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SV40 T Antigen *Alone* Drives Karyotype Instability That Precedes Neoplastic Transformation of Human Diploid Fibroblasts

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To define the role of SV40 large T antigen in the transformation and immortalization of human cells, we have constructed a plasmid lacking most of the unique coding sequences of small t antigen as well as the SV40 origin of replication. The promoter for T antigen, which lies within the origin of replication, was deleted and replaced by the Rous sarcoma virus promoter. This minimal construct was co-electroporated into normal human fibroblasts of neonatal origin along with a plasmid containing the neomycin resistance gene (neo). Three G418-resistant, T antigen-positive clones were expanded and compared to three T antigen-positive clones that received the pSV3neo plasmid (capable of expressing large *and* small T proteins and having two origins of replication). Autonomous replication of plasmid DNA was observed in all three clones that received pSV3neo but not in any of the three origin minus clones. Immediately after clonal expansion, several parameters of neoplastic transformation were assayed. Low percentages of cells in T antigen-positive populations were anchorage independent or capable of forming colonies in 1% fetal bovine serum. The T antigen-positive clones generally exhibited an extended lifespan in culture but rarely became immortalized. Large numbers of dead cells were continually generated in all T antigen-positive, pre-crisis populations. Ninety-nine percent of all T antigen-positive cells had numerical or structural chromosome aberrations. Control cells that received the neo gene did not have an extended life span, did not have noticeable numbers of dead cells, and did not exhibit karyotype instability. We suggest that the role of T antigen protein in the transformation process is to generate genetic hypervariability, leading to various consequences including neoplastic transformation and cell death.

Key words: immortalization, chromosome damage, SV40, simian virus 40, large T antigen, karyotype instability

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Despite years of extensive research, the role of the SV40 large T antigen in neoplastic transformation of human cells has not been clearly defined. It is called an immortalizing oncogene, and yet it is well known that immortalization of T antigen-positive human cells is a rare event [1–3]. T antigen-positive cells have been observed to senesce [4]. T antigen is reported to be necessary to maintain the immortal state in rodent cells when temperature-sensitive viral mutants have been used [5]. A similar result has recently been reported in human cells [42]. Chang et al. used a plasmid that should express only large T to extend the life span of human embryonic kidney cells (HEK). However, since the origin of replication was present in these plasmids, it was not clear if T protein was sufficient to extend the cellular proliferative potential [6].

Early investigators used virions to transform and immortalize human cells. Infectious virions express viral late proteins and replicate viral DNA, in addition to expressing both early viral proteins, large and small T antigens [2,7]. Cells that survived productive viral infection were reported to be T antigen positive and to have acquired neoplastic indicators, such as anchorage independence, but only rarely could immortal cell lines be derived [1]. These transformed mortal cells were said to be "abortively transformed" [7]. It was not clear that large T antigen alone was sufficient to cause any direct neoplastic change. Karyotype instability and cell death were observed in these cells but could not be attributed to T antigen per se owing to the presence of other viral proteins and viral infectivity [2,7]. Early events preceding transformation could not be studied because of the complexity of partial viral infection and the lack of selectable biochemical markers.

More recently, plasmid constructs such as pSV3gpt have been used to immortalize human fibroblasts. These constructs, however, contain the SV40 origin of replication and thus can exhibit autonomous replication of plasmid DNA in semipermissive human cells, again confounding attempts to study T antigen-induced change [8–16]. pSV3gpt and pSV3neo contain the entire early region of the SV40 genome, and thus small t can be expressed with large T [14,15]. Origin defective plasmids have been used but again were capable of expressing both T and t antigens [17–20]. T antigen binds to its own promoter and downregulates transcription, which may further complicate studies on its effect on human cells. No reported studies have systematically examined the role of T antigen sans small t and the viral replication and promoter mechanisms on precrisis human cells. Considerable evidence indicates that large T antigen is indirectly responsible for neoplastic transformation of human cells [1–4,6–12,16–20]. It is unclear what direct effects if any the T protein exerts on human fibroblasts. T antigen is known as an immortalizing oncogene, yet immortal human cell lines are derived at rare, mutation-like frequencies even when T antigen is retained by biochemical selective pressure [9]. The present study was undertaken to clarify the role T antigen may play in the transformation and immortalization of diploid human fibroblasts.

A minimal plasmid construct, containing only the T antigen DNA sequences of a small t deletion mutant virus of SV40 (dl884) was electroporated into diploid human fibroblasts. T antigen-positive clonal strains were derived after cotransfection with a plasmid containing the neo gene and selection of individual colonies in Geneticin (G418). Both input plasmids were transcriptionally driven by the Rous sarcoma virus (RSV) promoter in order to remove the SV40 origin and promoter region and prevent replication at the viral origin. For comparative purposes, pSV3neo was also electroporated, and G418-resistant clones were derived. Several in vitro neoplastic indicators were examined

immediately after clonal expansion of G418-resistant colonies. Not all G418-resistant colonies were T antigen positive, and these clones served as negative controls.

Newly expanded, G418^r clones that were T antigen positive in virtually 100% of the cells were assayed as soon as possible for karyotype instability, anchorage independence, morphology, colony formation in low serum, immortalization, and tumorigenicity. We found that metaphase chromosome spreads were aberrant numerically and structurally at a high frequency in three such clones. Chromosome number varied so much from cell to cell, it was difficult to establish modal numbers for the T antigen-positive cell strains. Structural aberrations were present in the chromosomes of the three clones at a high frequency with from 54 to 78% of the cells having at least one aberration. Chromatid breaks, gaps, and dicentrics were frequently observed. These changes were not observed in G418-resistant clones that were T antigen negative. Conversely, T antigen-positive clones were virtually negative for standard neoplastic markers such as anchorage independence and reduced requirement for serum growth factors. None of these cells were immortal at the time these initial assays were done, although rare variants have since emerged from cultures in crisis in two instances. We therefore suggest that the primary role of T antigen is to act as a mutagen, inactivating the finite proliferative life span genes and allowing variants with proliferative advantages typical of neoplastic cells to be selected.

MATERIALS AND METHODS

Plasmids

pRSVEdl884 was constructed by ligation of the 2,391 nucleotide pair (np) BamHI/HindIII (partial digest) fragment from the SV40 deletion mutant, dl884, to the BamHI/HindIII 3,388 np fragment of pRSVcat [21,22]. In this construct, the gene containing large T only is under the control of the RSV promoter; the SV40 promoter and origin of replication were removed. Similarly, pRSVneo was constructed by ligation of the same RSV promoter containing fragment to the BamHI/HindIII fragment with the neo gene from pSV2neo [15]. Therefore, neither plasmid (Fig. 1A) to be cotransfected should support autonomous replication, since neither plasmid contained an SV40 origin of replication (pSV3neo uses the SV40 origin and promoter region to drive transcription of the neo gene as well as T/t proteins). pSV3neo (Fig. 1B) has been described [15].

Cells and Culture Conditions

Human diploid fibroblast strain 43 (HSF43) (kindly provided by Ms. Anita Stevenson, LANL) derived from neonatal foreskin was cultured in alpha minimum essential medium (AMEM) containing 10% fetal bovine serum (FBS). All cells were negative for mycoplasma contamination by the method of House and Waddel [23]. Cells were counted using a Coulter counter, and doublings were calculated with the doubling formula $LnF - LnI/Ln2 = D$ where F is the number of cells per flask at subculturing and I is the initial number of cells per flask. Expansion of G418-resistant single cells to 1×10^6 cells thus equaled approximately 20 doublings. If a cell population failed to double in 3 months, the cells were counted and discarded. At least, 1×10^6 T antigen-positive cells of each clone were carried into crisis, but this number dropped as cell death exceeded the growth rate. If the cells went through a visible crisis and doubled the

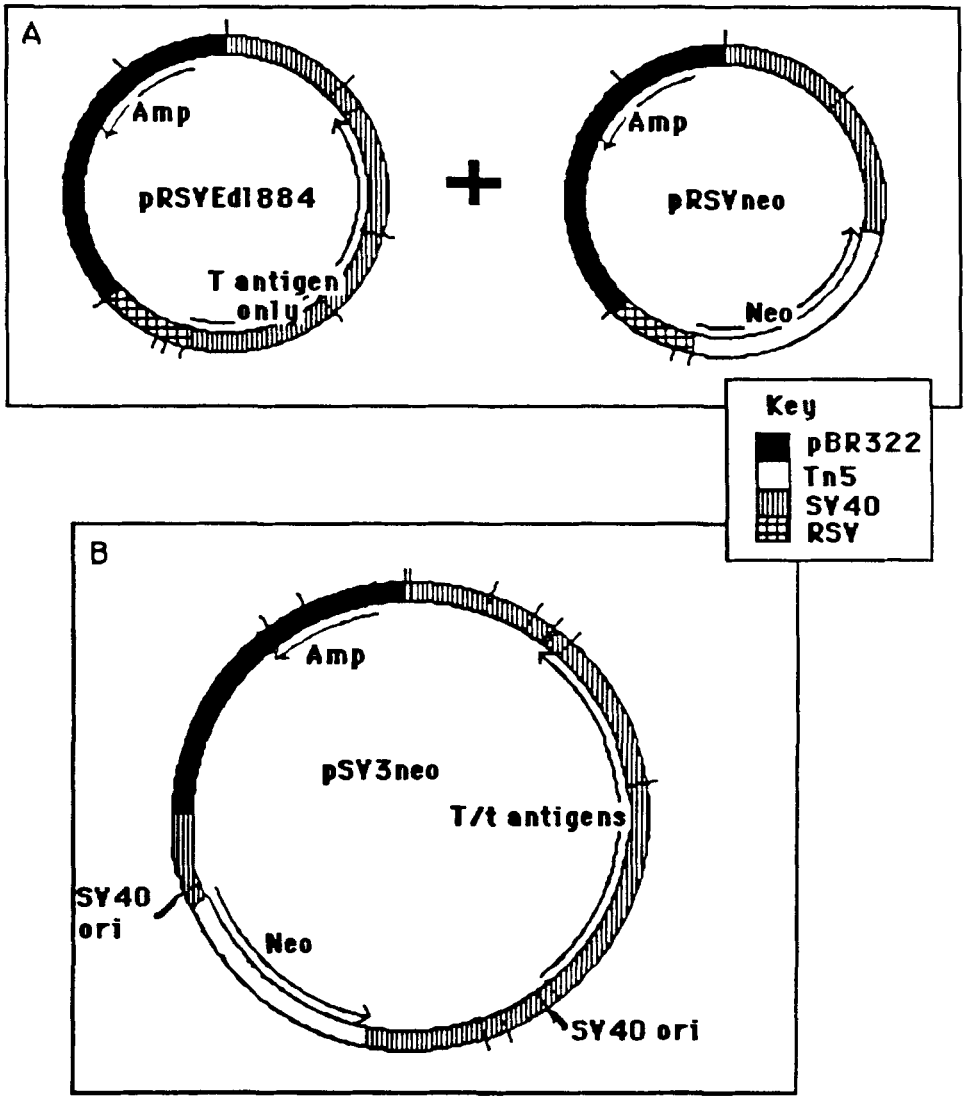

Fig. 1. HSF 43 cells were electroporated with either (A) pRSVEdl884 and pRSVneo whereby there were no SV40 origins of replication and most of the sequences unique to small t were deleted or (B) pSV3neo containing two SV40 origins plus small t antigen sequences.

cumulative population doublings (CPD) of the parental strain at senescence (~71 CPD), then they were scored as immortal.

For each electroporation reaction, 1.7×10^6 HSF43 cells (~24 CPD) were used. Electroporation was performed at 220 volts and 960 μF with the BioRad Gene Pulser [24]. Ten micrograms of plasmid DNA was used in the case of pSV3neo or when co-electroporation was done, 10 μg of pRSVEdl884 were always used, and pRSVneo was diluted. Therefore, a 1:1 co-transfection would contain 20 μg plasmid DNA. The electroporated cells were divided equally into ten 100 mm culture dishes containing

alpha MEM with 10% FBS and incubated at 37°C for 55 hours, at which time additional AMEM with 10% FBS containing Geneticin (G418, Gibco) was added to a final concentration of 400 μg/ml. At that time, two dishes from each set were trypsinized and counted in order to measure transfection frequency. After 12 days, when colonies were to be selected, four of the remaining eight dishes were stained for assessment of transfection frequencies, and four were used for cloning. Colonies were selected on the basis of size (>500 cells) and not morphologic transformation. No more than two colonies were ringed from any one dish. Picked colonies were incubated in T150 flasks and left undisturbed for 18 days.

After this period the cells were subcultured, and 10^4 cells were plated on LabTek slides to assay for expression of T antigen using indirect immunofluorescence. The cells were fixed for 3 minutes in acetone and stored at 4°C. A 1:5 dilution of monoclonal antibody in phosphate-buffered saline (PBS) was reacted with the fixed cells for 1 hour at room temperature, washed 2× for 5 minutes each in PBS, rinsed in distilled H_2O, and reacted for 20 minutes at 37°C in the dark with fluoresceinated goat anti-mouse antisera (CalTag) diluted 1:5 in PBS. The monoclonal antibodies that were used were antibody 2 (Oncogene Science), specific to the amino terminal half of large T, or antibody 1 (Oncogene Science), specific to the amino terminal third of large T and that also reacts with small t. Cells were scored positive if bright nuclear fluorescence was observed relative to cytoplasmic fluorescence and relative to cells that were reacted with PBS and then to second antibody.

Western Analysis of Nuclear Proteins

To determine the size and relative amount of T antigen protein, cells were grown on six 150 mm dishes, rinsed twice with PBS without Mg^{++} or Ca^{++} (PBSw/o), and removed with rubber policemen. The entire procedure was performed at 4°C. Cells suspended in PBSw/o were centrifuged at 200*g* and resuspended in 2–4 ml homogenization buffer (homo; 10 mM mannitol, 1 mM N-(2-hydroxyethyl) piperazine-n'-2-ethanesulfonic acid (Hepes), pH 7.4, and 2 mM $MgCl_2$). Cells were homogenized by passage once through an 18 gauge needle and twice through a 25 gauge needle. Triton-X100 (10% in H_2O) was added to a final concentration of 0.5%, while vortexing and nuclear pellets were collected and washed 3× with homo buffer by centrifugation. After the final wash 1/10 volume homo buffer was added, the $MgCl_2$ concentration was adjusted to 3 mM, and 30 units of DNase I was added (Promega RQ) and incubated at 20°C for 20 minutes. An equal volume of Western blot buffer (WB; 4.0% SDS, 125 mM Tris, pH 6.8 and 25% glycerol) was then added, and the nuclear extract was centrifuged at 430,000*g*. The supernate, representing soluble nuclear proteins, was collected and stored in liquid nitrogen.

Protein concentrations were determined by the BCA* protein assay (Pierce) and 50 μg of protein was loaded into wells of a 4% stacking gel (pH 6.8) and electrophoresed on a 5–15% polyacrylamide gradient gel containing 0.4% SDS at pH 8 [25].

Proteins were blotted onto nitrocellulose filters for 4 hours by electrotransfer at 500 mA [26]. These filters were then prehybridized in PBSw/o containing 0.5% Tween 20 (BioRad) and 2.5% nonfat dry milk (Carnation) for 2 hours at room temperature with agitation. Antibodies were then added at a 1:50 dilution to the above mixture and allowed to hybridize overnight. Antibodies used were either monoclonal antibody 2 described above or a polyclonal antisera to T antigen kindly provided by Dr. John

Lehman. After a series of 5 PBSw/o washes over a 30 minute period, blots were resealed with fresh prehybridization mixture containing 1×10^6 cpm I^{125}-labeled protein A per ml solution. The protein A reaction was allowed to procede for 2 hours, and the blots were washed 3×in PBSw/o for 15 minutes each. After drying the blots were placed on Kodak X-Omat XAR-5 Xray film in casettes with intensifying screens at −70°C.

Southern Analysis

Cellular DNA was prepared using standard techniques [27]. Ten micrograms of cellular DNA was electrophoresed in 0.7% agarose gels and transferred onto nylon membrane (Zetabind) under alkaline conditions. The DNA fragment used as probe was the 936 base pair HpaI/DraII (SV40 base pair #3733-2797) fragment at the carboxy end of the large T gene, which was excised from pSV3neo. Probes were labeled with alpha P^{32} nucleotide triphosphates by nick translation according to standard techniques.

Cell Death and Growth Rate

Growth rate of newly expanded clones was determined. First 1×10^4 cells were plated onto 60 mm culture dishes. At 24 hour intervals thereafter duplicate dishes of each cell type were trypsinized, and the cells were counted in a Coulter counter for 5 days.

The percentage of accumulated dead cells was also determined. For this 10^4 cells were plated onto 60 mm dishes and allowed to grow for 1 week. When the dishes were removed from the incubator they were examined, and an estimate of percent confluence and cell death was made. Subsequently, media containing the majority of dead cells was rinsed across the dishes to dislodge loosely attached cells and was placed into 15 ml centrifuge tubes with 100× Hoechst 33342 (final concentration 10 μM) and 100×propidium iodide (final concentration 5.0 μg/ml) at 4°C. Cells attached to the dishes were detached by trypsinzation and combined with the dead cell fraction. Cells were concentrated by centrifugation at 200*g* and resuspended in 0.5 ml PBS containing 1×Hoechst and propidium iodide. Cells were counted on a hemacytometer. Epifluorescent UV excitation allowed live Hoechst-stained cells (blue nuclei) to be discriminated from those cells having lost their membrane integrity and that contained pink nuclei.

Karyotype Analysis

Cells for chromosome analysis were blocked in mitosis with 0.1 μg Colcemid (Gibco)/ml media for <2 hours, and metaphase spreads were prepared and stained in 10% Giemsa stain. Fifty metaphase spreads were counted and scored for aberrations.

In Vitro Transformation Assays

To determine if T antigen-induced anchorage independent growth, 60 mm dishes were coated with 0.8% sterile agarose and 3×10^5 cells were suspended in 10 ml 1.5% methylcellulose in AMEM with 10% FBS and distributed equally to each of three gridded dishes, which were incubated at 37°C for 21 days. On the day following the initial plating, the dishes were examined for clumps. Those dishes with clumps were discarded. At day 21, one dish of each cell type was counted. Only colonies having an average diameter ≥100 μm were judged as positive. The cells were assayed immediately after clonal expansion of G418-resistant cells at 50-55 CPD.

To determine if T antigen increased the ability of cells to form colonies when serum growth factors were decreased, cells were plated (500 cells per 100 mm dish) at approximately the same CPD level as for anchorage independence in 10% FBS and allowed to attach 12 hours. After 12 hours, they were rinsed twice with serum free media and either 1% or 10% media was added. After incubation for 10 days, the dishes were rinsed with isotonic saline and stained 15 minutes with 1% crystal violet in 70% ethanol. Colonies were scored as positive if they contained $\geq$50 cells.

To determine if any of the T antigen-positive clones were immortal immediately after clonal expansion, a modified serial clonogenicity assay was performed [28]. Ten 100 mm dishes were plated with 500 cells/dish. After 12 days the dishes were examined. If visible colonies were present, then 5 dishes were trypsinized, and the cells were pooled and replated as above. If the cell count was not above the Coulter counter background, the remaining cells (80%) were all plated. The other 5 dishes were stained, and colonies $\geq$50 cells were counted. The cell count allowed an estimation of the number of doublings accumulated, at each serial step, to be made, and the colony counts allowed plating efficiency to be determined. Cells were scored as immortal if they had accumulated twice the number of CPD as their untransfected counterparts.

Tumorigenicity

Tumorigenicity was assayed by injecting a slurry of cells containing at least 1×10^7 cells into collagen sponges implanted 10 days earlier in nude mice [29]. The preimplanted sponge assay has been found to increase sensitivity of the tumorigenicity assay, by providing a vascularized substrate upon which cells can attach and progress in vivo. Cells that do not form tumors when injected subcutaneously may form tumors when injected into preimplanted sponges [29].

RESULTS

After electroporation with the various plasmids, 18 G418-resistant clones were ringed and transferred to T150 flasks containing selective media. The cells were incubated undisturbed for 18 days. After visual examination, the 18 independent clones of HSF 43 were then subcultured. Of the 18 clones, four had received pSV3neo and were designated SV3-1 through SV3-4. The remaining 14 clones were *Co-T*ransfected (electroporated) with pRSVEdl884 (expressing T only) and pRSVneo and were designated CT followed by the ratio of pRSVneo/pRSVEdl884 contained in the electroporation cocktail. For example, as shown in Table I, when the ratio of neo to T was 1:10, two of the resultant three G418-resistant colonies were T antigen positive. These clones were designated CT10-1,2 and 3 (10 representing the 1:10 ratio of neo to T). Since pRSVneo was diluted relative to pRSVEdl884 the number of G418-resistant colonies decreased, while the number of G418 resistant colonies that were also T antigen positive increased (Table I).

After expansion of the clones in T150s, they were subcultured and also plated into wells of LabTek slides to assay for the production of T antigen using indirect immunofluorescence. Three SV3s of four were T antigen positive, and 3 of 14 CTs were positive.

The number of cells per T150 flask ranged from 4×10^4 to 2.6×10^6 for the 12 T antigen-negative clones and 2×10^6 to 2×10^7 for the six T antigen-positive clones. Cell morphology was extremely variable from clone to clone with several of the T antigen-

TABLE I. Transfection Frequency and Frequency of T Antigen Expression

Plasmid introduced	Transfection freq.[a]	T antigen[b]
1. HeBS buffer only	NA[c]	NA
2. pRSVneo	2.4×10^{-5}	NA
3. pRSVneo/pRSVEdl884 1:1[d]	2.5×10^{-5}	0/7
4. pRSVneo/pRSVEdl884 1:2[d]	1.3×10^{-5}	1/4
5. pRSVneo/pRSVEdl884 1:10[d]	3.1×10^{-6}	2/3
6. pSV3neo	4.2×10^{-6}	3/4

[a]No. of large colonies (>500 cells) per cell present at the time of G418 addition.
[b]No. of large T antigen-positive colonies per large neo-resistant colony cloned. Scored by indirect immunofluorescence.
[c]Not applicable.
[d]pRSV neo was progressively diluted, and the concentration of pRSVEdl884 was kept constant. Thus, the No. of neo-resistant colonies decreased, but the percent T antigen-positive colonies increased accordingly.

negative clones growing in dense piled up "foci." The best example of this morphology in a T antigen-negative clone was CT1-6, which grew in colonies that grew upward rather than outward. Although, initially these cells grew at a moderate rate they did not cover the surface of the flask but piled up in a crisscross fashion. Conversely, T antigen-positive clones (90–100% positive) had morphologies ranging from normal fibroblast to epithelial-like.

Three T antigen-negative clones were subcultured as a negative control until they failed to double the population in 3 months (Fig. 2). They reached 51-56 CPD. This group includes SV3-2, transfected with pSV3neo yet T antigen negative. A T antigen-negative clone such as SV3-2 could have been caused by disruption of T antigen sequences during integration of pSV3neo into the cellular DNA. Two separate monoclonal antibodies that react with amino terminal epitopes of the large T antigen (see "Materials and Methods") were used in these assays, but the possibility exists that a T antigen-negative clone, such as SV3-2, expressed a smaller peptide or that the epitopes were otherwise altered.

The three pSV3neo-transfected, T antigen-positive clones (SV3-1, SV3-3, SV3-4) accumulated 69-83 CPD prior to crisis (HSF43, the parental strain reached 71 CPD) (Fig. 2). All entered crisis; SV3-1 and SV3-3 did not double in 3 months and were discarded (Fig. 2). A rapidly proliferating colony was observed in one flask of SV3-4. This colony was subcloned and was expanded to millions of cells; it is presently at 158 CPD, and is therefore scored as immortal. The 3 pRSVEdl884 transfected, T antigen-positive clones (CT2-2, CT10-1, CT10-2) entered crisis at 86-101 CPD (Fig. 2). CT 10-2 emerged from crisis and is also immortal, presently at 151 CPD.

Western Analysis of T Antigen

Soluble nuclear proteins were isolated and examined with the monoclonal antibody specific for large T protein (Fig. 3) and with a polyclonal antisera to T protein (not shown). T12 is a T antigen-positive human fibroblast line [30]. T12 cells were used as a positive control. The results were in total agreement with the indirect immunofluorescence data with the same six T antigen-positive clones showing a protein band corresponding to a molecular weight of approximately 89 kD (Fig. 3). Three T antigen-negative, G418-resistant clones were tested and were negative in this assay as was the parental, HSF 43.

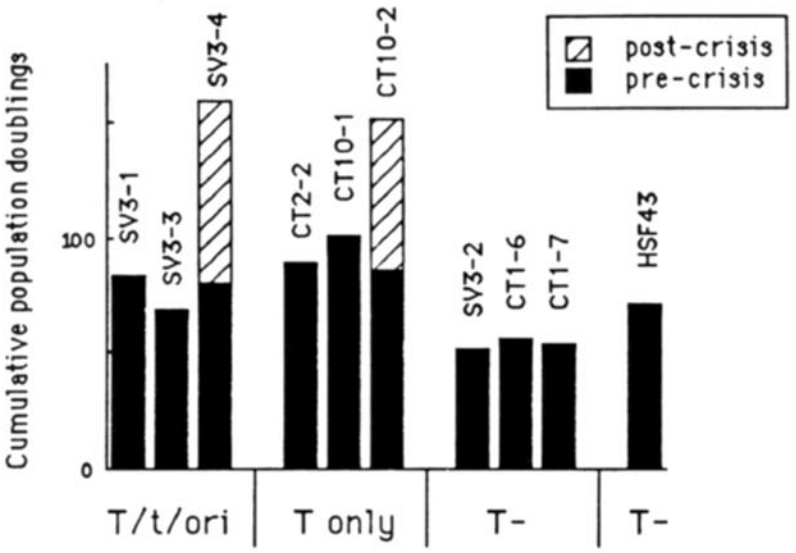

Fig. 2. The in vitro life span of T antigen-positive clones was compared to three T antigen-negative clones and to the parental mass culture.

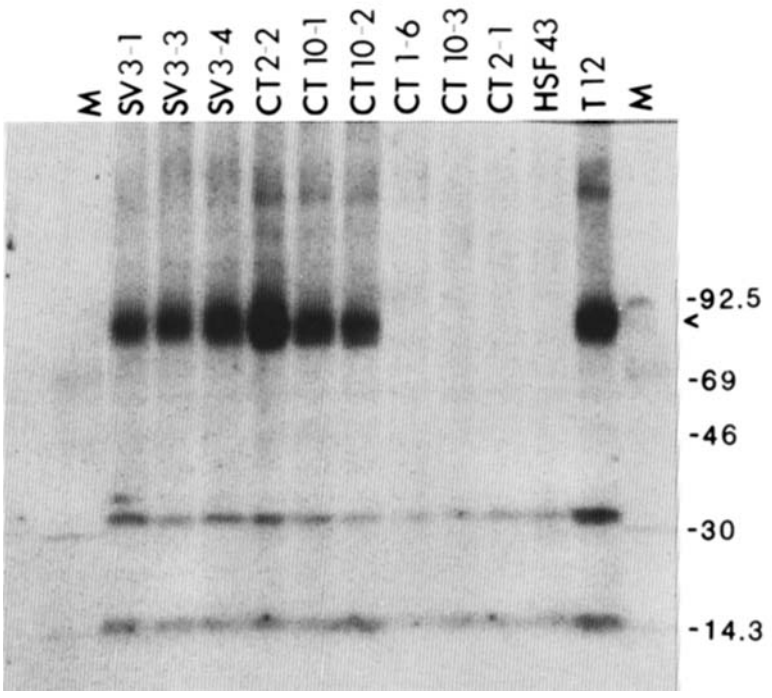

Fig. 3. Western blot analysis of soluble nuclear proteins using a monoclonal antibody specific to SV40 T antigen. T12 is an immortal tumorigenic human cell line that was known to be T antigen positive and therefore served as a positive control. M = molecular weight markers.

Southern Analysis

Southern analysis of the electroporated clones showed that the T antigen DNA sequences were integrated into the high molecular weight cellular DNA of the T antigen-producing, G418-resistant clones (Fig. 4A). The three clones that received pSV3neo plasmid show evidence of autonomously replicating DNA elements (AREs). The T antigen-positive CTs, lacking the viral origin of replication, did not contain these AREs.

When the cellular DNA from the T antigen-positive CTs was digested with Bgl I (which cuts only once in pRSVEdl884) and probed with T antigen-specific probe, one to five bands of varying intensities were observed (Fig. 4B). When the cellular DNAs were restricted with Sac I (which does not cut within the plasmid), single bands were observed for CT2-2 and CT10-2 (Fig. 4C). This result indicates a single integration site in both cell strains. However, within that single integration site, CT10-2 had multiple copies of the T sequences as shown by the Bgl I digest. CT10-1 went from 2 clear bands of equal intensity in the single cutter digest to a smeared band with the no-cutter, presumably due to an incomplete Sac I digestion.

Cell Death and Growth Rate

A large number of dead and dying cells were noted in all T antigen-positive clones and were not observed in control or parental cultures. To quantitate this observed phenomena, the percentage of accumulated dead cells relative to total cell number was measured daily for 3 days. The assay was performed soon after clonal expansion (CPD 51-54) and therefore well before the crisis observed for T antigen-positive cells and just prior to senescence of T antigen-negative clones. HSF43 parental cells at ~20 CPD were included for comparison. The assay was performed at the end of exponential growth in order to have enough cells to reliably score on the hemacytometer (see "Materials and Methods"). Cells left on ice for an additional hour and then recounted did not have significantly increased percentages of dead cells, indicating that the assay itself was not killing cells. The results are shown in Figure 5A. T antigen-positive cells generated large percentages of dead propidium iodide-stained cells relative to live Hoechst-stained cells. SV3-3 had the highest percentages of dead cells, which was consistent with microscopic observations.

Exponential growth rate was measured at the same CPD as cell death. The results shown in Figure 5B indicate that T antigen-positive SV3 clones grew more rapidly than the T antigen-positive CTs. None of the T antigen-positive cells appeared to grow as fast as young parental HSF43 cells, but this result is difficult to interpret in light of the observed cell death and the large difference in cumulative population doublings (>20). The growth of the T antigen-negative clones was initially close to the parental strain but rapidly plateaued, thus causing the large standard deviation seen in Figure 5B.

Neoplastic Phenotype

Immediately after clonal expansion the T antigen-positive clones and several T antigen-negative clones were assayed for three common descriptors of the neoplastic phenotype; anchorage independence, ability to form colonies in 1% FBS versus 10% FBS, and immortalization.

Cells that had received the plasmid pSV3neo had the highest percentage of cells that could form anchorage-independent clones in methylcellulose, which was still less than 1.0% in each case as shown in Table II. Chinese hamster ovary CHO cells from a

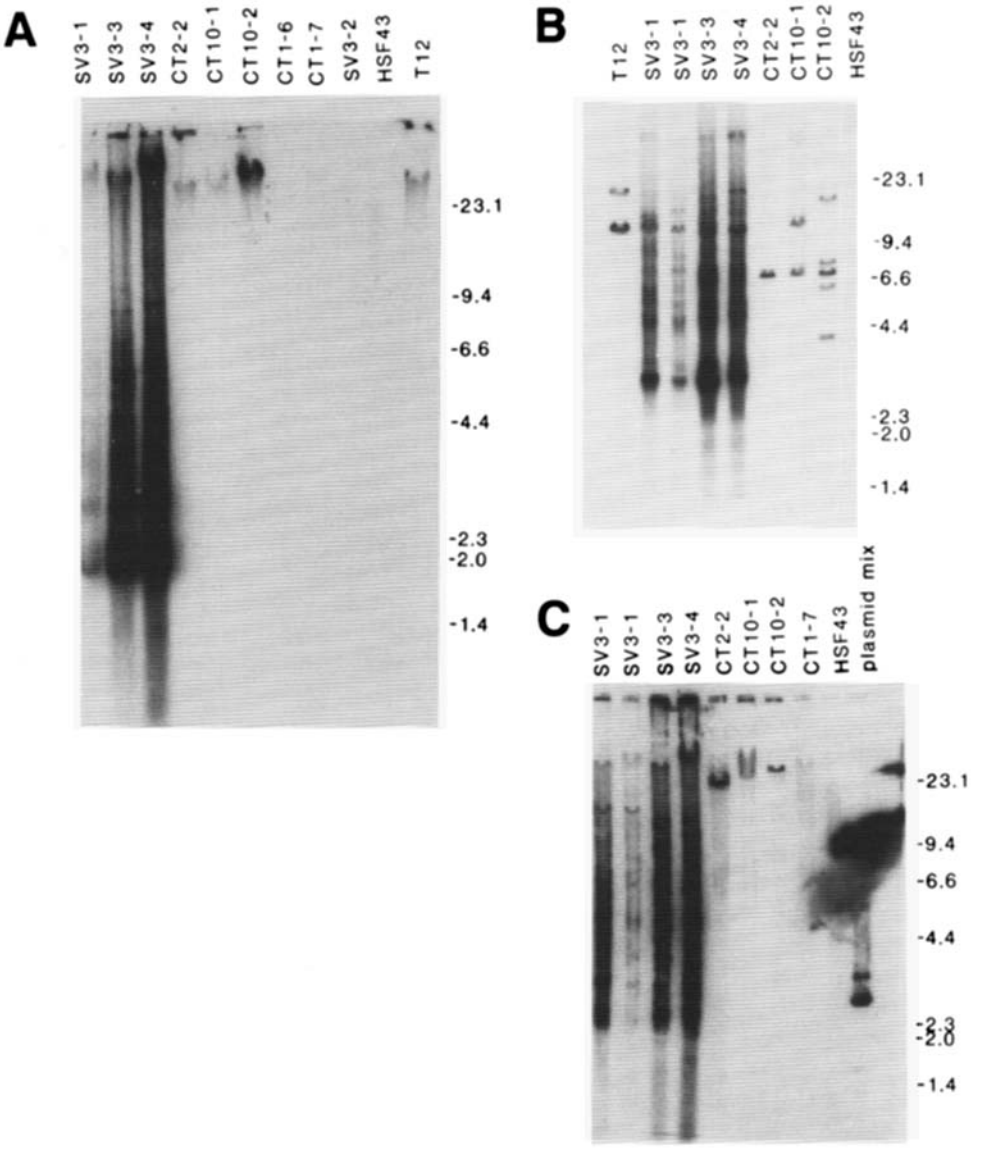

Fig. 4. Southern analysis of cellular DNAs with a probe specific to T antigen sequences. **A:** Uncut DNA (T12 DNA an immortal and tumorigenic, T antigen-positive cell line was included as a positive control). **B:** T12 through SV3-4 DNAs were digested with Bgl II (single cutter for pSV3neo), and CT2-2 through HSF43 DNAs were digested with Bgl I (single cutter for pRSVEdl884). **C:** Sac I digested DNAs (plasmid mix represents a mixture of pSV3neo restricted with Bam HI and pRSVEdl884 digested with Bam HI and Pvu I. Molecular weight markers in the three blots were Lambda DNA digested with Hind III and Phi X 174 digested with Hae III.

spinner flask grew with nearly 100% efficiency. Therefore, although virtually every cell at this CPD was T antigen positive in the indirect immunofluoresence assay, very few were anchorage independent. Still fewer were positive in the case of the three T antigen-positive CT clones. This result suggests that T protein is not sufficient to allow a cell to grow as an anchorage-independent clone. It is not clear at this time whether small

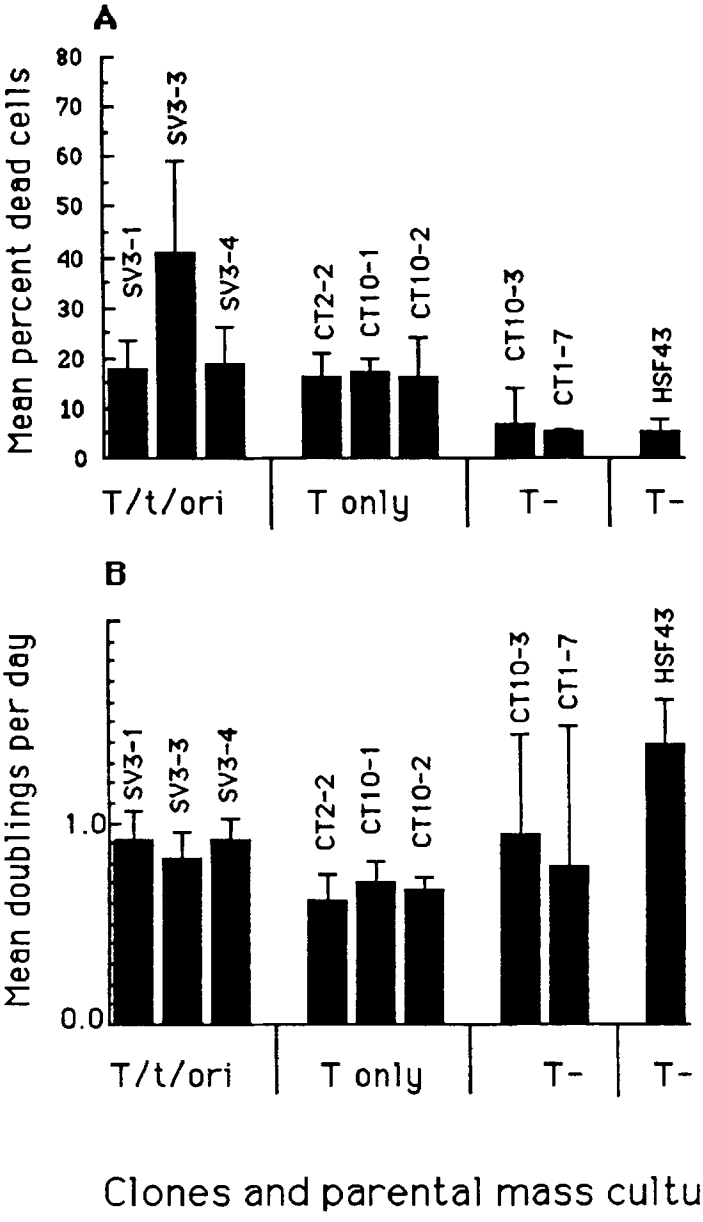

Fig. 5. **A:** The percentage of dead cells was determined daily for 3 days, and the mean was calculated. HSF43 cells were at ~20 CPD. **B:** The same cells at the same CPD were counted daily for 5 days, therefore n = 5.

t or the replication origin or both are responsible for the approximately 40-fold difference observed between the two sets of T antigen-positive clones.

Similarly, very few T antigen-positive cells were capable of colony formation in 1.0% FBS as shown in Table II. When this experiment was repeated 10–15 doublings later (data not shown), the T antigen-positive SV clones showed decreased plating efficiency in 1.0% serum and a concomitant decreased plating efficiency in 10.0% serum

TABLE II. Anchorage Independence and Plating Efficiency in 10.0% or 1.0% FBS

Characteristics	Clone (CPD)[a]	% AI[b]	Plating efficiency 10%[c]	Plating efficiency 1.0%[c]
G418^r/T+/t/ori+	SV3-1(54)	0.54	3.7	0.68
	SV3-3(51)	0.45	7.1	0.26
	SV3-4(55)	0.30	16.7	0.64
G418^r/T+	CT2-2(55)	0.00	2.4	0.00
	CT10-1(55)	0.01	5.2	0.02
	CT10-2(55)	0.02	2.0	0.00
G418^r/T−	CT2-1(51)	0.00	6.8	0.00
	CT10-3(51)	0.00	16.9	0.00
G418^r/T−/ori+	SV3-2(50)	0.00	6.3	0.00

[a]CPD at which anchorage independence assay was begun; plating efficiency assay was performed 3–4 CPD earlier.
[b]Percent of cells plated that could form colonies in 21 days in 1.5% methylcellulose. A colony had a diameter ≥ 100 μm.
[c]Percentage of cells capable of forming colonies when plated in either 10.0% or 1.0% FBS. A colony was ≥ 50 cells.

as they neared crisis. The T antigen-positive CTs showed increased plating efficiencies in 1.0% serum (0.1% to 0.4% PE) with no significant changes in 10.0% plating efficiency when repeated at the higher doubling level. Additionally, in the later assay, young (24CPD) HSF 43 cells were tested and found to have 1.0% and 10.0% serum plating efficiencies of 0.1% and 30.0%, respectively.

Three separate lines of evidence suggest that the T antigen-positive clones were not immortal immediately after clonal expansion. First, the clonally derived populations all entered a crisis stage where cell death exceeded cell growth and in most cases no further population doublings were achieved (Fig. 2). Second, although all of the clones, including T antigen-negative clones, could form colonies at sparse density (500 cell/100 mm dish), they all eventually phased out when passaged at these cell densities (as serial clones) (Table III). The serial clonogenicity assay measures those cells that are already immortal, as the cell number is much too low to observe events that occur at mutation-like frequencies. Therefore, none of the clones was immortal immediately after expansion. The life span of the serial clones closely paralleled the life span observed for the mass cultures (Table III). Third, when the T antigen-positive cells from the newly expanded clones were injected into preimplanted collagen sponges in nude mice ($n \geq 4$ mice) no progressive tumors were found within 6 months.

Chromosome Changes

When mitotic spreads of T antigen-positive cells were examined, the results were dramatic (Table IV; Fig. 6). Without G banding, only 3 cells were found of 300 counted and scored for aberrations that had 46 chromosomes and no visible aberrations. Aberrations included chromosome or chromatid breaks and gaps, rings, minute chromosomes, dicentrics, and complex rearrangements. Dicentric chromosomes were observed most frequently.

HSF43 and two G418-resistant T antigen-negative clones were also scored. All three T antigen-minus strains had low percentages of aberrations, and all three had a modal number of 46. Therefore T antigen alone is sufficient to severely destabilize the genome as evidenced by these chromosomal changes. These chromosome changes

TABLE III. Serial Clonogenicity

Clone	DMC[a]	DSC[b]	Average No. colonies/dish with serial passage[c]							
			1	2	3	4	5	6	7	8
SV3-1	83	23(74)	19.2	30.8	87.4	20.8	0.4	0.2		
SV3-3	69	16(64)	29.2	27.0	19.2	1.0				
SV3-4	80	26(77)	72.0	18.0	25.8	21.8	8.2			
CT2-2	89	34(86)	6.6	14.0	3.6	43.4	30.2	53.6	4.2	
CT10-1	101	40(92)	17.3	7.6	19.6	83.6	79.4	52.8	23.8	2.2
CT10-2	86	24(76)	5.8	14.6	9.8	29.6	14.0	5.6	1.4	
CT2-1	52[d,e]	22(70)	34.2	33.6	33.2	14.8	11.2			
CT1-6	56[e]	16(65)	99.4	101.2	7.6	12.8				
SV3-2	51[e]	5(53)	16.2	0.4						
HSF43	71[f]	ND[g]	ND							

[a]Cumulative population doublings of the mass culture at crisis or senescence.
[b]Population doublings as serial clones (CPD of serial clones, which equals CPD of clones prior to the assay plus the population doublings as serial clones).
[c]500 cells were plated/100 mm dish at each serial step; 5 dishes were used for serial passage, and the other 5 dishes were stained and colonies were counted. A colony represented ≥50 cells. Underlined values represent unknown No. of cells plated owing to decrease in cell No. below countable level. In these cases all cells remaining after attempted count were plated.
[d]This mass culture was terminated prematurely.
[e]G418[r], T antigen-negative clones.
[f]Parental cell strain.
[g]Not done.

TABLE IV. Chromosome Number and Percentage of Cells With Chromosomal Aberrations

Clone (CPD)	Chromosome No.		Percent aberrations[b]
	Mode[a]	Range	
SV3-1(59)	86/87/88(7)	33–114	84.0
SV3-3(60)	69/81/83(3)	22–88	94.0
SV3-4(62)	44(6)	24–416	52.0
CT2-2(59)	41(6)	27–100	56.0
CT10-1(59)	43/80(4)	27–162	64.0
CT10-2(59)	44(10)	32–108	78.0
HSF43(35)[c]	46(22)	21–47	6.0
CT2-1(55)[d]	46(12)	26–92	2.0
CT10-3(54)[d]	46(19)	28–86	4.0

[a]Modal No. (No. of cells with modal No.), n = 50.
[b]% cells with 1 or more structural aberration.
[c]Parental cell strain.
[d]G418[r], T antigen-negative clones.

preceded the aquisition of neoplastic indicators such as morphological transformation, anchorage independence, colony formation in low serum, and immortalization. It is also noteworthy that clone SV3-3, which had the highest percentage of cell death, also had the highest percentage of chromosome aberrations.

DISCUSSION

Human diploid fibroblasts were electroporated with a plasmid that contained DNA sequences that would allow the expression of SV40 large T antigen and at most a

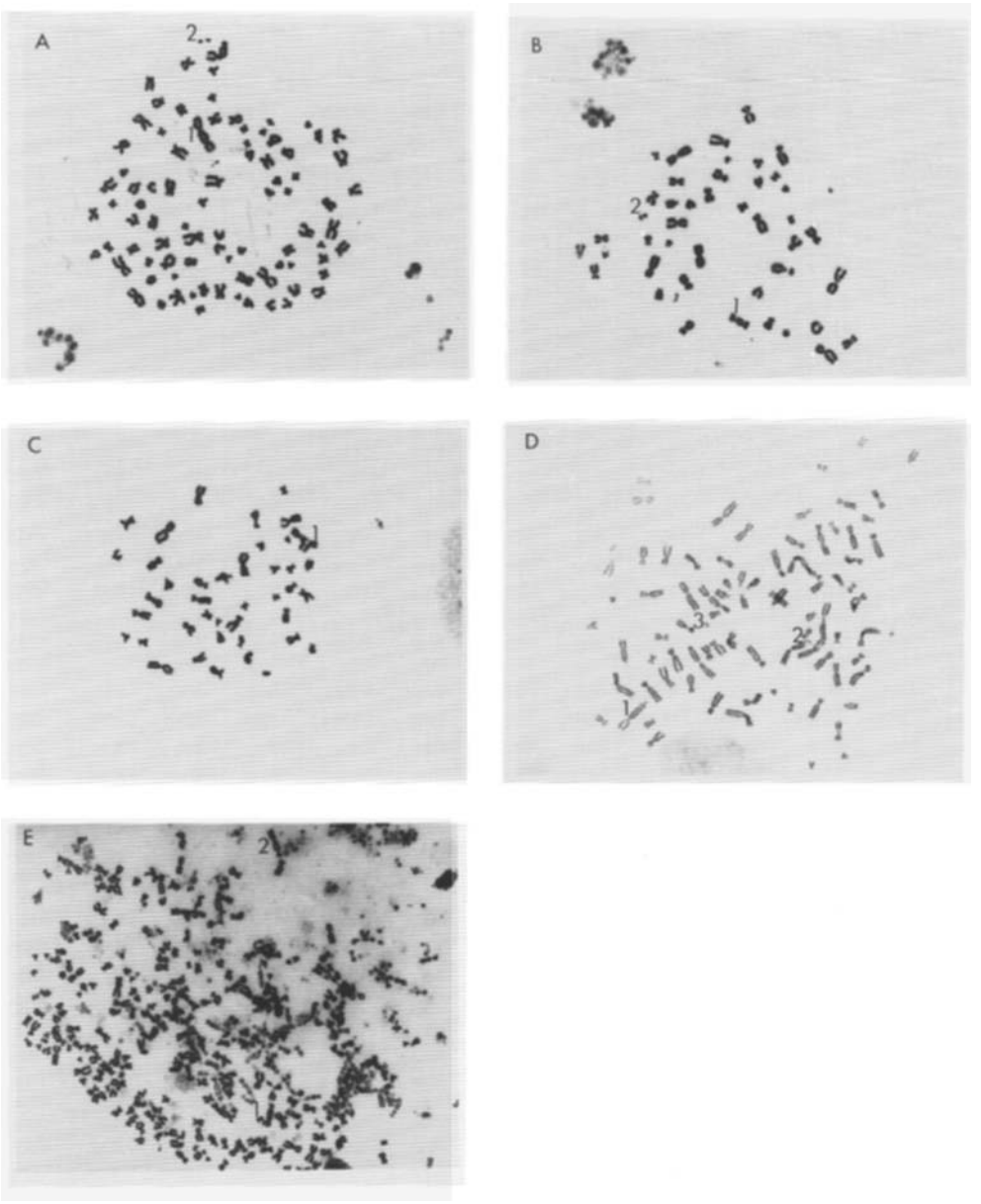

Fig. 6. Chromosome aberrations in T antigen-positive CT clones. **A:** CT10-2, 1) dicentric, 2) double minute. **B:** CT10-2, 1) dicentric, 2) double minute. **C:** CT2-2, 1) triradial. **D:** CT2-2, 1) dicentric, 2) chromatid break, 3) acentric fragment. **E:** CT2-2, 1) incomplete condensation, 2) dicentric.

severely truncated small t antigen [31]. These cells were co-electroporated with a second plasmid containing the neomycin resistance gene. Both of these plasmids had the SV40 origin of replication and promoter region replaced by the RSV promoter.Concurrently, the same parental human cell strain was electroporated with the plasmid pSV3neo. pSV3neo has the entire SV40 early region DNA sequences and both T/t antigens can therefore be expressed. In addition, there are 2 SV40 origins of replication and promoter

regions in the plasmid, one used as the promoter for the neo gene and the second serving as the promoter for T/t antigens. pSV3neo was transfected to compare to the results observed with T antigen alone.

We report here that T antigen caused direct effects when expressed in human fibroblasts. T antigen caused structural and numerical karyotype instability in almost every cell. These changes were similar to results reported for virions and pSV3 plasmids [8,32]. This indicates that T antigen itself is sufficient to cause gross karyotypic destabilization. Dicentric chromosomes were the most frequent structural aberration observed, which is consistent with earlier studies. It is not currently known if any of these observed dicentrics are transient structures such as observed by Nichols et al. [33].

An earlier study from our laboratory employed G-banding of pre-crisis and post-crisis human fibroblasts that had been transfected with pSV3neo and that were T antigen positive [30]. A large percentage of rearranged chromosomes were found in pre-crisis cells, but there were no frequent marker chromosomes observed. Later after crisis, stable marker chromosomes evolved, indicating the selection of clones with in vitro growth advantages. Those results combined with the results presented here indicate that SV40 T protein can drive these neoplastic changes through karyotypic scrambling. Those rare cells that become immortal can then acquire additional neoplastic traits.

Karyotype scrambling may allow ongoing genetic changes to occur by a number of mechanisms. Changes in ploidy of a given chromosome can cause changes in gene dosage [34]. Structural rearrangements and aberrations may disrupt regulatory elements, turning genes on or off inappropriately, or could interrupt coding sequences directly. We suggest that this may be the cause of immortalization, whereby putative finite life span genes are inactivated [3,4]. However, Wright et al. [43] have recently proposed that T antigen also has a direct phenotypic effect on one step in immortalization.

Furthermore, disruption of important enzymatic "housekeeping" functions could be partially responsible for the observed cell death. Cell death may also be partially contributed to by increasing percentages of cells entering crisis throughout their limited life span [35]. This concept is consistent with the observation that SV3-3, the clone with the shortest life span, consistently had the highest percentage of dead cells.

We suggest that T antigen is performing the role of a continually acting sublethal mutagen. This concept is reminiscent of Namba's transformation of human fibroblasts with repeated doses of Co 60 gamma rays [36]. Increased chromosome mutation may be a common mechanism of neoplastic transformation as evidenced by recent reports where karyotype instability was reported for myc, E1A, polyoma large T, human papillomavirus, and nickel sulfate [37–40].

In the two immortal clones reported here, cell death diminished after crisis. In our earlier work with T12, a lineage expanded after transfection with pSV3neo, cell death was observed early but disappeared in post-crisis culturing. This suggests that cells become T antigen resistant with time. Further support of this notion comes from the apparent loss of clastogenicity observed in post-crisis, T antigen-positive cell lines. For example, Canaani et al. reported 85% of G-banded chromosome spreads from their immortal lines had a uniform karyotype with stable marker chromosomes and these karyotypes were stable with passage [17]. Immortal human cells in which T antigen is mutated have been reported [20,41]. Such mutant T proteins may be less toxic to the

cells and therefore give them a selective advantage. Cellular mutations could also occur to detoxify T protein.

The third effect that is clearly demonstrated by the T antigen-positive CT clones is that of extended life span. The CTs exceeded the number of cumulative population doublings of the parental mass culture by approximately 20 doublings. This is necessarily a low guess owing to the number of unrecorded doublings of the T antigen-positive CTs, lost due to lack of accountability for cell death. The extended life span has been observed in previous studies but previously could not be attributed to T antigen alone [6,16,19]. When T antigen alone was expressed we were able to clearly observe this phenomena. Addition of extra population doublings may put more cells at risk of becoming immortalized. This advantage may be counterbalanced, however, by tetraploidization, which could provide extra alleles of the finite life span gene(s). In any case, the extended life span, which is a mass culture phenomenon, has not been linked to immortalization, which appears to be a rare mutation-like event of a single cell.

Autonomous DNA replication was observed in all instances when we used the ori containing plasmid, pSV3neo and has been reported previously [10–13,30]. Zouzias reported that these free viral DNA sequences may contain cellular flanking sequences and are probably derived from integrated viral DNA rather than persistent replicating plasmid [13]. He also reported this episomal DNA to be in circular supercoiled form. These episomal DNA molecules may also replicate as evidenced by mammalian shuttle vectors, where plasmids containing the SV40 ori have been shown to replicate when T antigen is provided in trans [12]. Interestingly, T12, which was derived by transfection with pSV3neo and was used in this study as a positive control for Southern analysis, no longer exhibited autonomous replication. Goolsby et al. reported that early passage T12 did show autonomous replication, which disappeared after the cells had become an immortal line [30].

In this study, there were higher percentages of cells with structural chromosome aberrations in the clones having received pSV3neo. The number of dead cells also appeared to be higher in these cells. Production of this extraneous DNA could be deleterious to the cell for two reasons. First, it expends cellular resources, including replicative enzymes and nucleotides. Second, reintegration of these molecules increases the chances of inactivating necessary housekeeping genes. A similar explanation could be invoked to explain why these cells consistently had greater (but still less than 1.0%) percentages of anchorage-independent cells than T antigen-positive CTs, when assayed immediately after expansion of the clones.

In this study, large G418-resistant colonies were ringed and cloned without bias to neoplastic phenotype. Frequently, in studies of the effect of SV40 constructs, transformed "foci" or anchorage-independent clones have been isolated and expanded for study [7]. Obviously this methodology enriches for those cells that have undergone a transformation step(s) and selects against those cells that are T antigen positive but have a more normal phenotype. Using G418 selection we observed the newly expanded CT clones had a fibroblast morphology and yet were virtually 100% T antigen positive. One such strain of cells, CT10-2, was capable of generating rare anchorage-independent variants but could not form colonies in 1.0% serum when first assayed. After crisis the cells had a flat epithelial morphology, and we are presently reassaying the other neoplastic indicators. The continual scrambling of genetic information as evidenced by

the observed gross karyotypic instability may allow selection of such variants, variants that have selective advantages within a tumor.

In summary, we report that SV40 T antigen alone caused karyotype instability, cell death, and extended life span of human diploid fibroblasts. T antigen did not *directly* cause cells to change morphology, become anchorage independent, grow in low serum, or to grow indefinitely. These traits may be acquired through mutation and accumulated through selective growth advantage.

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