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Detection and Quantitation of Simian Virus 40 Genetic Material in Abortively Transformed BALB/3T3 Clones

(mice/diploid cells/virus equivalents)

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ABSTRACT **Infection with simian virus 40 is known to induce many cells to synthesize DNA and to divide in a medium lacking serum protein growth factor(s) that is essential for growth of uninfected cells (factor-free medium). Cells infected under these conditions then go through several rounds of division, since colonies containing more than 100 cells are formed. Many of these colonies are abortively transformed since, upon subsequent passage of the cells in standard medium, they can no longer grow in factor-free medium and show no other properties of viral transformation. We have examined these abortively transformed cells for the presence of simian virus 40 DNA sequences. Of the three clones tested, two were found to contain viral genetic material despite the fact that they were phenotypically normal.**

The number of simian virus 40 genome equivalents present was determined by measurement of DNA reassociation kinetics on hydroxyapatite. Two of the abortively transformed lines contained approximately five viral genome equivalents per diploid cell, while the DNA from a third abortive transformant was indistinguishable from that of uninfected BALB/3T3 cells. A standard simian virus 40 transformant, isolated under similar conditions, contained two copies of the viral genome per cell. The abortive transformants also appear to contain the entire viral genome rather than multiple partial copies. Subclones of one abortively transformed line containing five copies per cell had 2.7–10 copies of viral genetic material per diploid cell.

Most cells that are transformed by simian virus 40(SV40), are characterized by their ability to form multiple cell layers under conditions where uninfected cells remain confined to a monolayer (1–3). Such transformants are also able to grow in agar suspension (4) and in medium lacking serum growth factors (factor-free medium) (5, 6). Differences between the cell surfaces of transformants and nontransformants can also be detected by use of plant lectins, such as conconavalin A (7) or wheat germ agglutinin (8). SV40-transformed lines also synthesize virus-specific tumor and transplantation antigens (9, 10) and most of them release infectious virions after fusion with permissive cell lines (11–13).

Some SV40-transformed lines are called "flat transformants". These are lines that synthesize SV40 tumor antigen, yield virus after fusion with permissive cells, and are able to grow in factor-free medium, but do not grow in multiple cell layers (14). Other cells, when infected under conditions where few cells become permanently transformed, are abortively transformed. These cells transiently express properties of viral transformation, such as synthesis of tumor antigen (15), agglutination by conconavalin A (7), and induction of DNA synthesis at confluence (16–18). Upon subsequent passage, the cells usually return to what appears to be the nontransformed state. These properties cannot be used to select abortively transformed cells, however, since assay procedures cause cell death. Smith *et al.* circumvented this problem by using the ability of transformed cells to grow in factor-free medium (6). When cells planted sparsely (1500–5000 cells/plate) in factor-free medium were infected with SV40, most were induced to form large colonies (greater than 100 cells). These colonies were then isolated and grown to mass culture in complete medium. After growing to mass culture, many of the abortively transformed clones were no longer able to grow in factor-free medium and had no other property usually associated with SV 40 transformation. They were negative for SV40 tumor antigen when tested by fluorescent antibody staining and complement fixation, did not yield virus after fusion with permissive cells, and remained contact inhibited (6). Since a functioning viral genome was required to induce colony formation (6), these clones must have contained the SV40 genome during their passage in factor-free medium.

How do these abortively transformed clones regain the growth properties of uninfected cells? One possibility is that they degrade or somehow eliminate the SV40 genetic material. Alternatively, the viral DNA may remain associated with the cells in a cryptic or nonfunctioning state. To distinguish between these hypotheses, we utilized the nucleic acid hybridization techniques used by Gelb *et al.* (19) to detect SV40 DNA sequences within abortively transformed cells. Our results indicate that two out of three abortively transformed lines continue to contain SV40 DNA. These phenotypically normal cells have more than twice the number of SV40 DNA equivalents than a standard SV40 transformant isolated under similar conditions. Furthermore, these abortively transformed lines appear to contain the entire SV40 genome.

MATERIALS AND METHODS

Cell Lines and Culture. BALB/3T3 clone A31 (14) and clones isolated from this line after SV40 infection in factor-

Abbreviation: SV40, simian virus 40.

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free medium (5, 6) were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.). The abortive transformants (Clones 12A1, 12A2, and 12A3) and the standard transformant (clone 11A8) were isolated as colonies containing about 200 cells, grown to mass culture, and frozen at a relatively early passage (about 50 generations in culture after cloning). The abortively transformed clones were contact inhibited, failed to yield virus after fusion with African green monkey cells, were negative for the SV40 tumor antigen, and did not grow in factor-free medium. These stocks were used to prepare 30–50 prescription bottles (32 oz [940 ml]) of each line. Reclones of line 12A3 were made by seeding 100 cells per 60-cm petri dish, isolating well separated colonies, and growing the cells to mass culture. The reclones were negative for SV40 tumor antigen and exhibited contact inhibition. ^{32}P-labeled SV40 virus was purified from infected VERO cells as described (19).

Detection of SV40 DNA Sequences in Abortively Transformed Lines. The preparation of normal, transformed, and abortively transformed cellular DNA has been described (19). These DNA solutions were sheared at 50,000 lbs/inch^2 in a Ribi cell fractionator in 0.01 M phosphate buffer (pH 6.8, equimolar parts Na_2HPO_4 and NaH_2PO_4) and 1 mM EDTA, precipitated in two volumes of cold ethanol, resuspended in distilled water, and dialyzed against 0.1 M NaCl before use. The preparation of SV40 [^{32}P]DNA I, sheared at 50,000 lbs/inch^2 has also been described (19). An approximately 10^6-fold excess of cellular DNA was mixed with the ^{32}P-labeled SV40 DNA, heat denatured at 100°C for 5 min, quenched in ice water, adjusted to a concentration of 0.6 M phosphate buffer, and incubated at 68°C. Aliquots were removed periodically and passed through columns of hydroxyapatite at 60°C for measurement of the fraction of ^{32}P DNA reannealed at each interval. Single-stranded DNA was removed in 0.14 M phosphate buffer and double-stranded DNA was eluted with 0.4 M phosphate buffer. Each fraction was precipitated in 5% trichloroacetic acid, filtered, and counted in a liquid scintillation spectrometer. These values were then plotted as a function of Cot, the product of the concentration of ^{32}P-labeled nucleotides in mol/liter and the time in seconds (20, 21). The magnitude of the reassociation rate increase produced by the addition of unlabeled cellular DNA then permits the calculation of SV40 DNA equivalents contained within the mammalian DNA (19).

RESULTS

Quantitation of SV40-like DNA sequences within normal and transformed cells was accomplished by comparison of the reassociation of ^{32}P-labeled SV40 DNA in the presence of unlabeled cellular DNA with that of a control mixture containing ^{32}P-labeled SV40 DNA and salmon-sperm DNA (added to control viscosity). Fig. 1 illustrates the reassociation curves obtained when ^{32}P-labeled SV40 DNA was mixed with salmon-sperm DNA, BALB/3T3 DNA (the parent cell), 11A8 DNA (the standard transformant), and DNA from two abortive transformants (12A1 and 12A3). BALB/3T3 DNA increased the rate of reassociation of ^{32}P-labeled SV40 DNA only slightly compared to the control reaction. This increase indicates the presence of 0.39 copies of SV40 DNA per diploid cell, a value similar to that reported in other

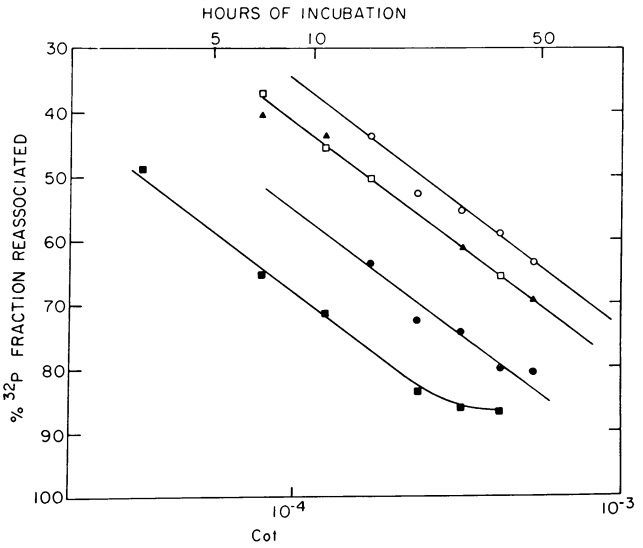

FIG. 1. Reassociation of ^{32}P-labeled SV40 DNA with abortively transformed murine DNAs. Each reaction mixture contained 1.2 ng/ml of sheared ^{32}P-labeled SV40 DNA (829,000 cpm/μg), 2.5 mM EDTA, 0.6 M phosphate buffer and 1.81 mg/ml of sheared DNA from salmon sperm (○), uninfected BALB/3T3 clone A31 (□), standard transformant 11A8 (●), abortive transformant 12A1 (▲), or abortive transformant 12A3 (■). The DNA mixtures were denatured at 100°C, incubated at 68°C, and periodically monitored for reassociation on hydroxyapatite columns. Cot, represents the product of the concentration of ^{32}P-labeled nucleotides in mol/liter and the time in seconds.

normal mammalian cell lines‡ (19). Clone 11A8 increased the reassociation of ^{32}P-labeled SV40 DNA 3.3-fold, equivalent to 1.96 copies of SV40 DNA per diploid cell. This value is similar to that which has been found in other virus-free transformed lines by use of this hybridization technique (19). The DNA from the two abortively transformed lines had markedly differing effects on the [^{32}P]DNA reassociation. The DNA from line 12A1 behaved like the uninfected BALB/3T3 DNA, while the DNA from the abortive line 12A3 increased the rate of [^{32}P] DNA reassociation by a factor of 7.1. This increase is equivalent to 5.2 copies of SV40 DNA per diploid cell.

A third abortively transformed line, 12A2, was also tested for the presence of SV40-specific DNA sequences. Fig. 2 shows that the DNA from this line, like the abortive line 12A3, increases the rate of reassociation of ^{32}P-labeled SV40 DNA 6.7-fold. This figure also shows that another preparation of line 12A1 DNA again had little effect on the reassociation of ^{32}P-labeled SV40 DNA compared to the control reaction.

Since the loss of viral genetic material with passage could account for the difference between clones 12A1 and 12A3, we next examined subclones of 12A3 for viral DNA sequences. Three independent isolates were cloned from abortive line 12A3. All three had growth properties similar to the original

‡ Calculation of the number of viral equivalents in the cellular DNA has been described in detail (19). Briefly, the number of viral equivalents in each of these cells is calculated by multiplying the amount of ^{32}P-labeled SV40 DNA added per mammalian diploid cell in the reaction mixture by the (factor of increased rate minus 1).

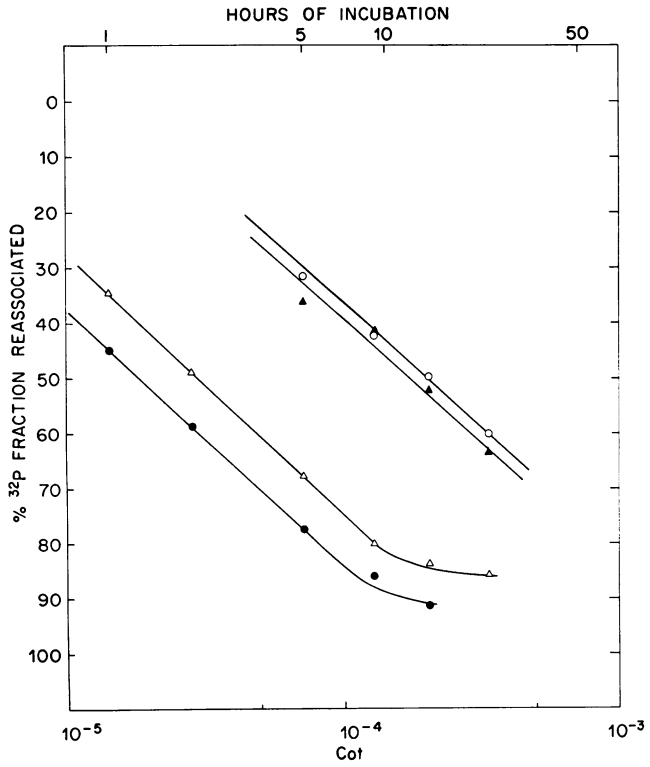

FIG. 2. Reassociation of ^{32}P-labeled SV40 DNA with abortively transformed murine DNAs. Each reaction mixture contained 1.42 ng/ml of sheared ^{32}P-labeled SV40 DNA (656,000 cpm/μg), 2.5 mM EDTA, 0.6 M phosphate buffer, and 1.91 mg/ml of sheared DNA from salmon sperm (○), abortive transformant 12A1 (▲), abortive transformant 12A2 (△), or abortive transformant 12A3 reclone 10 (●). The DNA mixtures were denatured at 100°C, incubated at 68°C, and periodically monitored for reassociation on hydroxyapatite columns.

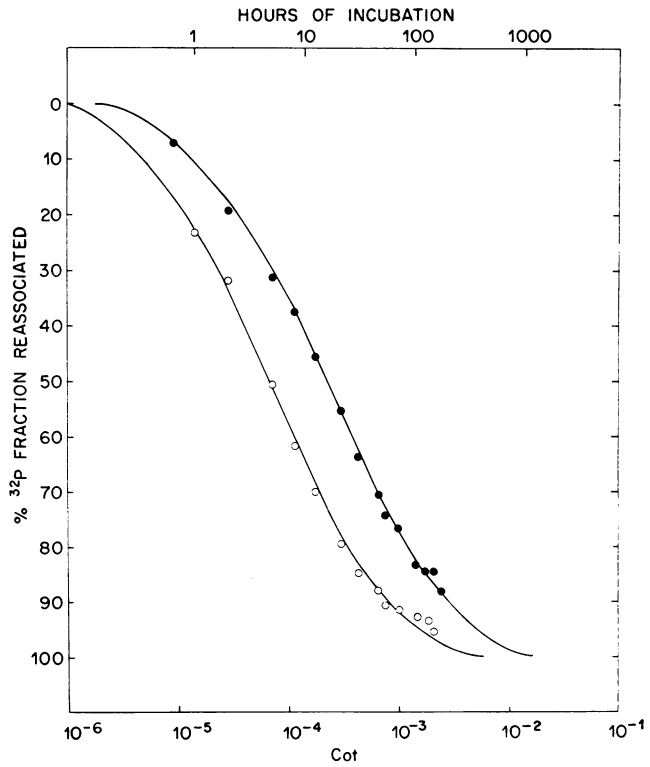

FIG. 3. Reassociation of ^{32}P-labeled SV40 DNA with 12A3 reclone 3 DNA. Each reaction mixture contained 1.42 ng/ml of sheared [^{32}P] SV40 DNA (656,000 cpm/μg), 2.5 mM EDTA, 0.6 M phosphate buffer, and 1.67 mg/ml of sheared DNA from salmon sperm (●) or 12A3 reclone 3 (○). The DNA mixtures were denatured at 100°C, incubated at 68°C, and aliquots were monitored periodically for reassociation on hydroxyapatite columns.

clone, 12A3, and were also negative for SV40 tumor antigen. DNA from 12A3 reclone 10 (Fig. 2) accelerated the reassociation reaction even more than the parent clone, 12A3 (Fig. 1). This reclone contained about 10 genome equivalents per diploid cell, while the original clone 12A3 contained only 5.2 copies. A second reclone, 12A3 reclone 3, contained only 2.7 SV40 genomes per diploid cell (Fig. 3), while a third reclone, 12A3 reclone 8, contained 4.8 copies (data not graphed).

The presence of only partial copies of SV40 DNA within these abortively transformed cell lines could explain the inability to detect any of the characteristics associated with viral transformation. If the DNA preparations from abortively transformed cells contain only partial copies of SV40 DNA, there would be a disproportionate amount of some viral sequences compared to others, when this DNA is incubated with the labeled SV40 DNA probe. The reassociation curve obtained with such a mixture would then be biphasic, reflecting the greater concentration of some sequences (those shared by the cellular DNA and the labeled probe) over the remainder. The reassociation of such a mixture of DNAs (12A3 reclone 3 and ^{32}P-labeled SV40) was examined in detail (Fig. 3). The curve obtained was similar to that of the control reaction without any evidence of a biphasic response. Therefore, at the present level of detection, these abortive lines appear to contain the entire viral genome.

Table 1 summarizes our findings indicating the increase in the rate of reassociation produced by each of the lines tested and the calculated number of SV40 DNA equivalents per diploid cell. A standard transformant, 11A8, contains two viral DNA equivalents per diploid cell. Two out of three abortive transformants contain more viral DNA, while one abortive transformant shows no increase over uninfected BALB/3T3 cells. The values for three independent reclones of 12A3 ranged from 2.7 to 10 viral DNA equivalents per diploid cell. The two values indicated in Table 1 for 12A3 reclone 3 were obtained from entirely separate preparations of DNA. The variation in the number of viral DNA equivalents may indicate a real biological phenomenon rather than technical error, as standard SV40 transformants consistently contain 1.5–2.0 SV40 DNA equivalents per diploid cell when this technique is used.

DISCUSSION

Our experiments indicate that two of three abortively transformed lines contain SV40 genetic material even though they are phenotypically indistinguishable from uninfected mouse cells. These two positive abortive transformants (which we will call cryptic transformants) contained 3–10 copies of viral DNA per diploid cell. A standard SV40 transformant, isolated under similar conditions, contained two viral DNA equivalents per cell, a value very similar to that reported (19) previously. Our data (Fig. 3) also suggest that within the

limits of detection, most of the SV40 genome, rather than multiple partial copies, is present in these clones.

How does the viral DNA in the cryptic transformant differ from the viral DNA associated with standard SV40 transformants? One possibility is that the viral DNA exists in different physical states in cryptic and standard transformants. Sambrook *et al.* (22) have shown that SV40 genetic material in virus-free transformed cells is linked to mammalian DNA by an alkali-stable (covalent) bond. The viral DNA in the cryptic transformants may be associated with mouse DNA by a less stable linkage, such as hydrogen bonding of homologous nucleotide sequences. Under these conditions, the SV40 DNA may not be capable of responding to host cell or endogenous viral control mechanisms that regulate gene expression. Two observations suggest that the SV40 DNA may indeed be less-stably associated with host cell DNA in cryptic transformants. First, three subclones of one cryptic transformant contain different amounts of SV40 DNA. Second, there was more variation in the number of genomes per cell in two different DNA preparations of the same line (12A3 reclone 3) than we usually see in standard transformants (19). Further experiments are needed to determine the stability of the SV40 DNA in these lines and the nature of the linkage between viral and host DNAs.

An alternative explanation is that the SV40 DNA detected in these cells is integrated at a different chromosomal site than in standard transformants. If adjacent mammalian-cell DNA sequences play an important role in modulating the transcription of integrated viral genetic material, the presence of an inappropriate host gene near the integration site could account for the lack of any SV40 gene function. This SV40 DNA could then not be transcribed.

All of the abortive transformants initially displayed evidence of SV40 infection, such as tumor antigen and the ability to grow in factor-free medium. Sometime after the cryptic transformants were removed from factor-free medium and propagated in complete medium, viral gene activity ceased. When and why did this cessation occur? The absortive transformants were isolated from factor-free medium after they had grown to colonies containing more than 200 cells; therefore, phenotypic expression of viral transformation must have continued for at least seven or eight cell divisions after infection. Since the cells were initially infected with a multiplicity of about 50 plaque-forming units/cell (6), it is not clear whether the SV40 gene products that transiently appeared during abortive infection were transcribed from the same input viral DNA that became stably associated with the cryptic transformants.

The presence of nunfunctioning SV40 genomes in mammalian cells suggests that integration of viral DNA may be a frequent occurrence. Previous methods that have been used to detect those cells containing viral genetic material have depended on evidence of viral DNA function, such as tumor antigen synthesis or loss of contact inhibition. The selection of cryptic transformants relies only on the ability of cells to grow in factor-free medium immediately after virus infection. The vast majority of these cells appear phenotypically normal, yet two of three lines tested carry SV40 DNA. Cryptic transformants may represent the most common cell type harboring the viral genome, but have remained unrecognized until now because the methods used for selection have depended on those properties characteristic of the transformed state.

TABLE 1. *SV40 genome equivalents in BALB/3T3 cells*

Cell line	SV40 DNA equivalents per diploid cell
Parent:	
BALB/3T3 clone A31	0.39
Standard transformant:	
11A8	2.0/2.3
Abortive transformants:	
12A1	0.39/0.17
12A2	5.7
12A3	5.2
12A3 reclone 3	2.7/6.2
12A3 reclone 8	4.8
12A3 reclone 10	10.0

Even though Fig. 3 suggests that the entire viral genome is present in 12A3 reclone 3, the measurement of DNA reassociation on hydroxyapatite columns is not sufficiently sensitive to detect the absence of up to 15% of the SV40 DNA sequences in this cell. If such sequences are involved, for example, with the binding of RNA polymerase, transcription of SV40 DNA could never occur and this would also explain the absence of any viral gene function.

The simplest interpretation of the one abortively transformed line, that did not contain SV40 DNA is that a stable association between viral genetic material and host cell DNA never occurred. One cannot rule out the possibility that this clone arose from a rare BALB/3T3 cell that spontaneously gained the ability to grow in factor-free medium. This seem unlikely, however, since there were no colonies as large as 12A1 in any mock-infected control plate (ref. 6 and H. S. Smith, unpublished observations).

It has been proposed that viral genetic material, which is carried by mammals in a latent form throughout life, becomes derepressed and results in cancer (23). The cryptic transformants provide and *in vitro* model system to study this kind of virus–cell interaction. SV40 DNA, which is capable of transforming cells, is being carried in these lines in a latent form. Experiments designed to test whether this latent SV40 can be induced to again express transformed properties may provide some insight into the ways in which viruses and cells interact to cause a loss of cellular growth control.

Finally, our experiments suggest a method by which new genetic information can be added to cells. Since the virus neither kills the cells nor permanently transforms them into tumor cells, it is possible that the viral genes could remain present in the genetic pool of a species for long periods in evolutionary history. These viral genes may be the initial source of the new gene families present in eucaryotic cell DNA (20).

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