, 14).

Mutants defective for transformation of both rodent cell lines retained transactivation activity in monkey cells; conversely, mutants defective for transactivation in monkey cells retained the ability to transform both rodent cell lines. This is reminiscent of results obtained in studies on the adenovirus E1A protein, which showed that the transcriptional activation activity of E1A maps to the unique portion of the 289-amino-acid 13S mRNA product of the E1A gene, a region known to be completely dispensable for E1A immortalization and transformation activities (42). Nevertheless, transactivation of some cellular genes may be important for transformation of rodent cells, and mutants which failed to transform might not be able to transactivate these genes.

The full transformation of cells, and the formation of tumors in animals, is a multistep process. Each may result from alterations in multiple proto-oncogenes, the loss of multiple tumor suppressor genes, or some combination thereof. Many studies indicate that expression of multiple proto-oncogenes, activated by overexpression or mutation, can immortalize and fully transform primary rodent embryo fibroblasts (26, 48; for a review, see reference 20). The multifunctional SV40 large T antigen, acting alone, is able to both immortalize and transform primary cells. T antigen forms complexes with two tumor suppressor proteins, $p105^{Rb}$ and p53, and is thought to inactivate the growth suppression properties of these proteins. That T antigen might interact with still other tumor suppressor proteins is possible. This suggests that the interactions between T antigen and tumor suppressor proteins is likely to lie at the heart of the mechanisms of immortalization and transformation by SV40 large T antigen. Furthermore, we suspect that sequestration of tumor suppressor proteins by large T antigen results in changes in patterns of cellular gene expression critical for establishing the transformed phenotype.

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