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Cell Lines Established by a Temperature-Sensitive Simian Virus 40 Large-T-Antigen Gene Are Growth Restricted at the Nonpermissive Temperature

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The thermolabile large T antigen, encoded by the simian virus 40 early-region mutant *tsA58*, was used to establish clonal cell lines derived from rat embryo fibroblasts. These cell lines grew continuously at the permissive temperature but upon shift-up to the nonpermissive temperature showed rapidly arrested growth. The growth arrest occurred in either the G_1 or G_2 phase of the cell cycle. After growth arrest, the cells remained metabolically active as assayed by general protein synthesis and the ability to exclude trypan blue. The inability of these cell lines to divide at the nonpermissive temperature was not readily complemented by the exogenous introduction of other nuclear oncogenes. This finding suggests that either these genes establish cells via different pathways or that immortalization by one oncogene results in a finely balanced cellular state which cannot be adequately complemented by another establishment gene.

When cells are removed from an embryo or an adult animal and cultured in vitro, they proliferate initially but stop after a finite number of divisions even if supplemented with fresh growth medium. For example, human embryonic fibroblasts double about 50 times before ceasing growth. Similarly, rodent embryonic fibroblasts (REFs) progress through about 30 generations before a gradual decrease in the growth rate; the cultures then undergo crisis, and the cells senesce (48). Analysis of human fibroblasts suggests that the decrease in growth rate is due to a lengthening of the G_1 phase of the cell cycle and that the cells probably arrest in late G_1 or at the G_1-S boundary rather than G_0 (5, 16, 40). In addition, there is some evidence suggesting that growth arrest may also occur in the G_2 phase (12). Moreover, by using propidium iodide (PI) cytofluorimetry, it has recently been shown that when REFs cease growth in vitro, growth arrest occurs in either the G_1 or G_2 phase (T. E. W. Riley, S. C. Barnet, and P. S. Jat, manuscript submitted for publication). Even though extensive analysis of both pre- and postsenescent cultures has defined many biochemical changes that have been incorporated into numerous explanations for the finite life span, the underlying basis is not known (for reviews, see references 1, 32, 34, and 42).

A subgroup of viral and cellular oncogenes has the ability to establish the continuous proliferation in culture of primary cells. Therefore, introduction of one of these oncogenes into a primary cell can yield a transformant that does not senesce upon continuous passage. Such immortalized cells have acquired one property of tumor cells: subsequent introduction of an activated *ras*-type oncogene into these cells can result in fully transformed or malignant cells. Introduction of the same activated *ras* oncogene into most primary cells typically results in cell death (19). All establishment-type oncogenes can complement an activated *ras* oncogene in transformation (28, 46). The viral oncogenes which display establishment activity are adenovirus E1a, simian virus 40 (SV40), and polyomavirus large-T-antigen genes and the E7 gene of human papillomavirus (21, 23, 36–38, 43). The cellular oncogenes with establishment activity are *myc*, *fos*, and *p53* (7, 8, 22, 33). All of these oncogenes have the common feature of a nuclear subcellular localization, and each has the ability to stimulate or suppress transcription of a subgroup of cellular and viral promoters (24). The mechanism by which oncogenes enable cells to continuously proliferate in culture has been difficult to investigate. In fact, it is not clear whether all oncogenes establish cells by a single biochemical pathway or by multiple pathways.

Analysis of conditionally lethal mutants has been valuable in the characterization of many complex processes. The same approach could be taken in studying the process of establishment and senescence. Previous studies have shown that the SV40 large T antigen alone is capable of efficiently establishing primary fibroblasts without crisis (21). Earlier characterization of a few cell lines suggested that primary cell lines established by using temperature-sensitive (*ts*) mutants of SV40 and polyomavirus large T antigen were restricted in growth potential at the nonpermissive temperature (36, 39). These virus mutants encode a large T antigen that behaves as wild type at the permissive temperature (33°C) but is inactive at the nonpermissive temperature (39°C; 10, 47, 49).

If, as the previous results suggested, the large-T-antigen gene is essential for maintenance of the proliferative state, then cell lines established with a *ts* mutant of SV40 might be conditional for growth in the establishment pathway. Here, we present a systematic isolation and analysis of a number of cell lines derived from REFs by infection with a retrovirus recombinant encoding a *ts* mutant of SV40 large T antigen. These cell lines are temperature sensitive for growth, and this conditionally lethal phenotype cannot be complemented by most other viral or cellular oncogenes.

MATERIALS AND METHODS

Cells and cell culture. NIH 3T3 (48) and Ψ_2 (31) cells were maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) calf serum plus penicillin and streptomycin. REFs prepared from 12- to 14-day-old Fischer rat embryos and COS M6, a subclone of COS-1 cells (13)

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isolated by M. Horowitz, were maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum plus penicillin and streptomycin.

Construction of pZipSVtsa58. The recombinant pZip SVtsa58 was constructed from the plasmid pZipSV40, which contains the wild-type SV40 early region (nucleotides 5235 to 2666) inserted at the unique *Bam*HI site of the pZipNeoSV(X)1 shuttle vector (4, 20), by replacing the region of the insert corresponding to nucleotides 5235 to 3204 with the identical region from the SV40 mutant *tsA*58. Successful replacement of the wild-type fragment with that derived from the mutant was verified by DNA sequence analysis. Once again, the insert was in the sense orientation with respect to retrovirus transcription.

Plasmids were propagated in both small and large scales in *Escherichia coli* JS4, a *recA1* derivative of MC1061 (3, 44). All manipulations involved in the construction and maintenance of plasmids were carried out by standard procedures (30) under the conditions recommended by the suppliers.

DNA transfection and isolation of stable cell lines. Cells that had been passaged the previous day were transfected with 10 to 15 μg of plasmid DNA by the calcium phosphate procedure of Graham and van der Eb (15) as modified by Parker and Stark (35). The glycerol shock was generally omitted in all transfections; in the transfection of the *tsa* cell lines, it was replaced by addition of chloroquine diphosphate (50 to 100 μg/ml) to the transfection mix for 12 h (29). Cotransfection was done by using a 10:1 ratio of nonselectable to selectable DNA. At 48 h after transfection, the Ψ_2-transfected cells were passaged 1:15 and selected in 1 mg of G418 (GIBCO Laboratories, Grand Island, N.Y., or Bethesda Research Laboratories, Inc., Rockville, Md.; 6) per ml. The medium was changed every 3 to 4 days until distinct colonies were visible; then at least five colonies were isolated and expanded, and virus titers were determined. To assay for complementation by other oncogenes by a continuous-growth procedure, cultures of tsa cell lines that had been transfected 48 h previously were passaged 1:5 and selected in 100 μg of hygromycin B (Calbiochem-Behring, La Jolla, Calif.) per ml at 33°C. After 7 to 10 days, during which the medium was changed every 4 days, the hygromycin B was removed and the cultures incubated at 33°C in normal growth medium until colonies were clearly visible; at this point, representative cultures were shifted to 39°C. Incubation was continued for an additional 7 to 10 days, at which time plates were stained and scored for densely growing colonies. Whenever possible, representative colonies were isolated from each transfection, expanded into a cell line, and analyzed for growth potential.

Retrovirus infections and isolation of stably infected cell lines. Retroviruses were prepared as a 12-h supernatant from a 90 to 100% confluent dish of an appropriate Ψ_2 producer cell line; before use in infections, the viral supernatant was filtered through a 0.45-μm-pore-size filter. Cells for infection were passaged the day before. Infections were done on 10-cm-diameter dishes at 37°C for 2 h in 2 ml of growth medium containing the virus and 8 μg of polybrene (Aldrich Chemical Co., Inc., Milwaukee, Wis.) per ml, after which 8 ml of fresh medium was added. At 48 h after infection, NIH 3T3 cells were passaged either 1:10 or 1:20, and G418-resistant colonies were isolated by growth in medium containing 1 mg of G418 per ml (6). The cultures were refed every 3 to 4 days for about 14 days; the dishes were then stained with 2% methylene blue in 50% ethanol, and the virus titer was determined. Similarly, infected cultures of REFs were passaged either 1:10 or 1:20, and G418-resistant colonies were isolated by selection at 33°C in growth medium containing 0.25 mg of G418 per ml. The medium was changed every 4 days until distinct colonies were clearly visible, at which time the number indicated in Results was selected and isolated on microdilution dishes. Clones that grew were expanded into cell lines and passaged at 33°C at least 10 times before further analysis.

Analysis of protein expression. The level of large T antigen in the various cell lines was measured by Western blot (immunoblot) analysis. Extracts were prepared by the procedure of Garrels (11) and stored frozen at −70°C. When all samples had been prepared, the protein concentration was determined by using a kit from Bio-Rad Laboratories (Richmond, Calif.). The volume of extract corresponding to 35 μg of protein for each sample was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel (27). The fractionated proteins were transferred to a 0.45-μm-pore-size nitrocellulose filter and probed with pAb419, a monoclonal antibody specific for the SV40 large T antigen (17). ^{125}I-labeled goat anti-mouse immunoglobulin G (4×10^6 cpm per blot) was used as the reagent to visualize the specific antigen-antibody complexes.

To analyze whole-cell protein synthesis, the cells were labeled with 125 μCi of [^{35}S]methionine for 25 h at the times and temperatures indicated in Results. Labeling was done in medium prepared as a mixture of methionine minus minimal essential medium (Flow Laboratories, Inc., McLean, Va.), 10% dialyzed fetal calf serum, 2% Dulbecco modified Eagle medium, and glutamine. Extracts were prepared by lysing cells in 1 ml of cold lysis buffer (15% [vol/vol] glycerol, 100 mM Tris hydrochloride [pH 6.8], 7 mM EDTA, 2% [wt/vol] sodium dodecyl sulfate, and 5 μg of aprotinin per ml) at 4°C for 30 min. The lysate was then boiled for 3 to 5 min and stored frozen at −70°C. The level of whole-cell protein synthesis was measured by trichloroacetic acid precipitation of 10 μl of each extract. The profile of major proteins synthesized was examined by fractionating a volume of lysates corresponding to an equal number of trichloroacetic acid-precipitable counts per minute on 5 to 10% sodium dodecyl sulfate-polyacrylamide gradient gels.

Analysis of cell cycle. Cell cycle analysis using PI cytofluorimetry, followed by computer determination of the percentage of cells in the various phases of the cell cycle, was done by standard protocols provided by Ortho Instruments, the manufacturers of the cytofluorograph used for the analyses.

RESULTS

We have previously described the efficient establishment of cell lines by infection of primary REFs with a defective retrovirus vector encoding the SV40 large T antigen. An equivalent retrovirus recombinant (ZipSVtsa58; Fig. 1) that transduces the early region of the SV40 mutant *tsA*58 was constructed (47). This mutant is particularly thermolabile for large-T-antigen activity at the nonpermissive temperature, 39°C. A number of stable producer cell lines were isolated by transfection of the Ψ_2 packaging cell line with plasmid pZipSVtsa58. Virus supernatant collected from each of these cell lines was assayed for transmission of resistance to G418 by infection of NIH 3T3 cells. Although the virus titers were very similar, cell line *tsa*58-3 gave the highest titer ($\sim 10^5$ G418-resistant colonies per ml) and was thus the source of virus for all subsequent experiments.

Cell lines were established by infection of tertiary REFs prepared from 12- to 14-day-old Fischer rat embryos with a

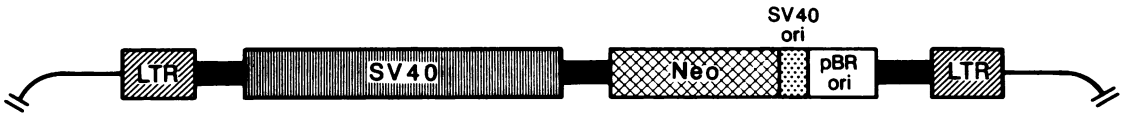

FIG. 1. Structure of the provirus transduced by the pZipSVtsa58 retrovirus recombinant. Shown is a schematic representation of the various segments of DNA (drawn to scale) that constitute the recombinant provirus. The recombinant was constructed from the shuttle vector pZipNeoSV(X)1, whose structure has been described previously (4).

stock of ZipSVtsa58 recombinant virus (see Materials and Methods). As a control, REFs from the same preparation were either mock infected, infected with virus representing the backbone retrovirus vector virus [ZipSV(X)1; 4], or infected with virus that transduces the wild-type SV40 large T antigen (SV40-6; 20). Forty-eight hours after infection, the cultures were passaged and subjected to selection for resistance to the drug G418. After approximately 3 weeks, when discrete colonies were visible, the number of G418-resistant colonies was counted. The typical titer of virus was about 2,500 resistant colonies per ml. This is approximately 40 times lower than the titer of the same stock on NIH 3T3 cells and reflects, as observed previously, a lower efficiency of infection of primary cells.

Seventeen colonies were isolated after infection with the *tsA*58 recombinant retrovirus. Even though all of the colonies initially expanded, only 11 could be established into cell lines. Of these, nine (tsa4, -6, -7, -8, -9, -11, -12, -14, and -17) grew rapidly. No colonies were observed on the mock-infected dishes, whereas one colony was found in the control (backbone only) virus infection. However, this colony did not expand when isolated, in agreement with our previous observations indicating that the virus specifying the backbone vector is not capable of immortalization (21). For use as temperature-insensitive controls in subsequent experiments, four colonies from infection with a retrovirus recombinant transducing the wild-type large T antigen were isolated. All four colonies expanded without crisis into the cell lines designated SV1, SV2, SV3, and SV4.

All cell lines were expanded through at least 10 passages before they were characterized. It has previously been observed that during this period of growth, primary fibroblasts that do not contain an establishment gene enter crisis and senesce. None of the 11 cell lines entered crisis, even though 2 of these lines, tsa10 and -15, continued to grow slowly. All of the cell lines were morphologically similar to and not significantly different from the parental REFs or the wild-type large-T-antigen-established cell lines (data not shown).

Temperature sensitivity for growth. The effect of temperature on growth was analyzed by culturing about 10^3 cells at 33 or 39°C. The control cell lines SV1, -2, -3, and -4 were not significantly reduced in growth during incubation at 39°C (Fig. 2A). In fact, these lines grew more rapidly at the higher temperature and therefore yielded larger colonies than did the corresponding 33°C cultures (data not shown). In contrast, the cell lines derived by infection with the *tsA*58 large T antigen clearly had lower growth upon the shift to 39°C (Fig. 2A). The colonies that developed at the higher temperature were typically flat and grew poorly. The degree of reduction in cell viability was variable; of the eight cell lines analyzed, tsa9 efficiently generated colonies at 39°C, whereas all others were reduced in growth; tsa8 was the most severely restricted (at least 200-fold). More quantitative experiments extending over longer periods of time have shown that these initial experiments underestimated the reduction in growth potential of all of the tsa cell lines and that tsa8 is reduced at least 10^4-fold (data not shown; E. Ruley, personal communication). Even though the *tsa* cell lines failed to grow at 39°C, the cells did not die but were metabolically active (see below) and retained the ability to exclude trypan blue for more than 6 days.

The rate at which the cells lost the ability to divide upon shift to the nonpermissive temperature was also determined. This involved plating about 10^3 cells at 33°C, shifting them to 39°C for the indicated periods of time, and then shifting them back to 33°C for 15 days. The reduction in growth potential upon the shift to 39°C occurred very rapidly (Fig. 2B); for the tsa8 cell line, a 50% decrease occurred in less than 40 h.

In view of these results, four tsa cell lines (tsa8, -12, -14, and -17) and one control cell line (SV4) were chosen for further analysis. tsa8 and -12 were chosen as examples of cell lines that were highly temperature sensitive; tsa14 and -17 represented cell lines with much lower temperature sensitivity.

Cellular DNA from tsa8, tsa12, and SV4 was analyzed by Southern blot analysis to determine the structures and numbers of integrated proviruses. The three cell lines each contained a single provirus insert located at different positions in the genome (data not shown). These results again showed that the recombinant retrovirus produced by the *tsA*58-3 cell line transduced only the large-T-antigen gene as well as the neomycin phosphotransferase gene, even though the original pZiptsa58 construct carried sequences that specified both the large and small T antigens. Thus, the viral RNA serving as the template for reverse transcription during passage must be accurately spliced, resulting in the loss of sequences specifying the small-T-antigen slicing pattern.

Analysis of protein expression. The effect of shift to the higher temperature on the level of large T antigen was determined by Western blot analysis. The results of a representative experiment, in which tsa8, -12, -14, and -17 as well as SV4 were analyzed, are shown in Fig. 3. Cell line COS M6, which constitutively expresses a functional large T antigen, was used as a positive control. At the permissive temperature (lanes f, j, m, and q), all of the tsa cell lines expressed large T antigen at different levels. Upon shift to the nonpermissive temperature, this level rapidly diminished and decreased to marginally detectable levels after 25 h of incubation at 39°C. By comparison, the level of T antigen in the control cell line SV4 did not change significantly, which indicated that the decrease in the level of T antigen observed in the four tsa cell lines was specific for the thermolabile large T antigen. In fact, mutant *tsA*58 was chosen as the source of large T antigen for this property of rapid degradation at the nonpermissive temperature (47).

The effect of growth at the nonpermissive temperature on whole-cell protein synthesis was also examined. Cell lines tsa8 and SV4 were continuously labeled for 25-h periods after incubation at 33 or 39°C for 0, 24, 48, 72, or 96 h. After incubation with radioactive methionine, extracts were prepared and the total amount of protein synthesized was measured by trichloroacetic acid precipitation. The results obtained were very surprising; even though the tsa cells no

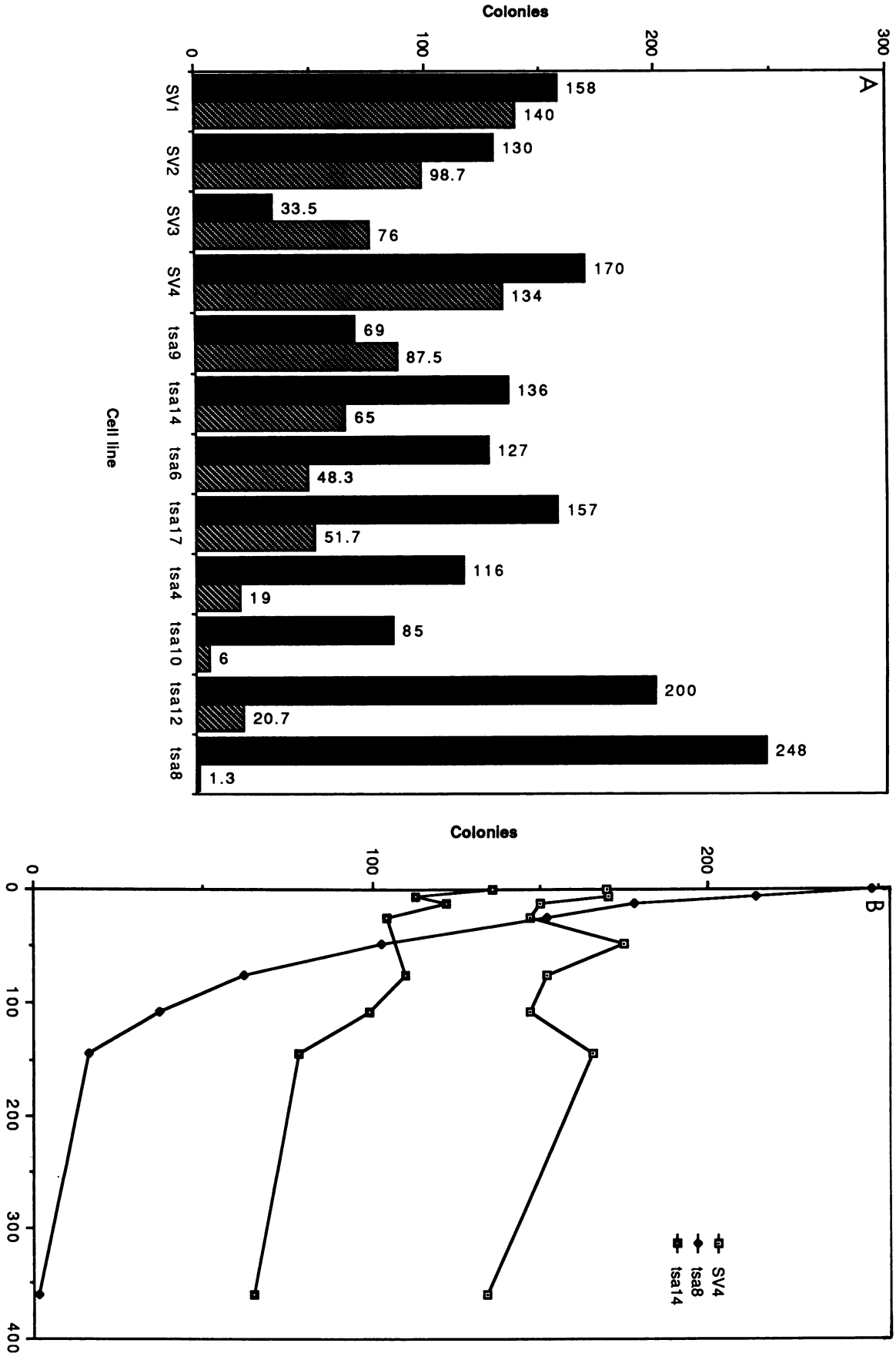

FIG. 2. Cell growth. (A) Histogram of the difference in growth potential at the permissive and nonpermissive temperatures. The total number of colonies was determined after culture at either 33°C (■) or 39°C (▨). (B) Effect of time of incubation at the nonpermissive temperature on growth potential. Cells (10^3) from each cell line were plated at 33°C, shifted to 39°C for the indicated time periods, and then shifted back to 33°C for 360 h. Each datum point is an average of the number of colonies obtained on two dishes.

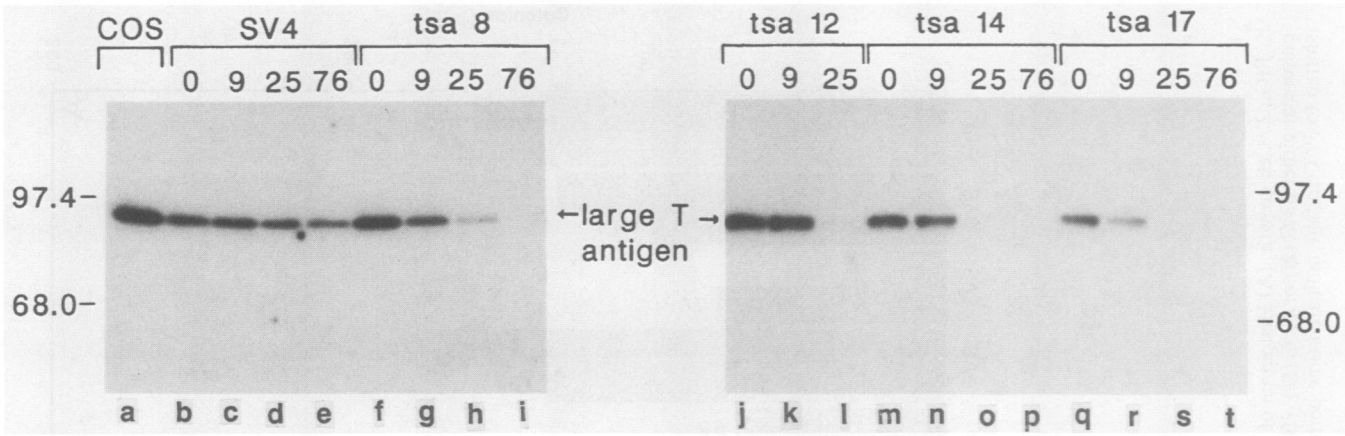

FIG. 3. Effect of shift to 39°C on the level of SV40 large T antigen. Extracts were prepared from the cell lines shown after the shift to 39°C for 0, 9, 25, or 76 h and fractionated on a 10% polyacrylamide gel. Proteins were transferred to 0.45-μm-pore-size nitrocellulose filters by electroblotting. The COS cell extract was prepared from freshly subpassaged COS M6 cells that had been grown at 33°C for 18 to 24 h. The level of large T antigen was monitored by using pAb419, a monoclonal antibody specific for the SV40 large T antigen. Numbers on the left and right indicate the relative positions of prestained molecular weight size markers (in kilodaltons).

longer divided at the nonpermissive temperature, they still synthesized proteins at levels comparable to those found at the permissive temperature (data not shown). Moreover, when extracts prepared from cells grown at different temperatures were analyzed on a one-dimensional gradient sodium dodecyl sulfate-polyacrylamide gel, no significant differences in the synthesized proteins were detected (data not shown). Although it is possible that a more sensitive two-dimensional gel analysis would resolve proteins that are differentially expressed, these experiments clearly demonstrated that the protein-synthesizing capacity of the tsa cells was not significantly altered at the nonpermissive temperature.

Cytofluorimetric analysis of cell lines. The stages of the cell cycle in which cells accumulated upon shift to 39°C were investigated by PI cytofluorimetry. Since cells in the M and G_2 phases contain twice as much DNA as do those in G_1 and cells in the S phase contain intermediate levels of DNA, it is possible to use PI fluorescence coupled with computer analysis to determine the percentage of cells in each phase of the cell cycle. Representative results for one such experiment with cell lines tsa8, tsa14, tsa17, and SV4 are presented in Table 1 and Fig. 4.

The control cell line SV4, which proliferated at both temperatures, had a normal cell cycle profile at both temperatures (compare Fig. 4C with Fig. 4A and B). The population had a high percentage of cells in the S phase, but this percentage decreased with time at both temperatures as the density of cells increased and they became contact inhibited. The initial high proportion of cells in the S phase was expected, since the cells had been passaged in fresh medium 12 h earlier. By contrast, the tsa cell lines yielded markedly different results. Cell cycle analysis at 33°C produced results analogous to those obtained with the SV4 line. However, upon transfer of these cells to 39°C, initiation of new rounds of DNA synthesis was markedly diminished within 25 h and was insignificant by 75 h, as manifested in the depletion of cells in the fluorimetric distributions between the G_1 and G_2/M populations (compare Fig. 4E and F). Moreover, the percentage of cells in the G_2/M phases increased markedly after transfer to 39°C; the levels attained were 18.5, 20.8, and 36.2% for tsa8, -14, and -17, respectively (Table 1). In another experiment, incubation of the tsa8 cell line at 39°C resulted in a population in which 35% of the cells were in the G_2/M phases. Since the level of large T antigen significantly diminished within 9 h after transfer to 39°C, the tsa8 cell line was analyzed at a period of less than 24 h. Entry of cells into the S phase did not significantly decrease after 9.5 h at 39°C, but an additional 14.5 h of incubation at the high temperature clearly decreased the number of cells entering the S phase and increased the number of cells in the G_2/M phases (data not shown).

Unfortunately, PI cytofluorimetry does not differentiate between cells in the G_2 and M phases. This ambiguity was overcome by immunofluorescence analysis, using a mouse monoclonal antibody, RT97, that was isolated for recognition of neurofilaments (50). This antibody also cross-reacts with phosphorylated histones and thus stains the nuclei of all cells in the M phase. Indirect immunofluorescence analysis of the tsa8 cell line by using RT97 revealed that the number of cells positive for staining actually decreased with time of incubation at 39°C (data not shown). This finding indicated that growth arrest did not occur in the M phase. These

TABLE 1. Cell cycle analysis[a]

Cell line	Incubation time (h)	% of cells in given phase after growth at:					
		33°C			39°C		
		G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
tsa8	0	61.3	36.2	2.5			
	25	75.1	18.9	6.0	63.4	9.1	27.4
	75	76.5	10.4	13.2	81.5	0	18.5
tsa14	0	60.6	32.3	7.1			
	25	55.5	28.2	16.3	61.7	16.0	22.3
	75	63.0	15.8	21.2	76.3	3.0	20.8
tsa17	0	48.6	49.8	1.6			
	25	59.4	38.4	2.2	53.9	12.2	33.9
	75	64.3	20.1	15.6	59.0	4.8	36.2
SV4	0	53.1	45.7	1.2			
	25	65.6	31.3	3.1	61.9	29.8	8.3
	75	69.7	22.5	7.9	65.7	29.3	5.0

[a] After the indicated periods of growth, cells were harvested, fixed, and stained with PI. The stained cells were analyzed on a cytofluorograph, using a doublet discrimination protocol to eliminate clumps of two or more cells. Values were determined by computer analysis.

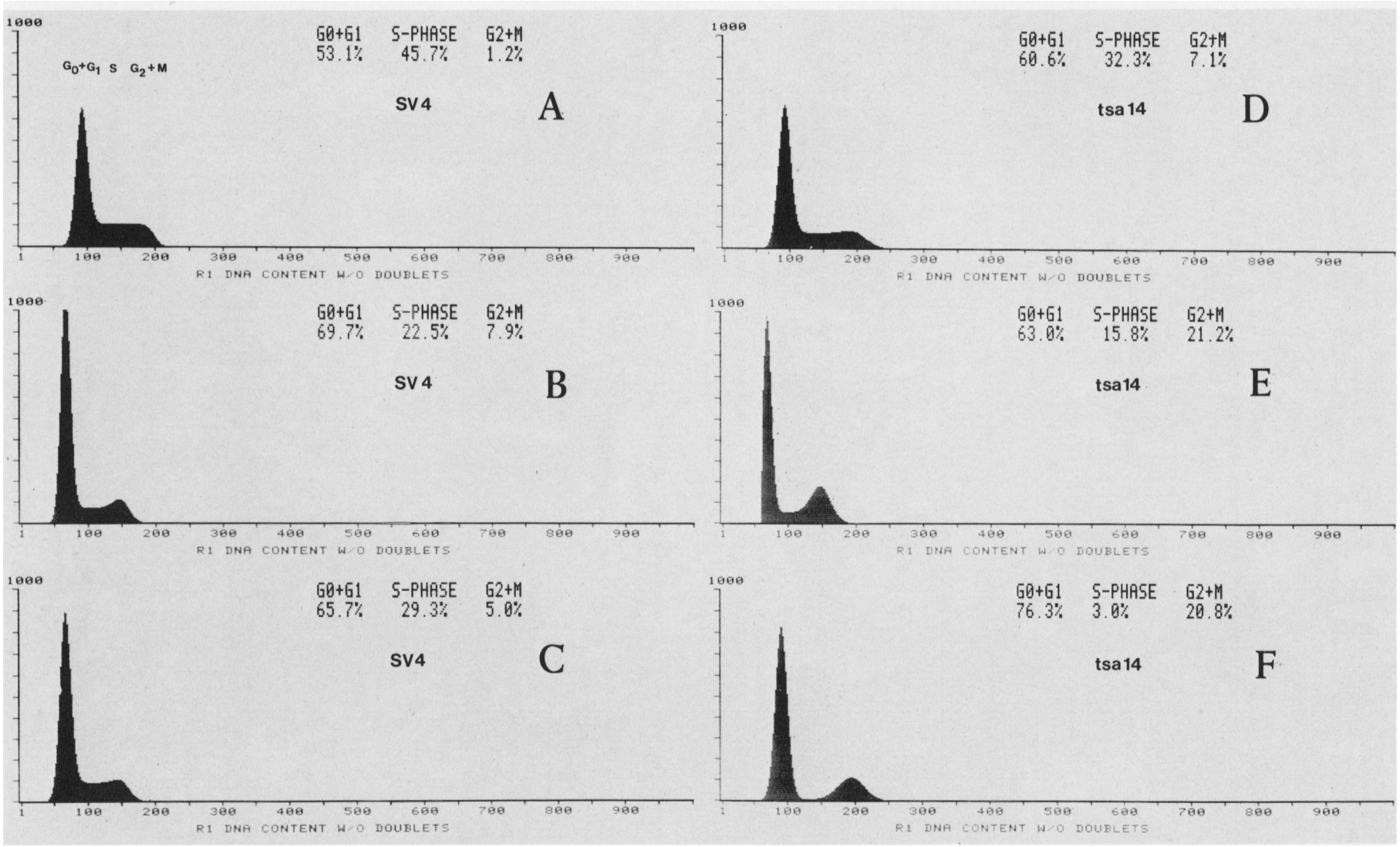

FIG. 4. Cell cycle analysis of data from which the results in Table 1 were derived. Three datum points are shown: zero time (panels A and D) and analysis after incubation for 75 h at 33°C (panels B and E) or 39°C (panels C and F). A doublet discrimination protocol was used to discard clumps of two or more cells. Data show cell number plotted versus DNA content (R1 DNA content without doublets).

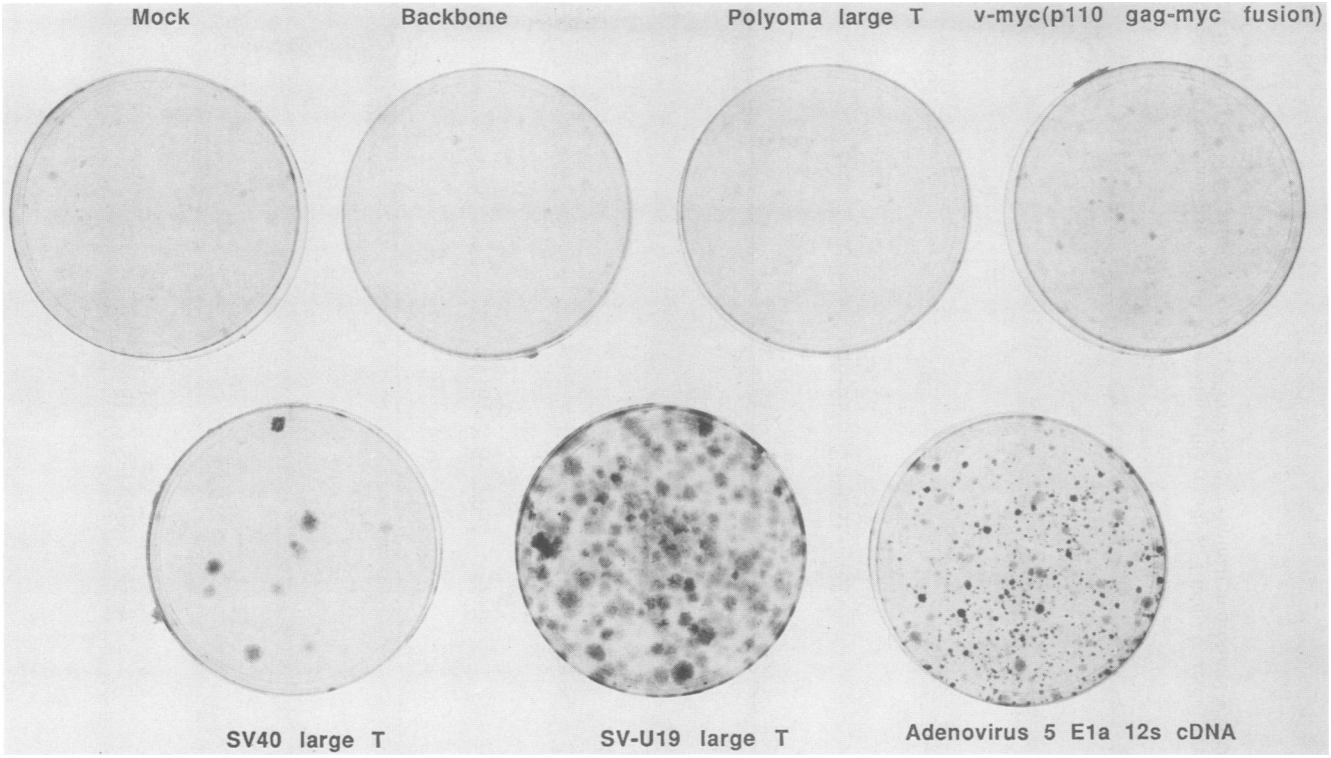

FIG. 5. Rescue of the temperature-sensitive growth defect by superinfection with retroviruses that transduce other oncogenes. Representative dishes obtained after growth at the nonpermissive temperature are shown. See Materials and Methods and the footnote to Table 2 for details.

results have also been verified by electron microscope analysis (L. M. Franks and P. S. Jat, unpublished results). Taken together, these analyses demonstrated that upon shift to the nonpermissive temperature, the tsa cells did not initiate new rounds of DNA synthesis and accumulate in either the G_1 or G_2 phase.

Suppression of the growth defect by introduction of other known establishment genes. Many oncogenes encoding nuclear proteins have the ability to establish primary cells in culture. To determine whether one or more of these establishment genes were capable of suppressing the thermolabile growth defect in the tsa cell lines, experiments were initially performed by superinfection with recombinant retroviruses that transduce these immortalizing genes and later by introduction of appropriate constructs encoding these genes by cotransfection with a plasmid that encodes resistance to the antibiotic hygromycin B. In the transfection experiments, the cultures were initially selected for resistance to hygromycin B as described in Materials and Methods. All four of the tsa cell lines were extensively analyzed. Since tsa8 and tsa12 yielded results that were mutually identical but different from those obtained with tsa14 and -17, only the results for tsa14 and -8 are presented.

The thermolabile growth defect of the tsa14 cell line was analyzed by superinfection with recombinant retroviruses, each of which has previously been shown to be capable of immortalizing REFs (7, 21). This defect was readily overcome by superinfection with retroviruses that transduce the large T antigen derived from both the SV40 wild type or the SV40 mutant U19 (Fig. 5, Table 2). It was also complemented by the adenovirus type 5 E1a 12S gene product. In contrast, both v-*myc* and polyomavirus large T antigens were unable to complement. Rather surprisingly, v-*myc* appeared to induce focus formation at the permissive temperature, albeit at a frequency that was very low in comparison with the actual virus titer. A few of these foci were isolated and expanded into cell lines; all exhibited a similar transformed morphology that was clearly different from that of the parental cells. Moreover, when cells derived from one

TABLE 2. Complementation of the growth defect by superinfection[a]

Virus	Protein transduced	No. of tsa14 foci	No. of NIH 3T3 G418^r colonies (10^4)
Mock		9	
ZipNeoSV(X)1	Backbone only	4	7.0
SV40-6	SV40 large T antigen	50	0.5
SV40 mutant 19-5	SV40 mutant U19 large T antigen	800	14.0
ZipE1a12S	Adenovirus type 5 E1a 12S cDNA	1,800	25.0
LT-4	Polyomavirus large T antigen	6	5.5
v-*myc*	p110$^{gag\text{-}myc}$ fusion	10	14.8

[a] Cultures of tsa14 cells (5×10^5) that had been split the day before were infected with each virus at 33°C, passaged the next day, and maintained at 33°C until the following day, when they were shifted to the nonpermissive temperature for 14 days. Simultaneously, a smaller volume of each virus stock was used to infect NIH 3T3 cells to determine the virus titers as G418-resistant colonies. Construction and isolation of the recombinant retroviruses have been described [ZipNeoSV(X)1, 4; v-*myc*, 7; SV40 mutant U19-5, 21; SV40-6 and LT-4, 20; ZipE1a12S, 41]. The mutant large-T-antigen gene in SV40 mutant U19-5 encodes a protein defective for sequence-specific binding but is fully active in immortalization (21).

of these foci were shifted to the nonpermissive temperature, they showed rapidly arrested growth; PI cytofluorimetry has shown that the growth arrest still occurred in either G_1 or G_2/M. Further preliminary experiments have shown that levels of polyomavirus large T antigen higher than those obtained with the LT-4 viral construct may be able to complement the growth defect (Z. Ikram and P. S. Jat, unpublished data).

The results obtained with tsa8 are very perplexing. This cell line cannot be rescued by superinfection with the wild-type SV40 T-antigen-only retrovirus but is rescued by the SV40 mutant U19 large T antigen. Similarly, transfection of tsa8 with pZipSV40, a plasmid in which expression of the SV40 early region is regulated by Moloney murine leukemia virus long-terminal-repeat sequences, did not rescue the growth defect. However, transfection with pSE, a plasmid in which expression of the SV40 early region is controlled by SV40-regulatory sequences, readily complemented the growth defect. In contrast to tsa8 and -12, tsa14 and -17 were rescued by both pZipSV40 and pSE but more efficiently by the former. Since tsa8 and -12 contained a higher level of T antigen than did the tsa14 and -17 (Fig. 3), it is possible that they required different levels of T antigen for efficient rescue, which may explain the differences observed with the two plasmids. In addition, none of the other nuclear establishment genes, v-*myc*, adenovirus E1a proteins, and polyomavirus large T antigen, were able to complement growth of tsa8 and -12 (data not shown). Colonies were occasionally obtained at 39°C, but they were rather flat and unhealthy in appearance and did not readily expand into cell lines upon isolation.

Since it was possible that the direct-rescue procedure was too harsh, the effect of the different immortalization genes was also assayed by isolating colonies and expanding them into cell lines at the permissive temperature. The ability of these cell lines to rescue the growth defect was then monitored by comparing the growth potential of these clonal cell lines at 33 and 39°C. The results (Table 3) were the same as those obtained from the direct transfection. Expression of the SV40 early region from its normal promoter complemented much better than when expression was regulated by a long terminal repeat (compare growth of SV40 lines A through C and D through F in Table 3). Analysis of the polyomavirus large-T-antigen lines A and B and the adenovirus E1 region line B showed that even though the loss of growth was not fully overcome, there was a slight alleviation of the defect. In addition, constitutive expression of the human *myc* protein did not overcome the growth defect. In contrast to the results presented above for the establishment-type oncogenes, transfection of the EJ c-Ha-*ras*1 gene gave rise to rapidly growing, highly refractile, and morphologically transformed colonies at both 33 and 39°C. However, if the colonies obtained at 39°C were isolated and expanded, they grew for only a few passages and then detached from the substratum. Similarly, colonies initially isolated at 33°C grew for a while after the shift to 39°C and then detached. It is not clear whether these findings were due to a partial complementation of the growth defect by the activated EJ c-Ha-*ras*1 gene or, more likely, to simply a nonspecific reduction in temperature sensitivity as a result oncogenic transformation. This partial rescue could not be augmented by cointroduction of the human *myc* gene. Introduction of a plasmid capable of simultaneous expression of the human *myc* and EJ c-Ha-*ras*1 proteins gave results identical to those obtained for EJ c-Ha-*ras*1 alone (Table 3).

TABLE 3. Complementation of the growth defect after cotransfection[a]

DNA used for isolation	Protein transduced	Cell line	No. of densely staining colonies at:	
			33°C	39°C
Control (pY3)		A	328	15
		B	264	0
		C	237	1
pSE	SV40 large and small T antigens	A	221	90
		B	265	189
		C	290	187
pZipSV40	SV40 large and small T antigens	D	220	29
		E	246	36
		F	178	5
pZipPyLT	Polyomavirus large T antigen	A	303	59
		B	317	63
		C	190	2
pHindIIIG	Adenovirus type 5 E1a and E1b19k	A	135	2
		B	344	62
		C	245	3
pSV-human *myc* Manca	Human *myc*	A	200	0
		B	230	14
		C	155	17
pH06T1	EJ c-Ha-*ras*1	A	181	105
		B	167	87
		C	158	91
p-human *myc*/T24-24 (pHmrn-24)	Human *myc* and EJ c-Ha-*ras*1	A	106	37
		B	81	20
		C	110	17

[a] Plasmids were transfected into the tsa8 cell, using hygromycin B as a coselectable marker, as described in Materials and Methods. Construction and characterization of the plasmids used in this experiment have been described previously (pY3, 2; pZipSV40 and pZipPyLT, 20; pHindIII G, 25; pSV-human *myc* Manca and pHmrn-24, 26; H06T1, 45). These plasmids are fully functional and capable of expressing the appropriate protein.

DISCUSSION

We have isolated a number of clonal cell lines derived from REFs by infecting tertiary REFs with a recombinant retrovirus which simultaneously transduces the thermolabile large T antigen encoded by the SV40 mutant *tsA*58 (47) as well as resistance to the antibiotic G418. These cell lines were established for continuous proliferation in culture at 33°C but rapidly stopped dividing upon shift to 39°C in either the G_1 or G_2 phase of the cell cycle. These results demonstrate unequivocally that the SV40 large T antigen is required for both initiation and maintenance of the immortal phenotype.

The groups of Cuzin and Feunteun have previously shown that introduction of thermolabile large-T-antigen mutants of polyomavirus and SV40 into primary cells can produce cell lines that are restricted in growth at the nonpermissive temperature (36, 39). The study described here greatly extends these findings. Fourteen cell lines were isolated after infection of REFs with the retrovirus recombinant. Each line was initially selected on the basis of resistance to G418 and had the properties of an established cell line of continuous passage without crisis. Therefore, the properties of these cell lines should reflect the typical range of effects of the SV40 large T antigen on cell division. Essentially, all cell lines infected with the *tsA*58 large-T-antigen gene were highly thermolabile for growth. Many of the cell lines stopped dividing within 24 h of transfer to 39°C. After growth arrest, the cells remained metabolically active, as assayed by general protein synthesis, for more than 6 days. However, many

of the cells quickly lost the ability to commence growth upon transfer to 33°C. For example, if the tsa8 cell line, which failed to divide after 24 h at 39°C, was shifted to 33°C after this 24-h period, a large proportion of the cells efficiently resumed proliferation. However, incubation of the cells for 48 or 72 h at 39°C destroyed the ability to commence growth at 33°C. Upon transfer to 39°C, growth arrest occurred in either of two phases of the cell cycle, G_1 or G_2, which suggests that the cells depend on large-T-antigen activity to proceed through these decision points in the cycle. This dependence could be direct (i.e., the large T antigen is an immediate regulator of a process) or indirect (i.e., the large T antigen stimulates the synthesis of one or more activities that regulate these transitions).

It is difficult to determine whether the tsa cell lines recapitulate senescence upon transfer to 39°C. Little is known about the processes that occur during senescence. Cells in a senescent culture may be undergoing differentiation to a nonproliferative cell type or may be responding to an internal biological clock-type process that restricts their life span. Some of the tsa cell lines described above may be undergoing normal differentiation upon transfer to 39°C. If so, we have been unable to identify the final differentiated cell type. In parallel experiments, we have found that loss of renewal capacity is coupled to differentiation for cells derived from the central nervous system. By using identical retrovirus recombinants and procedures, a bipotential neuroepithelial precursor cell was established in continuous culture. Upon transfer to 39°C, these cells lose a marker characteristic of rapidly dividing cells and display markers characteristic of either astrocytes or neurones (9; R. McKay, K. Frederikson, P. S. Jat, and D. Levy, Prog. Brain Res., in press). Together, these results suggest that growth arrest of some of the tsa cell lines may be coupled to differentiation.

It is possible that the tsa cell lines that rapidly arrest upon transfer to 39°C are not undergoing normal differentiation. One potential indication of this is their accumulation upon transfer to the nonpermissive conditions in both the G_1 and G_2 phases of the cell cycle. However, arrest of cells at the G_2/M transition is not a unique feature of viral transformation, since a fraction of normal cells during senescence also arrest in the G_2 phase (Riley et al., submitted). In cell lines established by the activity of large T antigen, the normal processes regulating the synthesis of components essential for transitions between stages of the cell cycle must be partially supplemented by the oncogene. Rapid removal of oncogene activity should then leave the cell paralyzed at the point in the cycle where the component is essential. The fact that the tsa cells arrest in at least two points in the cycle suggests that the large T antigen may supply multiple signals.

Results of the oncogene complementation experiments are particularly interesting. Most of the tsa cell lines were not readily rescuable by other oncogenes that have previously been shown both to establish primary cells in culture and to transform these cells in conjunction with an activated *ras* gene. For example, the v-*myc* gene could readily immortalize the primary REFs from which the tsa cell lines were produced. However, this oncogene was totally defective in complementing the temperature-sensitive growth restrictions in the tsa cell lines. There are many interpretations of the failure of other oncogenes to complement, but only two will be discussed for illustrative purposes. First, it is possible that different oncogenes establish or immortalize cells by distinct mechanisms and that once a state is established, the cell can no longer respond to other types of oncogenes. This may be the correct interpretation for the failure of some oncogenes, such as v-*myc*, to complement but certainly is not an adequate explanation for the lack of complementation in some tsa cell lines by genes encoding wild-type SV40 large T antigen. The second possibility probably explains this latter case. It is likely that the cellular state developed in the tsa cell line is uniquely adapted both qualitatively and quantitatively to the range of activities of the synthesized large T antigen. According to this scenario, withdrawal of these activities by temperature shift produces a number of different deranged states, some or all of which cannot be efficiently complemented by a homologous oncogene with a different level of activities. By extrapolation from the latter situation, this interpretation suggests that tumor cells in general may be uniquely adapted to the activities of their activated oncogenes and that even slight modulations in the levels of expression of these oncogenes may specifically kill the tumor cells.

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