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Identification of a Region of Simian Virus 40 Large T Antigen Required for Cell Transformation

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A series of replication-competent simian virus 40 (SV40) large T antigens with point and deletion mutations in the amino acid sequence between residues 105 and 115 were examined for the ability to immortalize primary cultures of mouse and rat cells. The results show that certain mutants, including one that deletes the entire region, are able to immortalize. However, consistent with previous data, the immortalized cells are not fully transformed, as judged by doubling time, sensitivity to concentrations of serum, and anchorage-independent growth. The region from 106 to 114 has structural features in common with a region involved in transformation by adenovirus E1a protein (J. Figge, T. Webster, T. F. Smith, and E. Paucha, J. Virol. 62:1814–1818, 1988) and influences the binding of the retinoblastoma gene product to large T (J. A. DeCaprio, J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston, Cell 54:275–283, 1988). Together, these results imply that the sequence from 106 to 114 forms part of a domain that is essential for transformation of established cells, is dispensable for immortalization, and is not required for SV40 replication. The results also indicate that the ability of SV40 large T to immortalize primary cells is independent of its ability to bind to the retinoblastoma gene product.

It has been known for some time that large T antigen, a product of the early region of simian virus 40 (SV40), is capable of transforming both primary and established rodent cells in culture (47). Typically, following viral infection, dense foci of morphologically distinct cells appear on monolayers of unaltered cells. When plated in semisolid medium, cells derived from such foci are able to grown in an anchorage-independent manner. They also form tumors when injected into susceptible hosts.

Studies with other viral oncogenes, however, have shown that transformation of primary and established cells may not be carried out by the same viral functions. Land et al. (17) and Ruley (35) showed that certain classes of oncogenes were able to rescue primary cells from senescence without conferring upon them a typical transformed phenotype, as measured by focus formation or anchorage-independent growth. This ability to confer on primary cells the capacity for continued proliferation has been referred to as establishment or immortalization. Earlier work suggested that immortalization is distinct from the subsequent process of transformation and tumorigenesis and that the two processes occur in the nucleus and cytoplasm, respectively. Most recent work has indicated that the division between immortalization and transformation is not absolute in that certain immortalizing oncogenes, like c-*myc* (14, 42), can render cells tumorigenic when expressed at high levels. Similarly, expression of moderately high levels of the prototype transforming oncogene Ha-*ras* can itself immortalize cells (15). For SV40 large T, it is not known whether immortalization of primary cells and phenotypic transformation of established cells are carried out by the same or different functions.

Some of the known biochemical activities of large T have been mapped to the amino-terminal region of the protein. Most notably, the domain required for binding of large T to sequences at the viral replication origin has been localized to a region which begins at amino acid 149 and extends past residue 200 (24, 28, 32, 38). However, mutants which lack origin-binding activity retain the ability to transform both primary and established cells phenotypically, indicating that origin binding is dispensable for full transformation (22, 23, 29, 33, 44).

A region required for the nuclear localization of large T has been defined between amino acids 126 and 132. Analysis of mutants has shown that these sequences are not required for transformation of established cells in culture (8, 12, 18). However, their role in immortalization remains controversial. Initial studies suggested that mutants which failed to localize to the nucleus were unable to establish primary rat embryo fibroblasts (REF; 19), a result which supported the notion that the establishment and transformation activities of large T are separable and possibly take place at different subcellular locations. More recent results suggest that nonnuclear large T can immortalize. Vass-Marengo et al. (48) showed that a cytoplasmic mutant could effectively immortalize REF when expressed together with the neomycin selectable marker. Similarly, a cytoplasmic variant of large T constructed by Segawa and Yamaguchi (37) was able to immortalize primary rat brain cells efficiently. A third line of evidence was provided by the studies of Michalovitz et al. (25), which showed that cytoplasmic mutant large T was able to cooperate efficiently with an activated Ha-*ras* in the transformation of early-passage REF. These studies all argue that large T need not be in the nucleus to perform either establishment or transformation functions. While it is difficult to rule out the possibility that small amounts of large T may reach the nucleus by leakage, the observed efficiency of the effects described is less consistent with their being caused by a very minor population of large T than with their being caused by a nonnuclear species of the protein.

The only other known biochemical function which appears to be associated with the amino-terminal portion of large T antigen is the ability to stimulate DNA synthesis in quiescent

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cells (9). This activity has not been localized to any particular region within the amino-terminal half of the protein, although the finding that a large T-related protein, D2, which lacks residues 1 to 82 of the authentic sequence, was active in this regard suggests that sequences shared between the large and small T antigens are not essential for this function (41). In adenoviruses and the E5 protein of bovine papillomavirus, the ability to transform has not been separated from the ability to stimulate host cell proliferation (20, 43, 49), indicating that the two activities are tightly linked. No T antigen mutants have been reported which are defective in the stimulation of cellular proliferation. Therefore, the role of this activity in transformation by SV40 remains unexplored.

There is evidence indicating that the amino-terminal segment of large T extending from residues 1 to ~150 has transforming activity in selected cell lines in the absence of additional T sequences (1, 4, 5, 31, 39, 40, 46). Thus, one or more functions unique to this region appear to be sufficient for maintenance of neoplastic phenotype, at least in some cells. In keeping with this contention, a number of laboratories have generated mutants within the amino-terminal region of large T which are defective in the ability to transform cells (12, 36, 37, 45). In particular, Kalderon and Smith (13) isolated a group of such mutants with alterations in the residues between amino acids 105 and 115 of large T. These mutants were unusual in that many of them were fully competent in viral replication. This result suggested that the stability of large T was not grossly altered by the mutations and that transformation and viral replication are separable functions. We have extended the characterization of these mutants and show here that the region between amino acids 105 and 115 of large T forms a domain whose integrity is required for full transformation of both primary and established cells in culture. By contrast, some mutants in this region retain at least partially the ability to immortalize early-passage rodent embryo fibroblasts, providing evidence for the separation of these functions.

MATERIALS AND METHODS

Cells and viruses. All cells were grown in Dulbecco modified Eagle minimal essential medium containing 10% newborn calf or fetal serum in a 10% CO_2 atmosphere. Primary mouse embryo fibroblasts (MEF) were obtained from C57BL/6 embryos. Recombinant retroviruses were generated by using the pZipNeo backbone vector (3) by established techniques (21). Wild-type SV40 early-region sequences cloned into the pZipNeo backbone vector and called Ψ2WWTS were a gift from M. Brown (2). Since all of the mutants used were present in exon 2 of the SV40 early region, the *Bst*XI-*Bam*HI fragment of the wild-type WWTS vector was replaced with the corresponding fragment derived from the plasmids generated by Kalderon and Smith (13). The fragment from pPVU-0 which is the parent of the mutants was similarly inserted such that Ψ2WWTS and Ψ2pPVU-0 are both wild-type early-region genomic vectors. Tex is a similar vector into which large T cDNA was inserted (10). This vector therefore codes for only large T, whereas all of the others code for both large T and small T.

Ψ2 cells were transfected and subjected to G418 selection by methods described previously (3, 21). Typically, viral harvests were 24-h collections of freshly changed medium taken from atop the cultures of interest. These fluids were then filtered through 0.22-μm-pore-size membranes and used almost immediately. To assess the capacity for anchorage-independent growth, cells were suspended in 10% calf serum-containing Dulbecco modified Eagle minimal essential medium containing 0.3% Methocel and then incubated at 37°C. Agar growth was measured by light microscopy.

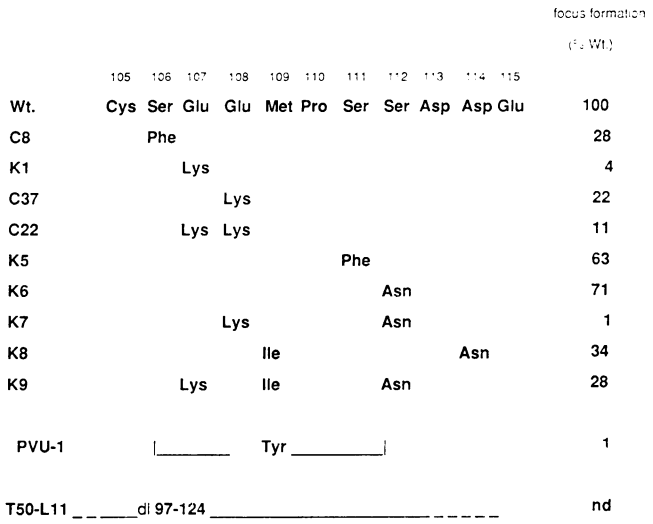

FIG. 1. Positions of mutations in the amino-terminal region of large T antigen. The positions and predicted amino acid alterations of the mutant antigens used in this study are shown. The data summarized are those of Kalderon and Smith (13). The efficiencies of transformation shown in the right-hand column are averages of results reported by those researchers calculated on the basis of experiments in which 10 μg of plasmid DNA was used to transfect monolayers of Rat-1 cells. Wt., Wild type; nd, not done.

DNA transfection. DNA transfection was performed by the $CaPO_4$ method with accompanying glycerol shock, as described previously (6).

[^{35}S]methionine labeling, immunoprecipitation, and SV40 T antigen immunofluorescence measurements. [^{35}S]methionine labeling, immunoprecipitation, and SV40 T antigen immunofluorescence measurements were performed by methods reported previously (28). The radioactive amino acid was from Dupont, NEN Research Products.

RESULTS

Characterization of mutants that map between amino acids 105 and 115 of large T antigen. The predicted amino acid sequences of the mutants used in this study are summarized in Fig. 1. The transformation efficiency of each mutant in focus assays performed with an established rodent line, Rat-1, was described by Kalderon and Smith (13). Transformation was significantly reduced, in most cases, by comparison with a wild-type T antigen-producing culture. To determine whether the Rat-1 foci which arose on transfection with these mutant DNAs contained fully transformed cells, a number were picked, expanded into lines, and tested for the ability to grow in suspension. This assay was used as the most stringent proof of the transformed phenotype (34). Cells were suspended in semisolid medium at various densities and incubated for up to 1 month. Our results showed that all Rat-1 cells bearing large T antigen containing a mutation between amino acids 105 and 115 failed to grow in an anchorage-independent manner. An example is shown in Fig. 2. Fig. 2A shows colonies formed in Methocel by cells that express wild-type large T antigen, while Fig. 2B contains the same number of cells carrying the K1 mutation,

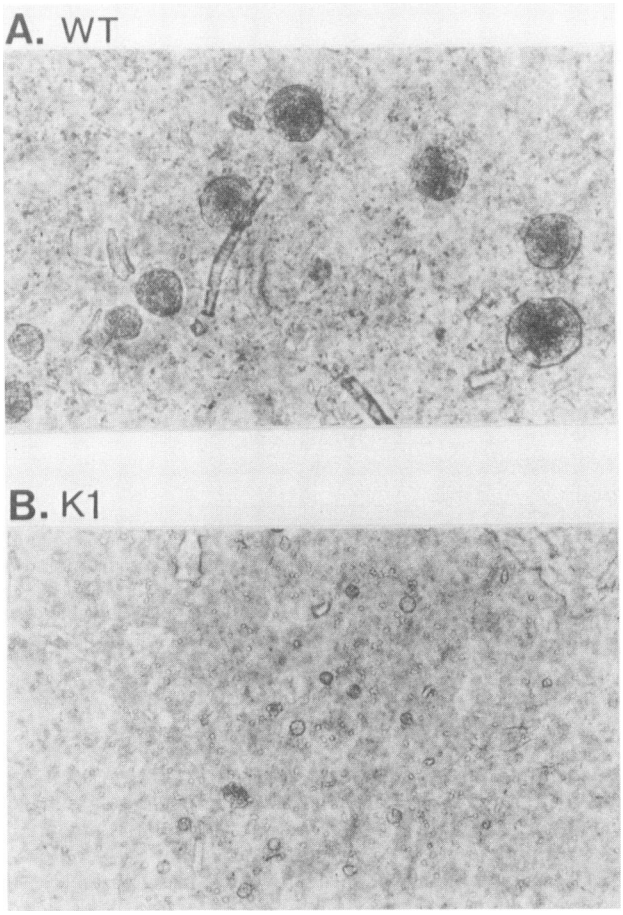

FIG. 2. Growth in suspension of cells expressing wild-type and mutant large T antigens. (A) Colonies formed in Methocel by wild-type (WT)-transformed Rat-1 cells. (B) Colonies formed by cells transformed by mutant K1. The calculated efficiencies of colony formation were 3.6% for the wild type and 0.029% for the mutant antigen.

predicted to contain Lys in place of Glu-107. Of the mutants that retain the ability to replicate at wild-type levels (13), K1 is the most defective for focus formation. Surprisingly, all of the mutant lines examined failed to grow in suspension, regardless of the efficiency with which the foci arose. The failure of lines that carry mutant large T to grow in suspension indicated that although they were originally derived from foci, these Rat-1 cells were not fully transformed. These data suggest that the integrity of the region between amino acids 105 and 115 of large T antigen is required for full transformation of this established rat cell line.

Ability of transformation-defective mutants to establish MEF. It has been reported that in some cases, transformation of primary cells in culture is a multistep process in which immortalization or establishment can be separated genetically from expression of the phenotypic properties of transformation (17, 35). Since wild-type SV40 large T alone can fully transform primary cells (11, 30), we next asked whether mutants defective in transformation retain the ability to immortalize early-passage MEF. The sequences that encode mutant large T antigens were inserted into the pZipNeo retroviral vector of Cepko et al. (3) as described in Materials and Methods. Helper-free retroviral stocks were generated and used to infect second-passage MEF. Following 2 weeks of selection in G418, colonies were picked and expanded. Cells were considered immortal if they grew to confluence in 90-cm^2 plates following successive passaging on plates of increasing surface area. In the experiments which were performed, this degree of expansion is estimated to correspond to about 17 cell doublings. None of the colonies that were infected with control retroviruses and expressed only neomycin resistance established themselves as defined by this criterion. Their proliferation ceased after about 14 doublings, that is, they failed to grow to confluence in 60-cm^2 plates. Results obtained with some of the mutant large T species are summarized in Table 1. As expected, large T from the wild-type genomic constructs WWTS and PVU-0 and from the cDNA-containing construct Tex all efficiently established MEF. The results indicate that large T bearing the K1, K8, PVU-1, and T50-L11 mutations can also establish MEF by this assay.

TABLE 1. Establishment of secondary MEF by mutant SV40 large T antigens[a]

Mutant antigen[b]	Expt no.	No. of colonies picked	No. surviving	% Survival
Zip	1	6	0	0
	2	12	0	0
	3	9	0	0
	4	12	0	0
PVU-0	1	5	4	80
	2	12	7	67
WTTS	3	14	14	100
	4	7	7	100
Tex	1	5	5	100
	2	12	12	100
PVU-1	1	5	2	40
	2	12	0	0
	4	12	11	92
T50-L11	3	7	6	86
	4	12	6	50
K1	1	6	5	83
	2	11	8	73
	3	17	12	71
K8	3	13	13	100
	4	12	12	100

[a] Secondary MEF cells were infected with helper-free retroviruses that encode the indicated large T antigen sequences. Following 2 weeks of G418 selection, colonies were picked and expanded. Percent survival indicates the percentage of colonies picked which survived more than 17 cell doublings.

[b] Retroviral vectors used to establish the mouse cell lines. Zip is the host vector lacking inserted SV40 sequences. PVU-0 and WWTS contain the wild-type SV40 early region. Tex contains large T antigen cDNA, and the rest contain SV40 early-region sequences bearing the designated mutations with the sequences shown in Fig. 1.

Mutants K7, K9, C8, and C22 did not immortalize cells in the experiment described in Table 1 for unknown reasons. One possibility is that the inherent variability in this assay, as reflected by the results obtained with mutant PVU-1 in different experiments (Table 1) masked their activity. Another possibility is that certain mutations in this region adversely affect an independent immortalization domain located elsewhere in the molecule. In view of the facts that mutations that delete part or all of this region (PVU-1 and T50-L11) retained the ability to immortalize mouse cells and

TABLE 2. Properties of MEF established by mutant SV40 large T antigens[a]

Cell line[b]	Saturation density (doubling time [h]) in 10% serum	Saturation density (doubling time [h]) in 2% serum	Growth in agar
Tex	3.0×10^6 (24)	2.0×10^6 (34)	+
PVU-0	2.5×10^6 (24)	1.4×10^6 (34)	+
WTTS.13	3.0×10^6 (19)	1.0×10^6 (29)	+
WTTS.14	5.0×10^6 (19)	1.5×10^6 (24)	+
WTTS.15	3.5×10^6 (19)	1.8×10^6 (36)	+
K1.1	1.4×10^6 (29)	2.8×10^5 (77)	−
K1.2	1.3×10^6 (29)	3.0×10^5 (77)	−
K1.6	1.1×10^6 (29)	3.5×10^5 (53)	−
K1.13	1.3×10^6 (19)	3.0×10^5 (72)	−
K1.14	1.4×10^6 (19)	3.0×10^5 (60)	−
K8.13	2.5×10^6 (19)	1.1×10^6 (29)	−
K8.15	1.8×10^6 (24)	6.0×10^5 (60)	−
K8.16	1.1×10^6 (24)	4.5×10^5 (43)	−
T50-L11.4	1.2×10^6 (24)	0.4×10^5 (144)	−
T50-L11.7	0.8×10^6 (24)	1.0×10^5 (72)	−
T50-L11.9	1.2×10^6 (24)	1.5×10^5 (53)	−
PVU-1.2	ND[c]	ND	−
PVU-1.3	ND	ND	−
PVU-1.5	ND	ND	−

[a] Growth properties of the different lines in 10% or 2% fetal bovine serum medium are shown. Cells were plated at a density of 10^4/60-mm^2 dish in the appropriate serum and then counted every other day. For measurement of growth in agar, cells were plated at the same initial density in medium containing 10% serum and 0.3% agar.

[b] The SV40 sequence present in the infecting retrovirus precedes the period, and the clone number of each cell line follows the period.

[c] ND, Not done.

all of the mutants immortalized rat cells (see below), further work would be necessary to make certain that K7, K9, C8, and C22 are overly defective.

We then determined whether the mouse cells established by the aforementioned mutant large T antigens were fully transformed. To minimize phenotypic differences which might be due to variations in the rates of large T synthesis, lines were screened for expression of large T antigen by indirect immunofluorescence. The cells were grown on cover slips, fixed, and stained for large T with monoclonal antibody PAb419. All lines exhibited nuclear fluorescence, although intensity was variable (data not shown). Cells with the brightest fluorescence were selected for further study.

The selected lines were tested for the ability to grow in low concentrations of serum (2% was used in this study) and for the ability to grow in an anchorage-independent manner (Table 2). Since the growth properties of clonal lines of both normal and transformed cells vary, the results can be interpreted only in broad terms. In general, all of the lines established by mutant large T antigens grew at about the same rate in medium containing 10% serum as did wild-type-expessing cells, although their number at saturation was generally lower. However, in 2% serum, although all of the cells grew more slowly, the effect was more marked for mutant-expressing lines than for their wild-type-expressing counterparts. The doubling times of mutant-expressing lines increased from two- to threefold, while wild-type-expressing lines were not so seriously affected. The saturation densities of the mutant-expressing lines were also decreased in low serum concentrations. In the most extreme case, T50-L11, growth virtually ceased under these conditions.

The morphology of the different lines appears to reflect their growth properties. Figure 3 shows representative phase-contrast photomicrographs of cells fixed for immunofluorescence. Cells that express mutant large T antigens

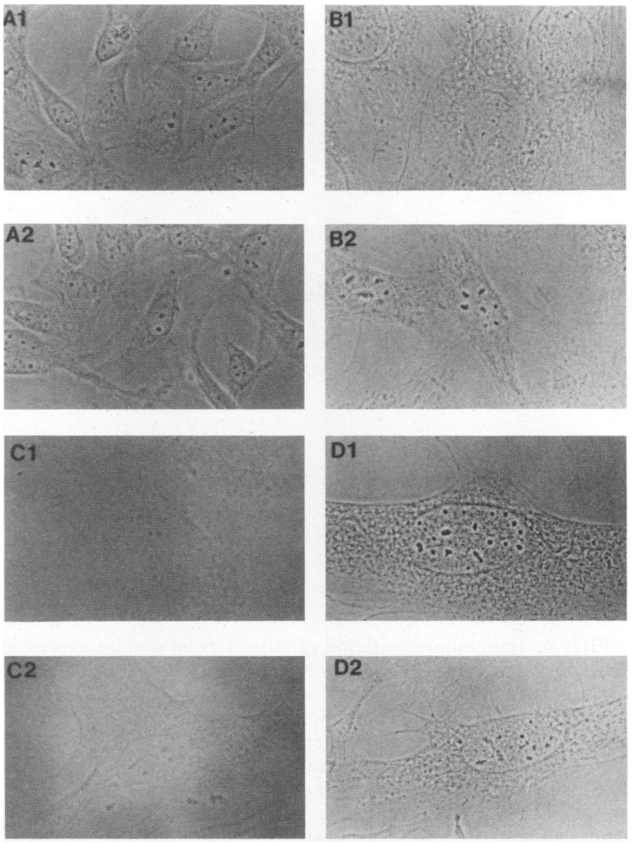

FIG. 3. Photomicrograph of MEF lines that express mutant large T antigens. Cells growing on glass cover slips were fixed in −20°C methanol and then permeabilized by washing with phosphate-buffered saline containing 1% Nonidet P-40. They are shown photographed by phase contrast at the same magnification. Panels: A1, U24 clone 1, a mutant which predicts the alteration of Ala-149→Val (this mutant is defective in the ability to bind with high affinity to sequences at the origin of SV40 DNA [25], but it transforms Rat-1 cells more efficiently than does the wild type [11]; U24 functioned like the wild type in all of the assays described here); A2, Tex clone 11; B1, K1 clone 13; B2, K1 clone 6; C1, PVU-1 clone 3; C2, PVU-1 clone 5; D1, T50-L11 clone 7; D2, T50-L11 clone 9.

have larger nuclei and appear larger and more adherent than do wild-type-expressing cells. This effect is particularly marked in cells that carry deletion mutant T50-L11, which lacks sequences between residues 97 and 124. This line showed the greatest inhibition of proliferation in medium containing 2% serum. Taken together, these results suggest that mutant large T antigens are defective in the ability to stimulate cell proliferation, particularly under stringent conditions. Moreover, in other experiments, all of the mouse cell lines that express these mutants failed to grow in suspension, regardless of their growth rates and sensitivities to serum. We conclude that although these lines are immortalized by the criteria summarized in Table 1, they are not fully transformed.

To ensure that the effects observed did not result from expression of different amounts of large T, cells were labeled for 4 h with [^{35}S]methionine and extracts were prepared. Equal numbers of trichloracetic acid-precipitable counts were then incubated with monoclonal antibody PAb419, which is directed against the amino terminus of the T antigens. Immunoprecipitates were collected on the surface

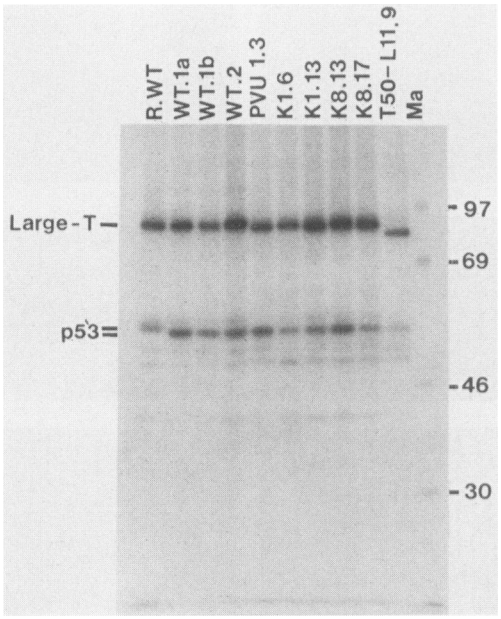

FIG. 4. Immunoprecipitation of large T from mutant-expressing MEF lines. Samples of [^{35}S]methionine-labeled cell extracts containing equal numbers of trichloroacetic acid-precipitable counts were immunoprecipitated with monoclonal antibody PAb419, which is directed against the amino terminus of the T antigen. R.WT, Control extract from wild-type-transformed Rat-1 cells. The central lanes show results obtained with extracts of MEF lines established by the various mutant antigens. WT.1a and WT1.b refer to lines generated with the wild-type construct, and WTTS.WT.2 refers to a line generated with PVU-0 (13). The numbers on the right indicate the molecular sizes (kilodaltons) of markers (Ma).

of protein A-Sepharose beads, washed, eluted, and then analyzed by polyacrylamide gel electrophoresis. Representative results are presented in Fig. 4. The data show that large T was present at roughly equivalent levels in all of the lines tested.

The apparent molecular weight of the protein encoded by the T50-L11 mutant is estimated to be about 82,000, compared with 90,000 for full-sized large T. The difference is somewhat larger than might be expected, since a deletion of 28 amino acids should reduce the molecular weight by ~3,000. PVU-1 large T always migrated slightly faster in this gel system than did the wild-type protein, although its deletion spans no more than 5 amino acids and might not be expected to alter the size of the protein greatly (see Fig. 5). One possible explanation for the aberrant migration of both of these proteins might be that the state of phosphorylation of the mutant T antigens is significantly different from that of the wild type, thereby leading to greater migrational effects than would be expected as a result of the deletion alone. The deletions in PVU-1 and T50-L11 affect residues which are known to be phosphorylated in large T, that is, Ser-106, Ser-111, Ser-112, Ser-123, and Thr-124.

All of the mutant large T antigens bound to p53, although the amounts of p53 detected in these immunoprecipitates varied more than did the amounts of large T. The significance of this observation is unclear, since the levels of p53 do not correlate with any of the properties measured here.

The results of this analysis strongly suggest that the failure of cells that express mutant large T antigens to exhibit a fully transformed phenotype is due to a defect in the function of large T itself rather than to variations in the level of expression. The data support the conclusion that the integrity of the region between amino acids 105 and 115 of large T is required for full transformation, although large T-bearing mutations in this region still retains its ability to establish early-passage MEF.

TABLE 3. Establishment of secondary REF by mutant large T antigens[a]

Mutant antigen	No. of surviving colonies[b]			Growth in suspension
	Expt 1	Expt 2	Expt 3	
PVU-0	17	21		+
C8	3	1	6	
K1	7	4	24	−
C37		22	26	−
C22	22	6		−
K5		13		−
K6		23		−
K7	3	8	12	−
K8	18	5		−
K9[c]		1	14	−
PVU-1	7	6	13	−
T50-L11	1	9	5	−

[a] Samples (10-μg) of plasmid DNAs that encode the indicated mutant large T antigens were cotransfected with 2 μg of a plasmid that encodes the neomycin resistance gene. Colonies were evident following 2 weeks of selection. In experiments 1 and 2, colonies were picked and expanded; the lines tested for expression of large T antigen and for growth in suspension were derived from these experiments. Remaining colonies were stained for counting. In experiment 3, no colonies were picked and all plates were stained.

[b] The number of colonies larger than 3 mm in diameter which survived the selection process is given.

[c] Immunoprecipitation indicated that this line contains no full-length large T antigen.

Abilities of transformation-defective mutants to establish REF. Since the putative transforming region of large T between residues 105 and 115 was originally defined by using established rat cell lines rather than mouse cells, we tried to confirm our results by using early-passage REF. Initially, we infected REF with retroviruses in experiments which paralleled those described using MEF. However, we found that with few exceptions, the colonies we selected expressed extremely low levels of full-sized large T. Because of this difficulty, the infection approach was abandoned and cotransfection of plasmids carrying the different large T-coding sequences and a plasmid encoding the neomycin resistance marker was performed. Following 2 weeks of selection with the antibiotic G418, colonies were picked and expanded as before. This approach permitted examination of a reasonable collection of mutants. Specifically, all of those denoted in Fig. 1 were tested this way. Table 3 presents the results of three independent experiments. The number of colonies that survived the selection process with each mutant is presented, together with the number of wild-type colonies detected in the same experiment. Each mutant was able to establish REF in this assay, although the efficiency of immortalization by each mutant varied from experiment to experiment and, in some cases, appeared to be less than that observed with mouse cells. On average, mutant large T antigens were less effective than the wild type, but in light of the variability in the assay, it was not possible to conclude that any mutant was strikingly defective in the ability to establish early-passage REF. As with mouse cells, rat cell lines expanded from colonies that survived transfection with mutant large T antigens all failed to grow in suspension (data

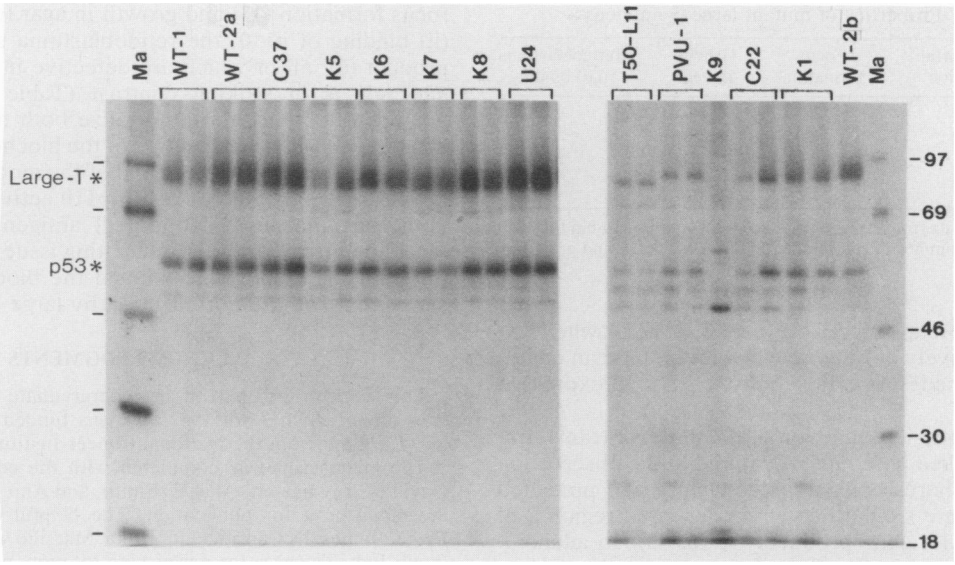

FIG. 5. Immunoprecipitation of large T from mutant-expressing REF lines. Samples of [^{35}S]methionine-labeled extracts containing equal trichloroacetic acid-precipitable counts were immunoprecipitated with monoclonal antibody PAb419 and then analyzed in sodium dodecyl sulfate-containing 10% polyacrylamide gels. Each pair of lanes derives from immunoprecipitation of two independently generated lines of cells. Lanes: Wt-1, control extracts, i.e., two separately prepared extracts of a wild-type-transformed Rat-1 cell line; WT-2a and WT-2b, two lines of cells transformed by wild-type T antigen-encoding plasmid PVU-0. The other lanes contained the extracts indicated. The numbers on the right indicate the molecular sizes (kilodaltons) of markers (Ma).

not shown), also indicating that the established REF were not fully transformed.

The levels of expression of the large T antigen were tested in the REF lines by immunoprecipitation following 4 h of labeling with [^{35}S]methionine as before. Large T was present in most of the lines examined at levels roughly equivalent to those of the wild type, with three exceptions (Fig. 5). The levels of large T encoded by deletion mutants T50-L11 and PVU-1 seemed to be decreased relative to the wild type in these experiments, and no full-length large T was detected in the line of cells established by mutant K9. Two fragments of 60 and 50 kilodaltons appeared to be immunoprecipitated instead. These were not investigated further, nor was the stability of the large T encoded by the deletion mutants examined. Although a number of factors may have contributed to the observed phenotype of the cell lines, the data obtained with the point mutants generally support the conclusion drawn from the results described earlier, i.e., that the failure of the cells to grow in suspension was due largely to a defect in large T itself. The position of the mutations tested localized the defect to the region between amino acids 105 and 115 of large T antigen.

DISCUSSION

Much research in recent years on SV40 large T antigen has attempted to correlate biological actions of the protein with known biochemical activities. A powerful method to do this has been the use of specific mutations introduced in the protein at known positions, followed by analysis of the resulting biological and biochemical phenotypes. Much effort has centered on mapping of the regions of DNA associated with replication and transformation and on establishing the corresponding biochemical activities. Here, we examined a group of mutants which had been reported to be wild type for replication but defective in transformation of Rat-1 cells as judged by focus formation but for which no known biochemical lesions were reported. The data we obtained show that the integrity of the region between amino acids 105 and 115 of large T is required for full transformation of both primary and established cells in culture. The close clustering of the point mutations, all of which result in a transformation defect, suggests that this region forms at least part of a functional domain. The observation that, in most cases, transformation-defective mutants retained the ability to immortalize early-passage fibroblasts indicates that the function of large T that enables it to establish cells and the function responsible for its ability to promote anchorage-independent growth are separable.

Consistent with this, J. Tevethia and co-workers (personal communication) have mapped a domain responsible for immortalization to a region outside that mutated in the variants reported here. It cannot be concluded on the basis of the data reported here, however, that the functions responsible for immortalization and transformation are the result of different biochemical activities. It is also possible that immortalization and transformation result from expression of a single function which is quantitatively altered but not inactivated by the mutations described here. Immortalization might result from residual expression of this activity at a level which is not sufficient to promote growth in suspension. This idea is consistent with the results of Risser and Pollack (34), who showed that the phenotypic characteristics exhibited by cloned transformants of a 3T3 cell line correlated well with the level of expression of large T. Cells which reached high saturation densities on plastic and grew well in suspension generally expressed higher levels of large T than did cells which exhibited an intermediate phenotype. However, most transformants grew well in low concentrations of serum, regardless of the level of expression of large T. These data can be interpreted to mean that a single biochemical activity of large T is responsible for all of these effects, with most extreme properties resulting from expression of the highest level of activity. This behavior is reminiscent of that of other nuclear oncogene products. In

TABLE 4. Properties of mutant large T antigens[a]

Cell line	Immortalization	Focus formation	Growth in agar	Retinoblastoma p110 binding
Wild type	+	+	+	+
PVU-1	+	−	−	−
T50-L11	+	−	−	−
K1	+	−	−	−

[a] Focus formation data from reference 13, retinoblastoma p110-binding data from reference 6, and immortalization data on REF and MEF and growth in agar are shown.

particular, c-*myc* can effectively establish cells when expressed constitutively at relatively low levels but can confer certain transformed properties only when overexpressed (14).

A clue as to a possible function of the putative transforming domain identified here might be found in the observation that this region shares considerable primary and predicted secondary structure similarities with conserved region 2 of the adenovirus E1A gene products (7, 16, 43). In adenoviruses, mutations within the domain 2 sequence have been shown to impair the ability of the E1A products to cooperate with an activated *ras* oncogene and to induce proliferation of quiescent primary baby rat kidney cells (27).

The known biological effects of large T antigen are consistent with the idea that the sequences between amino acids 105 and 115 might provide similar functions within the large T molecule. It has been reported that amino-terminal fragments as short as 130 amino acids can efficiently complement polyomavirus middle T antigen in the transformation of secondary REF (48). Furthermore, analysis of a number of mutants has suggested that the ability of large T to stimulate DNA synthesis in quiescent cells resides within the amino-terminal portion of the molecule (41). In addition, Sompayrac and Danna (40) and Pipas et al. (31) have observed that the N-terminal ~125 residues of T antigen has clear transforming activity in C3H10T1/2 cells, a continuous line of otherwise untransformed murine fibroblasts.

The notion that the amino terminus of large T antigen possesses some of the functions of the E1A region of adenoviruses is an intriguing one which has recently been tested. (i) Other evidence from our laboratory suggests that mutations within the 105-to-115 region of large T antigen can lead to defects in its ability to cooperate with *ras* in the formation of foci on monolayers of secondary REF (E. Marsilio and E. Paucha, unpublished data). (ii) Moran (26) has recently found that the 101-to-118 region of T antigen can substitute functionally for the E1A domain 2 sequence in a suitable chimeric protein. Specifically, the recombinant was able to cooperate with *ras* in a cotransformation assay on primary cells. (iii) Since completion of this work, it has also been shown that large T antigen interacts with the product, p110, of the retinoblastoma susceptibility gene and that this interaction is inhibited by mutations with the sequences between 105 and 115 (6). This correlation suggests that at least part of the contribution of T antigen, and of the 106-to-114 segment therein, to the viral transforming process is to bind to and, possibly, modulate the function of a known suppressor of neoplastic growth. Two questions that arise from such a conclusion are how p110 works and how T antigen might lead to its functional perturbation. As part of the examination of these questions, it will be interesting to learn whether the 106-to-114 sequence is the only segment of large T antigen which contributes to p110 binding.

The correlation between (i) transformation as judged by focus formation (13) and growth in agar (reported here) and (ii) binding of p110, the retinoblastoma susceptibility gene product (6), all of which are defective in PVU-1, T50-L11, and K1, is in striking contrast (Table 4) to the ability demonstrated here to immortalize both mouse and rat primary cells. Thus, irrespective of the biochemistry associated with the 106-to-114 domain and of the modulatory effect that large T antigen binding has on p110 activity, it is clear that immortalization by SV40 large T antigen is independent of these functions. Another interesting issue is identification of the domains responsible for and the biochemical activities associated with immortalization by large T antigens.

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