J. Biochem. 125, 531-536 (1999)

The Introduction of Dominant-Negative p53 Mutants Suppresses Temperature Shift-Induced Senescence in Immortal Human Fibroblasts Expressing a Thermolabile SV40 Large T Antigen[1]

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Received October 7, 1998; accepted December 7, 1998

Immortal human fibroblasts, SVts8 cells, which express a heat-labile SV40 large T antigen, induces a senescence-like phenomenon in response to upward shift in temperature. Cells with arrested division show strong induction of senescence-associated β-galactosidase. We examined how p53 and pRB are involved in this phenomenon since they are major targets of the T antigen. Transfection of cells with plasmids encoding the wild-type T antigen or human papilloma virus type 16 E6/E7 proteins completely abolished the arrest in cell division, a plasmid encoding the E6 protein suppressed it markedly, while a plasmid encoding E7 had no effect. Plasmids encoding dominant-negative p53 mutants also suppressed the arrest in cell division to various degrees. Upon temperature shift, p21 mRNA was upregulated 10-fold in SVts8 cells, but only slightly in clones expressing the wild-type T antigen or dominant-negative p53 mutants. These data demonstrate that p53 plays a major role in this senescence-like phenomenon.

Key words: dominant-negative p53, p21 mRNA, senescence, senescence-associated β-galactosidase, SV40 T antigen.

Normal human diploid fibroblasts lose their potential to divide in culture (*1*), a phenomenon referred to as cellular senescence. Transfection with a DNA tumor virus such as simian virus 40 (SV40) or human papilloma virus type 16 (HPV16) has been shown to abrogate this phenomenon (*2, 3*). Since these viral effects are brought on by their oncoproteins (*e.g.*, SV40 large T antigen or HPV16 E6/E7 proteins) and they inactivate p53 and pRB tumor suppressor proteins (*4*), one or both of them is thought to mediate a signal of senescence (*5-8*).

Virally transformed cells cannot, however, divide indefinitely and enter a state called crisis in which the cells eventually die (*2, 3*). Occasionally, virally transformed cells give rise to a clone that has acquired indefinite division potential (*9, 10*). Genetic analyses of such immortal clones have indicated that the functional loss of one of several unknown genes is sufficient for the appearance of immortality (*11, 12*). Recently, it is suggested that the genes have some relevance to telomere maintenance mechanisms (*13*).

For virally transformed immortal cells to maintain their immortal state, functional viral oncoproteins are required. Inactivation of thermolabile SV40 T antigen by temperature shift (*14, 15*) or elimination of the wild-type T antigen in an inducer-dependent expression system (*16*), has been shown to lead to an arrest in cell division. Under such restrictive conditions, cultures can be maintained for long periods without the loss of cells, but they undergo morphological alterations, becoming enlarged and flat, similar to normal senescent human cells (*15*).

The immortal human cell lines SVts7-1 and SVts8 (*15*), both isolated spontaneously from embryonic lung fibroblasts after transfection with a plasmid encoding a thermolabile SV40 T antigen, can enter a senescence-like state upon an upward shift in temperature (*15, 17*). Consistent with this findings, a mortality marker, mortalin, a member of the HSP70 family (*18*), was shown to undergo a change in its distribution pattern from the immortal perinuclear type to the cytosolic mortal type (*19*). Inhibitors of cGMP-dependent protein kinase have also been shown to suppress the induction of the senescence-like phenomenon (*17*). Here we assess the involvement of p53 and pRB in this phenomenon.

MATERIALS AND METHODS

Plasmids—pSV2neo encoding *Escherichia coli* neo under the control of the SV40 early promoter, and pSV3neo encoding both neo and the SV40 large T antigen (*20*) were obtained from Japanese Cancer Research Resources Bank. pC53-SCX3, pC53-175, pC53-248, and pC53-273 encoding dominant-negative p53 mutants with a substitution of valine to alanine at residue 143, arginine to histidine at

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: HPV16, human papilloma virus type 16; *neo*, neomycin resistance gene; pRB, retinoblastoma susceptible gene product; SV40, simian virus 40.

residue 175, arginine to tryptophan at residue 248, and arginine to histidine at residue 273, respectively, under the control of the cytomegalovirus promoter were obtained from Dr. B. Vogelstein of Johns Hopkins University School of Medicine (*21*). pSVneoE6E7 encoding both HPV16 E6 and E7 proteins under the control of the SV40 promoter, and pSRaE6 encoding the E6 protein and pSRaE7 encoding the E7 protein under the control of the SRa promoter were obtained from Dr. T. Kanda of the National Institute of Infectious Diseases (*22, 23*).

Cell Lines and Culture Conditions—SVts8 cells were isolated spontaneously from human embryonic lung fibroblasts (TIG3) after transfection with plasmid pMT-1ODtsA encoding a temperature-sensitive mutant form of the SV40 large T-antigen (*15*). Cells were maintained at the permissive temperature of 35°C in plastic Petri dishes (NUNCLON, Denmark) containing Dulbecco's modified Eagle's medium (Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (Moregate, Adelaide, Australia) under 5% CO_2 and 95% humidity (*17*).

Transfection with Plasmids—Samples of cells ($2\text{-}3\times10^5$ cells) were plated onto 60 mm dishes and cultured overnight at 35°C. When a test plasmid contained the selective marker neo, 5 μg of DNA was co-precipitated with calcium phosphate and added to the cells as described previously (*24*). When a test plasmid did not contain neo, 5 μg of pSV2neo DNA was mixed with 5 μg of the test plasmid DNA, co-precipitated with calcium phosphate, and added to the cells. After incubation for 8 h, the precipitated DNA was removed and the cells were cultured overnight in normal growth medium. The cells were then harvested by trypsinization and aliquots of $3\text{-}10\times10^4$ cells were plated onto dishes. Half of the dishes were shifted to 39.5°C and the rest were maintained at 35°C to determine the transfection efficiencies in each experiment. After culture at 35 or 39.5°C for approximately 2 weeks in the presence of 300 to 400 μg/ml G418 (Geneticin, Sigma, St. Louis, MO), the colonies formed were stained with Giemsa (GIBCO BRL, Grand Island, NY, USA) and counted.

Growth Curve—Aliquots of 1×10^4 cells were plated in 35 mm dishes and cultured overnight at 35°C. The cells were transferred to 39.5°C and cultured for 4 days, after which the cells were returned to 35°C and cultured for the times indicated. At intervals, cells were collected by trypsinization and counted with a hematocytometer.

β-Galactosidase Assay—Cells were stained for β-galactosidase as described previously (*13*). Cells were fixed in 2% formaldehyde/0.2% glutaraldehyde at room temperature for 5 min, and incubated at 37°C in fresh staining solution [1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactoside, 40 mM citric acid-sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM $MgCl_2$].

Northern Blot Analysis—Total RNA was extracted from cells by the acid guanidium-phenol-chloroform method (*25*). RNA samples were resolved by electrophoresis on 1% formaldehyde-agarose gels, transferred onto a nylon membrane (Hybond N^+), and hybridized with a ^{32}P-labeled probe at 65°C in a mixture consisting of 0.5 M sodium phosphate buffer (pH 7.2), 7% SDS, 1% bovine serum albumin, and 2 mM EDTA for 16 h. The membrane was washed twice at 65°C in 2×SSC and 0.1% SDS and twice with 0.1×SSC and 0.1% SDS for 30 min. The membrane was autoradiographed on a FUJI X-ray film backed with an intensifying screen.

RESULTS

Assay for Senescence Associated β-Galactosidase—We examined senescence-associated β-galactosidase since this enzyme is most frequently used as a reliable marker of cellular senescence. When SVts8 cells were cultured at 39.5°C for 2 weeks, they became enlarged and flat as in the case for normally senesced human fibroblasts. In these cells, more than 95% of them were heavily stained blue (Fig. 1), whereas control cells cultured at 35°C were not stained. These results favor the use of SVts8 cells as a model system for cellular senescence.

Effects of Viral Proteins on Colony Formation—SVts8 cells stop dividing within 3-4 days in response to an upward shift in temperature. Therefore, the cells can not form colonies at the nonpermissive temperature. The cells were transfected with plasmids encoding a viral oncoprotein(s) and pSV2neo if necessary (Fig. 2A), and allowed to form colonies at 35 or 39.5°C in the presence of G418. At the permissive temperature (35°C), transfection efficiencies were 3×10^{-4} to 10^{-3} per cell plated in each experiment. As expected, plasmids encoding the wild-type SV40 large T antigen or both HPV16 E6 and E7 proteins enabled the cells to form colonies normally at 39.5°C. A plasmid encoding the E6 protein markedly stimulated colony formation, whereas a plasmid encoding E7 had no effect (Fig. 2B). The numbers of colonies were counted to determine the colony forming abilities of the plasmids and the results are expressed

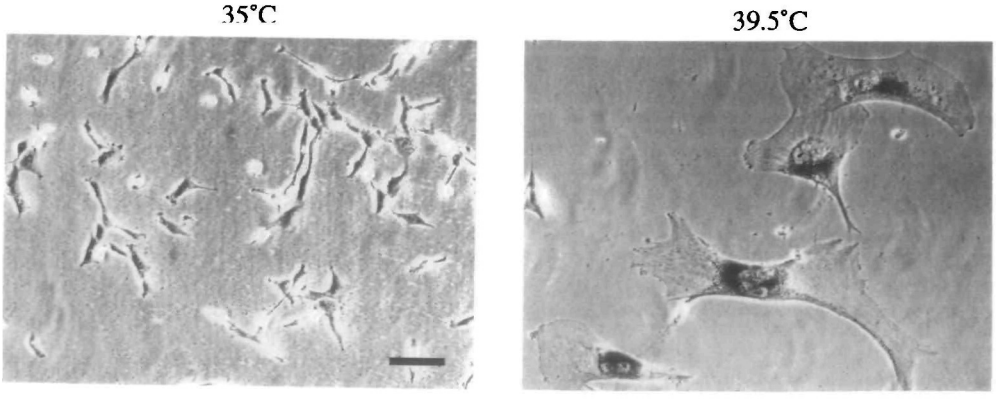


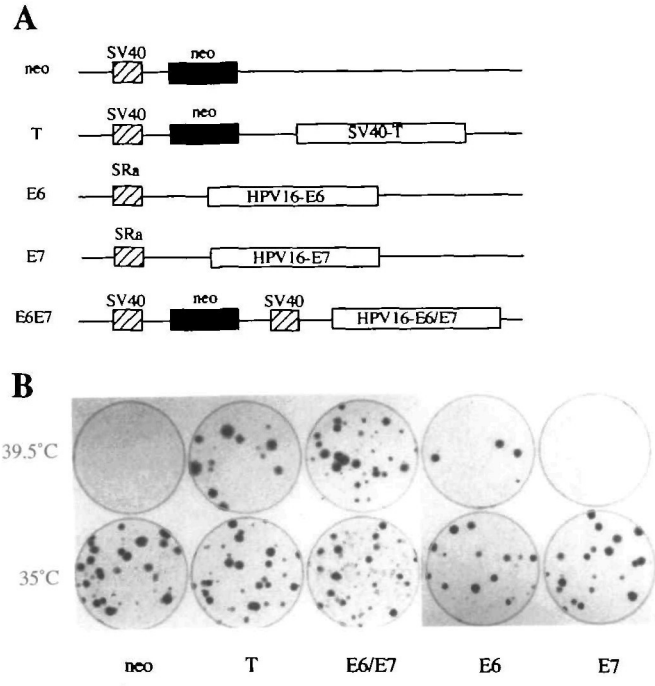
Fig. 1. **Induction of senescence-associated β-galactosidase in SVts8 cells.** Cells were cultured at 35°C (left) or 39.5°C (right) for 2 weeks and assayed for β-galactosidase as described in "MATERIALS AND METHODS." Scale bar, 100 μm.

relative to colony formation at 35°C (Fig. 2C).

These results suggest that viral proteins that inactivate p53, but not those that inactivate pRB, are effective in promoting colony formation under nonpermissive conditions.

Effects of Dominant-Negative p53 Mutants on Colony Formation—To confirm the role of p53 more directly, we transfected plasmids (Fig. 3A) encoding dominant-negative p53 mutants (*21*) into SVts8 cells and observed their colony-forming abilities (Fig. 3B). These p53 mutants have mutations in their DNA binding domain and potent tumorigenic activity arising from their inactivation of endogenous p53 in cells (*26, 27*).

The plasmids 175His, 248Trp, and 273His (Fig. 3A) all formed colonies at 39.5°C at the same frequently as at 35°C (Fig. 3, B and C). Plasmid 143Ala showed a weaker activity than the other three plasmids. The colony forming abilities of these plasmids seems to be higher than that of the



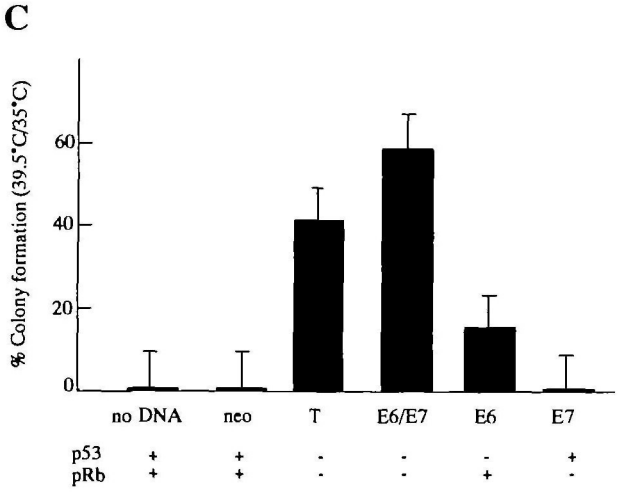
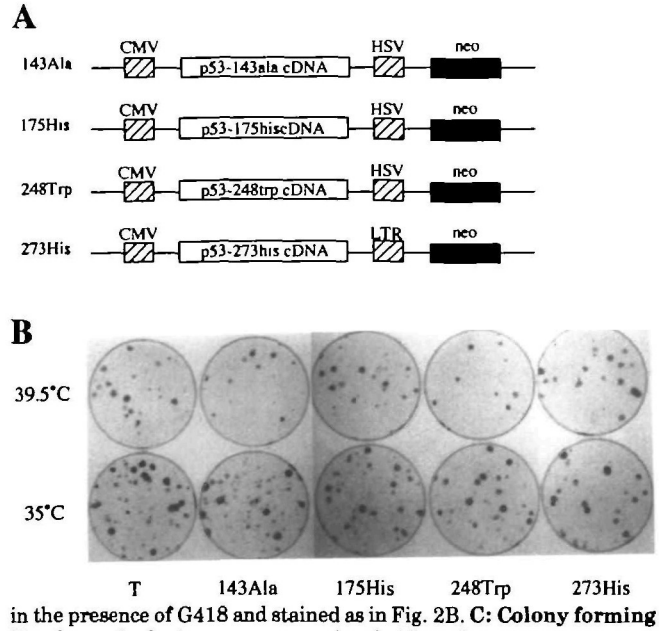


Fig. 2. **A: Plasmids used.** neo, pSV2neo; T, pSV3neo; E6, pSRaE6; E7, pSRaE7; and E6E7, pSVneoE6E7. ▨: promoter; □: gene or cDNA; ▩: *neo*. **B: Colony formation by transfection with plasmids encoding a viral oncoprotein(s).** Following transfection with the plasmid as indicated, colonies were formed at 35 or 39.5°C in the presence of G418 and stained with Giemsa as described in "MATERIALS AND METHODS." **C: Colony forming abilities of the plasmids encoding a viral oncoprotein(s).** The numbers of colonies formed at 39.5°C are expressed as percentages of those formed at 35°C. Error bars represent the standard deviation for four to five experiments. At the bottom, the status of p53 and pRB in the colonies formed at 39.5°C is shown: +, active; −, inactive.



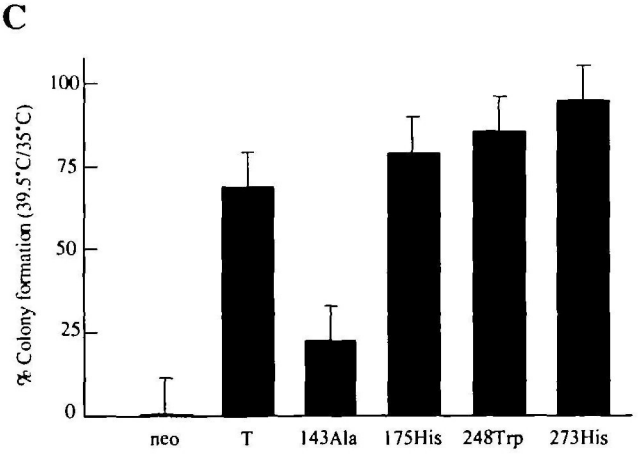


Fig. 3. **A: Plasmids used.** 143Ala, pC53-SCX3; 175His, pC53-175; 248Trp, pC53-248; and 273His, pC53-273. Symbols are as in Fig. 2A. **B: Colony formation by transfection with the plasmids encoding a dominant-negative p53 mutant.** Following transfection with plasmid as indicated, colonies were formed at 35 or 39.5°C in the presence of G418 and stained as in Fig. 2B. **C: Colony forming abilities of the plasmids encoding a dominant-negative p53 mutant.** Numbers of colonies are expressed as in Fig. 2C.

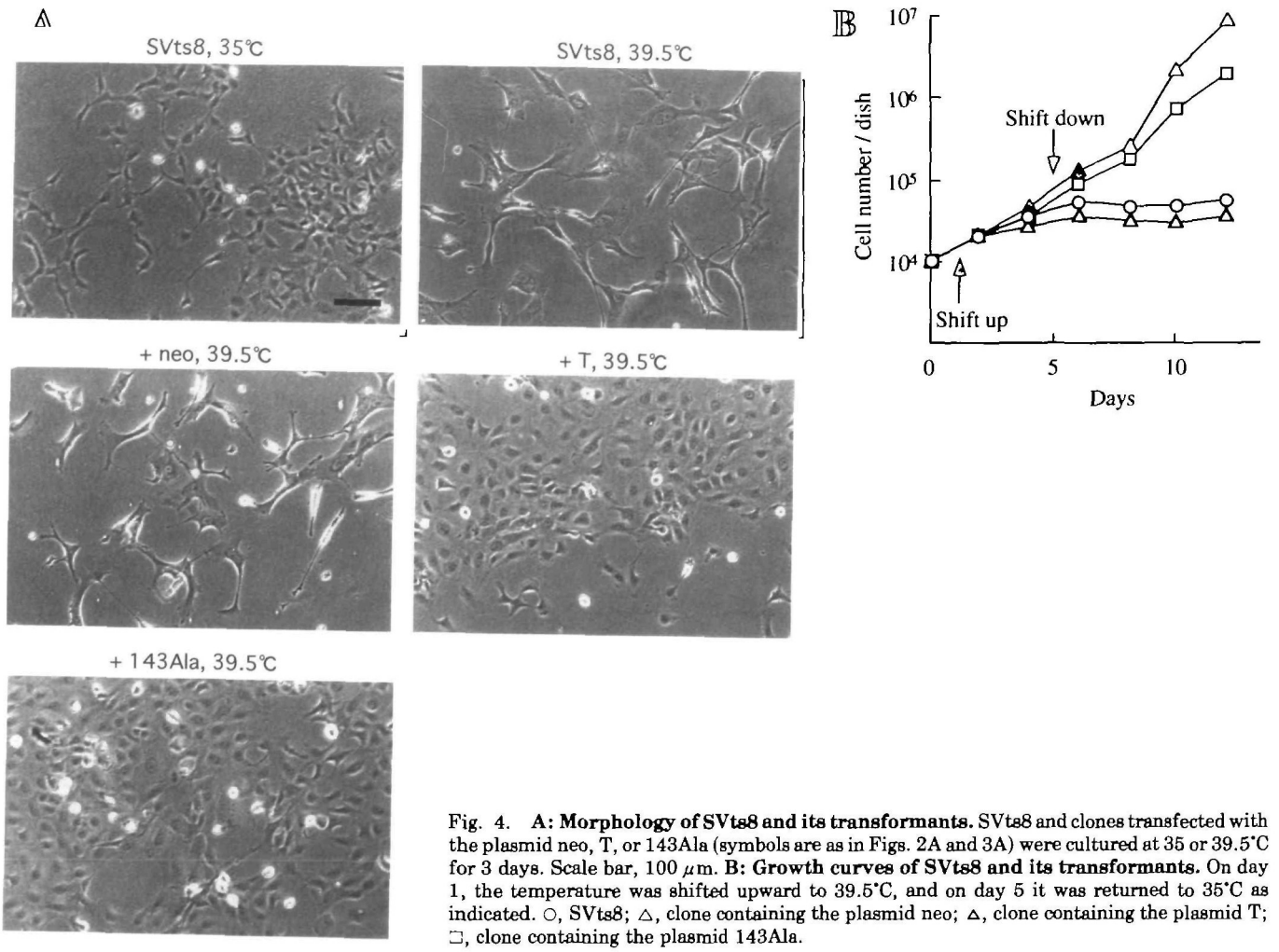


Fig. 4. **A: Morphology of SVts8 and its transformants.** SVts8 and clones transfected with the plasmid neo, T, or 143Ala (symbols are as in Figs. 2A and 3A) were cultured at 35 or 39.5°C for 3 days. Scale bar, 100 μm. **B: Growth curves of SVts8 and its transformants.** On day 1, the temperature was shifted upward to 39.5°C, and on day 5 it was returned to 35°C as indicated. ○, SVts8; △, clone containing the plasmid neo; ▲, clone containing the plasmid T; □, clone containing the plasmid 143Ala.

plasmid encoding the wild-type T antigen (Fig. 2C). However, it should be noted that the promoter activity of these plasmids is significantly stronger than that of the plasmid encoding the wild-type T antigen. Further, the abilities of the p53 mutants to stimulate colony formation seems to parallel their tumorigenic activities. In contrast, transfection of SVts8 cells with a plasmid encoding wild-type p53 did not enable colony formation under nonpermissive conditions (not shown).

These results indicate that p53 plays a major role in inducing the senescence-like phenomenon in SVts8 cells.

Growth of Clones Expressing SV40 T Antigen or a Dominant-Negative p53 Mutant—We examined the morphology of clones isolated with plasmids encoding the wild-type T antigen or the p53 mutant 143Ala. These clones appeared normal when cultured at 39.5°C, whereas SVts8 cells or clones isolated with pSV2neo became enlarged and flat within 3–4 days after the upward shift in temperature (Fig. 4A).

Next we examined the growth properties of the above clones. SVts8 cells and clones containing pSV2neo stopped growing within 4 days after temperature shift, and did not resume growth when the temperature was returned to 35°C (Fig. 4B). Clones containing the wild-type T antigen grew normally at 39.5°C. Clones containing the p53 mutant 143Ala grew slightly more slowly at 39.5°C than clones containing the wild-type T antigen. These results demonstrate that the inactivation of p53 alone is sufficient for SVts8 cells to grow normally under nonpermissive conditions.

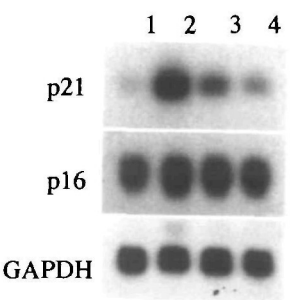


Fig. 5. **Northern blot analysis of SVts8 and its transformants.** The mRNA levels of p21 (top), p16 (middle), and glycerol-3-phosphate dehydrogenase (GAPDH) (bottom) were determined as described in "MATERIALS AND METHODS." Lane 1, SVts8 cultured at 35°C; lane 2, SVts8 cultured at 39.5°C for 3 days; lane 3, clone transfected with the plasmid T cultured at 39.5°C for 3 days; lane 4, clone transfected with the plasmid 143Ala cultured at 39.5°C for 3 days (symbols are as in Figs. 2A and 3A).

Expression of the p21 and p16 Genes—The cyclin dependent protein kinase inhibitors p16 (*28*) and p21 (*29*) are thought to play some roles in cellular senescence as their levels increase in some senescent cells. We investigated the expression of the p16 and p21 genes by Northern blot analysis in SVts8 cells and transformant clones.

In SVts8 cells, the level of p21 mRNA increased approximately 10-fold when the cells were cultured at 39.5°C for 3 days (Fig. 5). In clones expressing the dominant negative p53 mutant 143Ala, the level increased by approximately 2-fold. The clones expressing the wild-type T antigen showed only a slight increase in p21 mRNA level. These results are in good agreement with the observation that the plasmid 143Ala shows weaker colony forming ability than the plasmid encoding the wild-type T antigen.

The levels of p16 mRNA did not change significantly (less than 2-fold) upon temperature shift in SVts8 cells or their derivatives.

DISCUSSION

The introduction of a plasmid encoding HPV16 E6 protein (targeting p53) blocks loss of division potential induced in SVts8 cells by an upward shift in temperature, whereas a plasmid encoding HPV16 E7 (targeting pRB) fails to do so. Further, plasmids encoding various dominant-negative p53 mutants clearly suppressed the loss of division potential. Although there are small differences in the promoter activities of the plasmids used, the above results demonstrate that the inactivation of endogenous p53 is sufficient to suppress the senescence-like phenomenon in SVts8 cells. The introduction of plasmids encoding dominant-negative p53 mutants is also shown to extend the lifespan of normal human fibroblasts (*6, 8*). Taken together, p53, but not pRB, is shown to play a major role in cellular senescence.

At present, there seems to be disagreement concerning the roles of p53 and pRB in cellular senescence in human fibroblasts. In normal human diploid fibroblasts, the inactivation of both of p53 and pRB with antisense oligonucleotides is shown to extend their lifespan (*7*), whereas the abrogation of p53 function alone by HPV16 E6 protein or dominant-negative p53 is also sufficient (*6, 8*). In human fibroblasts expressing an SV40 T antigen in an inducer-dependent expression system, the inactivation of both p53 and pRB has been shown to be required for the suppression of cellular senescence (*5, 16*). These growth arrested cells can resume growth after a shift to permissive conditions, whereas SVts8 cells do not recover. It should also be noted that the cellular senescence observed in normal human fibroblasts and the senescence-like phenomenon observed in SVts8 cells do not necessarily represent the same phenomenon; however, we believe it represents a simple and convenient model system in which to study the mechanism of cellular senescence, especially regarding the roles of p53 and its down-stream targets.

More than half of all tumor-derived immortal human cell lines lack functional p53. Transfection of p53-negative cell lines with a plasmid encoding a conditionally functional p53 has been shown to induce an arrest in cell division and, in some cases such as SVts8 cells, lead to cellular senescence. In other experiments, we introduced a plasmid encoding a temperature-sensitive p53 into the p53-defective cell lines KMST-6 and SUSM-1. A majority of the resulting clones stopped cell division and became enlarged and flat upon a downward shift in temperature similar to senescent cells. However, these clones did not necessarily show an induction in senescence-associated β-galactosidase to a significant level (not shown). We do not know the reason why β-galactosidase is not induced. As the genetic backgrounds differ greatly among immortal cell lines and genetic heterogeneity also exists in each cell line, different consequences may result from the restoration of p53 function. In KMST-6 and SUSM-1, mutant forms of p53, but no functionally active form, are expressed (Dr. Namba, M., personal communications). The existence of such mutant forms might affect the functions of the introduced p53, although we did not examined the interaction between endogenous p53 and introduced p53 in the transformant clones of these cell lines.

In addition to acting as a transcription activator, p53 binds to various proteins to modulate their functions (*30, 31*). p53 is recognized as a guardian of the genome (*4, 30, 31*) that acts by responding to damage on DNA and arresting cell cycle progression to allow repair. In this context, an immortal cell might have already accumulated stress to a level sufficient to induce senescence. In support of this interpretation, p21, which is induced by p53 in response to DNA damage, is up-regulated in SVts8 cells upon temperature shift. In normal human fibroblasts, telomere shortening is thought to be one type of damage recognized by p53 to lead to senescence.

In the process of immortalization, we postulate that two types of genetic events occur. One is thought to interrupt a signaling pathway leading to senescence, and can be replaced by the introduction of the oncoprotein(s) encoded by DNA tumor viruses. Our data demonstrate that p53 is a major target of such oncoproteins. We have shown that a particular kind of protein kinase inhibitor blocks the commitment to growth arrest in SVts8 cells induced by temperature shift (*17*). The characterization of the target(s) may help to identify a downstream kinase that is activated by p53 and induces senescence.

The other type of genetic event is thought to represent mutations in novel genes on particular chromosomes (*32*), *e.g.*, human chromosomes 1, 4, 6, and 7 (*33, 34*). SVts8 most probably arose by a loss of function of a gene on chromosome 6 following the transfection of normal human fibroblasts with the plasmid encoding the thermolabile SV40 T antigen (*3, 15*). Although these genes remain to be identified, we have indirect evidence that they negatively regulate a telomere maintenance mechanism (*13*). The cloning of these genes will facilitate an understanding of the role of p53 in senescence and immortalization in human cells.

We thank Dr. Bert Vogelstein of Johns Hopkins University School of Medicine for providing us with the plasmids.

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