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Isolation and Characterization of T Antigen-Negative Revertants from a Line of Transformed Rat Cells Containing One Copy of the SV40 Genome

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Summary

Negative selection with FUdR produced revertants from the transformed rat line 14B, which contains one insertion of the SV40 viral genome (Botchan, Topp and Sambrook, 1976). 14B contains nuclear T antigen, grows to a high density, grows in low serum and is anchorage-independent. The revertants fall into three classes with regard to viral DNA sequences: the SV40 DNA is retained; the SV40 DNA is retained but has undergone a deletion; and the SV40 DNA is lost, generating a cured cell. This heterogeneity is not a result of long-term passage. The revertants arise with a frequency of one in 8.4×10^5 cells after as few as 12 passages. All three classes of revertants are T antigen-negative, density-sensitive, more serum sensitive than 14B and anchorage-dependent. These data argue for a direct role of the functioning viral genome in the maintenance of the transformed state, and that with 14B, the phenotypes of transformation are not virus gene dosage-dependent.

Introduction

Nonpermissive cells, infected with SV40, give rise to transformed cell lines. These cells lose some or all of the normal growth controls, they may grow in low serum concentrations, grow beyond confluence in high serum, grow when suspended in methocel and form tumors when injected into animals.

SV40-transformed cells also display a number of antigenic differences from the parent cell. Most notable of these is the presence of T antigen(s) in the nucleus. One of these proteins has been shown to be the product coded for by the A complementation group of SV40 (Tegtmeyer et al., 1975; Tenen, Baygell and Livingston, 1975). The SV40 T antigen (or A gene product) is a polypeptide of approximately 94,000 daltons and is expressed both before and after the onset of viral DNA replication in the viruses lytic cycle (Tooze, 1973). The presence of this T antigen is believed necessary for the establishment of transformation (Tegtmeyer, 1975; Martin and Chou, 1975) and may also be necessary for the continued expression of the transformed phenotype (Tegtmeyer, 1975; Brugge and Butel, 1975; Martin and Chou, 1975; Osborn and Weber, 1975). Based in part on the observation that temperature-sensitive mutations in the A gene render the initiation of viral DNA replication temperature-sensitive in its lytic cycle (Tegtmeyer, 1972) and in part on changes observed in the initiation of cell DNA synthesis in SV40-transformed cells, many investigators have proposed that the SV40 T antigen acts directly as an initiator of host cell DNA synthesis (Tegtmeyer, 1972; Butel, Brugge and Noonan 1974; Osborn and Weber, 1975; Martin and Oppenheim, 1977).

At the present time, we do not know whether all the aspects of transformation require a direct viral role for maintenance, or whether the virus is needed only for the initiation of some of the transformed phenotypes. Moreover, we do not know which genes of the virus are required for the various transformation characteristics. Conditional mutations in the A gene, which is required for transformation, have been of some use in this respect. Revertants should allow us to probe these questions further. Furthermore, if viral genes are required to maintain a particular transformed phenotype, one class of revertants should include cell lines whose viral DNA sequences and viral gene expression have been altered. Our understanding of the mechanisms of reversion and of transformation itself has been limited, however, by the fact that most transformed cells contain multiple copies of the virus. Clearly then, if a transformed cell contains multiple copies of dominant viral genes, reversion from transformation must frequently occur by mutations in other cellular functions. It has therefore not been possible to determine with certainty whether reversion resulted from an alteration in a viral genome, the host genome or some interaction between the two. It has also not been possible to determine whether a reduction in the number of copies of *active* SV40 DNA in the revertants is a major factor in the phenomenon of reversion. Previous studies have not detected a significant loss of viral DNA sequences from revertant cells containing multiple copies of viral DNA (Ozanne, Sharp and Sambrook, 1973).

Revertants from transformed cells have been isolated by exposing the transformed cells to a variety of killing agents, including FUdR (Pollack, Green and Todaro, 1968; Nomura et al., 1973), concanavalin A (Ozanne and Sambrook, 1971; Culp and Black, 1972), colchicine (Vogel, Risser and Pollack, 1973) and BUdR (Vogel and Pollack, 1973), under conditions where only transformed cells could grow.

One such group of revertants from SV40-transformed cells was selected for density inhibition. These density revertants, isolated independently

by Pollack, Green and Todaro (1968), Pollack and Burger (1969), Grimes and Black (1971), Culp and Black (1972), Wicker et al. (1972), Risser and Pollack (1973) following treatment with a variety of killing agents, have a number of characteristics in common. All the cells grow to a much lower density than the transformed parent. At confluence, the number of cells in mitosis is greatly reduced. They generally have an organized cytoarchitecture, as revealed by indirect immunofluorescence, similar to nontransformed cells (Pollack, Osborn and Weber, 1975). Most of these revertants have a marked reduction in their ability to grow in methocel and are less tumorigenic. Most will still grow in low serum. They still contain SV40 DNA, however, and infectious virus can be rescued from the cells. Moreover, the revertants generally exhibit a marked increase in their DNA content and increase in ploidy. Perhaps most important of all, they still express the SV40 T antigen.

At the time this work was initiated, all revertants not only contained SV40 DNA, but also synthesized T antigen. The persistence of T antigen(s) in revertants can be interpreted in several different ways. It can mean that T antigen(s) is necessary but not sufficient for the transformation phenotype, and that the cell can revert by a cellular alteration. Alternatively, it can mean that T antigen(s) is not involved in maintenance of the transformed cell. A third explanation is that the antigenicity is present but the product is not functional.

There are reports in the literature which support the concept that reversion can correlate with a loss of viral T antigen. Basilico and Zouzias (1976) have reported a temperature-sensitive revertant which ceases T antigen synthesis at confluence at high temperature, when a temperature-sensitive cellular mutation permits the cells to block in G_0. Furthermore, Kelly and Sambrook (1974) isolated cytochalasin B-resistant variants from a cell line containing multiple copies of SV40. These variants had lost one copy of the SV40 genome and were transiently T-negative. They did not, however, maintain the properties characteristic of revertants. Finally, Marin and MacPherson (1969) isolated revertants from a polyoma transformant which lacked viral antigens, and it was suggested by these investigators that the cell reversion was initiated by the loss of the viral genome.

An established rat embryo line fully transformed by SV40 DNA, line 14B, has been reported to contain only one copy of the SV40 genome per cell (Botchan et al., 1976). In this paper, we describe density-sensitive revertants, isolated from this cell line with FUdR, which completely lack T antigen. In some of these revertants, the region of the integrated viral DNA that codes for T antigen(s) has been mutated.

Results

Expression of T Antigen in Revertant Cells

Seven density-sensitive colonies were isolated by FUdR selection from 14B using the following protocol. The transformed cells were cloned 3 times—first by picking isolated colonies, then by isolating single cells from this colony in microwells—grown for 20 doublings, recloned once by picking an isolated colony and then grown again for approximately 20 doublings. The transformed cells were then exposed to FUdR, and seven surviving colonies with flat "revertant" morphologies were picked as described in Experimental Procedures. All the colonies that were selected, which were subsequently found to be revertants with respect to many of the properties of SV40 transformation (see below), have in common a lack of T antigen expression. This SV40-coded protein is absent from all seven revertant cell lines as measured by immunofluorescence, and is below the limits of detection when labeled cell proteins are immunoprecipitated with anti-T sera.

Figure 1A displays the parent transformed cell line 14B, stained for T antigen by indirect immunofluorescence. The nuclei show the characteristic bright fluorescence of SV40-transformed cells. In contrast, the revertant varients of 14B, line FL[1] 1–3, FL[1] 3–8 and FL[1] 3–5, are shown in Figures 1B, 1C and 1D, respectively. These cell lines and the other four are negative for T antigen expression under a variety of conditions. Basilico and Zouzias (1976) had shown that a cellular mutation could render T antigen expression temperature-sensitive when the cells were confluent. We therefore tried varying incubation temperature and cell density testing 14B, Rat-1 (the parent of 14B) and three of the revertants for T antigen immunofluorescence. The results of this experiment are shown in Table 1. The transformed cell line, 14B, remained T antigen-positive under all conditions, while Rat-1 and the three revertant lines never expressed T antigen.

These observations were extended by immunoprecipitating the T antigen with anti-T sera (provided by Dr. R. Tjian) after labeling total proteins with either ^{32}P- or ^{35}S-methionine. Figure 2A is an autoradiogram of such ^{32}P-labeled immunoprecipitates after gel electrophoresis. From line 14B, a major band at 95,000 daltons is seen. This protein has a similar mobility to the phosphorylated protein immunoprecipitated from lytically infected monkey cells. T antigen is known to be a phosphorylated protein (Tegtmeyer, Rundell and Collins, 1977). No phosphorylated protein could be detected in any of the revertant cell lines. That this immunoprecipitated protein with an apparent molecular weight of 95,000 daltons is T antigen is substantiated by

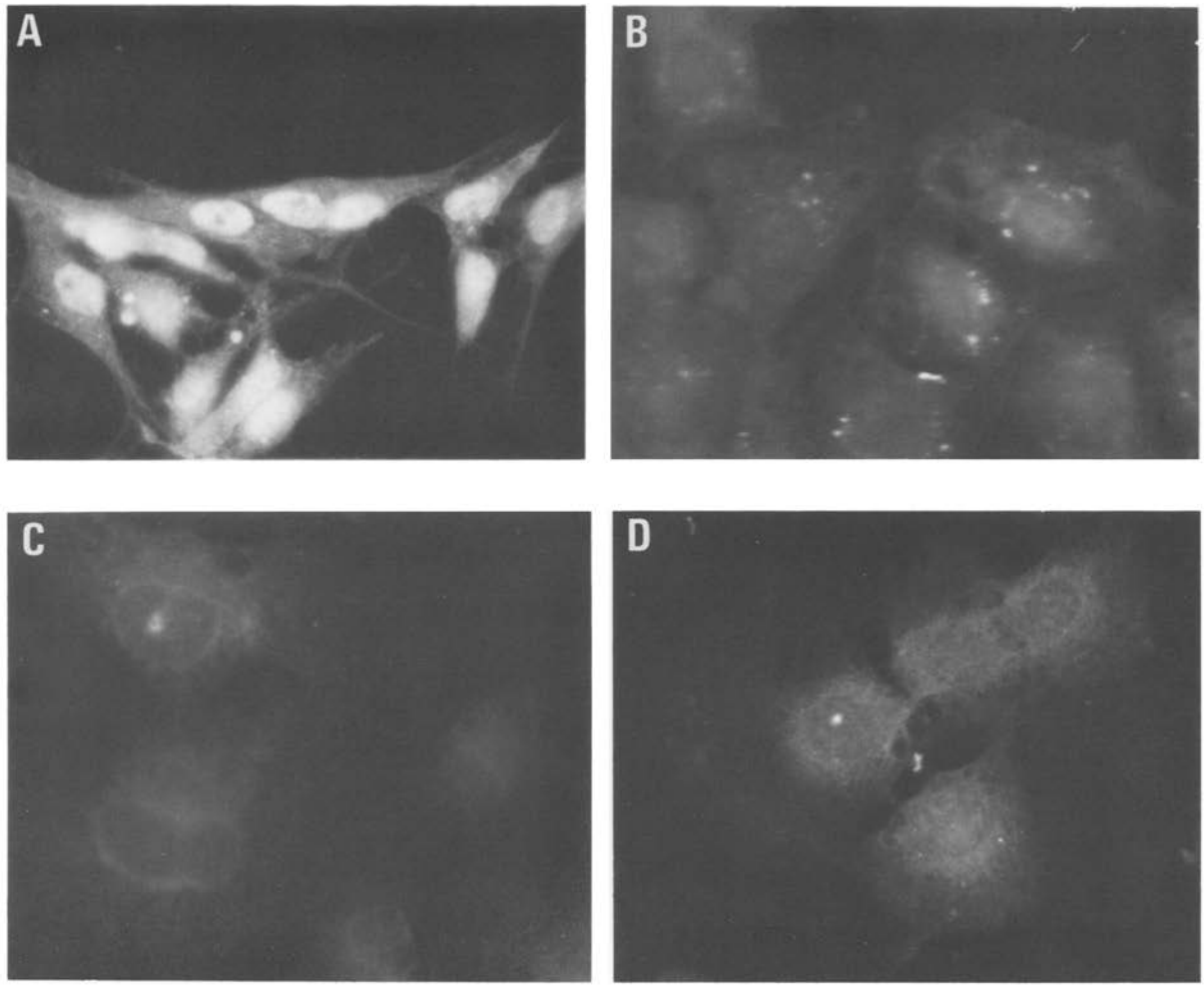

Figure 1. Immunofluoroescent Assay for SV40 T Antigen

The SV40 T antigen was detected by indirect immunofluorescence as described in Experimental Procedures. (A) is a photograph of the fluorescence seen from the transformed parent 14B. (B, C and D) represent fluorescent images of revertant cell lines FL^1 1-3, FL^1 3-8 and FL^1 3-5, respectively.

the finding that it is not immunoprecipitated by preimmune sera. When total cell proteins were labeled with ^{35}S-methionine, other protein bands were seen after immunoprecipitation with anti-T sera and subsequent gel electrophoresis and autoradiography (Figure 2B). No T antigen-specific bands could be detected in the revertant cell lines. The pattern of labeling shown for the revertant FL^1 3-5 is identical to that seen in its untransformed grandparent Rat-1 (data not shown). Several other protein bands could be detected in these ^{35}S-methionine-labeled immunoprecipitates from cells of line 14B. Presumably some of these represent proteolytic degradation products of the 95,000 dalton protein and/or other independent SV40 T antigen-containing polypeptides (see Experimental Procedures).

SV40 DNA in Revertants

The loss of T antigen expression in the revertant cell lines could be the result of an epigenetic change in the cells' regulatory machinery, or a genetic change in either the structural gene(s) coding for T antigen or its production. Analysis of the integrated viral DNA in the revertants and in the parent transformed cell line 14B was therefore performed by hybridization techniques previously described (Botchan et al., 1976). A continuous stretch of DNA sequences extending from position 20 clockwise to position 98 on the SV40 chromosome has been detected in line 14B by these techniques. Furthermore, there has been no indication of deletion or duplication of these viral sequences in this cell DNA. It has been inferred from these data that the SV40 DNA is joined to cell DNA by sequences mapping somewhere between position 98 clockwise to position 20 on the viral chromosome.

The restriction enzyme Bal I, an endonuclease isolated from Brevibacterium albidum, is of special

Table 1. Effect of Culture Conditions on T Antigen Expression

	Fraction of Cells Expressing T Antigen			
	Confluent Cells[a]		Sparse Cells[b]	
Line	37°C	32.5°C	37°C	32.5°C
Rat 1	<0.001	<0.001	<0.001	<0.001
14B	0.96	0.96	0.96	0.96
FL[1] 1-4	<0.001	<0.001	<0.001	<0.001
FL[1] 3-3	<0.001	<0.001	<0.001	<0.001
FL[1] 3-8	<0.001	<0.001	<0.001	<0.001

[a] Cells plated at 2×10^4 cells per cm^2 and grown at desired temperature until confluence.
[b] Cells plated at 10^3 cells per cm^2 and grown at desired temperature for 3 days.

use in this type of analysis because its recognition sequence is not present in SV40 DNA. Thus when high molecular weight chromosomal DNA is extracted from cell line 14B and this DNA is hydrolyzed by Bal I, only one SV40 DNA fragment is created. This fragment contains the entire SV40 DNA insertion and the chromosomal DNA that is proximal to the virus on both sides of the insertion. The resulting fragment can be detected by the Southern blotting technique (Southern, 1975; Botchan et al., 1976; see also Ketner and Kelly, 1976). Analysis of the SV40 fragment created by Bal I restriction enzyme hydrolysis of revertant cell DNA is shown in Figure 3. The results of two separate experiments are shown in this figure. The bottom panel shows one band in the slot marked "14B" and a band at the same position for cell lines FL[1] 1-3 and FL[1] 1-4. In one lane, the equivalent of one copy per cell of SV40 DNA (2×10^{-6} μg per 2 μg of cell DNA) was mixed with FL[1] 1-4 DNA. Bands corresponding to forms II and III SV40 DNA are visible, as well as a very faint band at the position of form I DNA. In two other lanes, 2 μg of 14B DNA were mixed with 2 μg of revertant cell DNAs from lines FL[1] 1-3 and FL[1] 1-4. Only one intense band was seen in these lanes. In contrast to these results, no detectable SV40 DNA could be found in cell DNA from lines FL[1] 3-8 and FL[1] 3-2. That FL[1] 3-8 and FL[1] 3-2 had lost their copy of the SV40 DNA has been verified by independent experiments with several other enzymes including Eco RI, Hpa I and Pvu II (data not shown). We have not been able to detect any mutation in the SV40 DNA of cell lines FL[1] 1-3 and FL[1] 1-4 with the same set of enzymes. This, of course, does not rule out the possibility that small deletions and/or point mutations in the viral DNAs of these cells had occurred (see Figure 4). The results of a similar experiment are illustrated in the upper right panel of Figure 3. The panel in the upper left of this figure is a picture of the total cell DNA in each slot stained with ethidium bromide before the transfer of these DNAs to nitrocellulose was executed. The slot labeled "R" contained 0.2 μg of Ad-2 DNA which serve as markers and 5×10^{-6} μg of SV40 DNA. Forms I, II and III SV40 are visible in the autoradiogram. Several other faint bands are also visible in the autoradiogram. These represent marker fragments of phage Mu DNA that were added to the DNA samples to ensure that transfer and detection efficiency were satisfactory and that the migration of DNA in the different lanes was equivalent. The slot labeled "3-5" also contained one tenth equivalent of free SV40 DNA. These various controls make it quite certain that cell lines FL[1] 3-8 and FL[1] 3-2 can contain no more than a fragment of SV40 DNA of length equivalent to 10% of the viral genome. In cell line FL[1] 3-5, a new DNA fragment is detected which has a faster mobility than that fragment seen in cell line 14B. The difference in mobilities between these bands indicates a difference of molecular weight between these two fragments of approximately 300,000 daltons. This result can mean that the SV40 sequence has translocated its chromosomal location or there has been a deletion of DNA. To learn more about the difference between the SV40 genomes of these two cells, the restriction enzyme Pvu II (from Proteus vulgaris) which cleaves SV40 DNA at positions 98.5, 32 and 72 was used. The results of this experiment are shown in Figure 4. Digestion of 14B DNA with endonuclease Pvu II yields a fragment of molecular weight 2.3×10^6 daltons, which is probably the fragment which contains the fusion sequences of cell DNA with SV40 DNA. Two other fragments with mobilities identical to the A and B fragments of SV40 cleaved with Pvu II are seen. The C fragment is missing in this digestion, presumably because it contains within it the host cell DNA-viral DNA recombination joint. This result, taken together with our previous analysis of this cell line (Botchan et al. 1976), must mean that the recombination joints map very close to position 98 on the viral chromosome, on both sides of the viral insertion. The DNA samples from revertant cell lines FL[1] 1-3 and FL[1] 1-4 show a pattern of fragments indistinguishable from their parent 14B. In the case of revertants FL[1] 3-5 and FL[1] 3-3, the A fragment is missing and a new fragment appears. In view of the results shown in Figure 3, the appearance of a new fragment in FL[1] 3-5 DNA with a mobility indicative of a size approximately 230,000 daltons smaller than the Pvu II A fragment of SV40, we must conclude that there has been a deletion of SV40 DNA within this cell. The new fragment in cell line FL[1] 3-3 is consistent with a deletion in the SV40 DNA sequences of this cell of

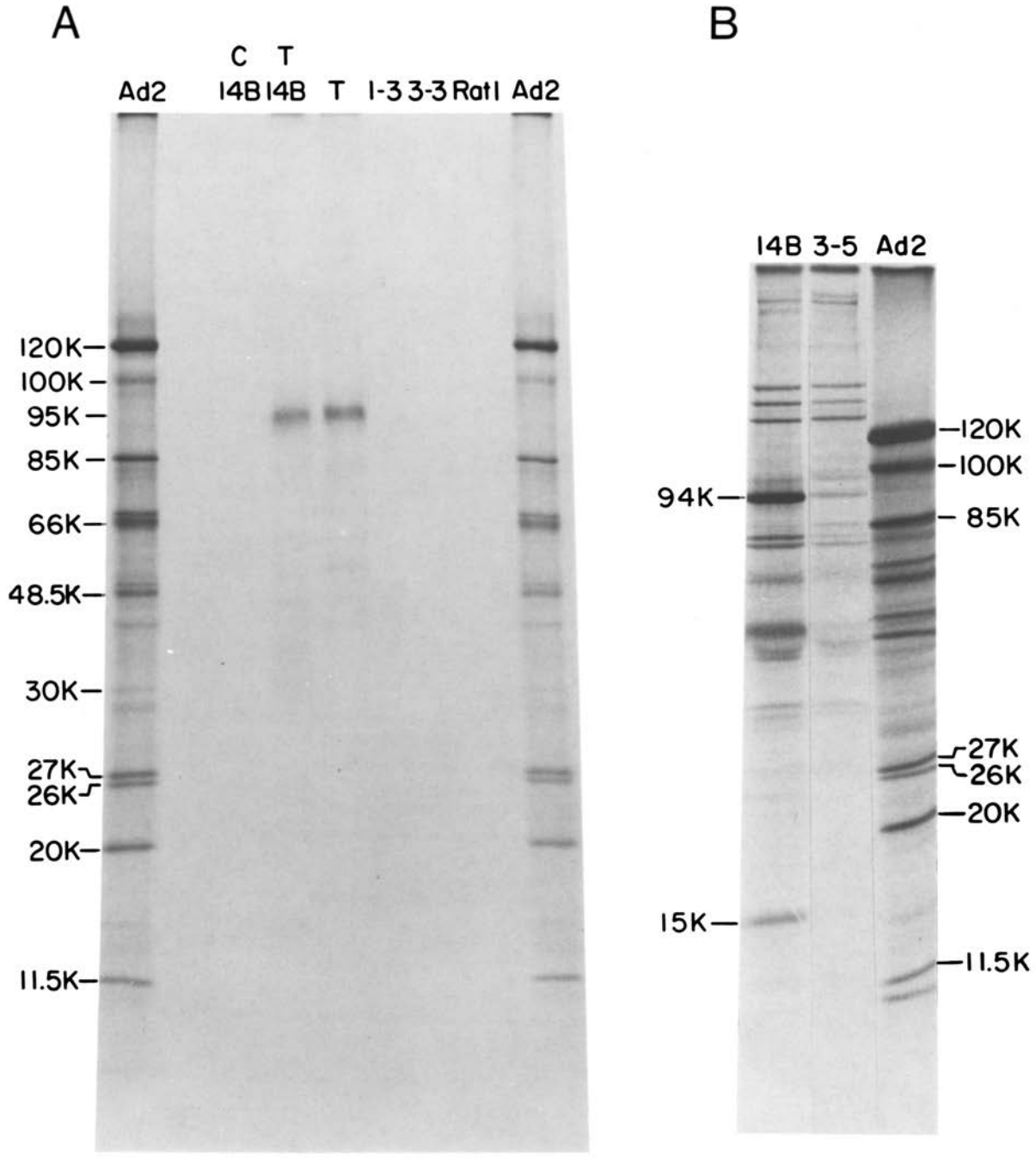

Figure 2. Autoradiographic Detection of SV40 T Antigen

Total cell proteins were pulse-labeled with either ^{32}P-phosphate (A) or with ^{35}S-methionine (B) as described in Experimental Procedures. Immunoprecipitated T antigens were prepared and fractionated by electrophoresis on 7-15% (A) or 5-20% (B) gradient polyacrylamide gels (Tjian and Pero, 1976).

(A) The slot labeled C/14B was a control immunoprecipitate prepared with preimmune anti-T sera and labeled 14B proteins. T/14B shows the protein band detected in transformed cells of 14B; this is to be compared to that band detected in SV40 lytically infected CV-1 cells which is shown in the slot labeled T. No phosphorylated protein could be detected in cell lines 1-3, 3-3 or Rat-1 after immunoprecipitation. Marker proteins are total adenovirus 2 proteins (Ad2) labeled (with ^{35}S-methionine) late in the lytic infection of Hela cells.

(B) Two SV40-specific protein bands could be detected in ^{35}S-methionine proteins of transformed cell line 14B. They are labeled 94K and 15K. These bands could not be detected in the proteins from cell line FL[1] 3-5.

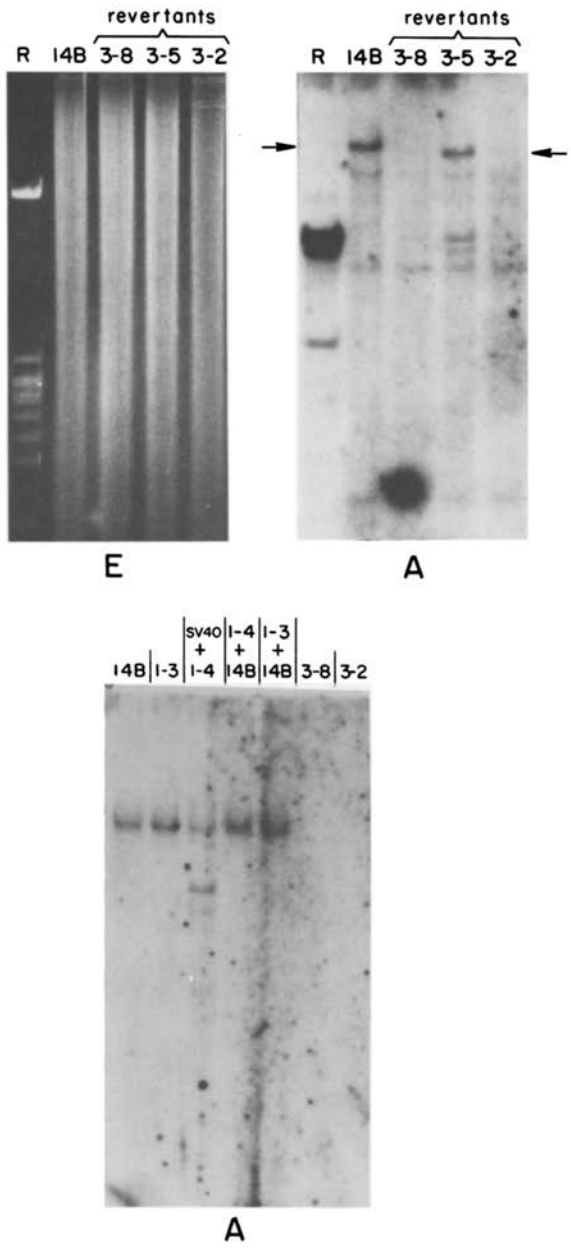

Figure 3. Detection of Fragments That Contain SV40 Sequences after Hydrolysis of Cell DNA with Endonuclease Bal I

(E) is a photograph of the total DNA in each slot stained with ethidium bromide after fractionation of the fragments on 0.7% agarose gels. Slot R is a control experiment which was a digestion of 0.2 of Ad2 DNA and 5×10^{-6} μg of SV40 DNA. Each cell DNA lane contained 2 μg of the various cell DNAs as well as ^{32}P-labeled marker Mu-phage fragments. Slot 3-5 also contained 2×10^{-7} μg of SV40 DNA.

The upper right panel (A) is an autoradiogram of the fragments detected on the nitrocellulose sheet after transfer of the fragments and hybridization with "nick-translated" SV40 DNA. The arrows point to the integrated DNA fragments seen in cell DNAs from line 14B and FL[1] 3-5.

The bottom panel (A) is also an autoradiogram of a similar experiment. Various mixtures of SV40 DNA and cell DNAs were made as indicated in the figure and text.

690,000 daltons. This has been confirmed by Bal I digestion of this DNA (data not shown). The deletions in FL[1] 3-5 and FL[1] 3-3 are 6 and 18%, respectively, of the SV40 genome and map within the coordinates of positions 32 and 72 on the viral chromosome. Although further work will be necessary to refine the map coordinates of these deletions, it is clear that they fall within that part of the inserted SV40 DNA which must code for the messenger RNA of T antigen (Khoury et al., 1975; Prives et al., 1977).

In summary, three different types of revertants have been isolated: those which seem to have lost their SV40 DNA; those which have suffered deletions within that region of the SV40 DNA which codes for T antigen mRNA; and those which show no gross rearrangements at all. Because only seven independent revertant clones were analyzed, we do not know the actual frequencies with which these different classes of revertants arise. We have chosen to examine the biological properties of one representative of each class in what follows.

Fluctuation Analysis

FUdR treatment may either induce the conversion of 14B to the revertant phenotype, or it may simply select for revertants which had appeared spontaneously in the population, or both. To distinguish among these possibilities, we did a fluctuation analysis, as originally described by Luria and Delbrück (1943).

Line 14B was originally transformed by a DNA infection of Rat-1, cloned through microwells, replated and repicked and grown for 20 generations. It was then recloned, grown for approximately 200 doublings (12 passages) and used for the fluctuation analysis, described below.

Two confluent plates of 14B were prepared by plating 10^5 cells per plate and growing the cells to a final concentration of approximately 7×10^6 cells per plate. These plates were treated with FUdR, and the surviving cells from each plate were diluted 1:40 and replated (Table 2A). The number of survivors in this part of the experiment reflects the average number of flat colony-forming cells in a large population of 14B. The standard deviation reflects the statistical variability in plating aliquots of this population. The reversion frequency, calculated as that fraction of cells in the original large population capable of generating flat colonies, is 1.5×10^{-4}. In a parallel experiment, the FUdR survivors formed colonies on glass coverslips and were stained for T antigen. 56% of the flat colonies were T antigen-negative. Thus the frequency of T-negative revertants is approximately 8×10^{-5}.

In the second part of the experiment, 20 separate plates of 14B were grown to confluence from very small numbers of cells (< 100 cells per plate).

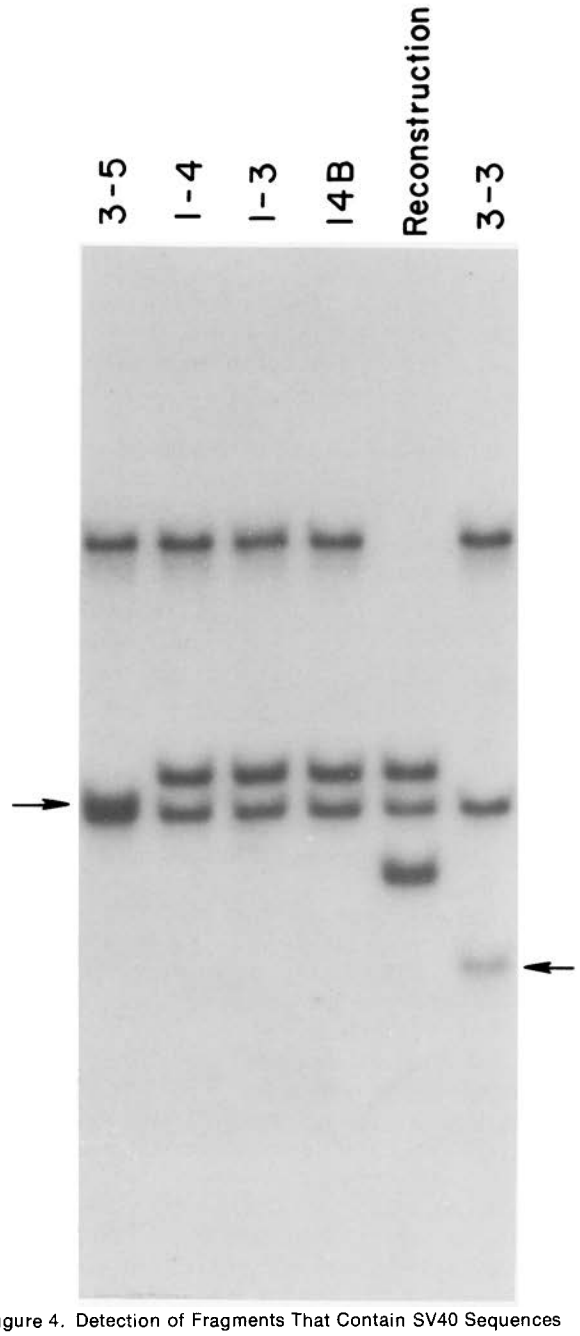

Figure 4. Detection of Fragments That Contain SV40 Sequences after Hydrolysis of Various Cell DNAs with Endonuclease Pvu II
50 μg of cell DNA were cleaved with endonuclease Pvu II, and the products were fractionated on alkaline-1.4% agarose gels (McDonnell et al., 1977). The slot labeled Reconstruction contained 50 $\times$ 10^{-6} μg of SV40 DNA and 1 μg of Ad2 DNA also cleaved with endonuclease Pvu II. The arrows point to bands seen in revertant cell lines FL[1] 3-5 and FL[1] 3-3 which are not present in the parent cell line 14B.

Each plate was then treated with FUdR. The survivors were diluted 1:25, and one aliquot from each plate was replated (Table 2B). If the appearance of cells capable of forming flat colonies is a random event, it will occur in some of the populations at an earlier time than in others. Those populations in which a cell capable of forming a flat colony arose early or was present in the original small population will accumulate more flat colony-forming cells than those populations where the event occurred shortly before drug treatment. Such random events will lead to a very large variance in the distribution of flat colonies per dish. Table 2B shows such a variance. The deviation we observe in Table 2B is much higher than could be accounted for by plating variability in Table 2A. FUdR treatment therefore selects for preexisting revertants in the 14B population and does not induce them. The high degree of heterogeneity observed in Table 2B after 16 doublings of the population reflects the high frequency of reversion shown in Table 2A.

Cellular Morphology

The cellular morphology of 14B, Rat-1 and the three revertants, growing in colonies, is shown in Figure 5. Cells in Figures 5a, 5c, 5e, 5g and 5i were located at the edge of the colony, while those in Figures 5b, 5d, 5f, 5h and 5j were in the center of the colony.

It is apparent that 14B (Figures 5a and 5b) is not contact-inhibited, and that the cells pile up in the center of the colony. At the bottom of Figure 5a, the cells are only one cell thick, and are oriented in such a way that the longitudinal axis of each cell tends to radiate from the center of the colony (seen at the top of Figure 5a). In Figure 5b, it is possible to see the dense multilayer morphology at the center of the colony.

Rat-1 (Figures 5c and 5d) and FL[1] 1-4 (Figures 5e and 5f) look very similar. The cells grow as a monolayer, are approximately the same size and change shape from an elongated form to a more cuboidal shape when they are at confluence for a long time (that is, in the center of a colony). Lines FL[1] 3-3 (Figures 5g and 5h) and FL[1] 3-8 Figures 5i and 5j) also form flat monolayers. They contain larger cells than Rat-1 or FL[1] 1-4. Note that the center of the FL[1] 3-3 colony (Figure 5h) contains a few very large cells with two nuclei. This is the only revertant that commonly contains such cells.

Growth Properties

Table 3 summarizes a number of growth characteristics of the transformed cell line and the revertants. These properties are described individually.

Effects of Serum Concentration on Growth Rate and Saturation Density

Cell increase was followed for 2 weeks for each line with concentrations of fetal calf serum from

Table 2. Fluctuation Analysis of 14B Treated with FUdR

Plate Number	Number of Flat Colonies (A1)	(A2)	Number of Flat Colonies (B)
1	24	20	25
2	21	34	77
3	17	30	72
4	39	22	20
5	22	35	700
6	33	39	14
7	23	29	300
8	31	27	64
9	30	31	65
10	25	35	118
11	18	36	28
12	28	40	89
13	22	28	40
14	25	28	700
15	29	40	22
16	28	41	23
17	30	40	12
18	32	36	30
19	36	30	75
20	16	32	100
Average	26.45	32.65	124.9
Variance	37.05	35.87	40079
Standard Deviation	6.08	5.99	200.2
Frequency of Flat Colonies	1.5×10^{-4}		
Frequency of Tag-Negative Colonies	8×10^{-5}		

(A) 14B was plated on two 60 mm plates at 10^5 cells per plate, grown for approximately six doublings, treated, diluted 1:40 and plated on 40 plates.
(B) 14B was plated on twenty 35 mm plates at 200 cells per plate, grown for 8 days, trypsinized and transferred to 60 mm plates, grown to confluence, treated, diluted 1:25 and replated.

0.1–10%. Rat-1 and the revertants did not increase significantly in cell number at serum concentrations below 1.0% (data not shown). The doubling time for each cell population at 10 and 1% was calculated from the linear portion of the growth curves (Table 3).

In 10% FCS, the transformed cells have a doubling time of 14 hr, while the normal cell line has a doubling time of 22 hr. All three revertants have doubling times similar to, but longer than, Rat-1. The differences among the lines are greater in 1% FCS. All the lines will grow in 1% serum, but the revertants have doubling times almost twice that of Rat-1, while the doubling time of the transformed cells is only two thirds that of Rat-1. The revertants are more like secondary rat embryo fibroblasts than like Rat-1 in their inability to grow in low serum.

Saturation densities were calculated from the number of cells per cm^2 when there was no further increase in growth curves. Line 14B grows very densely in 10% FCS, with the cells growing on top of each other to form a tangled mass. The morphology of the culture is similar in 1% serum, but the cells grow to a lower density. Rat-1 and the revertants cease to grow at confluence in both 10 and 1% FCS. The cells spread to a larger diameter in 1% FCS, which could contribute to the lower saturation density in low serum. With all three revertants, the saturation density is lower than for Rat-1. As with doubling times, the revertants are "more normal" than Rat-1. Experiments are currently in progress to determine whether Rat-1 and the revertants enter a stage of G_0 at confluence, in a manner similar to normal cells.

Plating Efficiency on Plastic

Plating efficiencies of the various lines are also shown in Table 3. They vary from 1–100% on plastic dishes. The cured cells, FL^1 3–8, have the highest plating efficiency on plastic (100%), although Rat-1 and FL^1 1–4 plate with reasonably high efficiency (80 and 62%, respectively). A growing culture of FL^1 3–3 contains many cells with two nuclei (Figure 5h). If these are cells which are no longer capable of division, their presence would contribute to both the slow doubling time of the population and the low plating efficiency of this revertant line.

Growth without Anchorage

Line 14B grows in methocel (Table 3). Within 2 weeks after inoculation, most of the 14B colonies were 0.15–0.3 mm in diameter. After 3 weeks, each of the other cell lines had only a few colonies larger than 0.15 mm on plates containing 10^5 cells. In addition, Rat-1, FL^1 3–3 and FL^1 3–8 had numerous tiny colonies, approximately 0.05 mm in diameter, while FL^1 1–4 had a few of these small colonies. These colonies presumably arose from cells that were only able to divide a few times in methocel.

The plating efficiency on methocel relative to that on plastic was calculated (Table 3). 14B had an RPE of 15%, more than three orders of magnitude higher than Rat-1, FL^1 1–4 and FL^1 3–8. FL^1 3–3 had an RPE 10 times higher than Rat-1 and the other revertants, because it makes colonies on plastic with such low efficiency. Methocel growth was the only culture condition where FL^1 3–3 grew as well as the other revertants. It is apparent that the growth characteristics of the revertants

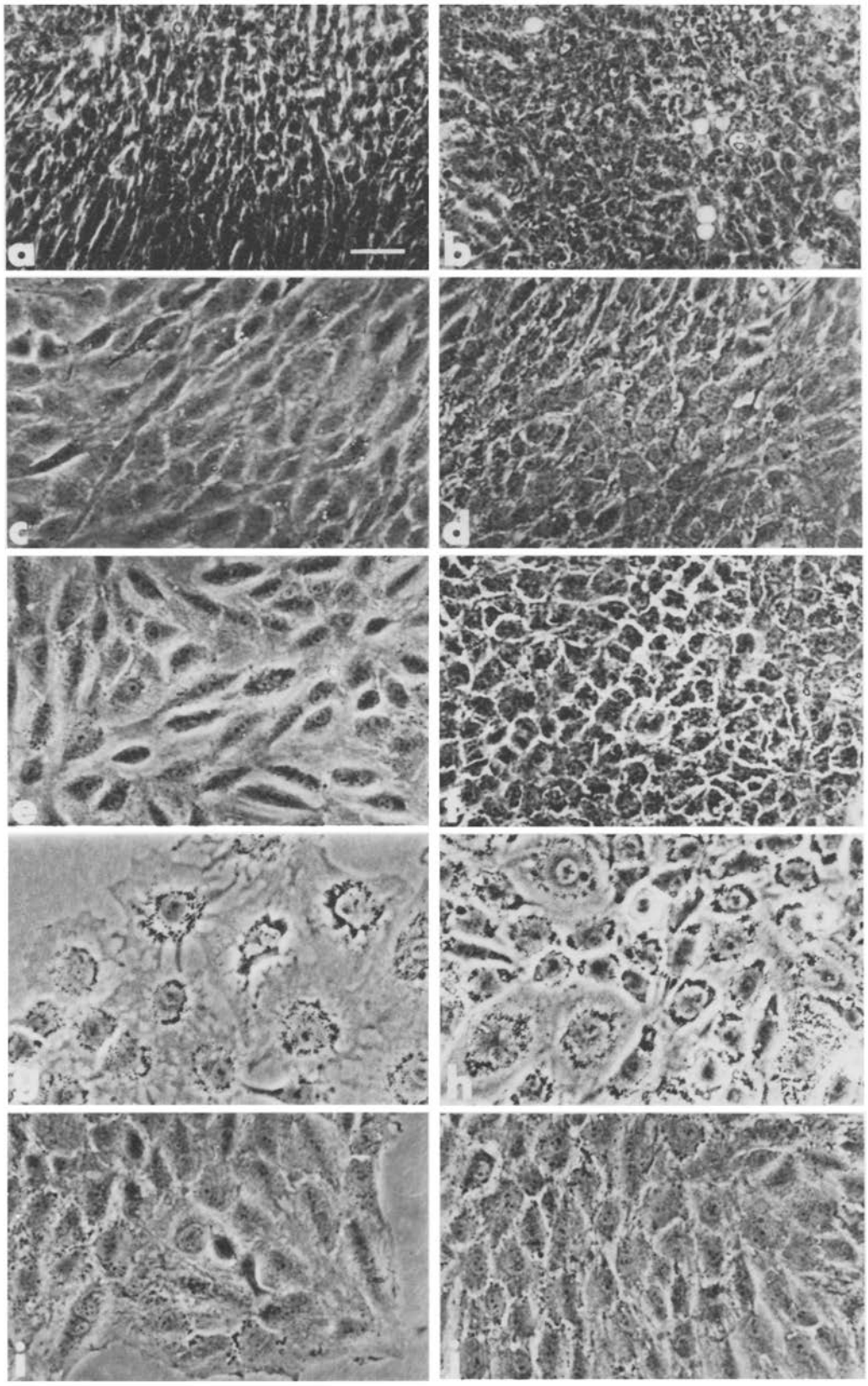

Figure 5. Cellular Morphology of 14B, Rat-1, FL¹ 1-4, FL¹ 3-3 and FL¹ 3-8

(a and b) 14B; (c and d) Rat-1; (e and f) FL¹ 1-4; (g and h) FL¹ 3-3; (i and j) FL¹ 3-8. (a, c, e, g and i) are from the edges of colonies, while (b, d, f, h and j) are from the centers of colonies. Bar=13 μ.

Table 3. Growth Properties of Cell Lines Derived From Rat-1 Cells

Cell Line	Doubling Time (Hr)		Saturation Density[a]		Plating Efficiency on Plastic[b] (10% FCS)	Plating Efficiency Methocel[b] (10% FCS)	Relative Plating Efficiency Methocel/Plastic
	10% FCS	1% FCS	10% FCS	1% FCS			
Rat-1	22	32	23	6.5	0.80	6×10^{-5}	7×10^{-5}
14B	14	22	83	28	0.35	0.05	0.15
FL[1] 1-4	26	56	16	4.9	0.62	3×10^{-5}	5×10^{-5}
FL[1] 3-3	35	68	3.4	0.4	0.11	5×10^{-5}	4.5×10^{-4}
FL[1] 3-8	27	56	8.4	6.3	1.0	4×10^{-5}	4×10^{-5}

[a] Cells per $cm^2 \times 10^{-4}$.
[b] Number of colonies formed per number of cells inoculated.

are more like Rat-1 than like the transformed line 14B.

Cytoarchitectural Proteins

There appear to be differences in the distribution, localization and packing of actin cables between SV40-transformed 3T3 cells and normal 3T3 cells (Goldman, Yerna and Schloss, 1976; R. D. Goldman and R. Pollack, manuscript in preparation). This results in a reduction in the number of transformed cells containing cables which can be visualized by immunofluorescent staining. 9% of the cells in line 14B contained visible cables. Rat 1 (55% cables), FL[1] 1-4 (60% cables), FL[1] 3-3 (57% cables) and FL[1] 3-8 (81% cables) approach the value for REF (84% cables).

Chen, Gallimore and McDougall (1976) have reported that adenovirus-transformed cells lack LETS (Hynes, 1974). In contrast, all our cell lines, including 14B, had large amounts of LETS over the surface of the colonies.

FUdR Sensitivity of Revertants

The possibility existed that the FUdR treatment selected for variants which were resistant to FUdR, rather than ones which were growth-controlled. To test this, sparse cultures of three revertant lines plus 14B and Rat-1 were exposed to concentrations of FUdR ranging from 3×10^{-3} μg/ml to 30 μg/ml. With all the cell lines, increasing the concentration of drug decreased the number of cells capable of forming colonies (Figure 6). Cells were treated for 4 days to ensure that all cells might undergo DNA synthesis since FL[1] 3-3 grows with such a slow doubling time.

Lines 14B, Rat-1 and FL[1] 1-4 all show a similar response to increasing drug concentration. Lines FL[1] 3-3 and FL[1] 3-8 are slightly more sensitive. Clearly, revertants are not FUdR-resistant cells.

Discussion

We have isolated stable nonconditional revertants from an SV40-transformed cell line, 14B, which contains one copy of the viral genome in a pseudodiploid cell. These revertants are T antigen-negative, density-sensitive, anchorage-sensitive and nontumorigenic, and arise with a relatively high frequency. One group of revertants contains genome length SV40 DNA, one group has deletions in the SV40 DNA, while another group contains no detectable SV40 DNA at all. These results argue for a direct role for a functioning viral genome in the maintenance of the transformed state. The evidence for reversion through loss of an early SV40 gene function is, of course, circumstantial based upon the correlation that in all seven revertants selected, all had lost the ability to express the T antigen gene. Furthermore, in four out of seven examples, either the SV40 genome was lost or had suffered a deletion in the early region of the viral DNA. These results, coupled with the high frequency of reversion, make it seem rather probable that any *one* of a series of different events can result in the initiation of reversion.

The fact that some of these revertants can be retransformed at normal frequencies reinforces this notion (see below). On the other hand, it should be clear that we do not know what function of the early SV40 gene product(s) has been selected against. It seems probable that the T antigen may have functions other than that for which temperature-sensitive mutations are available. In addition, there exists the possibility that another SV40 early protein is made whose expression may be coordinate with T antigen expression; baroque possibilities which involve splicing of two separate genes or overlapping coding sequences which would fit into a scheme involving coordinate expression of the genes are well beyond the scope of this discussion. Suffice it to say that there is a considerable amount of data which shows that there are two early complementation groups, both of which are involved in transformation for the sister virus polyoma of SV40 (for example Eckhart, 1977; Fluck, Staneloni and Benjamin, 1977).

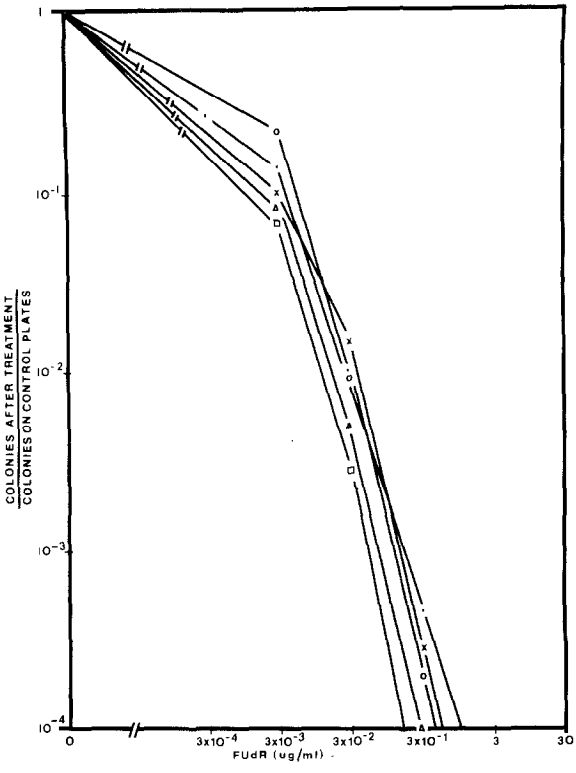

Figure 6. FUdR Sensitivity of 14B, Rat-1, FL¹ 1-4, FL¹ 3-3 and FL¹ 3-8

(●——●) 14B; (×——×) Rat-1; (○——○) FL¹ 1-4; (△——△) FL¹ 3-3; (□——□) FL¹ 3-8.

14B contains only one copy of SV40 DNA, grows in low serum, grows to a high density, forms colonies in methocel, and forms rapidly growing tumors in nude mice and invasive malignant tumors in newborn Fisher rats (unpublished observations). Thus the maximal transformed phenotype need not require multiple copies of the SV40 genome, and the different phenotypes of transformation need not be a function of SV40 gene dosage.

The reverants isolated from 14B are unlike the density-inhibited revertants which have been reported previously (Pollack et al., 1968; Pollack and Burger, 1969; Culp and Black, 1972; Wicker et al., 1972). The class of revertants which have completely lost the SV40 genome are totally unable to express any SV40-directed modifications of growth control, and might be expected to resemble most closely the nontransformed grandparental line Rat-1. Instead, they are more like primary cells than the established line Rat-1. If the progenitors of 14B in the sojourn from a normal Rat-1 cell to a fully transformed cell became dependent upon viral functions, and in so doing dispensed with their normal regulatory pathways (in part), one might predict that loss of this viral gene would leave the cell unable to grow in the same manner as its unperturbed ancestors.

All the revertants we have described are T antigen-negative, while all earlier revertants were T antigen-positive. The T antigen-positive revertants were less growth-controlled than their normal grandparents (Culp and Black, 1972; Pollack and Vogel, 1973; Vogel et al., 1973), while these T antigen-negative revertants have regained all the normal growth regulation properties we have examined. Furthermore, a large percentage of the 14B colonies surviving even one FUdR cycle are flat, and of these a majority are T antigen-negative. This suggests that loss of early SV40 gene expression is a very efficient pathway to reversion, and that early SV40 genes therefore have a major role in the maintenance of the transformed state. While we do not know the mechanisms by which this loss of expression arose, our data suggest the following possibilities:

– The most obvious way for 14B to have lost T antigen is through the loss of the viral genome, generating the class of cured cells. This loss could be the result of excision of the viral genome or the loss of all or part of the chromosome containing SV40. The cured cell line FL¹ 3-8 has a modal chromosome number slightly lower than Rat-1, suggesting that these cells might have lost one chromosome. Banding studies are now in progress to answer this question.

– Alternately, there could have been a mutation in the SV40 genome which prevents the subsequent transcription or translation of a stable viral gene product.

– The cellular genome can be altered either genetically or epigenetically in such a way as to block viral gene expression.

Any of these possibilities would result in revertants if viral gene expression is required for the maintenance of transformation. At the present time, we cannot prove by direct arguments that any of the above mechanisms were the cause of reversion, but it seems probable the FL¹ 3-2, FL¹ 3-7 and FL¹ 3-8 were generated by the first category, FL¹ 3-3 and FL¹ 3-5 by the second category, and FL¹ 1-3 and FL¹ 1-4 by the third category. Superinfection of the revertants with SV40 DNA produces two very different sets of results (our unpublished data). Those revertants which had lost the SV40 genome (FL¹ 3-7 and FL¹ 3-8) and one of those with a deletion in the SV40 genome (FL¹ 3-5) were retransformed, as measured by focus formation and growth in methocel, as efficiently as Rat-1 (for example, 0.1% for the focus formation), presumably by the replacement of the lost or altered genome. The other revertants, which contain the SV40 genome, were not transformed at measurable frequencies. This would suggest that these cells may be unable to express the SV40 early region or are refractory to the SV40 functions.

Preliminary evidence shows, however, that when a large number of molecules of purified SV40 DNA are injected directly into the revertant nucleus, T antigen is synthesized (A. Graessman, personal communication). Additional experiments are currently being performed to define the relationship between T antigen expression, reversion and retransformation in these revertants.

A major difference between these lines and others (Pollack, Wolman and Vogel, 1970; Culp et al., 1971; Vogel, Ozanne and Pollack, 1975; Bradley and Culp, 1977) is their pseudodiploid chromosome number. Nearly all the revertants in the literature have grossly elevated chromosome numbers. In contrast, most cells of Rat-1, 14B, FL[1] 1-4 and FL[1] 3-8 have modal chromosome numbers very close to the normal rat diploid number of 42 (see Table 4). Chasin (1974) has shown that the frequency of mutant expression is a function of the number of positive alleles present within the cell genome which are involved with the phenotype in question. For example, the spontaneous mutation frequency from A^+/A^+ to A^-/A^- for APRT is the square of the frequency of mutation from A^+/A^- to A^-/A^-. He measured the spontaneous frequency of APRT loss in the heterozygote at 1.5×10^{-5}. The frequency of all T antigen-negative revertants from 14B is not too different from the heterozygote mutation frequency measured by Chasin. It may well be that the pseudodiploid character of Rat-1 and 14B, as well as the presence of a single copy of SV40 DNA per cell, allowed for mutation to a T-negative revertant state to occur in 14B at frequencies such that the revertants could be easily isolated. Thus pseudodiploid cell lines may more frequently spontaneously alter expression of cellular genes which are involved in viral gene expression than do cell lines which have multiple copies of these genes. Lines FL[1] 1-3 and FL[1] 1-4, revertants which show no detectable changes in their integrated viral DNA and are not readily retransformed, are putative examples of this sort of variant. In any case, it seems probable that the reversion to a T-negative state that we have measured at 8×10^{-5} is the sum of a few different processes.

This set of cell lines has made it possible for us to ask a number of questions about transformation which were difficult to ask with other transformed lines. We can now question the absolute requirements for viral products to maintain the transformed state. The reverants from this line should allow us to determine the cellular contributions to the maintenance of transformation, and the ways in which such cellular contributions can be modified to produce lines which appear even more normal than the original Rat-1 line. This type of system may allow us to develop genetic models for the regulation of viral DNA integrated into the eucaryotic genome.

Table 4. Chromosome Number

Cell Line	% Pseudodiploid	% Pseudotetraploid
Rat 1	86	14
14B	80	20
FL[1] 1-4	92	8
FL[1] 3-3	<1	100
FL[1] 3-8	90	10
REF	100	0

Experimental Procedures

Culture Conditions

All experiments were performed in an atmosphere of 10% CO_2, 90% air, with a relative humidity of 100%. Unless specified differently, the temperature was maintained at 37°C. Cultures were grown in 60 mm plastic dishes (Falcon) in Dulbecco's modified Eagle's medium (Gibco H 21) supplemented with 100 units per ml of penicillin and streptomycin (Gibco) and 10% fetal calf serum [FCS (Reheis Laboratories)], unless a lower concentration is specified.

Cells

Rat-1, an established rat embryo line previously called F2408, has been described (Mishra and Ryan, 1973; Botchan et al., 1976). The SV40 transformant, 14B, was isolated as a dense focus following transformation of Rat-1 by purified SV40 DNA (Botchan et al., 1976).

Reversion

Revertants were isolated by plating 14B at 10^5 cells per 60 mm dish and growing for 5 days to yield dense plates containing approximately 5×10^6 cells. The plates were exposed to either 0.3 μg FUdR or 30 μg FUdR plus a 10 fold excess concentration of uridine (Pollack et al., 1968) for 2 days. The medium was then changed, and surviving cells were suspended in 0.05% trypsin with 5×10^{-4} EDTA (Gibco) and replated without further dilution. After culturing for 9 days, isolated colonies were picked using sterile cloning rings. Strains FL[1] 1-3 and FL[1] 1-4 were derived from plates exposed to 0.3 μg/ml FUdR plus 3 μg/ml uridine. Strains FL[1] 3-3, 3-5, 3-5 and 3-8 were exposed to 30 μg/ml FUdR plus 300 μg/ml uridine.

Growth Curves

Cells were seeded at 1.2×10^4 cells per cm^2 in 10% FCS. After 6 hr, the medium was changed to the desired serum concentration. The number of cells per plate was counted daily in a Coulter counter after trypsinization. The medium was changed every 2 days.

Plating Efficiency

Cells were trypsinized, counted and diluted into 60 mm dishes at 10^2 and 10^3 cells per dish. The medium was changed twice weekly. After 14 days, the cells were fixed in 10% formalin in PBS and stained with hematoxylin, and the colonies were counted.

Growth in Methocel

The ability of cells to grow in the absence of anchorage was determined by plating 10^5, 10^4 and 10^3 cells per 60 mm dish in culture medium containing 1.3% methyl cellulose (Methocel, 4000 cps; Dow Chemical Co.) over a layer of 1% agar (Difco Bacto Agar) in the same medium (Risser and Pollack, 1974). Cells were fed twice weekly with an additional 2 ml of methocel

preparation and cultured for 3 weeks. Colonies > 0.15 mm in diameter (visible by eye) were scored using a dissecting microscope.

Immunofluorescent Assays

The cells were seeded on coverslips in 35 mm dishes at either 10^3 cells per cm^2 or 10^4 cells per cm^2 and grown for 2–3 days. At the end of this period, the dishes seeded at 10^3 cells per cm^2 were still sparse, while those seeded at 10^4 cells per cm^2 were confluent. The cells were then fixed in 100% methanol and stained with hamster anti-SV40 T antigen (NIH), followed by fluorescein-conjugated rabbit anti-hamster immunoglobulin G (Flow). Actin was determined by fixing sparse cells in 10% formaldehyde, post-fixing in acetone and staining with rabbit anti-actin, followed by goat anti-rabbit immunoglobulin G (Flow), as described by Pollack and Rifkin (1975). LETS was determined using rabbit anti-human cold-insoluble globulin, followed by fluorescein-conjugated goat anti-rabbit immunoglobulin G, as described by Chen et al. (1976). The rabbit anti-actin was a gift from Dr. K. Burridge, and the rabbit anti-human CIG was a gift from Dr. L. B. Chen, both of Cold Spring Harbor Laboratory.

FUdR Toxicity

The cells were plated at 2×10^3 cells per cm^2, incubated over night and treated with FUdR as previously described (Pollack et al., 1968).

Fluctuation Analysis

The frequency of appearance of "flat" colonies in clutures of 14B, following FUdR treatment, was determined by a fluctuation analysis as described by Pollack (1970), modeled on the procedure of Luria and Delbrück (1943). Confluent cultures of 14B (7×10^6 cells per 60 mm dish) were grown from either 10^2 or 10^5 cells. The cultures were treated for 2 days with 3 μg/ml FUdR + 30 μg/ml uridine, and then suspended with trypsin, diluted and plated. After 2 weeks, the colonies were fixed and stained, and the number of dense and "flat" colonies was counted.

In a parallel experiment, a confluent 60 mm plate was treated with FUdR ± uridine for 2 days, suspended with trypsin and diluted to a final volume of 16 ml. 2 ml aliquots were transferred to eight 35 mm dishes containing one 18 mm glass coverslip per dish. After 2 weeks, the coverslips were fixed and stained for T antigen, and mounted cell side up on slides. The number of T antigen-negative colonies per coverslip was counted. The slides were then stained with hemotoxylin, and the number of "flat" colonies was counted.

Immunoprecipitation of SV40 T Antigens

For labeling cell proteins with ^{32}phosphate (Ortho phosphoric acid carrier-free from New England Nuclear), transformed cells were grown to semiconfluence and starved for phosphate by incubation in phosphate-depleted media for 3–5 hr. The cells were then given 1 mCi of ^{32}P-orthophosphoric acid per ml in complete media and allowed to incorporate the label for 2 hr. Nuclei from these cells were then isolated, and the immunoprecipitates were prepared from extracts of these nuclei as described by Tjian (1978). The extraction buffer contained 0.01 M Tris-HCl, (pH 8.0) 1 mM EDTA, 0.4 M $LiCl_2$, 200 μg/ml PMSF, 0.5 mM dTT. Samples were analyzed on gradient 7–15% polyacrylamide gradient gels (Tjian and Pero, 1976). The ^{35}S-methionine protocol was as described by Prives et al. (1977), except that the cells were prestarved in methionine-free media and labeled for 2 hr in the presence of 250 μCi/ml of methionine in methionine-free media. The use of NP-40 in the extraction buffers increases the probability of proteolytic degradation of the 95,000 dalton protein (R. Tjian, personal communication). A predominant band, however, seen both in lytic infections and in transformed cells at an apparent molecular weight of 15,000 daltons which may be a second early SV40 protein is not seen without this detergent extraction (R. Hanich and M. Sleigh, personal communication).

Detection of Integrated Viral DNA in Chromosomal DNA of Cells

The blotting experiments described were performed according to procedures previously detailed by Botchan et al. (1976). For those results shown in Figure 4, the DNA samples were fractionated by electrophoresis in 1.4% alkaline-agarose gels. The conditions for this electrophoresis system are as described by McDonnell, Simon and Studier (1977). The capacity of this gel system is high. Each lane contained 50 μg of Pvu II-cleaved total cell DNA except for the slot labeled R, which contained the digestion products of 50×10^{-6} μg of SV40 DNA and 1 μg of Ad2 DNA. After transfer to nitrocellulose and hybridization with labeled SV40 DNA probe, the fragments were detected by autoradiography. The image was enhanced by the use of Dupont "Lightning-Plus" intensifier screens (P. Shank and S. Hughes, personal communication).

Chromosome Number

Determination of chromosome number was carried out by the method of Vogel et al. (1975).

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