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Expression of SV40 T Antigen in Finite Life-Span Hybrids of Normal and SV40-Transformed Fibroblasts

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Abstract—*The fusion of normal human fibroblasts with SV40-transformed human fibroblasts resulted in hybrid clones, 85% of which exhibited a finite in vitro life-span. Foci of rapidly dividing cells appeared in 15% of the hybrid clones. The cells within these foci repopulated the culture and could then be subcultured through more than 100 population doublings. One or two foci of dividing cells occurred per culture of 10^5 or more cells. The change to an indefinite life-span was, therefore, a rare event. All hybrid clones, including those that exhibited a finite in vitro life-span, expressed viral T antigen. Thus, even though viral DNA was present and being expressed in all hybrid clones, the senescent phenotype was dominant in these hybrids.*

INTRODUCTION

Normal human and chick cells have a finite proliferative potential in culture (1, 2). This is in contrast to tumor cell lines and virally or spontaneously transformed cell lines which can divide indefinitely in culture.

The limited division potential of normal cells has led to the proposal that they be used as a model for aging at the cellular level (1). Investigations in the field of cellular aging have attempted to determine the mechanisms which limit the proliferative ability of these cells and cause them to behave differently from transformed or tumor cell lines. One experimental approach to the problem has been to study DNA synthesis in hybrids formed after the fusion of normal fibroblasts with transformed cells and tumor cells and also to determine the in vitro life-span of proliferating hybrid cells. Early reports

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indicated that the transformed phenotype was dominant in fusions between transformed cells and senescent cells, because, following fusion, DNA synthesis was reinitiated in the senescent normal cell nuclei (3) and indefinitely proliferating hybrid clones were isolated (4–6).

Recently, however, Stein and Yanishevsky (7) reported that no reinitiation of DNA synthesis occurred in the senescent cell nuclei present in heterodikaryons formed after the fusion of senescent cells with either a human glioblastoma cell line T98G or a rabbit kidney cell line RK13. Instead, they observed that DNA synthesis was suppressed in the nuclei of these aneuploid cell lines, after fusion with a senescent cell. T98G and RK13 are unusual cell lines in that they exhibit an indefinite in vitro life-span but still retain some of the characteristics of normal cells, i.e., contact inhibition of growth and serum dependence for growth. Bunn and Tarrant (8) and Muggleton-Harris and DeSimone (9) have observed that the majority of the hybrids obtained after fusion of normal young and old cells with HeLa or SV40-transformed fibroblasts exhibited a finite in vitro life-span. Matsumura (10) has reported that viable proliferating hybrids were not obtained from fusions of normal fibroblasts, that were 6 months postsenescent, with UV-irradiated SV40-transformed fibroblasts.

The study reported here is an extension of the previous studies on hybrid cells formed between immortal and normal cells. This study was initiated to determine the in vitro life-span of hybrids capable of only a few doublings in vitro as well as hybrids that could be isolated and subcultured through more than 20 population doublings (PD). The distribution of the in vitro life-spans of hybrid clones could then be compared with the life-span distributions of the individual cells within the parent cultures used. Such a comparison would permit an analysis of the degree of dominance of either the senescent or transformed phenotype. In order to identify both short- and long-lived hybrid clones, a biochemical selection procedure was used. The basis of the biochemical selection procedure was the use of a normal parent line that was a double mutant, deficient in the enzyme hypoxanthine guanine phosphoribosyltransferase ($HPRT^-$) and resistant to ouabain (Oua^R), and wild-type SV40-transformed parent line that was $HPRT^+$ and ouabain sensitive (Oua^S). Only hybrid cells resulting from the fusion of such parental cells will proliferate in medium containing hypoxanthine, aminopterin, and thymidine (HAT) and ouabain. This selection system has not been applied previously in the study of in vitro cellular aging.

In addition, the possibility that the finite in vitro life-span, exhibited by hybrid clones in the earlier investigation (8, 9), was due to the loss of specific HeLa chromosomes or the SV40 viral DNA from the hybrids had to be considered. The presence and expression of the SV40 A gene can be readily determined by assaying for the presence of viral T antigen. The viral A gene

has been strongly implicated in the maintenance of transformation (11, 12). Thus the presence of SV40 viral T antigen in hybrid cells would clearly demonstrate that viral genetic material was present and expressed.

MATERIALS AND METHODS

Cell Cultures. The cells included the following:

GM 1662 Oua^R Clone. The diploid skin fibroblast cell line GM 1662 was obtained from the Institute for Medical Research, Camden, New Jersey. This cell line was derived from a white male with Lesch Nyhan syndrome and is deficient in the enzyme HPRT. A clone resistant to 10^{-6} M ouabain was isolated from this cell line after mutagenesis (13).

CSC-301 Oua^R Clone. A clone of normal human cells was derived in our laboratory that has two spontaneous mutations. It is resistant to 10^{-6} M ouabain and will not grow in HAT medium (i.e., HPRT enzyme deficiency). The clone was isolated from the cell line CSC-301, derived from human fetal lung in our laboratory.

VA13. This SV40-transformed human lung fibroblast cell line was kindly supplied by Dr. E. Schneider, G.R.C., Baltimore. It was found to be $HPRT^+$ and Oua^S. It stained intermediate for viral T antigen, i.e., staining was diffuse, perinuclear, and nonhomogeneous.

GM 639. This is a clone of an SV40-transformed human skin line, kindly supplied by Dr. R. Kucherlapati, Princeton. This clone was $HPRT^+$ and Oua^S and strongly positive for viral T antigen.

Cell Culture Procedures. All cultures were routinely maintained in T-25 tissue culture flasks in minimum essential medium with Hanks' balanced salts (HMEM) containing 10% fetal bovine serum (FBS) and 28 mM HEPES. For work done in tissue culture dishes, minimum essential medium with Earle's balanced salts (EMEM) and 10% FBS was used. The dishes were incubated at 37°C in an atmosphere of 5% CO_2–95% air, 98% relative humidity. For subcultivation the cells were dispersed with 0.25% trypsin.

To determine the in vitro proliferation potential of cultures, cells were subcultured at 5×10^3 cells/cm^2, at weekly intervals. The number of cells obtained at each subcultivation was determined by counting the cell suspension with a Coulter counter. The number of population doublings (PD) achieved between subcultivations was calculated by $\log_2$ (number of cells obtained at subculture/number of cells inoculated) and the cumulative doublings achieved by cultures calculated. A culture was considered to be at the end of its in vitro replicative life-span when it did not achieve at least 1 PD in 4 weeks. Matsumura et al. (14) had shown that a culture in phase III does not double in cell number in this time period and is indeed at the end of its in vitro replicative life-span. Population doublings remaining (PDR) to a culture

were determined by subtracting the number of PD achieved by a culture at the time of use from the total number of doublings achieved by the culture.

Matsumura et al. (14) have reported the successful maintenance of senescent cultures for periods greater than 6 months. In this study to obtain cultures that were 6 months postsenescent, the medium over the culture was replaced with fresh medium weekly and the cultures were subcultured whenever they appeared confluent. Such cultures achieved about 1 PD every 4 months.

Cell Fusion. Of each parental cell type, 1×10^5 cells were inoculated into a 35-mm tissue culture dish. Eighteen hours later they were treated with 45% polyethylene glycol (PEG) (Baker) in HMEM for 55 sec. Twenty-four hours later the cells were trypsinized and resuspended, and 60-mm tissue culture dishes were inoculated with 20–300 cells per dish. The PEG treated cells were inoculated directly into selective medium, EMEM + 10% FBS containing hypoxanthine (1×10^{-4} M), aminopterin (5×10^{-7} M), thymidine (1×10^{-5} M), and ouabain (2×10^{-7} M). The dishes were incubated at 37°C in 5% CO_2–95% air, 98% relative humidity for 2 weeks. The dishes were scanned with a phase-contrast microscope to locate clones of 100 cells or more. Some of these clones were subcultured by the use of cloning cylinders. The dishes were then fixed and stained, and the size of the remaining clones was determined microscopically. The isolated clones were subcultured in selective medium to the end of their in vitro life-span or until they had achieved at least 100 PD. As a control for these experiments, the parental cell types were individually treated with PEG at the same time that mixtures of the parent cells were so treated. Twenty-four hours later, each parent culture was trypsinized and resuspended, and the two cultures were mixed prior to inoculation at 20–300 cells per 60-mm tissue culture dish. These mixtures of PEG-treated parental cells were treated in the same manner as the fused mixtures of parents. Life-span distributions of hybrid clones comprised both small, microscopically scored clones and large isolated clones. At least 300 clones were scored in each experiment to obtain such distributions.

The distribution of the life-span potentials of the individual cells within each parent culture was determined for each experiment by the colony size distribution (CSD) assay (15) and by isolating 25–200 single cells on glass chips (16) and carrying each isolate to the end of its in vitro life-span.

Viral T Antigen Expression. To determine if small nonproliferating hybrid clones expressed viral T antigen, 3000 cells of fused and unfused mixtures of the strongly T antigen-positive cell line, GM 639, and normal cells were inoculated onto 12-mm round glass coverslips in 35-mm dishes in selective medium. At the end of a 2-week incubation, these dishes were scanned for small (<20 cells) hybrid clones, which were marked. The selective medium in the dishes was replaced by fresh selective medium, and

the clones were incubated for an additional week. The clones that remained unchanged in cell number were then tested by an immunofluorescence assay for T antigen (17). Large hybrid clones were tested for the expression of T antigen 4 weeks after isolation.

Assay for Live Virus. VERO (African green monkey kidney) cells obtained from ATCC were used for this assay. To determine that the VERO cells used were indeed permissive for the virus, VERO cells were fused to GM 639 cells with PEG, and cytopathological effect (CPE) was observed in the fused cultures. The growth medium from these fused cultures and from hybrid cell cultures (CSC-301 $O^RC1 \times$ GM 639) was removed and placed over other monolayers of VERO cells. These cells were incubated for 7–14 days and inspected daily for CPE.

RESULTS

In order to determine the distribution of in vitro life-spans of hybrid cells resulting from the fusion of normal and SV40 transformed cells, a biochemical selection system utilizing ouabain and HAT was used. This selection system had been used successfully by others to select hybrids after the fusion of transformed (18) and normal (19) cells. However, it was found that about 10% of the normal parent cells inoculated into the selective medium remained as 1 or 2 cell clones at the end of a two week incubation. This is because normal cells remain viable though nondividing under conditions that arrest DNA synthesis (20). Therefore, the life-span distributions of the hybrid clones were corrected for the survival of normal parent cells, using dishes inoculated with unfused mixtures of PEG-treated parental cell types, to determine the survival of nonhybrid cells in selective medium. The low cell inoculum used (1–12 cells/cm^2) eliminated metabolic cross-feeding which can occur when two unfused parental cells are in contact.

Figure 1 shows the result of a typical hybridization experiment involving SV40-transformed (VA13) and normal (GM 1662 O^RCl) cells. The normal parent cells used in this experiment had reached the end of their in vitro replicative life-span (PDR O) and were unable to undergo further doublings in culture. The life-span distribution of the VA13 cells was similar to that obtained with HeLa cells (21). Of the VA13 cells, 80% were capable of indefinite proliferation. All the hybrid clones studied were able to achieve at least one cell division. Thus the fusion of a virally transformed cell with a normal cell that has stopped dividing results in the initiation and completion of at least one cell division in the hybrid. Of the hybrid clones, 70% were capable of fewer than 7 PD, while 30% achieved from 16 to 62 PD in vitro. Thus a significant number of the hybrids were capable of many more divisions than the normal parent. However, cell proliferation eventually

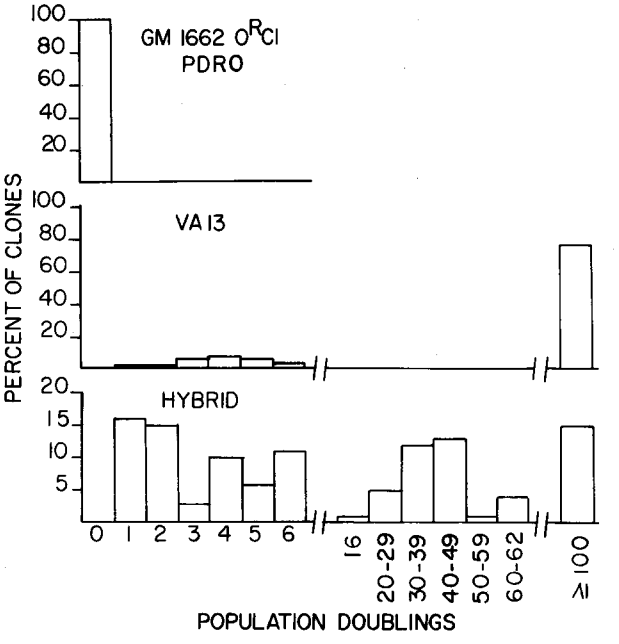

Fig. 1. Life-span distributions of parental cell cultures and hybrid clones. The life-span distributions of the parent cells were determined by methods explained in Materials and Methods. The distribution of hybrid life-span is derived from small hybrid clone sizes scored microscopically and the life-span achieved by extensively dividing isolated clones before they entered the nonproliferative state. In half the isolated clones, one or two cells would transform, form foci of dividing cells, and divide indefinitely. Thus 15% of all the hybrid clones eventually exhibited an indefinite life-span.

ceased in all of these hybrid clones. From a comparison of parental life-span distributions with the life-span distribution of hybrid clones, one must conclude that the senescent phenotype is temporarily reversed in all hybrids but that it is eventually reexpressed. The in vitro age of the normal parent did not appear to affect these results (Table 1). In addition, the results were not dependent on the parent cell lines used in the fusions (Table 2). The percentage of hybrid clones that could achieve more than 15 population doublings was the same whether the double mutant normal parent used had been isolated spontaneously (CSC-301 O^RCl) or after mutagenesis (GM 1662 O^RCl) or whether the SV40-transformed parent used was intermediate (VA13) or strongly positive (GM 639) for T antigen.

Twenty-eight hybrid clones were subcultured. These clones ranged in size from 100 to several thousand cells at the time of subcultivation. The clones were identified as hybrid on the basis of the fact that they could proliferate when kept continuously in the presence of HAT and ouabain. Karyological analysis of these clones was inconclusive because the chromo-

Table 1. Proliferative Potential of Hybrid Clones

In vitro age of normal parent[a]	Isolated hybrid clone	Population doublings achieved in vitro before crisis	Change to indefinite life-span[b]
PDR 6	1	28	–
	2	35	+
	3	35	+
	4	42	+
	5	58	–
	6	62	–
	7	62	+
PDR O	1	28	–
Senescent	2	32	–
	3	40	+
	4	41	–
	5	42	–
	6	43	–
	7	60	–
(1 month post senescent)	1	24	+
	2	30	+
	3	35	+
	4	45	+
	5	48	+
(6 months postsenescent)	1	16	+
	2	20	+
	3	30	–
	4	35	+
	5	36	+
	6	36	–
	7	40	–
	8	40	+
	9	43	–

[a] Parent used: GM 1662 O^RCl.
[b] Cultures achieved greater than 100 population doublings after the appearance of foci of dividing cells within nonproliferating cultures.

some complement of the cells within the SV40-transformed culture varied from 45 to 89 chromosomes. All these clones achieved at least 16 PD, irrespective of the parents used for fusion (Table 1) and went through two identifiable phases of growth (Fig. 2). An initial period of active division was followed by a period during which the division essentially ceased and the clones entered a phase which resembled the "crisis" phenomenon observed during viral transformation of normal cells in vitro (22). During this period a large number of cells lysed. Micronucleation, nuclear lobulation, and nuclear blebbing were observed in the cells. In approximately half the subcultured hybrid clones, foci of dividing cells appeared in the cultures. One or two foci occurred per 10^5 cells. Cells in these foci continued to divide until they had repopulated the culture. Such cultures then achieved more than 100 PD, at which time they were discarded. The growth rate of the "transformed"

Table 2. Effects of Different Parental Cell Lines on the Frequency of Hybrid Clones that Achieved ≥15 PD

SV40-transformed parent cell line	Normal parent cell line	Hybrid clones that achieved ≥15 PD (%)[a]
VA13	GM 1662 O^RCl PDR 6	25
VA13	GM 1662 O^RCl PDR O (6 months)	36
VA13	CSC-301 O^RCCl PDR 8	32
GM639	GM 1662 O^RCl PDR O (6 months)	40
GM639	CSC-301 O^RCl PDR O	25

[a]After 2-week incubation.

cultures was always greater than that of the original clone (Fig. 2). In the case of the clones that did not exhibit foci of dividing cells, the cultures were maintained for periods of up to 4 months with weekly feedings and never resumed division. Thus, in all experiments performed, most of the hybrid clones (85–90%) exhibited a finite in vitro life-span. About 70% failed to achieve as many as eight doublings and about 15% were "transformed" to indefinite life-span.

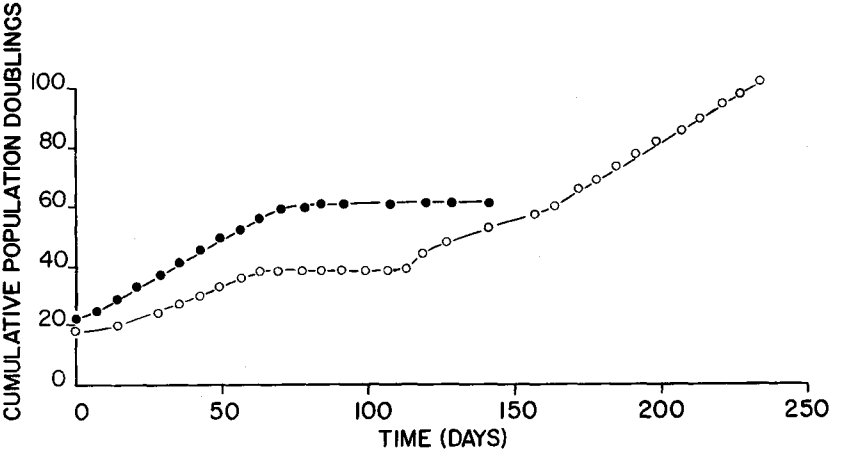

Fig. 2. The cumulative population doublings versus time for two isolated hybrid clones. Both clones show an initial period of rapid growth, followed by a period of nondivision. In one clone (●) the culture never resumed division. In the other clone (○), foci of dividing cells appeared, repopulated the culture, and went through more than 100 PD. The growth rate of the established transformant culture was much higher than that of the original clone.

It was necessary to determine if the senescent phenotype exhibited by the majority of these clones was the result of loss of viral DNA from these cells. Thirty hybrid clones that had ceased proliferation were tested for the presence of T antigen. All the cells within the nondividing hybrid clones that were tested were found to express T antigen irrespective of the fact that they had achieved 2 or 20 PD after fusion. This indicates that at least the viral A gene region of the SV40 genome was present and being expressed in these clones.

Another possible reason for the cessation of division of hybrid clones could be the production of live virus resulting in the lysis of most of the cells within the clone. The cells used in these fusions are human cells which are semipermissive for SV40 virus; therefore it is possible that live virus progeny are replicated and released from cells in the hybrid clones. Viral T antigen would be expressed simultaneously. Viable SV40 virus was not detected when the growth medium, removed from small and large hybrid clones that had stopped dividing, was placed over monolayers of VERO cells. However, lytic SV40 virus was released after fusion of the SV40-transformed parent cells with VERO cells. Cytopathological effect (CPE) was observed in such fused cultures, and the medium removed from these cultures also caused lysis of VERO cell monolayers.

DISCUSSION

When normal human fibroblasts that had ceased proliferation were fused with SV40-transformed fibroblasts, cell proliferation was reinitiated in the resultant hybrids. This occurred even when the senescent normal parent cells had ceased replicating and were nonproliferating for six months. In about 70% of the hybrids the induced proliferative activity ceased within seven PD. In the remainder of the hybrid clones the proliferation was much more extensive (16–62 PD). However, even these relatively long-lived hybrid clones eventually ceased proliferating. These results indicate that the processes that lead to in vitro senescence in normal cells can be reversed only temporarily by fusion with an SV40-transformed cell. It appears that the mechanisms limiting proliferation potential continue to operate effectively even when an integrated SV40 genome is present in the cell.

In rare instances (1 or 2 foci/10^5 cells) cells with indefinite growth potential occurred in hybrid cultures. These rare transformants could have arisen from cells that had lost the genetic information controlling the in vitro life-span (3, 7, 23) or from cells in which the viral genome had been relocated in a way such that the senescent information was rendered ineffective.

The results agree qualitatively with the results obtained by Muggleton-Harris and DeSimone (9) and Bunn and Tarrant (8). However, the percentage of extensively dividing hybrid clones (30%) obtained in this report was

much higher than that obtained by Muggleton-Harris and DeSimone (9). Furthermore, in this laboratory when HeLa cells were fused with GM 1662 O^RCl or CSC-301 O^RCl, the number of extensively dividing hybrid clones obtained was 1/100 of the normal parent cells inoculated as compared with 0.043/100 obtained by Bunn and Tarrant (8). The increased efficiencies of dividing hybrids obtained in this study could be due to the different parental cells and methodology used.

Matsumura (10) reported that no large proliferating hybrid clones were obtained after fusion of normal cells that were 6 months postsenescent with UV-irradiated SV40-transformed cells. The results of this report suggest that UV irradiation must have impaired the ability of the SV40-transformed cell to reinitiate cell division.

It is clearly demonstrated here that the hybrid clones which have limited division potential express viral T antigen. Thus these clones retain an actively transcribed viral A gene. Therefore the expression of a senescent phenotype is not a result of the loss of viral DNA. This also demonstrates that the expression of viral T antigen can occur in the absence of indefinite proliferative potential, indicating that there is more than one process involved in causing and maintaining cell transformation.

The entire viral genome is present in the SV40-transformed parent cells used. This has been demonstrated by DNA hybridization methods in other studies (24) and in this study by the production of lytic virus following fusion of the SV40-transformed parents with permissive VERO cells. However, the possibility that live viruses are being produced and thus causing a cessation of cell division in the clones exhibiting a limited in vitro life-span has been eliminated. Thus it can be concluded that the hybrid clones do contain viral DNA which is expressed, do not produce live virus, and continue to exhibit a limited life-span. If one considers the fact that the change from limited to unlimited (>100 PD) life-span is a rare event ($1–2/10^5$ cells), one must conclude that senescence and the control of long-term cell division is a dominant characteristic of cells. Since a great deal of nuclear morphological change is seen to be occurring in all hybrid clones, it is tempting to postulate that some part of the normal cell genome must be segregated or inactivated by rearrangement before a hybrid cell can divide indefinitely. This would explain why the change to unlimited life-span occurs so rarely.

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