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Rat Embryo Fibroblasts Immortalized with Simian Virus 40 Large T Antigen Undergo Senescence upon Its Inactivation

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Introduction of simian virus 40 T antigen into rodent fibroblasts gives rise to cells that can proliferate indefinitely but are dependent upon it for maintenance of their growth once the normal mitotic life span has elapsed. Inactivation of T antigen in these immortalized cells causes rapid and irreversible cessation of growth. To determine whether this growth arrest is associated with entry into senescence, we have undertaken a genetic and biological analysis of conditionally immortal (tsa) cell lines derived by immortalizing rat embryo fibroblasts with the thermolabile *tsA58* T antigen. This analysis has identified the following parallels between the tsa cells after inactivation of T antigen and senescent rat embryo fibroblasts: (i) growth arrest is irreversible; (ii) it occurs in G_1 as well as G_2; (iii) the G_1 block can be partially overcome by stimulation with 20% fetal calf serum, but the G_2 block cannot be overcome; (iv) 20% fetal calf serum induces c-*fos*, but c-*myc* is unaltered; and (v) fibronectin and $p21^{Waf1/Cip1/Sdi1}$ are upregulated upon growth arrest. These results suggest that T-antigen-immortalized fibroblasts are committed to undergo senescence but are prevented from undergoing this process by T antigen. Inactivation of T antigen removes this block and results in senescence of the cells. Thus, these cell lines may represent a powerful system for study of the molecular basis of entry into senescence.

Normal mammalian fibroblasts cultured in vitro undergo a finite number of divisions before undergoing a stable, irreversible arrest of cellular proliferation known as replicative or cellular senescence (21, 57). Cells from the same animal species undergo a relatively constant and highly reproducible number of population doublings for a given cell type, and it has been suggested that cellular senescence is a manifestation of ageing at the cellular level (reviewed in references 15 and 30). In addition, there is substantial evidence that senescent cells accumulate with age in vivo, where they might contribute to the ageing process and may represent a mechanism that protects against tumor formation (11). Senescent cells do not undergo apoptosis but remain viable and metabolically active for long periods of time (3). They can even respond to mitogens, as assayed by the induction of some immediate-early response genes, even though they cannot be induced to divide (54; reviewed in references 16 and 44). It is this property that distinguishes senescent cells from cells in quiescence, another state of growth arrest from which cells can be rapidly induced to reenter the cell cycle upon stimulation (38, 58).

Senescence is a dominant phenotype (4, 41, 42, 60; reviewed in reference 43). It has been shown that fusion of normal human diploid fibroblasts (HDF) with various immortal cell lines, including HeLa and simian virus 40 (SV40)-transformed cells, yields hybrids with a finite proliferative potential. This dominance is thought to be mediated via one or more proteins, because treatment of HDF with inhibitors of protein synthesis prior to fusion prevents senescence in the resulting hybrids. Moreover, microinjection of mRNA prepared from senescent HDF into proliferating cells inhibits DNA synthesis (28), indicating that senescent cells express genes able to inhibit DNA synthesis and progression through the cell cycle.

The genes that limit the replicative life span and those that induce growth arrest in senescent cells are not known, but a number of genetic differences between proliferating and senescent cells have been observed (14, 27, 29, 35, 44, 45, 49). In senescent cells, several late-G_1 genes, such as those for cyclins A, E, and D and cdc2 and cdk2 (12, 52), as well as genes encoding enzymes involved in DNA synthesis, such as dihydrofolate reductase and thymidine kinase (44, 45), are downregulated. In contrast, some growth inhibitors are overexpressed or activated. $p21^{Waf1/Cip1/Sdi1}$, a cdk inhibitor (13, 20), is overexpressed in senescent cells (33) and thereby prevents the phosphorylation of pRB, a tumor suppressor protein (51, 63). Thus, pRB is underphosphorylated and remains a constitutive growth suppressor (51). Prohibitin, a protein that inhibits DNA synthesis (35), as well as extracellular matrix proteins such as fibronectin and collagenase (32), is also highly expressed in senescent cells.

The progressive loss of telomeric DNA and other essential sequences from the ends of chromosomes has been proposed to contribute to senescence (19; for a review, see reference 10). HDF in culture lose about 50 bp of their telomeric DNA per population doubling. *Mus musculus* telomeres are about 10 times longer than human telomeres but do not show a higher rate of telomere shortening per population doubling (reviewed in reference 10). Therefore, it remains to be directly demonstrated that the finite life span is measured by the progressive shortening of the telomeres. When cells are passaged in vitro, they may also accumulate other forms of cellular damage, such as mutations, karyotypic changes, and/or loss of DNA methylation, which might lead to changes in the expression of both positive and negative regulators of cell growth (reviewed in references 6, 7, and 15).

Even though senescence has been extensively studied, the molecular basis for the entry into this state has not been elu-

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cidated. This has been largely due to the lack of suitable systems of study because the loss of proliferative potential in a heterogeneous culture of primary cells occurs asynchronously. Therefore, an important step in the investigation of this process would be to isolate clonal cell lines that could be induced to undergo senescence, a strategy analogous to the use of conditionally lethal mutants in the characterization of many complex processes in prokaryotes and yeasts. We have used the thermolabile large T antigen encoded by SV40 early region mutant *tsA58* (56) to isolate cell lines derived from rat embryo fibroblasts (REF; 23). These tsa cell lines grow continuously at the permissive temperature (33°C), but their growth rapidly arrests upon a shift up to 39.5°C, where the *tsA58* T antigen is rapidly denatured and inactivated. This cessation of growth is not associated with significant cell death, and the cells remain metabolically active as assayed by general protein synthesis and trypan blue exclusion. Growth arrest occurs rapidly within one to two generations and can occur in either the G_1 or the G_2 phase of the cell cycle. If such cultures are shifted to the permissive temperature within 24 h, the growth arrest is reversible, whereas if the cultures are shifted down after approximately 72 h, they cannot be induced to divide (23). Moreover, we have found that in mouse embryo fibroblasts, T antigen is required to maintain proliferation only when the normal mitotic life span has elapsed and the mechanism which measures the life span continues to operate in its presence (22). These results suggest that the regulation of the finite proliferative potential of mammalian fibroblasts may involve two processes: (i) a biological clock that measures the number of divisions that a cell undergoes and (ii) entry into the postmitotic state of senescence. The tsa cell lines may thus represent a model for studying the transition to the postmitotic state. However, since T antigen possesses a number of potent biological activities, prolonged exposure of cells to this molecule may profoundly alter their physiological state. Therefore, we have undertaken and present here a systematic analysis of the genetic and biological characteristics of representative tsa cell lines in comparison with those of normal REF.

MATERIALS AND METHODS

Cells and cell culture. tsa8, tsa14, tsa129, and SV4 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum (FCS), 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. REF were prepared from 12-day Sprague-Dawley rat embryos and maintained in the same medium. All media and components were obtained from Gibco BRL.

Analysis of cell cycle. Cell cycle analysis by propidium iodide (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 Becton Dickinson, the manufacturer of the cytofluorograph used for the analysis.

Estimation of fraction of cells in S phase. To determine the DNA synthesis labelling index for each cell line at both the permissive and nonpermissive temperatures, cells were seeded at a range of densities of 1.6×10^4 cells per 13-mm-diameter coverslip with doubling dilutions to 2×10^3 to accommodate the different growth rates at the different temperatures. This allowed coverslips with equivalent cell densities to be selected for pulse-labelling with bromodeoxyuridine (BrdU). Four replicate coverslips were used for each growth condition tested. All cells were initially seeded at 33°C and left to attach overnight before transfer to 39.5°C (this corresponds to 0 h). Cells were fed again at 48 h to ensure that they did not become quiescent. After the cells had been grown under permissive or nonpermissive conditions for 72 h, the medium was replaced with medium that contained either 10 or 20% FCS. At 84 h, 12 h after serum stimulation, coverslips with equivalent cell densities of each of the cultures were incubated for 2 h in complete growth medium containing 25 mM BrdU and 2 mM fluorodeoxyuridine to inhibit thymidylate synthetase. The cells were washed twice in phosphate-buffered saline (PBS) and fixed with ice-cold 2% acetic acid–98% ethanol at room temperature for 30 min. This was followed by three washes in PBS–0.1% Tween. Nonspecific binding was blocked with 3% bovine serum albumin (BSA) in PBS–0.1% Tween for 15 min at room temperature before incubation for 60 min with 20 μl of a solution containing a mouse monoclonal antibody against BrdU and a nuclease to cleave cellular DNA (Amersham). Cells were then washed three times in 3% BSA–PBS and incubated with 50 μl of a fluorescein-conjugated rabbit anti-mouse immunoglobulin G2a antibody diluted 1:100 in BSA-PBS (Southern Biotech Associates, Inc.) for 40 min at room temperature. The cells were then washed three times in PBS–0.1% Tween, rinsed in PBS, mounted on glass slides, and sealed with nail varnish. Stained cells were viewed with a 40× objective using epifluorescence optics. A total of 1,000 cells were counted in randomly selected fields from each coverslip to determine the proportion of cells that had incorporated BrdU.

Cell growth analysis. To determine whether stimulation with medium containing 20% FCS would cause an increase in cell number, cells were plated and left overnight at 33°C to adhere. Representative dishes from each set of cultures were counted to determine the cell number. Half of the dishes from each culture were then transferred to 39.5°C. Three days later, the cells in two dishes from each culture were counted. Half of the dishes were then transferred to medium containing 20% FCS and maintained there for the duration of the experiment; the remainder were maintained in normal growth medium. Cells were fed with medium containing the appropriate amount of FCS every 2 to 3 days, and at the times indicated in Results, two dishes for each culture from each experimental condition were harvested and the cell number was determined.

RNA isolation and Northern (RNA) blots. Total RNA was isolated by the guanidinium thiocyanate method (47) and purified by oligo(dT)-cellulose chromatography. Either 2 μg of mRNA or 10 μg of total RNA for each sample was electrophoresed on a 1% agarose formaldehyde gel and transferred to GeneScreen Plus (Du Pont). Probes were prepared by random priming of purified DNA fragments. Hybridization and subsequent processing of the filters were done by standard procedures (47).

Preparation of RNA probes. The following templates were used to generate RNA probes: pSP6-*fos*-5′ linearized with *Bss*HII, which yields two protected transcripts of 100 and 135 nucleotides which correspond to the major c-*fos* transcript in rat cells (59); pBS-R-*myc*-exon1 linearized with *Dde*I, which yields a protected fragment of 350 nucleotides from the rat c-*myc* transcript (40); and pBS-R-*GAPDH* linearized by *Dde*I, which protects bands of 72 and 70 nucleotides corresponding to rat *GAPDH* transcripts (40). RNA probes were synthesized with T3 RNA polymerase (for the c-*myc* and *GAPDH* probes) or SP6 RNA polymerase (for the c-*fos* probe) by using an RNA transcription kit (Stratagene) and 100 μCi of [α-^{32}P]GTP at 400 Ci/mmol per reaction. Probes were purified by fractionation on 8 M urea–6% polyacrylamide gels, elution in 0.5 M ammonium acetate–1 mM EDTA–0.1% sodium dodecyl sulfate at 37°C overnight, and ethanol precipitation.

RNase protection assays. A 10-μg sample of total RNA was hybridized overnight at 52°C with the appropriate RNA probe(s) (100,000 cpm per reaction for the c-*myc* probe, 200,000 cpm per reaction for the c-*fos* probe, and 10,000 cpm per reaction for the *GAPDH* probe) in 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–400 mM sodium acetate (pH 6.4)–1 mM EDTA. Samples were then treated with 0.5 U of RNase A and 100 U of RNase T_1 (Ambion) for 30 min at 30°C. The reactions were terminated by digestion with proteinase K (1 mg/ml) for 15 min at 37°C, and products were extracted with phenol-chloroform and ethanol precipitated. The protected fragments were fractionated on a 8 M urea–6% polyacrylamide gel.

RESULTS

Cell cycle profile of senescent REF. Since the growth of conditionally immortal tsa cell lines arrests in either G_1 or G_2 (23), a situation distinct from quiescence, in which cells arrest in G_0 (38, 58), we determined the phase of the cell cycle in which REF undergo growth arrest upon serial cultivation. Sprague-Dawley REF cultures were passaged at either low (Fig. 1A) or high (Fig. 1B) density and analyzed by PI cytofluorimetry. Although the low-density cultures proliferated less than the high-density cultures, both ceased dividing by passage 8, after which cell numbers no longer increased (Fig. 1A and B). The decline in growth rate was coupled to an increase in the proportion of cells with G_2/M DNA content and a decrease in the S phase and G_0/G_1 population (Fig. 1C and D). After passage 8, continued passaging of the cultures resulted in a small decrease in cell number, which was likely caused by losses incurred during passaging; senescent cells adhere very strongly to the tissue culture dishes and are very hard to remove by trypsinization. This continued passaging was associated with an increase in the proportion of cells with G_2/M DNA content and a decrease in the G_0/G_1 population and resulted in cultures in which over 35% of the cells had G_2/M DNA content (Fig. 1C and D).

The proportion of cells in the S phase declined rapidly within the first four to five passages and thereafter remained

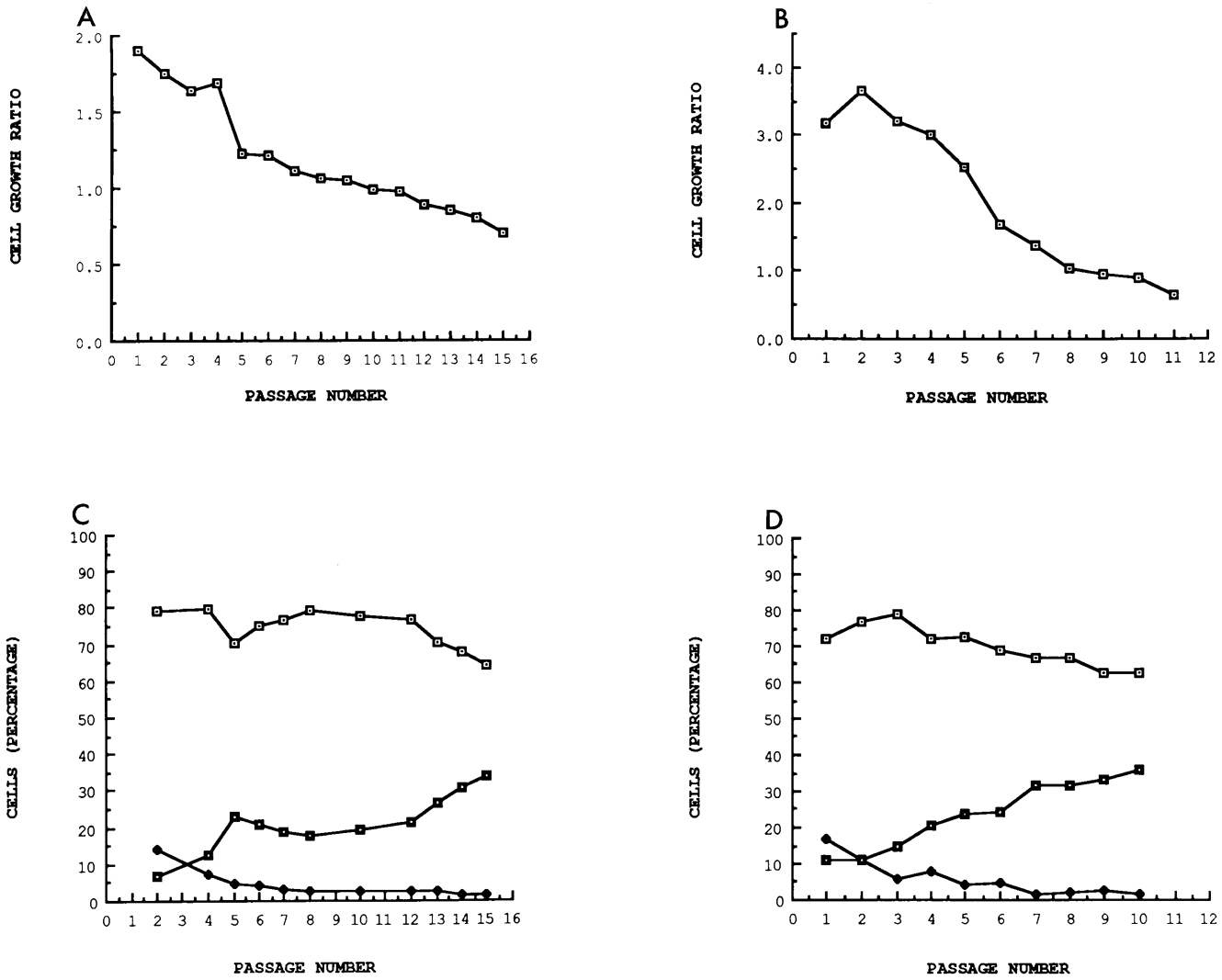

FIG. 1. Serial cultivation of REF passaged at different inoculation densities. REF were subcultured at a low density of 1×10^4 cells per cm^2 (A) and a high density of 1.9×10^4 cells per cm^2 (B) every 3 days until they became senescent. The cell growth ratio corresponds to the increase in cell number on day 3 relative to the number 12 h after initial plating, when the cells had attached. The proportions of cells in various stages of the cell cycle were determined for each culture at every passage by PI cytofluorimetry. For panels C and D, which correspond to the cultures in panels A and B, the percentages of cells in various stages of the cell cycle, as determined by cytofluorimetry, are plotted at each passage. Symbols: ⊡, cells in G_0/G_1; ◆, cells in S; ■, cells in G_2/M.

relatively constant at 1 to 2%, despite changes in the proportion of cells with G_0/G_1 and G_2/M DNA contents. The proportion of cells in the S phase was determined more accurately by BrdU and PI double staining followed by fluorescence-activated cell sorter (FACS) analysis (9, 55). In accordance with the results of PI staining alone, in early-passage REF, 79% of the cells were in G_0/G_1, 14% were in S, and 7% were in G_2/M (Fig. 2A). Interestingly, analysis of passage 8 cultures showed that 79.4% of the cells were in G_0/G_1, 2.6% were in the S phase, and 17.6% were in G_2/M (Fig. 2B). As before, continued passaging of this culture until passage 15 did not alter the S-phase population but the G_0/G_1 population was reduced to 64% and the G_2/M DNA population was increased to 34.1% (Fig. 2C).

To determine whether cells in the G_2/M phase were arrested in G_2 or in the mitotic phase, cultures were examined both microscopically and by indirect immunofluorescence with monoclonal antibody RT97, which is capable of differentiating between mitotic and nonmitotic cells as a consequence of binding to phosphorylated histones (61). This antibody stained mitotic cells among early-passage REF (Fig. 3A and B), but no appreciable staining was detected in the larger senescent fibroblasts (Fig. 3C and D). Further analysis showed that less than 2% of the cells arrested in the G_2/M phase were binucleated, suggesting that almost all of the cells arrested in this phase were in G_2 (data not shown). The binucleate cells that were present appeared to be arrested prior to cytokinesis, which may represent another point of regulation.

Since spontaneous increases in ploidy can be detected in senescent cells, albeit at a low level (4 to 7%; 57), it was necessary to eliminate the possibility that the cells with G_2/M DNA content were not tetraploid cells arrested in G_0/G_1. REF were prepared and passaged by using the same regimen, and the proportion of diploid and tetraploid cells was determined at each passage by counting the number of chromosomes in metaphase spreads. The results in Table 1 show that only a very small proportion of the mitotic cells were tetraploid. As expected, the proportion of cells in metaphase declined as REF

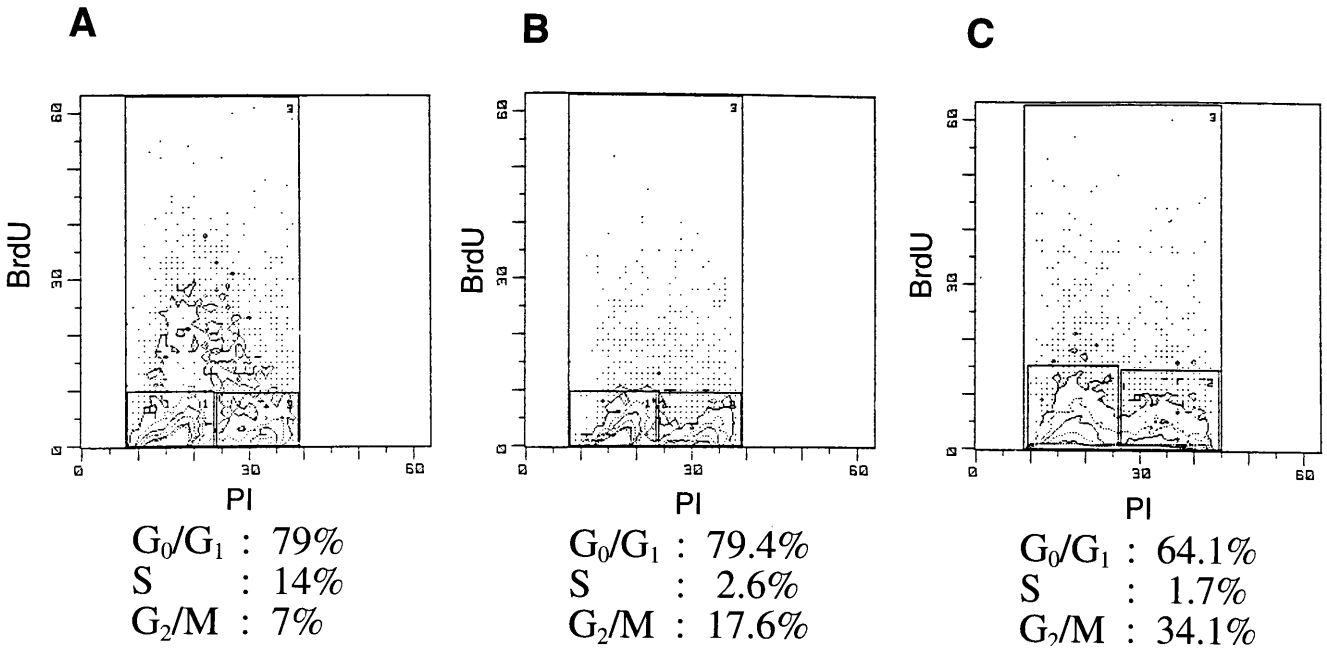

FIG. 2. More accurate determination of the proportion of cells in the S phase. Cells from passages 2, 8, and 15 of the low-density cultures in Fig. 1A were grown in the presence of BrdU prior to harvesting, stained with an anti-BrdU antibody and PI, and analyzed by FACS for PI and BrdU double staining. Cells were gated such that area 1 corresponds to cells in G_0/G_1, area 2 corresponds to cells in G_2/M, and area 3 corresponds to cells in the S phase. Analysis of cells from passages 2, 8, and 15 are shown in A, B, and C, respectively.

were passaged and the cultures slowed down. The proportion of tetraploid cells was very low (0.0 to 0.4%) and not very different between early-, intermediate-, and late-passage REF (Table 1). Thus, if senescent REF cultures do contain tetraploid cells arrested in G_0/G_1, then they constitute only a minor proportion of the G_2/M peak and the majority of the cells correspond to diploid cells arrested in G_2.

Stimulation of DNA synthesis with FCS. Since the property which distinguishes quiescent cells from senescent cells is that quiescent cells can be induced to divide, whereas senescent cells are postmitotic and cannot be induced to divide (38, 58), we determined whether cultures of growth-arrested tsa cells could be induced to enter the S phase or divide. Entry into the S phase was measured by BrdU incorporation. To determine whether stimulation with FCS would cause an increase in cell number, parallel cultures that had been treated identically were continuously maintained in medium with either 10 or 20% FCS after the initial 72 h and the cells were counted. To ensure that the differences observed were not due to the different growth temperatures, cells of the line SV4 were grown under identical conditions and used as a control. This cell line was derived from the same batch of REF as tsa8 and tsa14 cells by immortalization with the wild-type T antigen and thus proliferates at both temperatures (23). The tsa129 cell line was derived by infection of Sprague-Dawley REF with the same recombinant retrovirus that was used to isolate tsa8 and tsa14 cells (21a).

The results obtained are presented in Fig. 4 and Table 2; since the results for induction of DNA synthesis were the same with cultures maintained for 3 and 7 days at the nonpermissive temperature, only data from the 7-day cultures are presented. They show that the SV4 cell line both proliferates and can be induced to enter the S phase at both temperatures. In fact, SV4 cells grew faster at 39.5°C than at 33°C; the proportion of cells that incorporated BrdU after induction with 10 or 20% FCS was also higher at 39.5°C than at 33°C. In contrast, the tsa cell lines all had reduced growth at 39.5°C compared with 33°C, and as previously observed, the reduction in growth was less in tsa14 cells than in tsa8 and tsa129 cells (Fig. 4). However, cells from all three lines can be induced to enter the S phase upon induction at both temperatures; the induction was greater with 20% FCS than with 10% FCS. This was particularly striking for cultures under nonpermissive conditions, in which the proportion of cells in the S phase was increased from 11.4 to 29% for tsa8, from 23.8 to 28.5% for tsa14, and from 1.7 to 6.8% for tsa129. For tsa8 and tsa14 cells, the proportion of growth-arrested cells that entered the S phase with 20% FCS was not much lower than that of growing cells. In contrast, the proportion of growth-arrested tsa129 cells that entered the S phase was much lower (1.7% with 10% FCS and 6.8% with 20% FCS). However, the growth curves in Fig. 4 show that even when tsa cells were continually maintained at the nonpermissive temperature in media containing 20% FCS, there was no significant increase in cell number relative to that of cultures maintained in 10% FCS. Figure 5 shows the morphology and BrdU incorporation of representative cultures of growth-arrested tsa8, tsa14, and tsa129 cells and senescent passage 6 REF compared with those of proliferating SV4 cells. This shows that cells with the characteristic morphology of growth-arrested cells were able to incorporate BrdU and the pattern of staining was consistent with DNA synthesis rather than DNA repair (37). Thus, the S phase can clearly be induced in tsa cells after inactivation of T antigen but these cells cannot be induced to divide.

Even though senescent human fibroblasts have been shown to enter the S phase (17), we wanted to determine how senescent REF would behave under our experimental conditions. Therefore, REF at passage 2, when the cells were still proliferating, and at passage 6, when the cultures had essentially ceased dividing, were assayed for induction of DNA synthesis

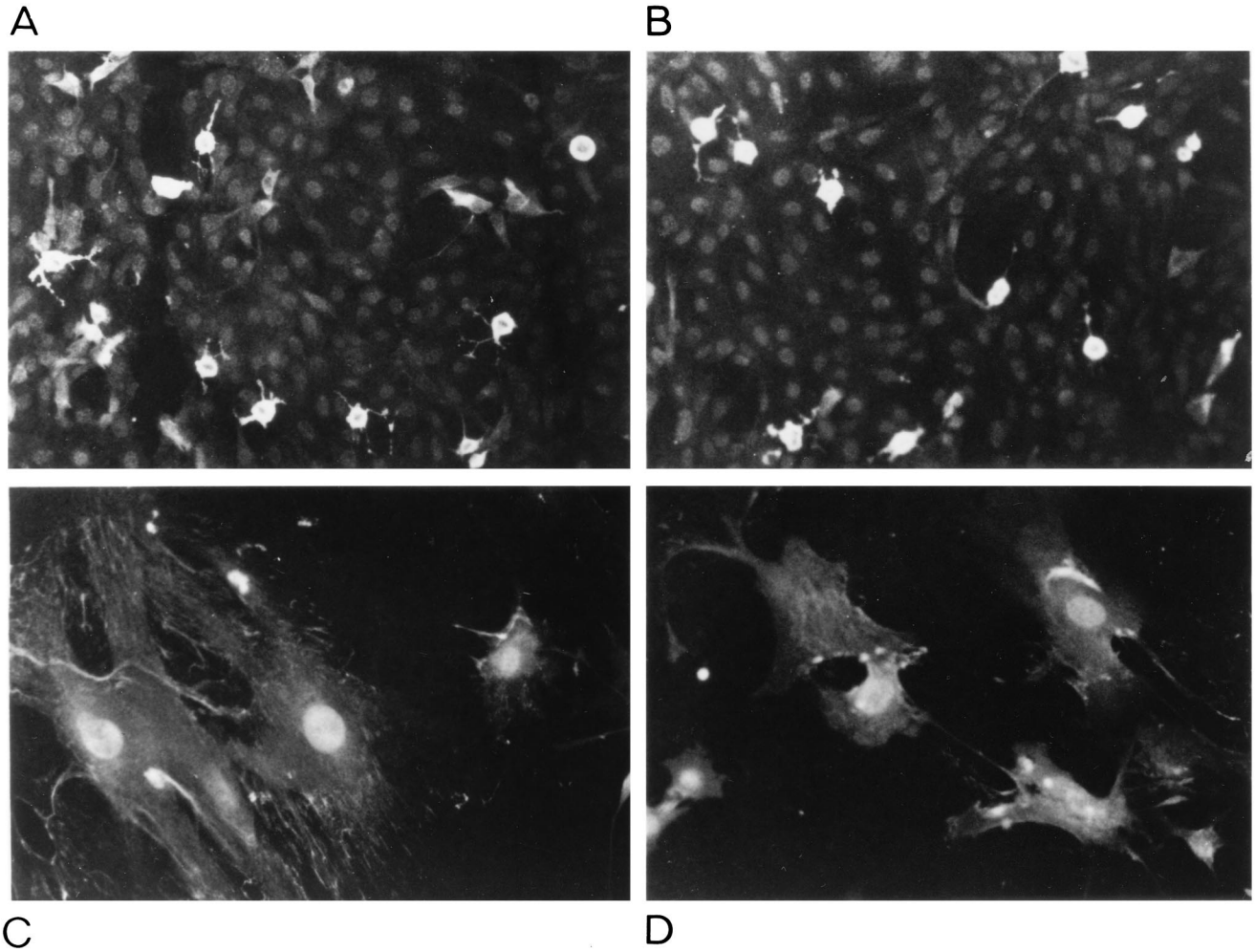

FIG. 3. Indirect immunofluorescence analysis. Representative samples of early-passage and senescent REF from the cultures of Fig. 1A and B were stained with monoclonal antibody RT97 and visualized by using an appropriate conjugated second antibody. A corresponds to early-passage REF from the low-density culture, B corresponds to early-passage REF from the high-density culture, C corresponds to senescent REF from the low-density culture, and D corresponds to senescent REF from the high-density culture.

and an increase in cell number following stimulation with 20% FCS. To permit comparison of the results of the different experiments, tsa8 and tsa129 cells were used as a control and the experiments were carried out at 33, 37, and 39.5°C. The growth curves presented in Fig. 4 show that the proliferation of passage 2 REF was greater at 39.5°C than at 33°C. This is in accordance with our observation that primary mouse fibroblasts have reduced proliferative potential at 33°C in comparison with that at 37 and 39.5°C (22). Exposure of these cultures at all three temperatures to fresh medium after 72 h resulted in the entry of cells into the S phase (Table 3); the induction with 20% FCS was greater than that with 10% FCS. Moreover, the proportion of REF that entered the S phase under the best conditions (24.7% at 37°C with 20% FCS) was not that much higher than that of growth-arrested tsa8 cells with 20% FCS (21.1%). When passage 6 REF cultures were assayed for growth, very little increase in cell number was observed (Fig. 4); growth at the different temperatures did not alter the growth potential, and neither did the continuous presence of 20% FCS. Cultures A and B were obtained by independent serial cultivation of cells from the same batch of primary REF. The BrdU incorporation results in Table 4 show that even in these cultures it was possible to induce cells to enter the S phase but the proportion of cells that underwent this transition was much lower than that of the growth-arrested tsa cultures.

Induction of immediate-early response genes with FCS. In addition to assaying the ability of FCS to induce DNA synthe-

TABLE 1. Number of tetraploid cells[a]

Passage no.	Total no. of cells scored	No. (%) of cells in metaphase	No. (%) of tetraploid cells
2	828	37 (4.4)	1 (0.1)
3	865	39 (4.5)	3 (0.3)
4	675	12 (1.8)	2 (0.3)
5	698	15 (2.1)	1 (0.1)
6	683	8 (1.2)	0 (0.0)
7	891	8 (0.9)	0 (0.0)
8	734	9 (1.2)	0 (0.0)
9	935	23 (2.4)	4 (0.4)
10	1,070	11 (1.0)	2 (0.2)
11	1,177	16 (1.3)	4 (0.3)

[a] REF prepared from day-12 Sprague-Dawley rat embryos were passaged every 72 h until they became senescent. At each passage, metaphase spreads were prepared from representative cultures and the proportion of tetraploid cells was determined by counting the number of chromosomes in each cell.

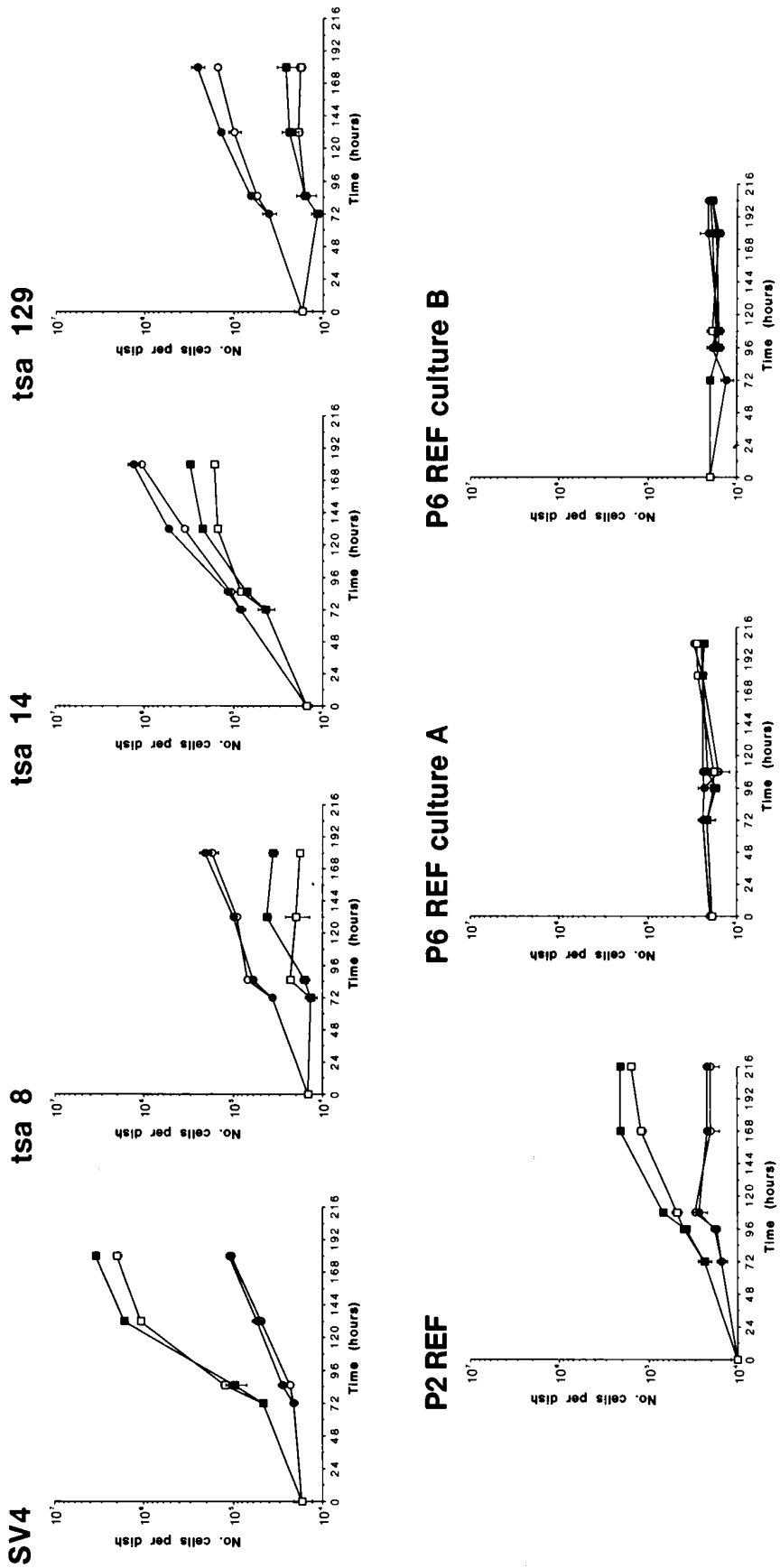

FIG. 4. Cell growth analysis. Approximately 4×10^4 cells per 6-cm-diameter dish from the indicated cultures were plated and left overnight at 33°C to adhere. Two dishes from each set of cultures were counted to determine the number of cells at the beginning of the experiment. Half of the dishes for each culture were then transferred to 39.5°C. Three days later, two dishes from each culture were counted. At that time, half of the dishes were transferred to medium containing 20% FCS and maintained in that medium for the duration of the experiment. At the indicated times, two dishes for each culture, from each of the experimental conditions, were harvested and the cell number was determined. The number of cells obtained in medium containing 20% FCS (filled squares and circles) in comparison with growth in 10% FCS (open squares and circles) at either 33°C (● and ○) or 39.5°C (■ and □) is presented.

TABLE 2. Induction of DNA synthesis[a]

Cell line	FCS concn (%)	Mean labelling index (% positive nuclei)	
		33°C	39.5°C
SV4	10	23.5	44.1
	20	27.8	45.0
tsa8	10	34.9	11.4
	20	35.9	29.0
tsa14	10	34.1	23.8
	20	34.0	28.5
tsa129	10	26.5	1.7
	20	21.2	6.8

[a] Cells that had been cultured at either 33 or 39.5°C for 7 days were stimulated with fresh medium containing either 10 or 20% FCS. The proportion of cells induced to synthesize DNA was determined by BrdU incorporation. One thousand cells from each of four coverslips were counted.

sis and cell division, we determined whether this treatment would induce expression of immediate-early response genes such as c-*fos* and c-*myc*. To explore this possibility, total RNA was prepared and analyzed from cultures of tsa8 and tsa14 cells grown at either the permissive temperature or 72 h after a shift up to the nonpermissive temperature. RNA was also extracted from parallel cultures after stimulation with fresh medium containing 20% FCS for 1 h. Stimulation for 1 h was chosen as a compromise for detection of expression of both c-*fos* and c-*myc*, which are known to be maximally expressed 30 and 120 min after induction with serum (18, 25, 31, 59). To control for changes in gene expression due to growth at different temperatures, RNA prepared from cultures grown under identical conditions from the nonconditional SV4 cell line was also analyzed. Total RNA from each sample was hybridized to a mixture of c-*fos*, c-*myc*, and *GAPDH* RNA probes and analyzed by RNase protection. Since the level of *GAPDH* mRNA was found to remain unaltered under the different conditions, the level of the *GAPDH*-protected band provides an internal control for experimental error. The results in Fig. 6A show that FCS induces a dramatic increase in the level of c-*fos* in both proliferating and growth-arrested cells (compare the minus and plus lanes for each set of conditions), whereas the c-*myc* gene level was increased only in the dividing cells. In the growth-arrested tsa8 and tsa14 cultures, the level of c-*myc* was not increased by FCS (compare the minus and plus lanes for the 33 and 39.5°C cultures). Even though, in the experiment shown in Fig. 6A, there appears to be a slightly higher level of c-*myc* at 39.5°C in tsa8 upon stimulation with FCS (see tsa8 minus and plus lanes at 39.5°C), this is due to experimental error because the *GAPDH*-protected band was lower in the minus lane than in the plus lane; in other RNase protection experiments, this increase was not observed. The lack of increase in the c-*myc* level was not due to the growth temperature, because c-*fos* and c-*myc* were both induced in SV4 cells at 39.5°C (Fig. 6A). Therefore, these growth-arrested cells were still capable of inducing c-*fos* but showed either a reduced or a severely delayed response for c-*myc*.

To determine whether these results were also a property of senescent REF, we analyzed RNAs extracted from cultures of early- and late-passage REF, both before and after stimulation with 20% FCS for 1 h, at 72 h after initial plating. A 10-μg sample of total RNA was analyzed for expression of c-*fos*, c-*myc*, and *GAPDH* by RNase protection. The results in Fig. 6B show that addition of 20% FCS for 1 h resulted in elevated levels of both c-*fos* and c-*myc* mRNAs in early-passage REF (E minus and plus lanes) but only c-*fos* in late-passage REF (L

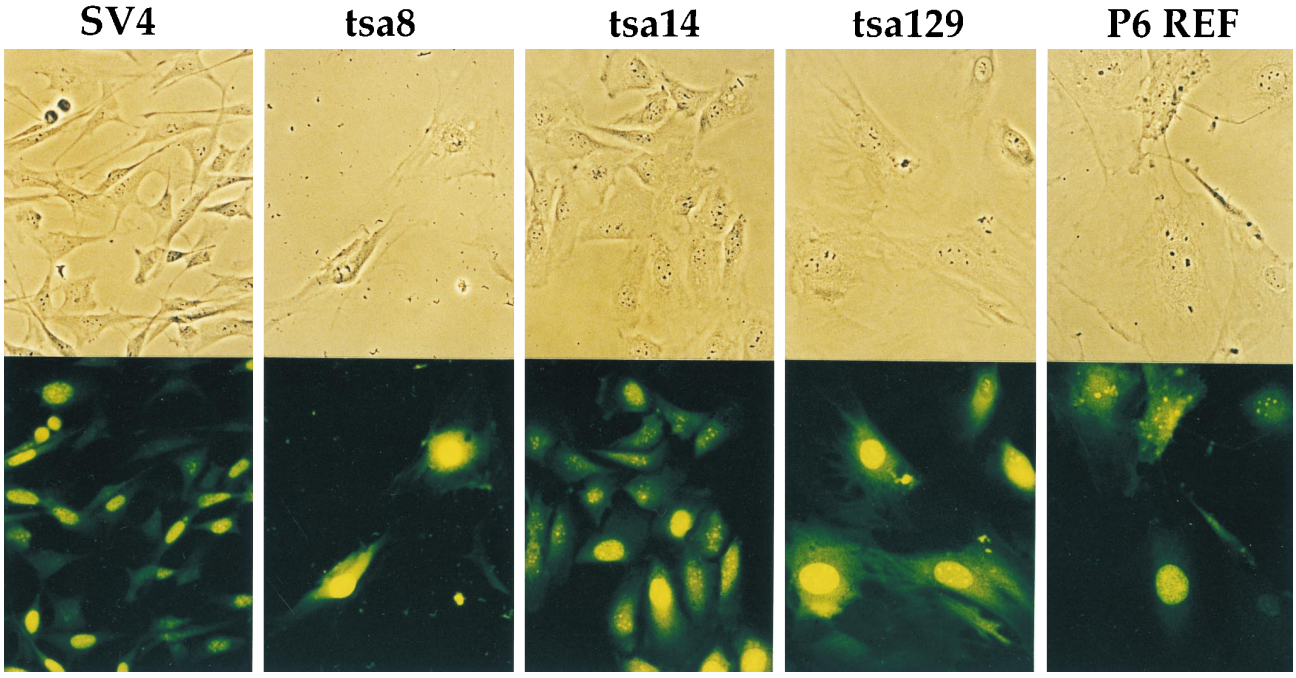

FIG. 5. Induction of DNA synthesis. Cells were grown in complete growth medium containing 25 mM BrdU and 2 mM fluorodeoxyuridine for 2 h. BrdU incorporation was measured by staining the cells with a mouse monoclonal antibody against BrdU, followed by a fluorescein-conjugated rabbit antimouse immunoglobulin G2a antibody. Representative cells derived from the experiments presented in Tables 2, 3, and 4 are shown to demonstrate the characteristic cell morphology and BrdU incorporation in growth-arrested tsa cells and senescent passage 6 REF. Cells of the SV4 line cultured under identical conditions are shown as a control.

TABLE 3. BrdU incorporation in early-passage (passage 2) REF and tsa8 cells[a]

Cells	FCS concn (%)	Mean labelling index (% positive nuclei)		
		33°C	39.5°C	37°C
REF	10	14.8	17.3	20.1
	20	15.2	19.4	24.7
tsa8	10	35.3	17.4	37.3
	20	36.5	21.1	37.8

[a] Early-passage (passage 2) REF cultured at three different temperatures for 3 days were stimulated with fresh medium containing either 10 or 20% FCS for 12 h, and the proportion of cells induced to synthesize DNA was determined by BrdU incorporation. tsa8 cells were used as a control.

minus and plus lanes); the level of c-*myc* mRNA was not elevated in response to FCS.

Gene expression. To further determine whether the loss of proliferative potential upon inactivation of T antigen resulted in senescence, we attempted to identify differentially regulated genes. If such genes could be identified, we could then analyze their expression when REF undergo senescence to further substantiate this hypothesis.

We first examined whether changes in the steady-state level of known cell cycle-dependent genes were involved. mRNAs from the tsa8, tsa14, and SV4 cell lines maintained at the permissive temperature, as well as at the nonpermissive temperature, for 72 h were analyzed by Northern blot analysis. To ensure that cells had not become quiescent, all cultures were refed with fresh medium 16 h prior to extraction of RNA. Equivalent loading of the samples was ensured by probing the blots for the β-actin and *GAPDH* genes. Representative results obtained from such experiments are shown in Fig. 7A. Even though it is the T antigen protein that is thermolabile (56), the level of T antigen mRNA was also found to be lower in growth-arrested rather than proliferating tsa cells (compare lanes 33 with lanes 39.5). In contrast, the SV4 cell line contained more T antigen mRNA at 39.5°C than at 33°C; interestingly, this cell line grew more rapidly at the higher temperature (Fig. 4). The immediate-early response genes c-*fos*, *krox-20*, and *krox-24* (2, 26) were not detectable in any cell line (data not shown). This was not surprising, since the RNAs had been extracted 16 h after refeeding, and it is known that these immediate-early genes are induced very rapidly, reaching a maximal level within 30 min, and then decline, becoming essentially undetectable after approximately 2 h (31, 59). In contrast, expression of the c-*myc* gene was detectable but remained unaltered upon a shift up to the nonpermissive temperature (Fig. 7A). Two mid- or late-G_1 genes, those for proliferating cell nuclear antigen (1) and thymidine kinase (45), were also not differentially regulated (data not shown). Since inactivation of T antigen requires shifting of cells to 39.5°C, expression of the heat shock protein 70 (*hsp70*) gene was examined to determine whether the temperature shift induced a heat shock response; the results showed that this did not induce higher levels of *hsp70* gene expression (data not shown).

Since genes known to be positive regulators of growth were not differentially expressed upon growth arrest, we investigated whether growth arrest-specific (*gas*) genes may be involved. Schneider et al. (48) have isolated six candidate *gas* genes (*gas-1* to *gas-6*) that are upregulated in NIH 3T3 cells upon serum starvation. Northern blot analyses with probes specific for the *gas* genes yielded surprising results: *gas-6* was upregulated in both tsa cell lines (Fig. 7A), *gas-1* and *gas-3* were detectable only in the tsa8 cell line but were upregulated upon a shift up (data not shown), and expression of the other three *gas* genes was not detectable in either of the cell lines analyzed. When expression of *gas-1*, *gas-3* and *gas-6* was analyzed in the SV4 cell line, they were upregulated upon a shift up (Fig. 7A). Since the growth of this cell line does not arrest upon a shift up, it suggested that the upregulation was due to the increase in the growth temperature rather than the loss of proliferative potential.

As we were unable to find any one gene whose expression correlated with the cessation of growth upon inactivation of T antigen, we undertook the isolation of genes differentially expressed in dividing versus growth-arrested cells (15a). The first such gene we identified was the fibronectin gene (Fig. 7B). Thus, we determined whether expression of the fibronectin gene is altered in REF upon senescence; the results in Fig. 7C show that it is expressed at a high level in late-passage senescent REF in comparison with early-passage REF.

We also examined expression of the $p21^{Waf1/Cip1/Sdi1}$ gene in our conditional cell lines, as well as in growing and senescent REF. The results in Fig. 7D show that it is upregulated upon inactivation of T antigen and that the upregulation is even greater than that observed in REF upon senescence.

DISCUSSION

Here we have presented a systematic analysis of some genetic and biological characteristics of a family of tsa cell lines that are conditionally immortal. These tsa cell lines were derived by immortalizing REF with the thermolabile *tsA58* T antigen and grow continuously at 33°C but rapidly cease dividing upon a transfer to 39.5°C, when the *tsA58* T antigen is inactivated. This analysis has identified the following parallels between the tsa cells upon inactivation of T antigen and senescent REF: (i) growth arrest is irreversible; (ii) it occurs in G_1 as well as G_2; (iii) the G_1 block can be partially overcome by stimulation with 20% FCS, but the G_2 block cannot be overcome; (iv) 20% FCS induces c-*fos*, but c-*myc* is unaltered; and (v) fibronectin and $p21^{Waf1/Cip1/Sdi1}$ are upregulated upon growth arrest. This suggests that REF conditionally immortalized with *tsA58* T antigen are committed to undergo senescence but are prevented from doing so by the T antigen and that when it is inactivated they undergo senescence.

TABLE 4. BrdU incorporation in late-passage, senescent REF and tsa8 and tsa129 cells[a]

Cells	FCS concn (%)	Mean labelling index (% positive nuclei)		
		33°C	39.5°C	37°C
REF (A)[b]	10	4.6	3.2	6.5
	20	3.4	5.2	9.8
REF (B)[b]	10	3.7	2.0	7.1
	20	3.7	3.2	6.0
tsa8	10	32.2	20.3	40.7
	20	34.9	27.0	47.3
tsa129	10	27.6	8.0	28.3
	20	34.4	12.6	28.5

[a] Late-passage, senescent REF cultured at three different temperatures for 3 days were induced with fresh medium containing either 10 or 20% FCS for 12 h, and the proportion of cells stimulated to synthesize DNA was determined by BrdU incorporation. Cells derived from the tsa8 and tsa129 cell lines were used as controls.

[b] Cultures A and B were obtained by independent serial cultivation of cells from the same batch of primary REF.

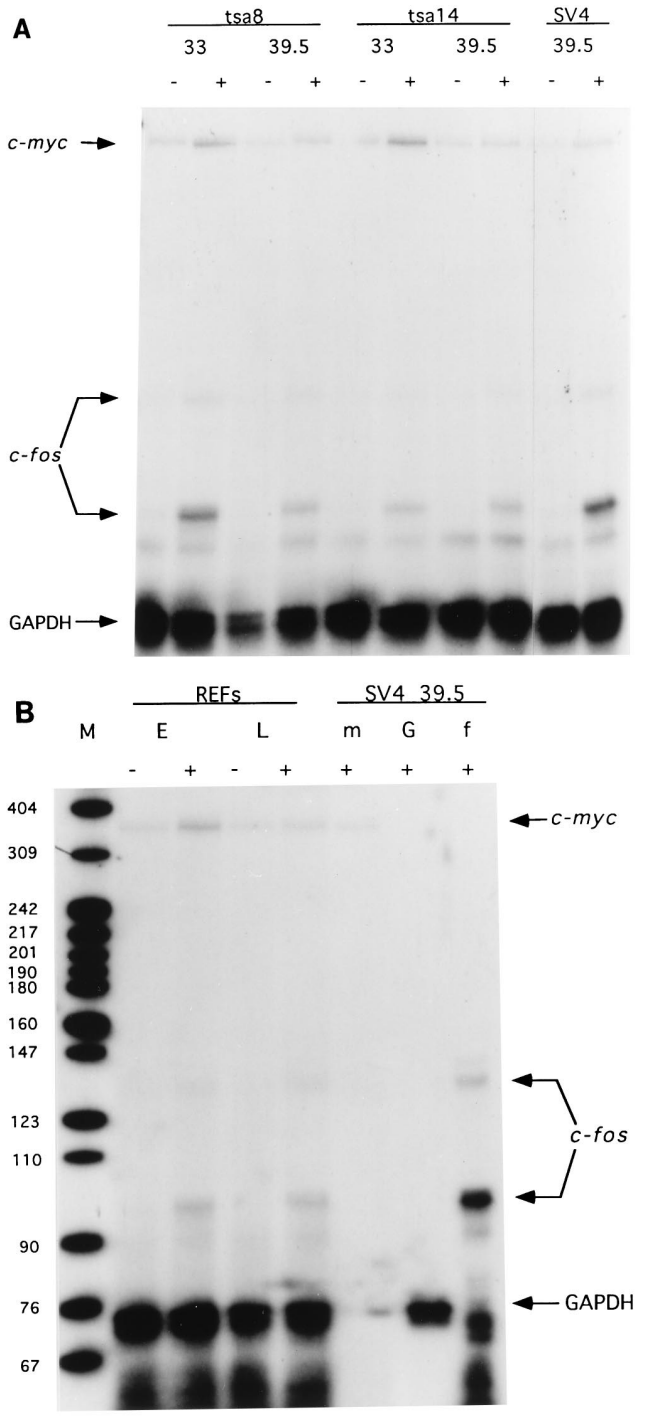

FIG. 6. Effect of FCS stimulation upon expression of immediate-early genes. (A) Ten micrograms of total RNA extracted from tsa8, tsa14, and SV4 cells grown at either 33 or 39.5°C for 72 h before (minus lanes) and after (plus lanes) stimulation with 20% FCS was analyzed by RNase protection with a mixture of c-*fos*, c-*myc*, and *GAPDH* RNA probes. The positions of migration of the RNase-protected bands corresponding to c-*fos*, c-*myc*, and *GAPDH* transcripts are indicated. (B) Ten micrograms of total RNA prepared from early (E)- and late (L)-passage senescent REF after 72 h of growth, both before (minus lanes) and after (plus lanes) stimulation with 20% FCS was analyzed with a mixture of the probes. The positions of migration of the protected bands corresponding to the different transcripts are illustrated by analysis of RNA prepared from cultures of SV4 cells grown at 39.5°C after stimulation with FCS by using the c-*fos* (lane f), c-*myc* (lane m), and *GAPDH* (lane G) probes separately. Lane M contained molecular size markers (sizes are in base pairs) prepared by ^{32}P labelling of an *Msp*1 digest of pBR322.

We have previously shown that these tsa cell lines grow continuously at the permissive temperature but rapidly undergo growth arrest upon a shift up to the nonpermissive temperature. The first observed consequence of the shift up was the loss of entry into the S phase which occurs within 12 to 24 h; the arrest in the G_2 phase occurs after about 48 to 72 h. When the cultures were shifted back down to the permissive temperature within 24 h, the growth arrest was reversible, whereas when they were shifted back down later, it was irreversible, suggesting that the irreversible loss of proliferative potential was associated with the arrest in the G_2 phase (23). Since it had previously been suggested that primary fibroblasts only undergo growth arrest in G_0/G_1, we investigated whether the biphasic arrest observed in the tsa cell lines is a peculiarity of these cell lines or also occurs during in vitro senescence of normal REF. The results show that when normal REF were passaged, the proportion of cells in the S phase diminished within the first few passages but the cell numbers of the cultures still continued to increase. As the cultures slowed down and eventually ceased dividing, there was an accumulation of cells in the G_2 phase which was further accentuated by continued passaging of these growth-arrested cultures. This increase in the proportion of cells in the G_2 phase was associated with an equivalent decrease in the G_0/G_1 population, suggesting that the continued serial cultivation caused cells to leak through from the G_0/G_1 phase into the S phase and become arrested in the G_2 phase. Interestingly, Sherwood et al. (50) have also analyzed senescence in HDF and suggested that the growth rate decrease was due to a lengthening of the G_1 phase and an eventual arrest in late G_1. In fact, they too had found that a proportion of HDF were arrested with tetraploid DNA content but, on the basis of karyotypic analysis, suggested that they were tetraploid cells arrested in G_1. In contrast, our karyotypic analyses detected very few tetraploid cells among the senescent REF, suggesting that most of the senescent REF with tetraploid DNA content were probably in the G_2 phase. Since it is difficult to karyotype a truly growth-arrested culture by metaphase spreads, it is possible that a proportion of the senescent HDF in the experiments of Sherwood et al. (50) were also arrested in the G_2 phase. This had already been suggested by Yanishevsky and Carrano (62), who used DNA cytophotometry and [^{3}H]thymidine autoradiography to suggest that arrested HDF may comprise G_1 diploid and G_2 diploid, as well as G_1 tetraploid, cells. However, the strongest evidence which supports this notion is provided by the work of Gorman and Cristofalo (17), who showed that SV40 infection of BrdU-selected, nondividing, senescent WI-38 cells caused reinitiation of DNA synthesis and resulted in cells with increased DNA content. This treatment did not, however, result in the infected population undergoing mitosis. Reinitiation of DNA synthesis in senescent cells can also be induced by fusion with SV40- or adenovirus type 5-transformed human cells (4, 34, 53). This suggests that in senescent cells, the G_1/S block can be overcome but there is a second block which cannot be overcome, which is consistent with our observation that continued passaging of growth-arrested normal REF cultures causes an increase in the G_2 population and an associated decrease in the G_0/G_1 population.

In accordance with the induction of senescent HDF to enter into the S phase, we have found that senescent REF can also be induced to enter the S phase by stimulation with 20% FCS, but the proportion of cells that underwent this transition was quite small. Nevertheless, this result was consistent with the passaging experiments in which continued passaging of REF after they had become growth arrested resulted in a gradual increase in the number of cells in the G_2 phase and a decrease

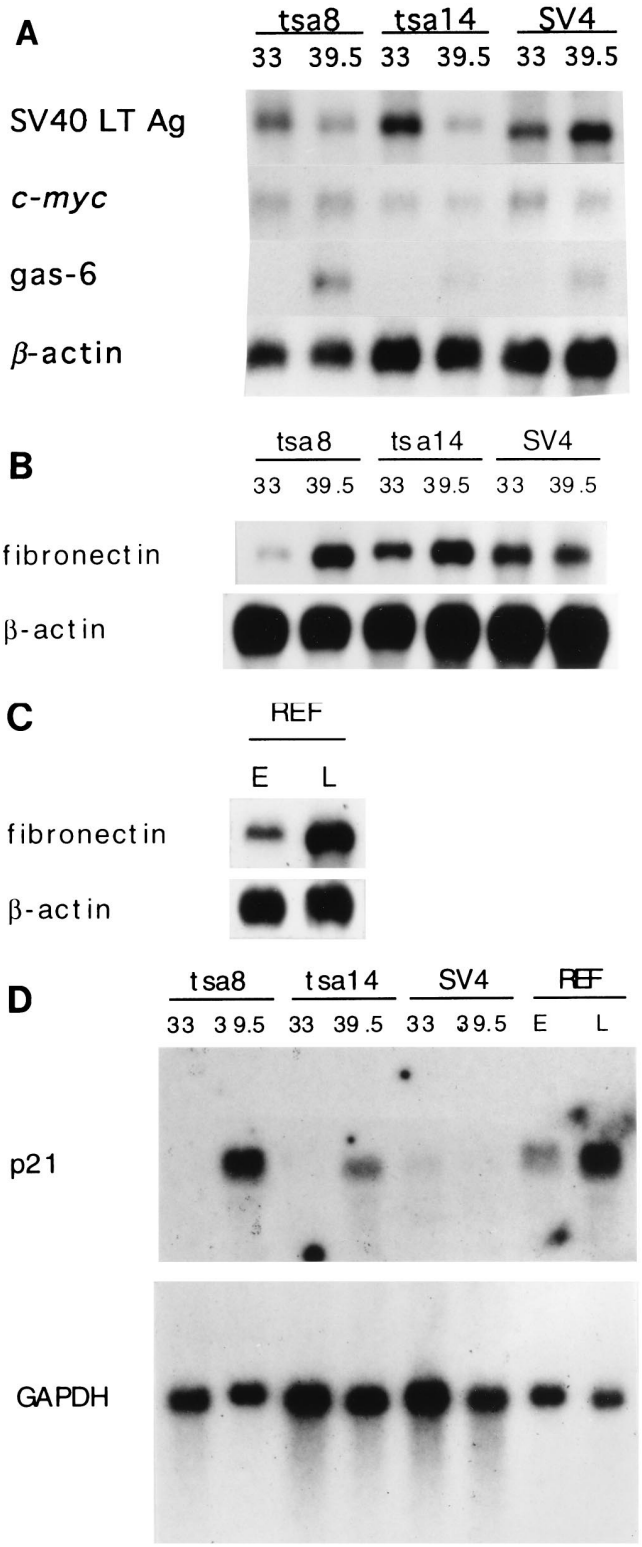

FIG. 7. (A) Northern blot analysis of mRNAs prepared from cultures of tsa8, tsa14, and SV4 cells grown at either 33°C (lanes 33) or after a shift up to 39.5°C for 72 h (lanes 39.5). Two micrograms of total RNA selected twice for poly(A)$^+$ RNA which had been extracted 16 h after refeeding with fresh medium was analyzed for each sample. Blots were probed with the indicated DNA probes. β-Actin was used to control for equal loading of mRNA. LT Ag, large T antigen. (B) Northern blot analysis of 2 μg of poly(A)$^+$ RNAs from tsa8, tsa14, and SV4 cells grown at two different temperatures for expression of fibronectin. (C) Analysis of 2 μg of poly(A)$^+$-selected total RNAs extracted from early (E) and late (L)-passage senescent REF for expression of fibronectin. (D) Northern blot analysis of 10 μg of total RNAs isolated from cultures of the indicated cell lines, as well as early (E) and late (L)-passage senescent REF for expression of p21.

in the number of those in the G_0/G_1 phase. In contrast, the results for the FCS induction of growth-arrested tsa cells were striking. They show that 20% FCS readily induces the S phase in cultures of tsa cells even 7 days after a shift up to the nonpermissive temperature; for tsa8 and tsa14 cells, the proportion of cells that can be induced to enter the S phase is not much lower than that of cells maintained at the permissive temperature. But the growth curves show that even if the tsa cells were maintained continuously in medium containing 20% FCS, there was no significant increase in cell number. Even though the proportion of senescent REF that can be induced to synthesize DNA is lower than that of growth-arrested tsa cells, these results suggest that growth-arrested tsa cells mimic senescent REF. Moreover, these results further show that senescence is associated with a block in both G_1 and G_2 and that the G_1 block can be overcome, whereas the G_2 block prevents cell division. The leakiness of the G_1 block in the tsa cell lines, especially tsa8 and tsa14, may be due to incomplete inactivation of the *tsA58* T antigen. Since the fraction of senescent REF that can be induced to synthesize DNA corresponds closely to that of growth-arrested tsa129 cells, it is also possible that the leakiness of the G_1 block in tsa8 and tsa14 cells is due to strain differences; tsa8, tsa14, and SV4 cells were derived from Fischer REF, whereas the tsa129 cell line was derived from Sprague-Dawley REF, the source of the REF used for passaging experiments.

In accordance with the ability to induce the S phase, it was also possible to induce changes in the expression of some immediate-early genes. The results obtained were rather surprising: serum increased the levels of c-*fos* and c-*myc* in growing cells, but in growth-arrested cells, there was a rapid induction of c-*fos* while c-*myc* levels remained low. These results are different from those of others (39, 45, 49) but correspond to the results obtained with growing and late-passage senescent REF; c-*fos* was induced in both early- and late-passage REF, whereas c-*myc* was induced only in early-passage REF. The most likely explanation for the differences between our results and those of others is that we did not induce quiescence in our senescent REF cultures before restimulation. Recently, inducibility of c-*fos* has also been shown to be retained in senescent Werner syndrome fibroblasts (36).

Analysis of mRNAs prepared from proliferating and growth-arrested cultures did not identify any changes in genes that are known to be positive regulators of cell growth. However, when expression of growth arrest-specific *gas* genes was analyzed, *gas-1*, *gas-3*, and *gas-6* were upregulated upon a shift up; since they were also upregulated in the nonconditional SV4 cell line upon the shift, it suggests that in this system, these three genes probably correspond to stress proteins. This upregulation of *gas* genes in the absence of growth arrest is consistent with the observation that microinjection of *gas-1* RNA into retrovirus-transformed 3T3 cell lines blocked entry into the S phase but was not effective in SV40-transformed 3T3 cells (8). Recently, Cowled et al. (5) have found that *gas* genes are regulated differently when murine cells undergo senescence; *gas-1* and *gas-6* were downregulated, whereas *gas-2*, *gas-3*, and *gas-5* were detectable at a low level that was not altered upon senescence.

Further verification that the tsa cell lines undergo senescence upon inactivation of T antigen was provided by the

analysis of expression of fibronectin and *p21$^{Waf1/Cip1/Sdi1}$* genes. The fibronectin gene has been found to be overexpressed in both normal HDF and Werner syndrome fibroblasts undergoing replicative senescence (32) and downregulated by SV40 transformation (24). We have found it to be similarly upregulated in the tsa cell lines upon inactivation of T antigen and in senescent REF. One of the ways in which the *p21$^{Waf1/Cip1/Sdi1}$* gene was isolated was as a gene that is upregulated in senescent HDF and would block DNA synthesis upon exogenous introduction into young HDF (33). We have found that it is upregulated in the tsa cell lines upon growth arrest and that the upregulation is even greater than that for senescent REF.

Initially, we showed that both rat and mouse embryo fibroblasts immortalized with SV40 *tsA58* T antigen grow continuously at the permissive temperature but rapidly undergo growth arrest upon its inactivation. Subsequently, we showed that mouse embryo fibroblasts become dependent upon the T antigen for growth only when their normal life span had elapsed. Here we have shown that when these cells undergo growth arrest, upon inactivation of T antigen, they undergo senescence. This suggests that fibroblasts immortalized by T antigen are committed to undergo senescence but are prevented from undergoing this process by its presence. However, when T antigen is inactivated, this block is removed and these cells synchronously senesce, and thus, such cell lines may represent a model system for studying the molecular basis for entry into this irreversible state in rodent cells. This is in contrast to human cells, in which it has been shown that T-antigen-containing cells exhibit an extended life span but the cells still enter the crisis phase and undergo senescence. Interestingly, Rubelj and Pereira-Smith (46) have shown that when SV40-transformed human cells enter crisis, they too exhibit changes that occur in normal senescence.

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