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Characterization of a T-Antigen-Negative Revertant Isolated from a Mouse Cell Line Which Undergoes Rearrangement of Integrated Simian Virus 40 DNA

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A transformation revertant has been isolated from an unusual line of simian virus 40 (SV40)-transformed BALB/c-3T3 cells in which rearrangements of integrated viral sequences are common. The revertant produces no SV40 T antigens, yields no virus on fusion with permissive cells, and can be retransformed by SV40 virions. SV40 DNA sequences are present within the cellular DNA, but interruption of the viral early transcription region by deletion and recombination with cellular sequences precludes the synthesis of T antigens. Analysis of this revertant lends further support to the notion that large T antigen plays an essential role in the maintenance of transformation in SV40-transformed BALB/c-3T3 cells. Examination of integration of SV40 DNA in this revertant, as well as in a temperature-sensitive A transformant, after retransformation by SV40 confirms that sequence homology plays little role in the insertion of SV40 DNA into cellular chromosomes.

The identification of viral and cellular functions involved in cellular transformation by simian virus 40 (SV40) and other tumor viruses may be facilitated by the analysis of cells which, once transformed, have reverted to a normal phenotype. Two classes of such transformation revertants are expected: one class consisting of cells no longer expressing those viral functions required for the maintenance of transformation, and another class consisting of cells defective in a cellular regulatory element with which the viral protein(s) interacts either directly or indirectly to produce the transformed phenotype. Many of the revertants described to date appear to be of the latter class. They continue to express viral products (T antigens) and to release infectious virus on fusion with permissive cells, and they are resistant to retransformation by exogenous SV40 (24). In contrast, there have been two reports characterizing revertants of the first class. Starting with a transformed rat cell line that contained a single inserted genome of SV40, Steinberg et al. (22) isolated revertants which had sustained deletions of viral DNA and consequently failed to produce T antigens. Although this class of revertants might be expected to occur most frequently in cells containing single copies of SV40 DNA, Maruyama et al. (17) have reported recently the isolation of T-antigen-negative revertants derived from mouse and rat transformed cells containing two and six genome equivalents of SV40, respectively.

These various revertants of SV40-transformed cells have been isolated by selective killing of growing cells under conditions in which only transformed cells could grow (6, 19, 21, 25, 26). However, we have recently reported the isolation under nonselective conditions of a revertant from the progeny of an SV40-transformed mouse cell line which displays an unstable pattern of integration of SV40 sequences (2). This revertant, while retaining some SV40 sequences, fails to express SV40 T antigens (5). In this communication, we characterize the properties of this revertant in detail and define the basis for its failure to express T antigens. We also examine the role of sequence homology in the integration of superinfecting SV40 DNA in the revertant and observe that as originally documented by Botchan, Topp, and their colleagues (3, 8) in rat cells, the presence of extensive regions of sequence homology does not influence the site of integration of the superinfecting DNA.

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MATERIALS AND METHODS

Virus and cells. Propagation of untransformed and transformed BALB/c-3T3 mouse cells was performed as previously described (2, 4). Infection of revertant

cells with wild-type SV40 strain 776 and selection of retransformed cells were performed as outlined for the transformation of BALB/c-3T3 cells (4, 11).

Transformation assays. The abilities to form colonies on monolayers of normal cells, to grow to high saturation density, and to form colonies in semisolid medium were assessed as described elsewhere (4, 5). Unless otherwise noted, cells were grown at 33°C in Eagle minimal essential medium supplemented with 10% fetal calf serum.

Cellular DNA content analysis. Flow microfluorimetric determination of cellular DNA content was performed as outlined previously (5).

Detection of T antigens. Indirect immunofluorescence and immunoprecipitation assays for SV40 T antigens were as previously described (4, 5). In addition to antitumor serum, a monoclonal antibody, clone 122 (10), against the 54,000-molecular weight (54K) nonviral T antigen (NVT), generously provided by Arnold Levine, was used in immunoprecipitation assays.

Determination of chromosome number. The procedure for chromosome number determination was based on that of Friend et al. (7). Growing cells were exposed to 0.1 μg of Colcemid (Sigma Chemical Co.) per ml at 37°C for 2 h, harvested, swelled at 37°C for 25 min in 0.075 M KCl, fixed in methanol-acetic acid (3:1, vol/vol), and spread onto slides. The cells were stained for 5 min in 1% Giemsa in 0.05 M Tris-hydrochloride (pH 7.5). Chromosomes were counted in 100 isolated metaphase spreads for each cell line.

Virus rescue. Fusion of transformed or revertant cells with BSC-1 monkey cells mediated by UV-inactivated Sendai virus was performed as described by Brockman (4). Fusion mediated by polyethylene glycol (PEG 1000, Sigma) was performed as outlined by Botchan et al. (3). Twenty-one days after fusion, cells were lysed by freezing and thawing, followed by disruption in a well sonicator. Virus yield was determined by plaque assay of lysates.

Analysis of SV40 DNA sequences in transformed and revertant cells. Cellular DNAs were prepared as described by Bender and Brockman (2) and were digested by a variety of restriction endonucleases (Bethesda Research Laboratories). The reaction conditions for digestion with a single enzyme were those specified by the supplier. Digestion with two restriction enzymes was performed either simultaneously (*Bam*HI/*Bgl*I, *Bam*HI/*Taq*I, *Hpa*II/*Bel*I) using the buffer specified for the first enzyme of each pair and incubating at 37°C overnight (followed by a 2-h incubation at 65°C for *Bam*HI/*Taq*I) or sequentially. In the cases of *Bgl*I/*Eco*RI and *Hpa*II/*Bam*HI, digestion was performed with the first enzyme of the pair for 3 h (37°C), NaCl was added to the concentration specified for the second enzyme, and digestion with the second enzyme was performed for 3 h (37°C). For *Bgl*I/*Taq*I, digestion with *Bgl*I (3 h, 37°C) was followed by ethanol precipitation and digestion with *Taq*I (3 h, 65°C). Electrophoresis and transfer of DNA to nitrocellulose followed by hybridization to ^{32}P-labeled SV40-plasmid DNA were performed as described previously (2), except that electrophoresis was through either 1.0 or 1.2% agarose gels.

TABLE 1. Growth properties of B1 cell lines

Cell line	Saturation density (cells × 10^4/cm^2)[a]		EOP on monolayer[b]		EOP in agarose
	MEM 10	MEM 1	33°C	39.5°C	
B1-0			47.5	0.08	37.0
B1-1d	60	15	17.0	0.04	40.1
B1-2a			0.05	<0.01	<0.001
B1-3	12	1.2	0.16	<0.01	<0.001
WT Bla[c]		16	30.0	22.5	38.2
3T3	13	2.1	<0.01	<0.01	<0.001

[a] Measured in Eagle minimal essential medium supplemented with either 10% (MEM 10) or 1% (MEM 1) fetal calf serum.

[b] EOP, Efficiency of plating, expressed as the number of colonies per 100 cells plated.

[c] A BALB/c-3T3 cell line transformed by wild-type SV40 (4).

RESULTS

Derivation and growth properties of the transformation revertant. The transformation revertant characterized in this report was isolated in the course of cloning cells derived from a single colony of SV40 *tsA*58-transformed BALB/c-3T3 mouse cells (2). The uncloned progeny (designated line B1-0) of the original transformation colony was sequentially cloned to generate three successive clones, B1-1d, B1-2a, and B1-3, in order of isolation. Analysis of SV40 sequences in these various cell lines revealed that the pattern of integration of SV40 sequences in the primary transformants and some of their descendants was not stable; alterations in the arrangement of viral sequences resulted in the appearance of new integration patterns in the first two generations, B1-1d and B1-2a, of subclones (2).

These four members of the B1 family of cell lines were assayed for expression of the transformed phenotype by assessing their ability to overgrow confluent monolayers of uninfected BALB/c-3T3 cells, to form colonies in semisolid medium, and to grow to high saturation density. Because B1-0 was transformed by SV40 virions which had a temperature-sensitive lesion (*tsA*) in sequences coding for large T antigen, the temperature dependence of the transformed phenotype was tested by conducting the monolayer overgrowth assay at both permissive (33°C) and nonpermissive (39.5°C) temperatures. Lines B1-0 and B1-1d were able to overgrow monolayers, form colonies in soft agarose, and grow to high saturation density at 33°C (Table 1). As indicated by the results of the monolayer overgrowth assay, these two lines expressed the transformed phenotype in a temperature-dependent fashion. Line B1-2a, in contrast, displayed growth characteristics similar to those of normal BALB/c-3T3 cells at both permissive and nonpermissive temperatures. This revertant phenotype was retained in the subclone B1-3.

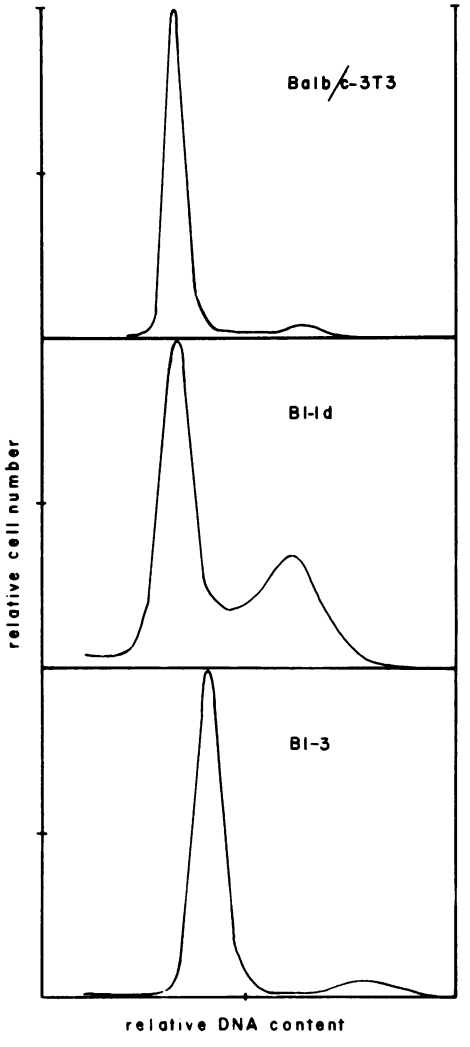

FIG. 1. Cell DNA content in BALB/c-3T3, B1-1d, and B1-3 cells. Flow microfluorimetry was performed on cells which had reached confluence.

As a further test of their growth properties, B1 cells were analyzed by flow microfluorimetric determination of DNA content. The distribution of cells at various stages of the cell cycle was determined when cells growing at 33°C had reached confluence, a condition under which 3T3 cells cease to grow. Transformed cells, in contrast, continue to synthesize DNA and divide at confluence. B1-1d cells behaved as transformed cells, continuing actively to synthesize DNA at confluence (Fig. 1); approximately 60% of the cells had the DNA content of G1 cells, whereas the remainder appeared to be in the process of, or to have recently completed, a round of DNA replication before cell division. In contrast, little DNA synthesis was seen in 3T3 cells at confluence, approximately 90% of the cells having the DNA content of G1-arrested cells. Cell cycling in B1-3 (Fig. 1) and B1-2a (data not shown) at confluence was dramatically decreased compared with that in their progenitor B1-1d. As noted previously (5), B1-3 cells when confluent or when plated below confluence in serum-deficient medium are not blocked in G1 as completely as are normal 3T3 cells. In this, as in the other assessments of the transformed phenotype, however, the growth properties of B1-3 more closely approximated those of untransformed 3T3 cells than those of the transformed progenitor cells. Expression of the transformed phenotype in B1 cells, then, has been altered coincident with the appearance of alterations in the arrangement of integrated viral DNA.

Chromosome number. Many revertants from SV40-transformed cells have been shown to have about a twofold increase in ploidy compared with their transformed progenitors (6, 9, 20). Such revertants continue to express T antigen and appear to have reverted by a cellular alteration. In contrast, because transformed cells frequently have a subtetraploid number of chromosomes (20), reversion resulting from loss of integrated SV40 sequences might be associated with a decrease in chromosome number. To determine whether reversion of the B1-1d transformant was associated with a change in ploidy, the chromosome number in the B1-3 revertant was compared with that in BALB/c-3T3 cells and in B1-1d cells (Fig. 2). Untransformed

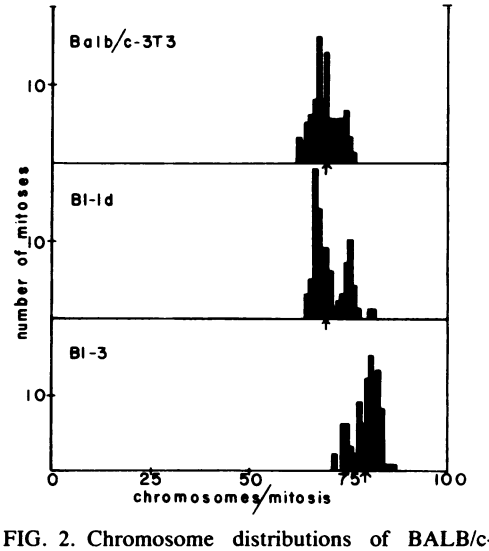

FIG. 2. Chromosome distributions of BALB/c-3T3, B1-1d, and revertant B1-3 cells. The mean number of chromosomes is indicated by the arrow.

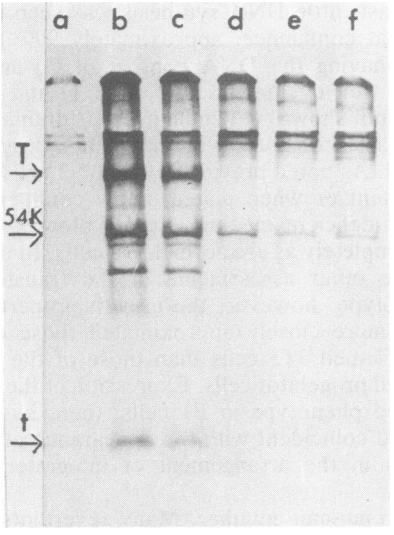

FIG. 3. SV40 T antigens in B1 cell lines; autoradiogram of ^{35}S-labeled immunoprecipitates separated by gel electrophoresis. The positions of large T, small t, and NVT antigens are indicated. Lane a, immunoprecipitate made with normal hamster serum; lanes a and b, B1-0; lane c, B1-1d; lane d, B1-2a; lane e, B1-3; lane f, normal 3T3 cells.

BALB/c-3T3 cells and the transformed cells had subtetraploid numbers of chromosomes (the mouse diploid number of chromosomes is 40), with modal numbers of 68 and 67 chromosomes per cell, respectively. Subtetraploidy is characteristic of normal, established—as well as SV40-transformed—mouse cell lines (20). The number of chromosomes in the revertant B1-3 had increased somewhat (modal number of 80) over that in the transformed progenitor, but not to the extent reported for T-antigen-positive revertants. This increase in chromosome number was probably responsible for the increase in DNA content of B1-3 cells compared with that of 3T3 and B1-1d cells (Fig. 1).

Expression of T antigens. Our previous studies have indicated that the presence of a functional SV40 large T antigen is required to maintain the transformed phenotype of most *tsA*-transformed BALB/c-3T3 cells (4, 5, 11). This phenomenon has been well documented in other cell lines by a number of investigators (reviewed in references 16 and 24). To determine whether the failure of the revertant cells to express a transformed phenotype correlated with a failure to express SV40 early proteins, each of the four B1 cell lines was examined for the presence of viral T antigens by indirect immunofluorescence and immunoprecipitation assays. In the immunofluorescence assay, the original transformant, B1-0, and its subclone, B1-1d, displayed the bright nuclear fluorescence of SV40-transformed cells. Neither of the subsequent clones, B1-2a and B1-3, stained positively for viral T antigens. Assays for immunoprecipitable T antigens, using antitumor serum (Fig. 3), revealed that both transformants, B1-0 and B1-1d, contained the 94K large T and the 21K small t antigens. Neither of these viral T antigens, however, was detected in the revertant B1-2a or its subclone B1-3. The antitumor serum was able to detect the host-encoded 54K NVT antigen in both the transformed and the revertant cells. The revertants, however, did not express NVT at levels above that in normal BALB/c-3T3 cells. Similar results for the relative amounts of NVT in the various cell lines were obtained when immunoprecipitation was performed with monoclonal anti-NVT antibodies. The difference in NVT levels in the transformed and revertant cells probably reflects a role for large T in the stabilization of NVT (15, 18).

Virus rescue and retransformation. One possible explanation for the revertants' lack of transformed properties and inability to express SV40 T antigens is that the integrated viral genome has been lost or altered so that T-antigen production is precluded (22). Alternatively, the reversion could be cellular in nature, i.e., an alteration of the cellular genome that affects viral T-antigen gene expression (1). To distinguish between these possibilities, virus rescue and retransformation assays were performed. Successful rescue of infectious virus from revertant cells would indicate the presence of complete viral genomes within these cells and would suggest a cellular alteration as the basis for reversion. Susceptibility to retransformation after superinfection of the revertant cells with SV40 would be consistent with the revertant having lost the transformed phenotype as a result of an alteration of the integrated viral genome. The presence of rescuable virus in the revertants and their progenitors was assessed by both Sendai virus-mediated and polyethylene glycol-mediated fusion with permissive cells. Neither revertant line yielded infectious virus in either assay (Table 2). In contrast, SV40 was rescued from

TABLE 2. Rescue of infectious virus from B1 cell lines

Cell line	Virus yield (PFU/ml) by fusion protocol	
	Sendai virus	PEG[a]
B1-0	2.0×10^7	1.4×10^6
B1-1d	1.3×10^7	6.0×10^6
B1-2a	<1	<1
B1-3	<1	<1

[a] PEG, Polyethylene glycol.

TABLE 3. Retransformation of revertant cells

Cell line	MOI[a]	No. of colonies per 10^5 cells		Frequency of transformation[b]
		Infected	Mock-infected	
B1-3	250	25	0	0.83
	1,000	55	0	0.45
3T3	250	115	0	3.8
	1,000	510	0	4.5

[a] MOI, Multiplicity of infection, in PFU per cell.
[b] Transformed colonies × 10^6 per PFU.

the transformed cells under both assay conditions.

In the retransformation assay, revertant B1-3 and untransformed 3T3 cells were infected by SV40, and transformation was assessed by colony formation in soft agar suspension. The revertant cells could be transformed (Table 3), though at a frequency 5 to 10 times lower than the transformation frequency of the BALB/c-3T3 cells. The basis for this reduced transformation efficiency is not presently understood. Four well-isolated retransformed colonies were picked from the agar, grown in liquid medium, and tested for the presence of T antigen by immunofluorescence. All four retransformants gave the positive nuclear fluorescence indicative of SV40 T-antigen synthesis. The absence of rescuable virus and the susceptibility to retransformation indicated that the untransformed phenotype and lack of T-antigen expression in the revertant could be attributed to a defect in the integrated viral DNA and not to an alteration of the cellular genome.

Arrangement of SV40 DNA in revertant cells. To define the nature of the alteration in integrated SV40 sequences which could account for reversion, an analysis of the viral sequences in the revertants was performed by restriction digestion and blot hybridization. Preliminary studies (2) revealed that, although lines B1-0, B1-1d, and B1-2a have certain arrangements of SV40 sequences in common, the overall pattern of integration in each of the three lines is unique. This indicates that during the establishment of the two lines B1-0 and B1-1d, certain viral sequences were rearranged in some cells. These rearrangements become apparent when the viral sequences in progeny clones are analyzed. Whereas the transformed lines B1-0 and B1-1d contain multiple inserts of SV40 DNA, the revertant B1-2a retains SV40 sequences inserted at a single site within the cellular genome. This single viral insert is present as one of several inserts in the B1-0 and B1-1d progenitor lines and persists as the only viral insert in the revertant subclone B1-3 (2).

Further characterization of the SV40 sequences retained in the revertant was performed by using a variety of restriction endonucleases. The rationale used for constructing a map of these sequences was that of Ketner and Kelly (12); if an SV40-specific restriction fragment in the revertant DNA corresponded to a fragment of viral DNA generated with the same enzyme, it was assumed that the revertant cells contained a segment of SV40 DNA colinear with the viral genome over at least the length of that fragment. Digestion of revertant DNA with restriction enzymes which cut the SV40 genome only once yielded no fragments corresponding to full-length linear SV40 DNA (Table 4). Thus, there was no indication of the existence of either partial or complete tandem duplications of the SV40 genome within the viral insert. More detailed mapping was performed by digestion either with pairs of restriction enzymes, each of which cut SV40 DNA once, or with individual enzymes which cut the SV40 genome two or more times (Table 4). For example, the presence in the revertant digests (Fig. 4) of fragments having the same electrophoretic mobility as SV40 *Hpa*I fragment A (0.758 to 0.169 map unit in a clockwise direction) and the SV40 *Bgl*I/*Bam*HI fragment B (0.66 clockwise to 0.14 map unit), respectively, indicated that the region of the SV40 genome from 0.66 clockwise to 0.169 map unit was inserted without interruption in the

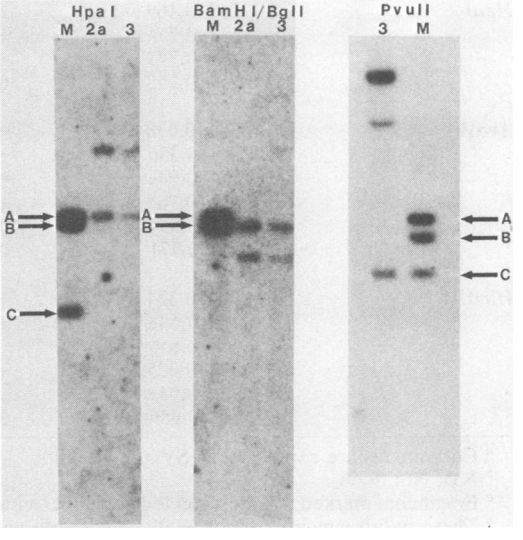

FIG. 4. Representative autoradiograms used in construction of the map of the SV40 DNA integrated in the revertant, B1-2a, or its subclone, B1-3. In each case, bands in lane M were derived from purified viral DNA digested in the presence of BALB/c-3T3 DNA, and bands in lanes 2a and 3 were derived from B1-2a and B1-3 cell DNA, respectively.

TABLE 4. Summary of restriction digestion–blot-hybridization of revertant cell DNA

Enzyme	SV40 genomic fragments and their coordinates[a]	SV40 genomic fragments in revertants		Additional SV40-specific fragments in revertants (kb)	
		B1-2a	B1-3	B1-2a	B1-3
*Eco*RI	A 0.000–0.000	–	–	>10, 3.2	>10, 3.2
*Bam*HI	A 0.144–0.144	–	–	10, 4	10, 4
*Taq*I	A 0.564–0.564	–	–	8	8
*Bgl*I	A 0.661–0.661	–	–	4.8	4.8
*Hae*II	A 0.818–0.818	–	–	>10	>10, 4
*Pst*I	A 0.271–0.039	ND[b]	–	ND	>10, 4.7
	B 0.039–0.271		–		
*Eco*RI/*Bgl*I	A 0.000–0.661	ND	–	ND	4.7, 3
	B 0.661–0.000		+		
*Bam*HI/*Bgl*I	A 0.144–0.661	–	–	2.2	2.2
	B 0.661–0.144	+	+		
*Bam*HI/*Hpa*II	A 0.144–0.725	–	–	4	4
	B 0.725–0.144	+	+		
*Bam*HI/*Taq*I	A 0.564–0.144	–	–	6.7	6.7
	B 0.144–0.564	–	–		
*Hpa*II/*Bcl*I	A 0.189–0.725	–	–	3.5	3.5
	B 0.725–0.189	+	+		
*Bgl*I/*Taq*I	A 0.661–0.564	–	–	4.2	4.2
	B 0.564–0.661	–	–		
*Pvu*II	A 0.330–0.710	ND	–	ND	5.7, 3.8
	B 0.987–0.330		–		
	C 0.710–0.987		+		
*Hpa*I	A 0.758–0.169	+	+	3.9	3.9
	B 0.373–0.754	–	–		
	C 0.169–0.373	–	–		
	D 0.754–0.758	0[c]	0		
*Ava*II	A 0.336–0.638	–	–	2.6	2.6
	B 0.044–0.336	–	–		
	C 0.854–0.044	+	+		
	D 0.638–0.765	+	+		
	E 0.771–0.854	0	0		
	F 0.765–0.771	0	0		
*Hin*dIII	A 0.986–0.324	–	–	1.7	1.7
	B 0.425–0.648[d]	+	+		
	C 0.648–0.859[d]				
	D 0.324–0.425	–	–		
	E 0.859–0.945	–	–		
	F 0.945–0.986	0	0		

[a] Coordinates are expressed as SV40 map units.
[b] ND, Not done.
[c] Fragments marked 0 were detected in neither viral nor cellular DNAs because of their small size.
[d] These two fragments cannot be distinguished under these conditions.

revertant cell DNA. Analysis of digests obtained with other restriction endonucleases extended the limits of the viral insert to include sequences proceeding from 0.638 map unit in a clockwise fashion on the viral genome to 0.189 map unit (Table 4 and Fig. 5). In contrast, the absence of a fragment corresponding to either *Bam*HI/*Taq*I fragment of SV40 and the absence of a fragment

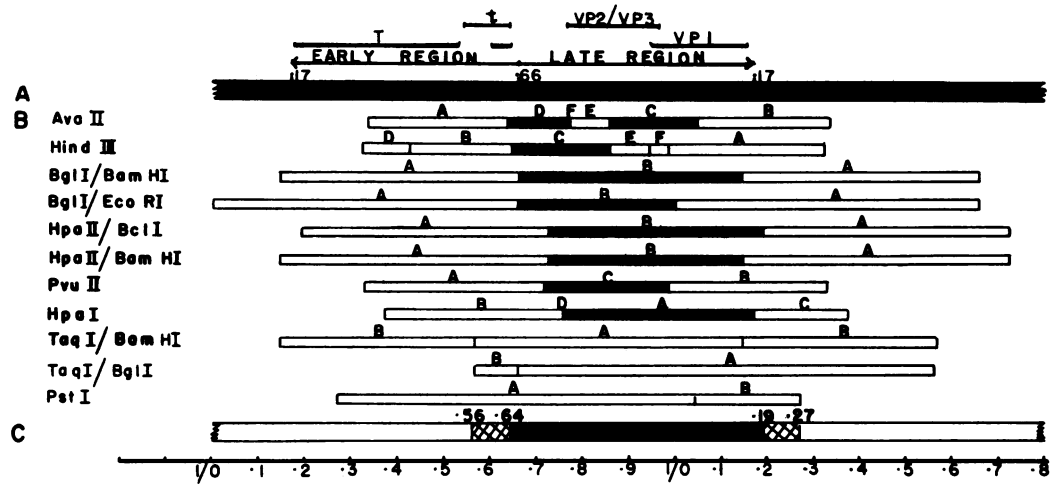

FIG. 5. Map of the SV40 DNA integrated in the revertant cell DNA. (A) Tandemly duplicated linear molecule of SV40 DNA. The regions coding for large T, small t, and virion proteins are shown. (B) Results of digestion with various restriction endonucleases. SV40 genomic fragments which are present in digests of cell DNA are drawn as solid bars; those not detected are drawn as open bars. (C) Map of SV40 sequences in revertant DNA. The integrated DNA comprises a single segment covering the region indicated by the solid bar and having ends which fall within the regions indicated by the crosshatched bars. The scale at the bottom is in map units of the SV40 genome.

corresponding to either *Pst*I SV40 fragment indicated that the *Taq*I site at 0.564 map unit and the *Pst*I site at 0.271 map unit were not present in the inserted DNA. Finally, digestion with many restriction enzymes generated two SV40-specific fragments which did not comigrate with fragments derived from the viral genome (Table 4, right column). These presumably contained host sequences which flanked the viral insert. That other enzymes produced only one such fragment is likely explained by the fact that fragments generated by endonucleases cleaving SV40 sequences near the junction with host sequences, as well as fragments smaller than 0.6 kilobases (kb), were not detected under the conditions of gel electrophoresis and blot hybridization utilized. In all digests, the SV40-specific restriction fragments present in revertant B1-2a were identical to those present in the subclone B1-3.

The viral sequences inserted within the revertant cell DNA, then, must include at least those sequences extending from 0.64 to 0.19 map unit and at most sequences from 0.57 to 0.27 map unit of the SV40 genome (Fig. 5). Thus, that region of the viral genome coding for the SV40 late proteins appeared to be retained intact in the revertant. The early transcription region of the viral genome, in contrast, was disrupted by a sizable deletion of viral sequences as well as by the recombination junctions between viral and host sequences. The viral insert contained only 2 to 10% of those sequences coding for the amino terminus of large T and 4 to 24% of sequences coding for its carboxyl terminus. It also lacked a portion of the region coding for small t antigen as well as both splice sites in small t messenger. Deletions which remove the proximal small t splice junction have been shown to produce no stable cytoplasmic small t mRNA (13). Disruption of the SV40 early transcription region, then, accounts for the lack of detectable large T and small t antigens in the revertant cell lines.

Arrangement of SV40 DNA in retransformed cells. The revertant cell DNA contains at least 55%, or approximately 2.9 kb, of the SV40 genome. As previously demonstrated by Graessmann et al. (8), analysis of transformants derived from such cells after superinfection with SV40 should provide information regarding the role that sequence homology plays in the integration of viral DNA into the cellular genome. It was, therefore, of interest to determine in the retransformed cells the relationship between the site of integration of the superinfecting viral DNA and that of the resident SV40 sequences. For this purpose, DNAs from the B1-3 revertant and its four retransformed derivatives were digested with *Bgl*II. This enzyme does not cleave the SV40 genome. Each virus-specific band present in the electropherogram of digested cell DNA, therefore, represents a unique site of integration. The revertant contained a single viral insert in a 6.3-kb fragment (Fig. 6A). An SV40-specific fragment of the same electrophoretic mobility was present in the digests of all four retransformants. In addition, each retans-

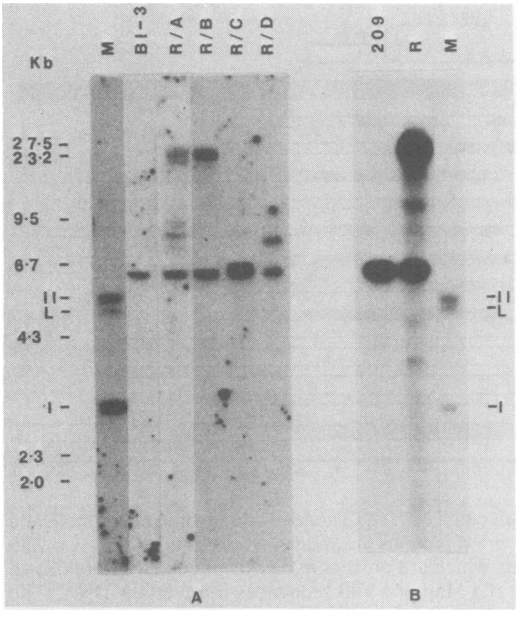

FIG. 6. Autoradiograms of SV40 DNA in retransformed cells. (A) SV40-specific fragments generated by *Bgl*II cleavage of revertant B1-3 and its four retransformed derivatives, R/A through R/D. Lane M contains closed circular (I), linear (L), and open circular (II) SV40 DNA as markers. The size and position of phage λ DNA *Hin*dIII fragments in the same gel are shown at the left. (B) SV40-specific fragments generated by *Sst*I cleavage of A209B4a and its retransformed (R) derivative.

formed line contained at least one new SV40-specific fragment.

The original viral insert within the revertant, then, does not appear to be involved in recombination with the superinfecting viral DNA. A similar observation has been reported by Botchan et al. (3) and by Graessmann et al. (8) in their analyses of rat cells retransformed by SV40. To extend this analysis to BALB/c-3T3 cells containing an SV40 insert distinct from that in the B1-3 revertant, we have compared the pattern of integration of viral DNAs in *tsA*209-transformed 3T3 cells with that in a retransformant derived from the *tsA*209 transformant. The *tsA*209-transformed cell line (A209B4a), which is temperature sensitive for transformation, was superinfected with wild-type SV40 virions, and cells were selected for acquisition of the transformed phenotype at the nonpermissive temperature (4). Digestion of the parental A209B4a cell DNA with *Sst*I (another restriction endonuclease which does not cleave the SV40 genome) produced one SV40-specific fragment, indicating the presence of a single viral insert within the cell DNA (Fig. 6B). Again, as in the case of retransformed B1-3 cells, the retransformed A209B4a cells retained the original insert and acquired new viral insertions at independent sites. Thus, as in the earlier studies of retransformed rat cells (3, 8), the presence of extensive regions of homology in the mouse cell DNA did not appear to influence the site of secondary insertions.

DISCUSSION

The revertant described in this communication produces no detectable T antigens, yields no virus on fusion with permissive cells, and can be retransformed by wild-type SV40 virions. A revertant displaying these properties would be expected to have sustained an alteration of the integrated viral genome rather than a cellular alteration. Analysis of the integrated viral DNA has confirmed this expectation. Two other groups of investigators (17, 22) have reported the isolation of T-antigen-negative revertants. In both of these cases, the revertants were selected under conditions in which transformed cells are killed. The B1 revertant was isolated under nonselective conditions from a transformed line in which the arrangement of integrated SV40 DNA sequences is not stable (2). This instability of sequence arrangement in the B1 cell series is unusual compared with other SV40-transformed mouse lines (unpublished data). The mechanism underlying the rearrangement of integrated viral DNA in the B1 cell series is not known, but it may be related to the nature of cell sequences at the primary site of integration; e.g., unstable arrangements may occur when viral DNA is inserted adjacent to reiterated sequences or movable elements in the cell DNA (2). Thus, the original transformant from which the revertant was derived may have contained viral DNAs inserted at each of two types of loci: one type which is conducive to rearrangement and another which is not. The stable viral insert which is present in the progenitor cells as well as the revertant (2) would then represent an insertion at the latter type of locus. Similarly, the relatively simple and stable patterns of viral integration observed in the retransformed revertant lines (as well as in the majority of SV40-transformed mouse cells) may be explained by integration at sites which do not predispose to rearrangement. A large T antigen capable of initiating viral DNA replication appears to be neither required nor sufficient for the observed rearrangements because (i) they occurred when B1 cells which were transformed by a *tsA* mutant were examined at the nonpermissive temperature (unpublished data), and (ii) the arrangements of viral DNA in other B1 derivatives stabilize while the cells continue to express T antigen (unpublished data). Of the various ar-

rangements of SV40 sequences present in B1 subclones, that of the stable revertant B1-2a and its subclone B1-3 is the simplest, the revertant having lost all but one viral insert. This remaining insert does not contain an intact SV40 early transcription region.

Loss of the transformed phenotype in B1 cells coincides with the loss of sequences coding for the viral T antigens while other SV40 sequences are retained. Retransformation occurs when reinfection of the revertant with SV40 results in acquisition of the T-antigen coding sequences. These observations complement the analysis by Steinberg et al. (22) of revertants of SV40-transformed rat cells and indicate that the mere presence of SV40 sequences is not sufficient to transform BALB/c-3T3 cells. Instead, these analyses of T-antigen-negative revertants point to the involvement of one or both of the viral T antigens in the maintenance of properties characteristic of SV40-transformed cells. This is consistent with the numerous observations (reviewed in references 16 and 24) that many cells, including B1-0 and B1-1d (see above) and other BALB/c-3T3 cells, transformed by SV40 mutants producing a thermolabile large T antigen, express many properties of the transformed phenotype in a temperature-dependent fashion. Moreover, we have been unable to detect any difference in the properties of 3T3 cells transformed by *tsA* mutants and those transformed by double mutants containing a deletion of the region unique to small t in addition to a *tsA* defect (5). We conclude from the analysis of the B1 revertant, as well as from the behavior of cells transformed by *tsA* and double mutants, that large T, but not small t, is required for continued expression of many parameters of the phenotype of SV40-transformed BALB/c-3T3 cells.

Although in the B1 revertant, the region of the SV40 genome which codes for early proteins has been disrupted by deletion as well as by recombination junctions with cellular DNA, the late region of the viral genome appears to be intact. Although the synthesis of small amounts of virion protein VP1 has been detected in mouse cells abortively infected wih SV40 (14), the presence of VP1 has not been demonstrated in mouse cells containing integrated SV40 sequences. In transformed cells containing an insert of a single SV40 genome, the need for expression of the early region would mitigate the presence of an intact late region. In an attempt to determine whether the presence of an apparently intact late region in the B1 revertant results in production of late proteins, [^{35}S]-methionine-labeled extracts were immunoprecipitated with anti-VP1 serum and analyzed by gel electrophoresis and autoradiography (data not shown). VP1 was not detected. Whether this reflects an undetected alteration of the late region of the integrated viral DNA or a relative inability of nonpermissive cells to express the late genes of SV40 is not known.

In addition to providing insight into the role of viral proteins in transformation, analysis of retransformed revertants and superinfected *tsA* transformants as described here and by Graessman et al. (8) and by Botchan et al. (3) provides information regarding the recombination events involved in integration of SV40 DNA into cell DNA. The results of these analyses indicate that sequences in the host cell homologous to those of the viral DNA play little role in integration. One suggestion that this is the case comes from the analysis of the frequency of retransformation of B1-3 cells. If the presence of extensive sequence homology facilitated integration, and if integration were the rate-limiting step in transformation, the frequency of transformation of B1-3 cells might be expected to be greater than that of transformation of 3T3 cells. The transformation frequency for B1-3 cells, however, was less than that for 3T3 cells. Furthermore, if integration proceeded by pairing of homologous DNAs, the superinfecting DNA would be inserted within the resident viral sequences. Analysis of SV40 sequences in the four B1-3 retransformants and the A209 retransformant, however, revealed that in each case, the superinfecting viral DNA was integrated at a site other than that of the original SV40 insert. The presence of extensive regions of sequence homology in the mouse cell DNA, then, does not appear to influence either the site of secondary insertion or the frequency of transformation. Analysis of the site of viral integration in retransformed rat cells has led to similar conclusions (3, 8). These findings are also consistent with the observations of Stringer (23) that transformed cell sequences flanking SV40 inserts do not share extensive homology with SV40 sequences.

Examination of T-antigen-negative revertants has provided information as to viral functions involved in transformation of rat (22) and mouse cells and the nature of recombination between SV40 and cell DNAs (8). Analysis of transformation revertants which continue to express T antigens should yield information regarding the nature of cellular functions involved in virus-induced transformation. For this reason, we are now characterizing T-antigen-positive revertants as well as other cells which produce SV40 antigens but do not manifest the transformed phenotype.

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