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NOTES

Growth of Immortal Simian Virus 40 *tsA*-Transformed Human Fibroblasts Is Temperature Dependent

R. L. RADNA,[1] Y. CATON,[1] K. K. JHA,[1,2] P. KAPLAN,[1†] G. LI,[1,2] F. TRAGANOS,[3] AND H. L. OZER[1,2*]

Department of Biological Science, Hunter College, City University of New York,[1] and Memorial Sloan-Kettering Cancer Center,[3] New York, New York 10021, and Department of Microbiology and Molecular Genetics, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, New Jersey 07103[2]

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Simian virus 40 (SV40)-mediated transformation of human fibroblasts offers an experimental system for studying both carcinogenesis and cellular aging, since such transformants show the typical features of altered cellular growth but still have a limited life span in culture and undergo senescence. We have previously demonstrated (D. S. Neufeld, S. Ripley, A. Henderson, and H. L. Ozer, Mol. Cell. Biol. 7:2794–2802, 1987) that transformants generated with origin-defective mutants of SV40 show an increased frequency of overcoming senescence and becoming immortal. To clarify further the role of large T antigen, we have generated immortalized transformants by using origin-defective mutants of SV40 encoding a heat-labile large T antigen (*tsA58* transformants). At a temperature permissive for large-T-antigen function (35°C), the cell line AR5 had properties resembling those of cell lines transformed with wild-type SV40. However, the AR5 cells were unable to proliferate or form colonies at temperatures restrictive for large-T-antigen function (39°C), demonstrating a continuous need for large T antigen even in immortalized human fibroblasts. Such immortal temperature-dependent transformants should be useful cell lines for the identification of other cellular or viral gene products that induce cell proliferation in human cells.

Human diploid fibroblasts (HDF) have been found in several studies to be rather resistant to transformation after introduction of activated cellular oncogenes; results have ranged from no phenotypic change in some cases (23) to expression in a minority of successful transfectants in others (13, 26). Even in the latter cases, the transformants failed to show the full spectrum of transformed phenotype, as they did not manifest indefinite life span in culture; i.e. they were not immortal. Rather, these cells ceased to proliferate after a variable number of generations, consistent with the nonreplicative phase, or senescence, observed with HDF (11). These results can be contrasted with those obtained with viral oncogenes, most notably simian virus 40 (SV40). Further investigation of SV40-mediated transformation, therefore, promises to shed light on the mechanism of transformation in general. Although the frequency of transformation of HDF by SV40 is significantly lower than that routinely obtained with established mouse cell lines such as 3T3 (25), part of the discrepancy arises from the fact that SV40 virus infection of HDF is semipermissive, with some associated cytotoxicity (20). Consistent with that observation, this laboratory (24) demonstrated in the case of DNA-mediated gene transfer that replication-defective SV40 genomes were more efficient in transformation than were wild-type genomes. Best results were obtained with a mutant (SVori$^-$) containing a 6-base-pair deletion at the *Bgl*I site (within the viral origin sequence [8]); other laboratories have also reported efficient transformation of HDF by this and other origin-defective constructs (2, 3, 5, 18). Furthermore, there was an excellent correlation between biochemical (G418 resistance) and morphological (SV40) transformation when both sequences were introduced on the same recombinant DNA (17). SVori$^-$ transformants showed an increased life span in culture. Such transformants were typically not immortal, however. Nonetheless, immortalized derivatives have been successfully isolated from these transformants, in contrast to the results with nonviral oncogenes (3, 5, 18, 19). In such studies, expression of the genes encoded by the SV40 early region (large T and small t antigens) has been observed in both the preimmortal and immortal derivatives (19). In an effort to clarify the role of large T antigen in maintenance as well as initiation of immortalization, we have isolated transformants after introduction of an origin-defective SV40 genome encoding a heat-labile large T antigen (*tsA58* transformants). Such transformants showed a temperature-dependent growth phenotype in both preimmortal and immortal transformants consistent with a role for large-T-antigen function in both states.

An origin-defective SV40 *tsA58* genome (pSVtsA) was cloned at the *Eco*RI site in a *Bgl*I- and *Bam*HI-resistant derivative of pMK16 in a multiple-step procedure by Y. Gluzman (unpublished data) and was kindly provided by him for these studies. The SV40 origin is defective because of a deletion that removes the *Bgl*I cleavage site. As expected, it was unable to replicate in permissive CV-1 cells at 35°C or in COS cells expressing a wild-type large T antigen (7) at 37°C as expected (data not shown). The early-passage human diploid fetal bone marrow fibroblast cell line HS74BM (HF) was transfected with 1 to 2 μg of closed circular pSVtsA DNA, with calf thymus DNA as the carrier, by the calcium phosphate DNA coprecipitation technique (9) as previously described (24). In a typical experiment, 5×10^5 cells were

* Corresponding author.
† Present address: Division of Cancer Genetics, Dana-Farber Cancer Institute, Boston, MA 02115.

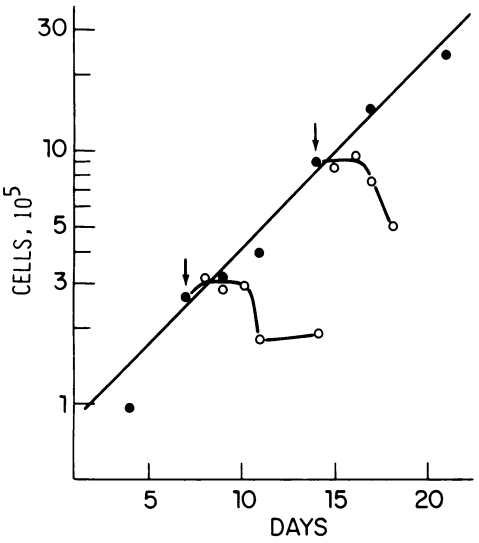

FIG. 1. Growth curve of immortal AR5 cells. Dishes (60 mm diameter) were seeded with 5×10^4 cells (passage 9) at 35°C. At the times indicated (←), replicate sets of dishes were shifted to 39°C. Cell number was determined as previously described (19). Symbols: ●, 35°C; ○, 39°C.

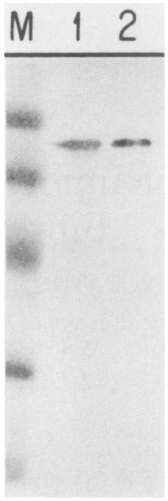

FIG. 2. Immunoblot for T antigen. AR5 cells were seeded at 35°C and incubated for 3 days at 35°C (lane 1) or 39°C (lane 2). Cells were harvested and extracted, and equivalent amounts of protein were immunoprecipitated with the monoclonal antibody PAb419 (10) as described elsewhere (22). The amount of large T antigen in the immune precipitates was quantified by immunoblot, using monoclonal antibody PAb419 and peroxidase-conjugated goat anti-mouse immunoglobulin G (19). Prestained markers of 200, 97, 68, 45, and 25 kilodaltons are in lane M.

transfected for 4 to 16 h at 2 days after being seeded on 100-mm-diameter dishes at 35°C. Three days later, the cultures were subcultured at a 1:3 ratio and maintained in growth medium. Transformed foci were observed at low frequency (one or a few on some but not all dishes) after 3 to 4 weeks at 35°C. The foci were generally fewer and smaller than those observed with HF transfected with origin-defective SV40 genomes encoding a wild-type large T antigen in parallel. Individual foci were picked, grown to mass culture in 100-mm-diameter dishes for frozen storage, and designated as reference stock.

The transformants (designated SVtsA/HF) were temperature dependent for growth as assayed by colony formation or repeated passage of mass cultures at 39°C, confirming the presence of *tsA58*-encoded large T antigen. The ratio of the number of colonies formed at 39°C divided by the number formed at 35°C (EOC ratio) was less than 0.0001. In contrast, HS74 and two SV/HF cell lines transformed by other origin-defective genomes (SV/HF-5/39 [19] and HS74 transformed with pSVori$^-$ at 35°C) grew at least as well at 39°C as at 35°C, as expected. In an effort to obtain immortalized derivatives, one of the transformants (SVtsA/HF-A) at passage 2 was subjected to colony isolation (see reference 19 for rationale); one clone, designated AR5, has been maintained in continuous culture for over a year. Passage of the uncloned mass culture of SVtsA/HF-A also generated a cell line which has been in continuous culture for more than 60 passages or 200 generations. (SVtsA/HF and SV/HF typically senesce at 60 to 90 generations [19].) They each contain two to three copies of integrated pSVtsA DNA, as determined by Southern blot analysis (data not shown).

SVtsA/HF-A (at late passage) and its clonal derivative AR5 are composed predominantly, if not exclusively, of immortal cells. They showed progressive improvement of the growth rate and the efficiency of colony formation on continued passage at 35°C, as previously noted for other immortalized cell lines (19). However, they did not reacquire the ability to grow at 39°C. They were defective in colony formation at 39°C (EOC ratio of 0.0006). Furthermore, when logarithmically growing cultures were shifted to 39°C, there was a rapid cessation of growth, independent of cell density over a severalfold range (Fig. 1). Cell cycle studies by flow cytometry of AR5 at 39°C showed a depletion of S-phase cells, consistent with the absence of an increase in cell number and inhibition of DNA synthesis as measured by incorporation of [^{3}H]thymidine (data not shown). However, cells accumulated with the G2 content of DNA (i.e., twice the diploid DNA content) rather than with the G1 content expected for a cell which had reverted to the untransformed phenotype and become growth arrested. Finally, AR5 showed a progressive loss of viability when cultures were maintained at 39°C for various periods of time and subsequently returned to 35°C to allow colonies to appear; there was a loss of approximately 50%/day over the first 3 days at 39°C. AR5 cells expressed immunoreactive large T (and small t) antigen at both 39 and 35°C (Fig. 2). As expected, the antigen was not functional at the higher temperature. pSV0, a plasmid containing a functional origin sequence encoded by wild-type SV40 (1), replicated when transfected into AR5 and incubated for 2 or 3 days at 35°C but not at 39°C (Fig. 3). These data demonstrate the persistent requirement for large-T-antigen function for growth and viability of the immortalized cell line.

These data do not, however, prove that dependence on large T antigen is solely responsible for the growth defects at 39°C, since abnormalities could have occurred and been masked during prolonged passage at 35°C. To rule out this possibility, we transfected AR5 with the plasmid pRNS-1 (17), encoding resistance to neomycin (and G418), and a wild-type large T antigen. Colonies isolated in G418 at 35°C had at least as good growth at 39°C as at 35°C (e.g., AR5/SN-11 cells had an EOC ratio of 2.5); colony size was also larger at 39°C. Revertants or pseudorevertants (e.g., AR5-R1) were isolated at low frequency (less than $1/10^5$ cells) when large numbers of AR5 were cultured at 39°C. They also acquired the ability to form colonies at 39°C;

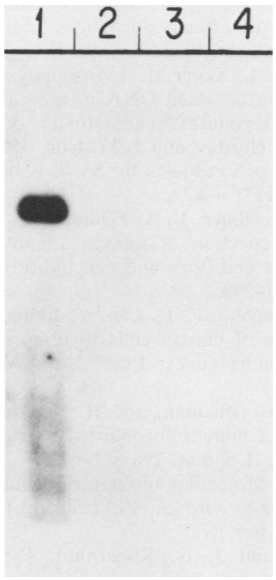

FIG. 3. DNA replication in AR5 cells. Cells were seeded at 35°C and transfected for 4 h on the following day by 1 μg of pSVO with carrier DNA (lanes 1 and 2) or by carrier DNA alone (lanes 3 and 4) as described in the text. Cultures were maintained at 35°C (lanes 1 and 3) or 39°C (lanes 2 and 4) for 3 days. DNA was extracted and analyzed for replicated SV40 sequences by the Southern blot procedure as previously described (15). DNA was digested with *Dpn*I and *Eco*RI (to linearize all forms) before electrophoresis. pSV0 labeled with [^{32}P]dCTP by the random-primer method (6) was used as a probe.

however, colony formation was poorer than at 35°C (EOC ratio of 0.16). The biochemical basis for partial restoration of growth of the AR5 revertants has not yet been resolved. Taken together, these data serve to further emphasize the full restoration of the growth properties upon introduction of a wild-type large T antigen.

SV40 transformation of human cells, especially fibroblasts, has been used for many years as a model for carcinogenesis in human cells and to obtain immortalized or continuous human cell lines for other purposes (see reference 4 for review). This study demonstrates that origin-defective *tsA58* transformants can be used to transform HDF. When immortalized derivatives obtained from one such transformant were used to assess the role of large T antigen in the maintenance of the immortalized phenotype, we found that the immortalized SVtsA/HF-A were temperature dependent for growth. In an independent series of experiments using transformants obtained with a recombinant DNA construct in which synthesis of SV40 large-T-antigen mRNA is hormone dependent, Wright and coworkers (27) have also observed conditional cell growth. Individually and together, these studies demonstrate that SV40 large-T-antigen function is required for the maintenance of the immortalization phenotype in human fibroblasts.

Our findings with immortal SVtsA/HF has parallels with those obtained with some viral transformants in rodent cells (14, 21). It should, however, be emphasized that significant differences in behavior between human and rodent fibroblasts with respect to immortalization are well documented. First, human fibroblasts do not spontaneously immortalize, whereas rodent fibroblasts commonly do, although the frequency and rapidity vary among the rodent species. Second, nonviral oncogenes (e.g., c-*myc* [16]) can be shown to immortalize primary or secondary rodent cells, as assayed by colony formation at low cell density, whereas they have not been found to have an effect on the cell growth phenotype when introduced into normal human fibroblasts (12). Finally, and most relevant to these studies, SV40-transformed HDF are not immortal upon isolation; rather, immortalization is most likely due to a change or changes within the cellular genome that occur at a variable and often quite low frequency even with replication-defective viral genomes, as previously shown for HS74 (19) and by others for other human fibroblasts.

Finally, it should be noted that although the immortalization phenotype was formally dependent on SV40 large T antigen in AR5 cells, this does not necessarily mean that the biochemical change responsible for immortalization is dependent on continuous large-T-antigen function. Alternatively, large T antigen could cause cellular proliferation in a manner similar to that involved in extending the life span of preimmortal cells (19). Inactivation of this mechanism upon shift of AR5 cells to 39°C would result in cessation of cell proliferation and apparent loss of immortality. This view is compatible with the model of at least a two-step basis for cellular senescence (27) and is supported by the common finding, already noted, that SV40-transformed human fibroblasts are typically not immortal. One might predict, therefore, that expression of other genes could also bypass the block in cell growth in senescent HDF. Such genes might also be expected to correct the temperature dependence of SVtsA/HF. We are currently testing this hypothesis by determining whether cellular or non-SV40 viral oncogenes can correct the growth defect at 39°C and provide an equivalent function.

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LITERATURE CITED

1. **Auborn, K. J., R. B. Markowitz, E. Wang, Y. T. Yu, and C. Prives.** 1988. Simian virus 40 (SV40) T antigen binds specifically to double-stranded DNA but not to single-stranded DNA or DNA/RNA hybrids containing SV40 regulatory sequences. J. Virol. **62**:2204–2208.
2. **Barbis, D. P., R. A. Schultz, and E. C. Friedberg.** 1986. Isolation and partial characterization of virus-transformed cell lines representing the A, G, and variant complementation groups of xeroderma pigmentosum. Mutat. Res. **165**:175–184.
3. **Canaani, D., T. Naiman, T. Teito, and P. Berg.** 1986. Immortalization of XP cells by SV40 DNA having a defective origin of DNA replication. Somatic Cell Mol. Genet. **12**:13–20.
4. **Chang, S. E.** 1986. In vitro transformation of human epithelial cells. Biochim. Biophys. Acta **823**:161–194.
5. **Daya-Grosjean, L., M. R. James, C. Drougard, and A. Sarasin.** 1987. An immortalized xeroderma pigmentosum group C cell line which replicates SV40 shuttle vectors. Mutat. Res. **183**: 185–196.
6. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Bioch. **132**:6–13.
7. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
8. **Gluzman, Y., J. Sambrook, and R. J. Frisque.** 1980. Expression of early genes of origin-defective mutants of simian virus 40. Proc. Natl. Acad. Sci. USA **77**:3898–3902.
9. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for

the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
10. **Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamsen.** 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. J. Virol. **39**:861–869.
11. **Hayflick, L., and P. S. Moorhead.** 1961. The serial cultivation of human diploid cell strains. Exp. Cell Res. **25**:585–621.
12. **Hurlin, J., V. M. Maher, and J. J. McCormick.** 1989. Malignant transformation of human fibroblasts caused by expression of a transfected T24 HRAS oncogene. Proc. Natl. Acad. Sci. USA **86**:187–191.
13. **Hurlin, P. J., D. G. Fry, V. M. Maher, and J. J. McCormick.** 1987. Morphological transformation, focus formation, and anchorage independence in human diploid fibroblasts with H-ras oncogene. Cancer Res. **47**:5752–5757.
14. **Jat, P. S., and P. A. Sharp.** 1989. Cell lines established by a temperature-sensitive simian virus 40 large-T-antigen gene are growth restricted at the nonpermissive temperature. Mol. Cell. Biol. **9**:1672–1680.
15. **LaBella, F., and H. L. Ozer.** 1985. Differential replication of SV40 and polyoma DNA in Chinese hamster ovary cells. Virus Res. **2**:329–343.
16. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:596–602.
17. **Litzkas, P., K. K. Jha, and H. L. Ozer.** 1984. Efficient transfer of cloned DNA into human diploid cells: protoplast fusion in suspension. Mol. Cell. Biol. **4**:2549–2552.
18. **Murnane, J. P., L. F. Fuller, and R. B. Painter.** 1985. Establishment and characterization of a permanent pSVori-transformed ataxia-telangiectasia cell line. Exp. Cell Res. **158**:119–126.
19. **Neufeld, D. S., S. Ripley, A. Henderson, and H. L. Ozer.** 1987. Immortalization of human fibroblasts transformed by origin-defective simian virus 40. Mol. Cell. Biol. **7**:2794–2802.
20. **Ozer, H. L., M. L. Slater, J. J. Dermody, and M. J. Mandel.** 1981. Replication of SV40 DNA in normal human fibroblasts and those from xeroderma pigmentosum. Virology **39**:481–489.
21. **Petit, C. A., M. Gardes, and J. Feuteun.** 1983. Immortalization of rodent embryo fibroblasts by SV40 is maintained by the A gene. Virology **127**:74–82.
22. **Radna, R., B. Foellmer, L. A. Feldman, U. Francke, and H. L. Ozer.** 1987. Restriction of human adenovirus replication in Chinese hamster cell lines and cell hybrids with human cells. Virus Res. **8**:277–299.
23. **Sager, R., K. Tanaka, C. C. Lau, Y. Ebina, and A. Anisowicz.** 1983. Resistance of human cells to tumorigenesis induced by cloned transforming genes. Proc. Natl. Acad. Sci. USA **80**: 7601–7605.
24. **Small, M. B., Y. Gluzman, and H. L. Ozer.** 1982. Enhanced transformation of human fibroblasts by origin-defective simian virus 40. Nature (London) **296**:671–672.
25. **Tooze, J.** 1980. Molecular biology of tumor viruses, 2nd ed., part 2. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Tubo, R. A., and J. G. Rheinwald.** 1987. Normal human mesothelial cells and fibroblasts transfected with the EJras oncogene become EGF-independent, but are not malignantly transformed. Oncogene Res. **1**:407–421.
27. **Wright, W. E., O. M. Pereira-Smith, and J. W. Shay.** 1989. Reversible cellular senescence: a two-stage model for the immortalization of normal human diploid fibroblasts. Mol. Cell. Biol. **9**:3088–3092.