Simian Virus 40-Transformed Human Cells That Express Large T Antigens Defective for Viral DNA Replication

WARREN R. GISH AND MICHAEL R. BOTCHAN*

Department of Molecular Biology, University of California, Berkeley, California 94720

Received 27 February 1987/Accepted 15 May 1987

Many types of human cells cultured *in vitro* are generally semipermissive for simian virus 40 (SV40) replication. Consequently, subpopulations of stably transformed human cells often carry free viral DNA, which is presumed to arise via spontaneous excision from an integrated DNA template. Stably transformed human cell lines that do not have detectable free DNA are therefore likely to harbor mutant viral genomes incapable of excision and replication, or these cells may synthesize variant cellular proteins necessary for viral replication. We examined four such cell lines and conclude that for the three lines SV80, GM638, and GM639, the cells did indeed harbor spontaneous T-antigen mutants. For the SV80 line, marker rescue (determined by a plaque assay) and DNA sequence analysis of cloned DNA showed that a single point mutation converting serine 147 to asparagine was the cause of the mutation. Similarly, a point mutation converting leucine 457 to methionine for the GM638 mutant T allele was found. Moreover, the SV80 line maintained its permissivity for SV40 DNA replication but did not complement the SV40 *tsA209* mutant at its nonpermissive temperature. The cloned SV80 T-antigen allele, though replication incompetent, maintained its ability to transform rodent cells at wild-type efficiencies. A compilation of spontaneously occurring SV40 mutations which cannot replicate but can transform shows that these mutations tend to cluster in two regions of the T-antigen gene, one ascribed to the site-specific DNA-binding ability of the protein, and the other to the ATPase activity which is linked to its helicase activity.

Simian virus 40 (SV40) has been studied in a wide variety of *in vitro* cell systems. SV40 infection of certain simian cells in culture induces a permissive response that leads to virus production and ultimately cell death, while infection of nonpermissive rodent cells is unproductive and leads to induction and maintenance of the transformed phenotype at low frequency. In the transformed cell, an integrated copy of viral DNA (vDNA) invariably persists. The integration process itself is a rather nonspecific event that, through transformation, immortalizes the apparently chance recombinational event between viral and cellular DNA (3). The viral DNA in these nonpermissive, SV40-transformed rodent cells usually cannot be induced to excise itself and replicate, but virus rescue can occur after cell fusion with permissive simian cells. Two essential viral components for this excision and lytic replication are a *cis*-acting origin of replication (6) and a replication-competent A gene product (large T antigen) acting in *trans* (13, 34). In this context, it has been proposed that the excision of viral DNA from the chromosomes of nonpermissive cells after cell fusion is mediated by *in situ* replication of vDNA initiated at a viral origin of replication, rather than by inactivation of virally encoded repressors (e.g., the bacteriophage λ lysogenic-to-lytic switch) and subsequent action of virally encoded factors specific for excision (4, 6). From the available biochemical and genetic data, it appears as if the unproductive response to viral infection in standard rodent test systems is due to a lack of factors which are present in the permissive host cell rather than to the activity of any virally encoded repressor (35).

The semipermissive response of human cells to SV40 infection appears more complex. Human cells can be productively infected by the virus, but the yield per infected culture is about 100-fold lower than that from simian cells (11, 12). Cells which survive the lytic infection may pass at

low efficiency through the crisis period to yield transformed cell lines which carry integrated copies of vDNA (26). A subpopulation of the cells within clonal lines of some human cell transformants appears to induce excision, and therefore the cultures persistently carry free, low-molecular-weight (lmw) vDNA (49). A variety of factors, including cellular modulating elements, may be involved in this switching phenomenon.

In consideration of the excision model mentioned above, several isolates of human cell lines that have been transformed by SV40 yet contain no free vDNA were of particular interest. If these cell lines were still permissive for viral replication and in fact contained integrated vDNA with *ori* elements and produced wild-type large T antigens, it would seem paradoxical not to find free vDNA in the cultures. However, the cells themselves could be interesting variants. For example, cellular factors might be responsible for repression of replicative elements of the integrated vDNA and thus block the excision process. From the data presented here, however, we suggest repression was not responsible in three out of four free vDNA-negative, SV40-transformed human cell lines examined—SV80, GM638, and GM639. Instead, the integrated SV40 A genes appeared to be mutated, which precluded excision. Furthermore, a cloned copy of the viral early region from the one cell line tested (SV80) maintained its ability to transform rodent cells at an efficiency comparable to that of wild-type SV40 DNA and lacked detectable replicative activity in permissive simian cells. The fourth free-vDNA-negative human cell line examined, GM2894, may not have carried a functional SV40 *ori*, as *trans* complementation by an exogenous viral A gene did not induce detectable free vDNA (data not shown).

Although the response to SV40 infection seems to be partly determined by the host cell factors present, our results provide further genetic evidence that the lytic replication and the transforming capacities of SV40 are separable by

* Corresponding author.

mutation of the viral A gene (7, 14, 31, 45). In addition, because no specific measures were taken to induce the spontaneous A gene mutations in the SV80, GM638, and GM639 cell lines, it is suggested that the continued growth of SV40-transformed human cells in culture imposes selective pressure against the replicative function and in favor of the transforming activity of the viral A gene.

MATERIALS AND METHODS

Nomenclature. The nucleotide numbering system used for the SV40 genome is that of Buchman et al. (46) and is based on the 5,243-base-pair (bp) genome, with the unique *Bgl*I palindrome centered on position 0/5243. Unless otherwise indicated, wild-type SV40 refers to SV40 strain 776. Specific nucleotide positions in the cloned inserts of plasmids pSV80-04, pSV80V, and pGM638V are frequently referred to by their analogous positions (APs) in the wild-type SV40 genome.

Cell lines. SV80, CV-1, and Rat-1 cell lines were obtained from the Cold Spring Harbor Laboratory Cell Culture Facility. A separate culture of SV80 cells was obtained from David Livingston (Harvard Medical School). Cell lines GM638, GM639, and GM2894 were obtained from the Human Genetic Mutant Cell Repository, Camden, N.J. The COS-7 cell line was provided by Yakov Gluzman, Cold Spring Harbor Laboratory; characterization of the COS-7 line has been described elsewhere (13). Unless otherwise indicated, cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GIBCO). Cell line GM2894 was cultured in Ham F12 medium (GIBCO) plus 10% FBS.

Bacterial strains and plasmids. All bacterial transformations and plasmid preparations used a *recA* derivative of *Escherichia coli*, DH1 (19). Plasmids pK1 (14), pSL1 (31), pJY1 (courtesy of Y. Gluzman [29]), and pML2 (29) have been described elsewhere. Plasmids pSTH1 and pSTH2 contained, respectively, the large and small SV40 *Taq*I-*Hind*III restriction fragments and were provided by Y. Gluzman. Plasmid pDL3, which contains a complete *Bam*HI-linearized copy of the SV40 strain dl1001 genome, a nonviable early-region deletion mutant, was provided by Daniel Nathans (Johns Hopkins University and Medical School).

Cell fusion. Cell fusions were carried out as previously described (4), with polyethylene glycol 1000 (J. T. Baker). Lmw DNA was isolated by Hirt extraction (22) 55 to 60 h postfusion. Fractions containing form I DNA were collected from ethidium bromide (EtBr) CsCl density gradients; the form I fractions were identified by using visible quantities of wild-type SV40 DNA banded in parallel gradients.

DEAE transfection. Subconfluent CV-1 cell monolayers were trypsinized and seeded onto 60-mm dishes to achieve approximately 70% confluence 12 to 20 h later, at the time of transfection. The indicated quantity of DNA (10 ng in replication assays) was transfected in 200 μ l of DEAE-dextran (500 μ g/ml; molecular weight, 200,000) for 30 min at 37°C by the method of McCutchan and Pagano (33) as modified by Kimura and Dulbecco (23). The transfected cells were then incubated at the temperatures and for the times indicated. In replication assays, Hirt extracts were collected and processed for blot hybridization as described elsewhere (29).

Cloning of lmw DNA. An SV80 $\times$ COS-7 cell fusion was carried out as described above. DNA (12 ng) from the form I fraction was digested with *Bam*HI restriction endonuclease

under standard conditions. This DNA was then ligated into the *Bam*HI site of pBR322 (0.3 μ g) which had been previously treated with bacterial alkaline phosphatase (Worthington). The ligation was carried out in a total reaction volume of 0.6 ml containing 1 Weiss unit (48) of T4 DNA ligase. DH1 cells were then transformed with the ligated DNA by an RbCl procedure of Hanahan (personal communication) (19). The resultant ampicillin-resistant colonies were screened for DNA sequences hybridizing to ³²P-labeled, nick-translated SV40 DNA by the method of Hanahan and Meselson (20). Greater than 1% of the colonies scored positive. Thirteen positive colonies were picked and screened a second time, followed by plasmid purification by the alkaline extraction method of Birnboim and Doly (1). Lmw DNA from GM638 $\times$ COS-7 and GM639 $\times$ COS-7 fusions were cloned similarly, except that a deletion derivative of pBR322, pML2 (29), was used as the bacterial vector.

Transformation. For each DNA transfection, 10 μ g of mouse LH7 carrier DNA was coprecipitated with 10 μ g of the indicated plasmid or viral DNA in a volume of 1 ml by the calcium phosphate coprecipitation method of Graham and Van der Eb (16). DNA-calcium phosphate coprecipitates were divided equally between duplicate 100-mm dishes each containing 1.8×10^6 Rat-1 cells (approximately 70% confluence) and 10 ml of medium. After 20 h at 37°C, cells were suspended at a density of 2×10^5 cells per 60-mm dish in 5 ml of DMEM containing 0.33% Noble agar (Difco) (30). Cells were fed at 1 and 2 weeks following transfection. Transformed colonies were scored at 3 weeks.

Marker rescue. Full-length SV40 genomes were constructed that contained the early region from pSV80-04 (pGM638-1) as follows. The viral early region was separated from the remainder of the cloned excision product in pSV80-04 (pGM638-1) by digestion with *Msp*I plus *Bam*HI (*Bgl*II plus *Bam*HI) and recloned with a wild-type copy of the SV40 late region in pBR322 (pK1) to produce pSV80V (pGM638V). Both SV40 chimeras, pSV80V and pGM638V, were constructed so that full-length, linear viral genomes could be separated from the bacterial plasmid vector by digestion with the single restriction endonuclease *Eco*RI.

For each marker rescue experiment, 0.5 to 1 μ g of *Eco*RI-digested pSV80V or pGM638V and 1 to 2 μ g of pSL1 digested with *Bam*HI plus *Msp*I were combined with 3 to 5 molar equivalents of the indicated wild-type SV40 restriction fragment in a total volume of 25 to 35 μ l of 3 mM Tris hydrochloride, pH 7.2. The DNA was denatured and renatured as described by Lai and Nathans (27, 28) except that renaturation was performed at 65 to 66°C. Transfection of 90% confluent CV-1 monolayers was performed by using DEAE-dextran as described above. After transfection, the cell monolayers were overlaid with 5 ml of DMEM supplemented with 5% FBS and containing 0.5% Noble agar. Cells were fed at 5 days posttransfection with 5 ml of DMEM containing 3% FBS and 0.5% agar. At 10 days posttransfection, cells were fed and stained with 5 ml of DMEM with 3% FBS, 0.5% agar, and 0.01% neutral red (Sigma); plaques were scored 12 to 48 h after staining.

Except for the *Bst*NI B fragment of SV40 DNA, which was gel purified from a digestion of pJY1, all marker rescue experiments reported here were done with fragments of SV40 DNA purified exclusively by prior cloning in bacterial vectors; each plasmid was then digested with the appropriate restriction endonuclease(s) before use. The use of cloned DNA fragments precluded gel purification, a procedure that in early attempts to map the SV80 lesion produced some inconsistent results, presumably due to cross-contami-

nation. Inconsistencies were never observed among the marker rescue experiments performed with cloned DNA fragments only.

DNA sequencing. All DNA sequencing was performed by the chemical method of Maxam and Gilbert (32). T4 polynucleotide kinase (P-L Biochemicals) and [γ - 32 P]ATP (Amersham) were used to label the 5'-hydroxyl groups of bacterial alkaline phosphatase-treated DNA restriction fragments of pSV80-04. Labeling of DNA restriction fragments from plasmids pSV80V and pGM638V was performed either by the above method or by labeling 3'-hydroxyl groups in a reaction mixture containing the Klenow (large) fragment of *E. coli* DNA polymerase I (Boehringer Mannheim) and α - 32 P-labeled deoxynucleoside triphosphates (Amersham). Labeled ends were separated by digestion with a second restriction endonuclease, followed by agarose gel electrophoresis and electrolution from gel slices.

The DNA sequence of the *A* gene lesion in pSV80V was determined by sequencing clockwise (see Fig. 3a) from the *Hind*III site at AP 4002 and from the *Rsa*I site at AP 4176 and by sequencing counterclockwise from *Taq*I at AP 4739. The precise lesion in pGM638V was determined by sequencing clockwise from *Pst*I at AP 3204 and counterclockwise from both the *Hind*III (AP 3476) and *Pvu*II (AP 3502) sites.

The complete sequence of the 352-bp *Bam*HI-*Hind*III fragment shown at the left end of the pSV80-04 map (see Fig. 3b) was obtained by sequencing from both ends inward. The recombinant joints which defined segment *D* near position 1000 on the pSV80-04 map were determined by sequencing rightward from the *Taq*I site near position 800. The sequence about the central *Bgl*II site (position 1700) was obtained by sequencing leftward from the proximal *Hind*III site. The silent point mutations around position 4200 (see segment X in Fig. 3b) in pSV80-04 were determined by sequencing from the *Pst*I site at AP 3204 to the *Bam*HI site at AP 2533.

RESULTS

Fusion of SV40-transformed human cells with simian cells. SV80, GM638, GM639, and GM2894 are nonproducer SV40-transformed human cell lines. Cells from the four human cell lines were all observed to carry integrated vDNA, their populations were greater than 99% T-antigen positive by immunofluorescence, and the cells produced large T antigens (96,000-molecular-weight [96K] proteins) of apparent molecular weight identical to that of lytic large T antigen (data not shown). Indeed, the SV80 cell line has been a common source for the biochemical purification of SV40 large T antigen (5, 17) as the cells are relative overproducers of this antigen (21). However, lmw SV40 DNA was not detected in either Hirt extracts from these cells alone (Fig. 1) (9, 44) or in the heterokaryons produced by their fusion with permissive CV-1 cells (Fig. 1). In view of the excision model presented in the Introduction, one possible explanation for the lack of detectable free vDNA in these experiments is that the viral *A* genes expressed by these human cells were defective.

To test the above hypothesis, cells from all four transformed human cell lines were fused with cells of the COS-7 line (CV-1 cells transformed by SV40 DNA). (Because of the engineered deletion in the origin of vDNA replication, the integrated SV40 DNA present in COS-7 cells does not excise at a detectable level [Fig. 1], but the cells do produce an *A* gene product that can complement SV40 *tsA* mutants [13] and support replication of bacterial plasmids which contain only the SV40 origin of replication [29, 37].) The harvested

FIG. 1. Southern analysis (43) of lmw DNA present in heterokaryons of SV40-transformed human cells and permissive simian cells. Cell fusions were carried out as described in Materials and Methods. Hirt-extracted lmw DNA was fractionated by electrophoresis through 1% agarose gels, transferred to nitrocellulose filters, and probed for vDNA species by hybridization with nick-translated pJY1. Photographs of the autoradiograms are shown in panels A and B. COS-7 $\times$ CV-1, control fusion of COS-7 cells with CV-1 cells; CV-1 $\times$ SV80, CV-1 cells fused with SV80 cells, etc. Samples of lmw DNA extracted from unfused GM638 and GM639 cells are also displayed in panel B. Reconstructions consist of SV40 DNA equivalent to about 100 copies of the viral genome per extracted cell. Bands corresponding to forms I (supercoiled), II (relaxed circle), and III (linear) of wild-type SV40 strain 776 DNA are indicated.

Hirt extracts from the resulting heterokaryons were examined for the presence of free vDNA by Southern blotting (Fig. 1). Intensities of the SV40-specific bands in the autoradiograms indicated that a considerable amount of viral DNA replication occurred in COS-7 fusions with three of the four human cell lines tested—SV80, GM638, and GM639. Similar fusion results with the SV80 cell line have been previously reported (18).

The data in Fig. 1 suggested that the *A* genes expressed in SV80, GM638, and GM639 cells were deficient in their ability to promote viral DNA replication and that the COS-7 *A* gene product complemented these deficiencies. Furthermore, if vDNA excision was inhibited in the unfused human cells by a host cell-encoded repressor of vDNA replication, one would have to hypothesize that this repressor was active in the fusions with CV-1 cells but not in the fusions with COS-7 cells, since the former set of fusions did not yield detectable free viral DNA. Evidence is presented below, however, that the SV80 cell line was in fact permissive for viral replication.

Replication of SV40 strain tsA209 DNA in SV80 cells. Gluzman (13) demonstrated that COS-7 cells, which express lytic SV40 large T antigen, can support the replication of SV40 *tsA209* virus at both the permissive and nonpermissive temperatures. In an analogous experiment, however, SV80

FIG. 2. Replication of SV40 strain *tsA209* DNA in transfected SV80 cells. SV80 and COS-7 cells were transfected at 70% confluence in 60-mm dishes with 10 ng of SV40 *tsA209* vDNA as described in Materials and Methods. The transfected cells were incubated at either 33 or 40°C for the indicated times (hours), after which Hirt extracts were collected and processed for blot hybridization as described by Lusky and Botchan (29).

cells only supported replication of *tsA209* DNA at the permissive temperature (Fig. 2). Furthermore, SV80 cells have been fused with cells from a nonpermissive Rat-2-derived cell line that expressed both large T and small t antigens promoted by Harvey murine sarcoma virus long terminal repeats; following this fusion, lmw vDNA species were detected, whereas a fusion with cells of the nontransformed parent line did not yield detectable lmw viral DNA (Kriegler, Gish, and Botchan, unpublished results). These results substantiated our conclusion from Fig. 2 that SV80 cells were permissive for SV40 replication.

The viral *A* gene expressed in SV80 cells thus appeared to be nonfunctional in at least one aspect of vDNA replication. The observed complementation of the SV80 *A* gene after fusion with COS-7 cells did not seem to depend on any permissive factors for vDNA replication from COS-7 cells, since transfected *tsA209* DNA replicated in SV80 cells alone and fusion of SV80 cells with nonpermissive rat cells that expressed both large T and small t antigens also yielded free vDNA.

Our findings seem at odds with those of Kriegler et al. (25), who observed complementation for replication of *tsA209* virus at the nonpermissive temperature in CV-1 cells that had been microinjected with purified SV80 *A* protein. A comparison of the SV80 cell line employed in our studies with an SV80 cell line given us by Livingston indicated that the two cell lines were identical, through extensive restriction mapping and Southern blot hybridization (data not shown). One interpretation of their results together with ours uses the observation that large T antigen assembles into oligomeric structures (38). Phenotypic mixing of defective SV80 and *tsA209* subunits at the nonpermissive temperature might produce an oligomeric form of the *A* protein with wild-type activity in CV-1 cells but not in SV80 cells. Alternatively, the amount of microinjected *A* protein may have been large enough to overcome the apparent defect in the SV80 T antigen.

Cloning of excised vDNA produced by fusions of SV80, GM638, and GM639 cells with COS-7 cells. Superimposed on the background smear of SV40-hybridizing material detected on the blot of the Hirt supernatant fraction from the SV80 ×

COS-7 fusion (Fig. 1) were several intense bands which corresponded to specific excision products from the heterokaryons. Such patterns were also observed from GM638 × COS-7 and GM639 × COS-7 fusions (Fig. 1). To define the structure of the *A* genes and other integrated vDNAs in SV80, GM638, and GM639 cells, several excision products from the fusions with COS-7 cells were cloned as described in Materials and Methods.

Of 13 clones isolated from the SV80 × COS-7 fusion (designated pSV80-01 through pSV80-13), 6 contained *Bam*HI inserts of similar electrophoretic mobility to the 4.4-kilobase (kb) linear form of the plasmid vector pBR322; by more detailed restriction analysis, the same 6 clones also appeared structurally identical to each other (data not shown).

Structural analysis of the pSV80-04 insert. Southern blots of genomic DNA presented below show that the 4.4-kb *Bam*HI fragment cloned in six of the pSV80 plasmids represented the one complete copy of SV40 early region DNA in the SV80 cellular genome. To study the biological and physical properties of the SV80 T-antigen allele, the 4.4-kb *Bam*HI insert from pSV80-04 was arbitrarily chosen. A physical map of the pSV80-04 insert is shown in Fig. 3b. In addition to a complete copy of the viral early region, pSV80-04 contained a partial tandem duplication of vDNA sequences. At least six recombinational events would be necessary to produce this insert's structure from wild-type SV40 DNA. All of the rearrangements were clustered in the repetitious left end of the 4.4-kb fragment. One rearrangement included a direct translocation of 28 bp of SV40 sequence (segment A), which permuted vDNA in the region of the SV40 early promoter. The other rearrangements produced novel vDNA-vDNA junctions that might have arisen via deletions from an oligomeric, head-to-tail array of SV40 DNA. All of the pSV80 clones but pSV80-05, -09, and -10 contained a *Hind*III-*Bam*HI fragment which comigrated with the approximately 352-bp *Hind*III-*Bam*HI fragment shown at the left end of the pSV80-04 physical map.

A contiguous stretch of viral DNA sequences that included an SV40 promoter region, origin of replication, and early region was present in pSV80-04. In addition, restriction endonuclease digestions of pSV80-04 and the five other plasmids that contained the 4.4-kb *Bam*HI fragment (pSV80-01, -02, -06, and -11) identified segment X, which is marked in Fig. 3b. A variety of restriction fragments of pSV80-04 that spanned segment X consistently exhibited a somewhat higher *M_r* than did their wild-type counterparts from both pJY1 and virion-extracted SV40 DNAs (data not shown). This region of the pSV80-04 insert was sequenced, and seven point alterations from the SV40 strain 776 DNA sequence were found (data not shown). The marker rescue results presented below, however, indicated that these sequence changes were not responsible for a lytic replication defect in the SV80 T antigen.

Structural analysis of pGM638 and pGM639 excision products. Four independent clones of GM638 × COS-7 excision products and two clones of GM639 × COS-7 excision products were isolated, designated pGM638-1, pGM638-2, pGM638-4, pGM638-6, pGM639-4, and pGM639-5. Restriction endonuclease digestions of the pGM638 and pGM639 plasmids were analyzed to identify an organization characteristic of wild-type SV40 DNA, particularly of the early region and origin-promoter sequences. *Bam*HI digestions showed that each plasmid contained but a single cloned insert, and unlike in pSV80, none of the inserts had the same size or structure (Fig. 4; data not shown).

FIG. 3. Physical maps of the SV40 genome and the insert cloned in pSV80-04. (a) Map of the SV40 genome adapted from Fiers et al. (8). Coordinates on the interior of the circle are in base pairs. Introns in the polyadenylated mRNAs coding for large T and small t antigens are denoted by zigzag lines. The small head-to-tail arrows demarcate the 72-bp long tandem repeats located on the late side of the origin of replication. Short DNA segments labeled A, B, C, and D are marked in their wild-type configuration and are referred to in panel b. Segment A includes nucleotides 203 through 229; segment B spans nucleotides 251 through 265; segment C covers nucleotides 2477 to the BamHI site at position 2533; and segment D includes nucleotides 3894 through 3930. (b) Map of the cloned BamHI insert in pSV80-04 derived from both restriction enzyme mapping and DNA sequence analysis, as described in Materials and Methods. Sequences derived from the SV40 late region are denoted by a bold line, while early region sequences are indicated by a thin line. In all cases but one, an inverted triangle demarcates a novel joint in the sequence which is not present in wild-type SV40 strain 776 DNA. The small 351-bp BamHI-HindIII fragment, which begins at pSV80-04 map position 1, contains a copy of segment C joined to segment A to create a junction between AP 2477 and AP 229. This copy of segment A is located on the opposite side of segment B from the wild-type orientation. The A/B junction in pSV80-04 brings AP 203 close to AP 265, with only the trinucleotide CGC between them. As drawn on the map and written in the sequence, the 3' end of segment B abuts a solitary copy of a 72-bp unit which is found repeated in both wild-type SV40 strain 776 DNA and pSV80-04 near position 1500. This isolated copy of the 72-bp unit harbors only one difference between it and the published SV40 DNA sequence, a G-to-A transition at AP 171. (In the listed sequence, the differing base is the first A in the third row.) In contrast, the nine bases immediately 5' to the BglI site in the listed sequence would be the result of extensive localized alteration. The rest of the small BamHI-HindIII fragment consists of wild-type SV40 sequence. DNA sequencing rightward from the TaqI site at position 800, through splice sites for SV40 early mRNAs, revealed two novel junctions apparently created by deletion of sequences flanking a retained D segment at position 1000. This copy of segment D is joined on the left to AP 4508 and on the right to viral late region sequence at AP 650. DNA sequencing leftward from the HindIII site at position 1800 did not uncover any mutations in the origin of replication there or in the adjacent early promoter sequence. Restriction analysis also showed that, except in the vicinity of segment X (see Results), the remainder of the cloned insert (from position 1800 through 4400) in pSV80-04 consists of an apparently intact copy of the SV40 early region.

FIG. 4. Restriction maps of the pGM638 and pGM639 cloned inserts. The pGM638 and pGM639 isolates were digested with restriction endonucleases *Bam*HI, *Hind*III, *Bgl*I, *Pst*I, *Bam*HI plus *Hind*III, *Bam*HI plus *Bgl*I, *Hind*III plus *Bgl*I, *Pst*I plus *Bam*HI, and *Bam*HI plus *Eco*RI and electrophoresed in agarose sizing gels (data not shown); the collated results are presented here. When a precise relative positioning of fragments could not be determined strictly from these data, the known map of wild-type SV40 DNA was referred to. References to the wild-type map were perhaps only of notable importance for the ordering of some of the *Hind*III fragments of pGM639-4, because the cloned insert in this plasmid contained a more cryptic arrangement of vDNA sequences, perhaps recombined with some cellular DNA. Restriction enzyme sites: B, *Bam*HI; H, *Hind*III; R, *Eco*RI; P, *Pst*I; L, *Bgl*I. Putative origin-promoter regions are shown as open circles centered above *Bgl*I sites. Zigzag lines are drawn that extend from putative origin-promoter regions only as far as the restriction analysis suggested that contiguous early region sequences continued. All of the pGM638 inserts, but neither of the pGM639 inserts, apparently carried an intact early region, from the origin-promoter region up to and including the early poly(A) addition site.

Each of the four pGM638 inserts contained an apparently intact copy of the SV40 early region, which neither of the two pGM639 isolates contained (Fig. 4). These results were consistent with a Southern blot of the GM638 $\times$ COS-7 and GM639 $\times$ COS-7 form I fractions, probed with 32 P-labeled pY1, which only detected a 2.7-kb, early region-sized, *Bgl*I-*Bam*HI fragment in the case of the GM638 $\times$ COS-7 fusion (data not shown).

Interestingly, the pGM638 and pGM639 inserts that included an otherwise intact SV40 *Hind*III C DNA fragment all exhibited a size for this fragment of approximately 1,050 bp, which compares with an 1,118-bp size for the wild-type *Hind*III C fragment. This size differential (approximately 70 bp) suggests that the strain(s) of SV40 used to produce the GM638 and GM639 cell lines may have carried only a single copy of the 72-bp enhancer, which is found duplicated in strain 776 DNA. Alternatively, the populations of virus used in the transforming infections may have predominantly carried two copies of the 72-bp enhancer sequence, but selection for transformation or survival of crisis may have selected for the deletion of one copy or selected for those cells infected with a rare virus particle that contained a genome with only one copy.

pSV80-04 also contained a mutated *ori* near position 300 (Fig. 3b) that was adjoined to only a single, point-mutated copy of the 72-bp enhancer sequence. As elaborated on in the Discussion, most of the stable virus-specific transcripts found in the nuclei of SV80 cells (9) may be initiated from this position 300 region, not from the promoter region adjoined to the duplicated 72-bp sequences near position 1700. It therefore seems plausible that the mutation in the

5'-most enhancer site in the SV80 DNA provided some selective advantage for the 5' promoter. By restriction mapping, pSV80-04 also appeared to contain the necessary complement of DNA for a tandem repeat of the 72-bp sequence near position 1700 (data not shown); it would therefore seem that tandem copies of this sequence are compatible with stable transformation and survival through crisis, although the possibility exists that the tandem repeat in pSV80-04 was mutated in a way not detected in the agarose sizing gels that were run.

As illustrated in Fig. 4, all of the pGM638 and pGM639 cloned fragments contained at least one *Bgl*I site closely associated (within approximately 75 bp) with an *Hind*III site, which suggested the presence of a viral origin of replication. Plasmid pGM639-4 actually contained two sets of closely associated *Bgl*I and *Hind*III sites, as well as a third *Bgl*I site located approximately 400 bp from its nearest *Hind*III site. Since *Bgl*I sites are found rather infrequently in mammalian DNA, in part because of the low abundance of the dinucleotide 5'-CG-3' which forms part of the enzyme's recognition sequence, it is plausible that the third *Bgl*I site was also located in a viral origin of replication.

None of the pGM638, pGM639, or pSV80 isolates contained an intact late region. It may be of some significance that most of the cloned excision products from GM638 cells carried complete copies of the SV40 early region but different arrangements of late-region sequences. While the precise integrated structure of viral DNA sequences cannot be inferred from these isolates alone, it seems most likely that GM638 cells harbor a single copy of the early region flanked on both sides by late region sequences.

FIG. 5. Comparison of the cloned insert in pSV80-04 with the organization of integrated vDNA in SV80 cells. The indicated restriction enzyme digestions were performed on 15 pg of pSV80-04 (lanes c, e, g, and i), 10 μ g of SV80 genomic DNA (lanes d, f, h, and j to n), lmw DNA isolated by Hirt extraction from the fusion of SV80 $\times$ COS-7 cells (lane b), and 15 pg of pJY1 (lane a). Each digestion of plasmid and lmw form I DNA was performed in the presence of 10 μ g of calf thymus DNA. Digests were electrophoresed in one 0.9% (lanes a to k) and one 0.7% (lanes l to n) agarose gel. The gel was subsequently blotted and probed with 32 P-labeled, nick-translated pJY1. Proceeding from left to right, the arrows point to the 4.4-kb species cloned in pSV80-04 which comigrated with linear pBR322 in lane a, the predominant hybridizing species present in the Hirt supernatant fraction from which pSV80-04 was cloned (lane b), and a *Bam*HI fragment present in SV80 genomic DNA (lane d); a 2.7-kb species (lanes e and f); a 1.4-kb species (lanes e to h); and 3.0-kb and 1.4-kb species (lanes i and j).

Comparison of the pSV80-04 insert with SV80 genomic DNA. In Fig. 5, evidence is presented that the 4.4-kb *Bam*HI fragment carried by pSV80-04 (i) was a bona fide copy of the SV80 genomic DNA encoding the viral A gene expressed in these cells and (ii) was contained within an abundant excised species observed in the SV80 $\times$ COS-7 Hirt supernatant fraction prior to cloning. First, when the lmw DNA extracted from the SV80 $\times$ COS-7 fusion was cleaved with *Bam*HI, a prominent band of 4.4 kb was detected by blot hybridization (Fig. 5, track b). Genomic blots of *Bam*HI-digested SV80 DNA revealed four DNA fragments that hybridized with SV40 DNA. Their sizes were 5.1, 4.6, 4.4, and 2.7 kb (Fig. 5, track d). The 4.4-kb *Bam*HI insert (the cloned excision product) in pSV80-04 (Fig. 5, track c) comigrated with both the genomic 4.4-kb fragment and the predominant *Bam*HI fragment contained in Hirt extracts of SV80 $\times$ COS-7 fusions.

Apparent structural identity between the 4.4-kb DNA fragments was also found. Of the four SV80 genomic *Bam*HI fragments, only the 4.4-kb fragment was cleaved by *Msp*I. In its place were two fragments of 3.1 and 1.3 kb (Fig. 5, track j), which aligned with the vDNA fragments produced by *Bam*HI plus *Msp*I digestion of pSV80-04 (Fig. 5, track i).

Thus, the novel 1.3-kb fragment (which spanned nucleotide positions 1 to 1300 on the pSV80-04 physical map) and the bona fide SV40 3.1-kb *Msp*I-*Bam*HI fragment were contained within the chromosomal 4.4-kb *Bam*HI fragment. Digestion of SV80 genomic DNA with *Bgl*II, as with *Msp*I, only cut the 4.4-kb *Bam*HI fragment. In its place was the expected 2.7-kb early-region fragment, which comigrated with the large *Bam*HI-*Bgl*II fragment of pSV80-04 (Fig. 5, tracks e and f). A 1.4-kb *Bgl*II fragment predicted from the mapping of pSV80-04 was also found in SV80 genomic DNA (Fig. 5, tracks g and h). A 300-bp *Bgl*II-*Bam*HI fragment, which would correspond to the *Bgl*II-*Bam*HI fragment at the left end of the pSV80-04 map, was not visible on this autoradiograph.

From the findings discussed above, we concluded that the SV80 genome carried integrated vDNA arranged in the same complex manner as the vDNA in pSV80-04 and contained only a single, apparently intact copy of the SV40 early region.

While the precise points of recombination that produced the pSV80-04 insert are unknown, something can be said about the probable genomic organization of duplicated viral DNA sequences which would promote production of the specific populations of excision products observed from the SV80 $\times$ COS-7 fusion. First, it is unlikely that integrated viral DNA sequences in the SV80 genome halt exactly at the *Bam*HI sites flanking the 4.4-kb *Bam*HI fragment, but rather that viral DNA sequences extend for some distance on each side. To the left of the 4.4-kb fragment (Fig. 3b), continuity of viral sequences would result in duplication of early region sequences found within the fragment at the right; to the right of the 4.4-kb fragment, late region sequences would naturally extend beginning with segment C, which is found duplicated at the left end of the pSV80-04 insert (Fig. 3b). Second, to obtain an excision product that is an accurate copy of the integrated 4.4-kb DNA fragment, recombination must take place between one point located internally to the 4.4-kb fragment and another point located externally, within duplicated flanking sequences on either side (2).

At the resolution of restriction enzyme mapping, the insert cloned in pSV80-08 appeared similar to the insert in pSV80-04, but without the 1.4-kb *Hind*III fragment (data not shown) which spans nucleotide positions 352 to approximately 1775 (Fig. 3b). Loss of this 1.4 kb of DNA to produce the pSV80-08 insert may have proceeded via homologous recombination across the duplicated sequences within the 4.4-kb *Bam*HI fragment. Thus, the pSV80-08 insert may have evolved from SV80 genomic DNA partially in the manner of the pSV80-04 insert, but with an additional homologous recombination event. Although the temporal order of the two recombinational events cannot be determined from their product, the structure of the pSV80-08 insert is consistent with a multiple initiation, or "onion skin," model for vDNA replication in the excision process (4).

Replication analysis of cloned excision products. The cloned excision products from pSV80-03, -04, and -08 were transfected into CV-1 cells and COS-7 cells to examine their ability to replicate. Control DNAs used were SV40 DNA extracted from virions; pJY1, a plasmid which contains a complete *Bam*HI linear of SV40 DNA; and pDL3, a pBR322 clone of a nonviable SV40 early region deletion mutant (see Materials and Methods). Prior to transfection, all of the form I DNAs were cut with *Bam*HI and ligated to produce form II molecules as described in the legend to Fig. 6. The measure of replication was then the observed accumulation of form I

FIG. 6. Replication of pSV80 cloned inserts. Prior to their use in transfection, samples of each DNA were digested with *Bam*HI and ligated with T4 DNA ligase at a concentration of 1 μ g of DNA per ml to recircularize cloned inserts separately from the bacterial vector, pBR322. The ligase reactions resulted in the *in vitro* formation of form II (relaxed, closed circular) molecules. The efficiency of conversion from linear form III molecules to the form II species was a function of the length of the DNA fragments, as the cloned inserts from pSV80-03 and -08 were completely converted to form II while the longer pBR322 sequences were only partially converted in the same reaction. Similarly, the cloned insert from pSV80-04, which is approximately the same size as pBR322, was only partially converted to form II. The processed pSV80-03, -08, -04, and control DNAs pDL3, pJY1, and SV40 were transfected into either CV-1 cells (panels a) or COS-7 cells (panels b) as described in the legend to Fig. 2. Transfected cells were incubated at 37°C, and Hirt extracts were made at 4, 16, 28, and 40 h posttransfection (from left to right in each panel). Samples were fractionated by electrophoresis through 1% neutral agarose gels, and vDNA species were detected by hybridization. Marker lanes (M) contained SV40 DNA roughly equivalent to 100 copies of the viral genome per extracted cell. Before alkaline denaturation, the gels were soaked for 30 min in 1 N HCl to increase the efficiency of blotting and hybridization of form I DNA relative to forms II and III through acid depurination and alkaline cleavage. The use of nick-translated pJY1 (specific activity, 2×10^7 cpm/ μ g) for the molecular probe allowed the detection of both pBR322 and SV40 DNA sequences. In all cases the fastest-migrating bands correspond to the form I replicated species of vDNA. The autoradiogram of the CV-1 transfection experiment (which was photographed and spliced for the figure to produce the a panels) represents a 2-week exposure of Kodak X-Omat AR film with two screens at -70°C. The COS-7 autoradiogram used for the b panels was prepared similarly, but exposed for only 1 week.

vDNA over time by Southern blot analysis of 1mw DNA extracted from the transfected cells.

While SV40 DNA from virions and from pJY1 replicated well in both CV-1 and COS-7 cells, the SV40 early region deletion mutant (from pDL3) and all of the excision products (from the pSV80 plasmids) were consistently observed to

replicate only in COS-7 cells (Fig. 6). Replication experiments performed with pSV80V showed the same apparent requirement for the COS-7 A gene product when the transfected cells were incubated at either 31.5 or 40.5°C (data not shown). In repeated experiments, SV40 DNA and pJY1 always replicated more than did either pDL3 or the pSV80 clones in COS-7 cells. We believe the additional A gene dosage provided by the transfected wild-type vDNA led to the increased replication observed (10, 29). Interference between a defective SV80 T and wild-type T may be indicated by the observation that the severely deleted mutant encoded in pDL3 replicated better than did the pSV80-04 insert in repeated experiments.

The above results show that the cloned inserts in pSV80-03, -04, and -08 contained responsive SV40 origins of replication but lacked A genes competent for replication. We also observed strong induction of nuclear T-antigen immunofluorescence in CV-1 cells transfected with pSV80-04 (data not shown), which led us to believe that the relevant lesion(s) in pSV80-04 was located in the structural portion of the encoded A gene and was not an expression defect.

Mapping of A gene mutations by marker rescue. To physically map the locations of mutations within the viral A genes expressed in SV80 and GM638 cells, full-length SV40 genomes were first constructed from the cloned copies of the early regions in pSV80-04 and pGM638-1 as described in Materials and Methods. The resultant viral DNA clones, pSV80V and pGM638V, were then used for mapping by the technique of marker rescue (27, 28). In several trials, transfection into permissive CV-1 cells of either reconstructed viral genome alone never produced plaques, which emphasized the completeness of the block in viral replication imposed by the A gene mutations. However, rescue of both viral genomes, as evidenced by plaque formation, was observed when the vDNA was annealed with specific early region fragments of wild-type SV40 DNA (Fig. 7). From overlaps of the rescuing DNA fragments, the mutation in pSV80V was localized between AP 4069 and AP 4741 (from *Bst*NI to *Taq*I); likewise, the pGM638V mutation was mapped between AP 2534 and AP 3208 (from *Bam*HI to *Pst*I).

Sequence determination of the mutations. The minimal DNA segments determined by marker rescue to span the mutations important for plaque formation in pSV80V and pGM638V were sequenced as described in Materials and Methods (Fig. 8). Only a single point mutation was found in each case. The SV80 mutation was found at AP 4377 (an A-to-C transversion on the late strand of vDNA), which is within the second exon predicted for large T antigen and converts the wild-type Ser-147 codon to an arginine; the GM638 mutation was found at AP 3449 (a G-to-T transversion on the late strand), also within the second exon, and converts the wild-type Leu-457 codon to methionine. Both mutations were in segments of the large T-antigen sequence which have a high degree of homology with the predicted sequence of polyomavirus large T antigen, and each was different from other SV40 mutants that have been found to exhibit similar phenotypes (Fig. 9).

pSV80V mutation present in the SV80 genome. The mutation found at AP 4377 in pSV80V fortuitously destroyed an *Hinf*I site (recognition sequence GATC, where N can be any nucleotide), and we could therefore readily test for the presence of this mutation at the same site in SV80 genomic DNA by Southern blotting and probing an *Hinf*I digest. The result of this experiment (Fig. 10) clearly indicated that SV80 genomic DNA lacked the same *Hinf*I site as pSV80V and

FIG. 7. Marker rescue results for pSV80V and pGM638V. The map shown is of the SV40 early region with the exons for large T antigen. Numbers at the sides of the figure are the plaque counts obtained when the corresponding early region fragments were used in the marker rescue assays of pSV80V (left side) and pGM638V (right side). The *Pst*I A fragment extends off the map to the left, while the *Pst*I B fragment extends off to the right. Assays of pSV80V were repeated to yield the number pairs shown for each fragment. pSV80V contained an *Eco*RI linearized copy of the complete SV40 genome cloned in pBR322 with the viral early region substituted by the full-length early region from pSV80-04 (from *Hpa*II to *Bam*HI); pGM638V contained the early region from pGM638-1 (from *Bgl*I to *Bam*HI) substituted for the wild-type early region of plasmid pK1. (See Materials and Methods for details.) The only uncloned fragment used in these assays was the *Bst*NI B fragment, which was gel purified from a *Bst*NI digest of pJY1. ND, Not done.

pSV80-04. In conjunction with the restriction mapping and marker rescue data described above, we were thus satisfied that pSV80V (and pSV80-04, from which it was derived) contained accurate copies of the viral A gene expressed in SV80 cells.

Transformation of rodent cells by cloned excision products. Our results show that the 4.4-kb *Bam*HI fragment cloned in pSV80-04 contained an unconditionally defective allele of the SV40 A gene. Nevertheless, pSV80-04 transformed Rat-1 cells to growth in agar as efficiently as wild-type SV40 DNA cloned in pBR322 (Table 1). In contrast, the carrier DNA alone, pBR322, and pSV80-03 (which lacks a complete viral early region) were each less than 1% as efficient as SV40 DNA at inducing this phenotype.

Six of the Rat-1 colonies induced by transfection of pSV80-04 were picked from the agar suspensions and propagated in petri dishes. Cells from all six cultures exhibited bright, homogeneous (>99% of the cells) nuclear fluorescence with nucleolar sparing when stained for T antigen by indirect immunofluorescence; all six were morphologically indistinguishable from Rat-1 cells transformed in the same assay by wild-type SV40 DNA (data not shown). Moreover, one line tested for tumorigenicity in nude mice was as effective in tumor formation as wild-type SV40-transformed rodent cells (P. Kahn, personal communication).

DISCUSSION

We have characterized four human cell lines transformed by SV40—SV80, GM638, GM639, and GM2984—that synthesized large T antigen at levels comparable to SV40-transformed Rat-1 cells and yet did not yield excised vDNA after heterokaryon formation with permissive CV-1 cells. For three of these cell lines, however, when a replication-competent A gene was provided *in trans* by an exogenous, integrated SV40 genome in COS-7 cells, heterogeneous

excision and replication of the vDNA sequences carried in the human cells was detected.

Thirteen excised vDNA fragments from the SV80 genome were isolated in pBR322. Six of the plasmids contained a 4.4-kb *Bam*HI fragment apparently identical in size and structure to the genomic SV80 A gene. This fragment was shown to be defective for vDNA replication after transfection into permissive CV-1 cells. Although the fragment encoded a full-length viral early region and could induce T antigen effectively in CV-1 cells, its replication was only detected when transfected into COS-7 cells, a permissive simian cell line which expresses a bona fide endogenous A gene product. The cloned 4.4-kb fragment therefore appeared to harbor an absolute A gene mutation, and conventional marker rescue experiments substantiated this point.

That the cloned 4.4-kb fragment transformed Rat-1 cells to anchorage-independent growth with an efficiency essentially equivalent to wild-type SV40 DNA can be conceptually understood in a number of ways. For example, the SV80 A gene might transform rodent cells through an activity pro-

TABLE 1. Transformation of Rat-1 cells to growth in agar

Transfected DNA	Avg no. of colonies/60-mm dish	Transformants/ μ g of DNA
Carrier DNA alone	4	3
pBR322	1.5	1
SV40	707 ^a	509
pJY1	314 ^a	226
pSV80-04	285 ^a	205
pSV80-03	8	5

^a Colony counts for entire dishes were determined from counts of several randomly selected 1-cm² areas on each dish. For this reason, these estimated values could be in error by as much as 10% in addition to the normal fluctuation expected. The colonies induced by pSV80-04 were generally smaller than those induced by either pJY1 or SV40 DNA.

FIG. 8. Sequencing the SV80 and GM638 mutations. The SV80 and GM638 mutations were determined from pSV80V and pGM638V as described in Materials and Methods. (Left) Part of an autoradiogram of DNA sequence obtained from a radiolabeled *Rsa*I site in pSV80V; (right) DNA sequence obtained from a radiolabeled *Pvu*II site in pGM638V. The specific Maxam-Gilbert reactions are indicated by the labels above each lane. Arrows point to the single point mutations identified; the corresponding wild-type nucleotide is shown in parentheses. For pSV80V, the autoradiogram reads cytosine at position 4377 on the late strand of viral DNA, where the wild-type sequence specifies adenosine. For pGM638V, the autoradiogram reads thymidine at position 3449, where the wild type is guanine.

vided by the small t protein—deletion mutations in the small t antigen coding sequences of SV40 do not interfere with lytic viral replication but do have an effect on the ability of the virus to transform cells. This effect is, however, dependent on the growth state of the cells at the time of infection. Rapidly dividing cells, such as were used in the transformation assays reported here, can be transformed by SV40 virus defective for small t protein with the same efficiency as the wild-type virus. It has therefore been postulated that the small t protein plays an indirect and conditional role early in the transformation process. Even if in some sense the small t protein may induce or maintain transformation, we would have predicted from experiments with *tsA* mutants a dramatic, quantitative decrease in transformation efficiencies with our cloned A gene mutants.

An alternative hypothesis, more consistent with the data that show direct involvement of the viral A gene in transformation, is the notion that large T antigen is multifunctional. Indeed, temperature-sensitive mutations in the viral A gene render the protein very sensitive to a high *in vivo* rate of turnover, presumably because of proteolytic degradation at nonpermissive temperatures. Thus, all activities possibly associated with the protein might show a temperature-sensitive phenotype. Culturing of SV40-transformed human cells may therefore select against the lytic replication function of the viral A gene while simultaneously selecting for the

protein's stability and preservation of its transforming activity, in order that the cells may survive crisis.

The lack of detectable free vDNA after fusion of GM2894 cells with COS-7 cells was reproducible and thus leaves open several plausible explanations of why replication of the vDNA present in the GM2894 genome was not observed. These include expression of a GM2894-encoded repressor of SV40 DNA replication, interference between the GM2894-resident viral A gene and the A gene expressed in COS-7 cells, and the absence of a functional SV40 *ori* in the GM2894 genome. Because the latter explanation is quite plausible, the results of our cell fusion experiments with GM2894 cells do not necessarily relate to the permissivity of the cell line or to the competence of the GM2894 A gene product for replication.

The notion that the A protein is multifunctional is not new, as several mutants which separate the replication and transformation functions of large T antigen have been identified previously (7, 14, 31, 40, 45). It is clear that an A gene activity that would have a wide pleiotropic effect on cells

FIG. 9. Large T-antigen mutations that inactivate replication but retain transforming activity. This figure is a map of the known SV40 early region mutations that inactivate the replication function of large T antigen and that were selected by their ability to transform permissive cells under conditions which normally result in lytic replication of the virus. Portions of large T antigen that encode the nuclear localization, SV40 origin-binding, and ATPase activities of large T antigen are bracketed. N denotes the amino terminus, and C denotes the carboxy terminus of the protein. Coordinates are in amino acid (aa) residues, and the bold lines signify the two exons which encode the protein. The precise mutations shown and their corresponding references are: C6-1 = Met to Ile (aa 30) and Lys to Asn (aa 51) (14); C6-2 = Asn to Thr (aa 153) (14); SVR9D = Lys to Glu (aa 214) (45); C2 = Lys to Arg (aa 516) (31); C11a = Pro to Ser (aa 522) (31); C11b = Pro to Arg (aa 549) (31).

(e.g., induction of cellular enzymes involved in DNA replication) would fit neatly into the economy of the viral replication apparatus. This activity in turn may, under the appropriate conditions, transform the growth properties of infected cells. It seems reasonable that this hypothetical activity be genetically separable from the direct role that large T antigen plays in initiation of vDNA replication.

Paucha et al. (39) reported an engineered mutant, D119, that contains the single point mutation of serine to asparagine at position 147. Like SV80, this mutant is reportedly negative in origin-binding activity and vDNA replication, and yet it can also efficiently transform Rat-1 cells. Myers et al. (36) showed that the SV80 large T protein does not bind to the SV40 origin of replication with the same affinity as does lytic T antigen. This defect may in turn explain not only why SV80 T is defective in catalyzing replication, but also why SV80 cells produce unusually high levels of SV40 early RNA: the protein cannot effectively autoregulate its synthesis. In addition, Flint and Beltz (9) found a predominant species of nuclear RNA from SV80 cells, which hybridized to radiolabeled pSV80-04 probe, that was 4 kb in size. This 4-kb size coincides well with the size we predict for a prepro-

cessed transcript that initiates near position 300 in the pSV80-04 insert and proceeds through to the poly(A) addition site at the right end of the 4.4-kb fragment (Fig. 3b). Splicing of this putative transcript could yield a normal-sized early mRNA for the mutant SV80 T antigen (as well as for a normal small t antigen); mutations in the 5' copy of the *ori* region in the pSV80-04 insert might prevent the SV80 T protein from autoregulating its synthesis via this alternate transcription unit. It would clearly be of interest to directly measure the transcriptional properties of the two SV40 promoters from pSV80-04 in both human and simian cells to probe this question further.

Interestingly, the GM638 mutation lies only four amino acids from the lesion in SV40 *tsA1642*, a mutant which replicates its DNA but is defective in a late lytic function at the nonpermissive temperature and, in some cell lines, is defective in transformation (7). The late lytic defect in *tsA1642*, which can be complemented by other *tsA* mutants, appears remarkably similar to a late lytic defect mapped to a short carboxy-terminal segment of large T-antigen coding sequences (47). This segment of the SV40 genome has been shown to function independently, in a necessary and sufficient manner, when linked to the carboxy terminus of VP1 coding sequences (47). It is tempting to speculate that the *tsA1642* mutation has an allosteric effect on the activity of the carboxy-terminal portion of large T antigen and does not strictly define a second late lytic function for the protein. Similarly, the GM638 mutation may interfere with an activity actually encoded distally within the molecule, perhaps in the vicinity of the SV80 mutation. While the GM638 mutation and the *tsA1642* mutation map extremely close to each other, and the former imparts a severe block to the early-to-late switch, these two mutations may actually define a narrow boundary between distinct functional domains of large T antigen. As summarized in Fig. 9, spontaneously occurring mutations which selectively inactivate the replication function of large T antigen do not fall within a single protein domain. Tertiary structure and high-resolution physical mapping of the protein will be needed to fully assess the relationships between these mutations.

Mutations either at the viral origin of replication or in the structural gene for the A protein can inactivate the lytic potential of SV40. It is clear that the A gene provides a much larger target for such a selective mutation and that the sorts of lesions we have described here will be more frequent than mutations around the viral origin of replication. Gluzman et al. (15) have shown that *ori* mutants of SV40 will transform cells with efficiencies equivalent to wild-type SV40. Our results extend this point to spontaneous mutations which occur in transformed human cells. Clearly, replication-competent T antigen is not required for transformation in a wide range of cell types. Standard *tsA* mutations may not be adequate viral materials for demonstrating this point, since these mutations, as suggested above, may render all functions of the A protein thermolabile.

In the context of the excision model outlined in the Introduction, it is now clear why the human cell lines SV80, GM638, and GM639 do not spontaneously induce vDNA. If a replication-competent A protein is required for viral DNA excision from chromosomes, which in permissive cells leads to further viral replication and cell death as we suggest, then this process is impossible in these cell lines. In addition, the GM2894 cell line may provide a complementary case in which it is the *ori* region that has been mutated instead of the A gene, although other explanations for the behavior of this cell line have not been ruled out.

FIG. 10. Verification of the pSV80V lesion in SV80 genomic DNA. *HinfI* digestions of the indicated DNAs were electrophoresed in a 1.5% agarose gel, Southern blotted, and probed with 32 P-labeled pJY1 (specific activity, 2×10^8 dpm/ μ g). In the digestions, 10 μ g of SV80 genomic DNA and single-copy equivalents of the other DNAs were used. Calf thymus DNA (10 μ g) was used as a carrier in the single-copy reconstructions. Arrows indicate the locations of *HinfI* restriction sites in the wild-type SV40 early region adjacent to the viral origin of replication. The *HinfI* site at 4377 is the one destroyed by the SV80 lesion, which resulted in the production of an 849-bp *HinfI* fragment from SV80 genomic DNA, pSV80V, and pSV80-04, compared with the wild-type 766-bp and 83-bp *HinfI* fragments.

A likely scenario for the establishment of human cell lines stably transformed by SV40 might then include the following steps: viral integration as a tandem unit; the integration site may dictate a level of T-antigen expression which falls between full lytic expression (10) with subsequent cell death on the one hand, and rapid proliferation and transformation on the other; continued culturing of this initially transformed focus may select for spontaneous mutations in the replication function of T antigen (or SV40 *ori*), which averts the excision and self-destruction pathway.

Results obtained initially by Girardi et al. (12) suggest that the A gene mutations identified here were selected for during crisis and recovery. In every case they examined, cultures of SV40-infected human cells that had been pampered through crisis no longer produced infectious SV40, although they had done so prior to crisis. Furthermore, survivors of crisis exhibited higher levels of T staining than they had prior to crisis. Since induction of both T-antigen expression and vDNA replication are correlated in human cells (49; unpublished observations), and high levels of T antigen may be necessary for survival of crisis through expression of a more highly transformed phenotype (41), we suggest that crisis and recovery select for mutations in the viral replication apparatus that leave the transforming activity of the SV40 A gene essentially intact. One may expect that host genes required for viral replication may also provide specific targets for this selection as well. A hunt for such cell types may provide interesting DNA primase or polymerase mutants incapable of, for example, interacting with T antigens (42).

The deeper question of why, in human cell lines transformed by SV40 which maintain a replication-competent A gene and origin of replication, only a fraction of the cells spontaneously induce vDNA replication remains unanswered. The amount of free vDNA in these induced cells (from 100,000 to 400,000 copies per cell) is equivalent to that found in fully permissive, infected simian cells (49; unpublished observations). Yet at any given time only a few of the cells are in the process of this induction. The virus-cell interaction that modulates this process remains a topic for further investigation.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant CA-30490 from the National Cancer Institute.

LITERATURE CITED

1. Birnboim, H. C., and T. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
2. Botchan, M., J. Stringer, T. Mitchison, and J. Sambrook. 1980. Integration and excision of SV40 DNA from the chromosome of a transformed cell. *Cell* 20:143-152.
3. Botchan, M., W. Topp, and J. Sambrook. 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. *Cell* 9:269-287.
4. Botchan, M., W. Topp, and J. Sambrook. 1978. Studies on simian virus 40 excision from cellular chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* 43:709-719.
5. Carroll, R. B., L. Hager, and R. Dulbecco. 1974. Simian virus 40 T antigen binds to DNA. *Proc. Natl. Acad. Sci. USA* 71:3754-3757.
6. Conrad, S. E., C.-P. Liu, and M. Botchan. 1982. Fragment spanning the SV40 replication origin is the only DNA sequence required in *cis* for viral excision. *Science* 218:1223-1225.
7. Cosman, D. J., and M. J. Tevethia. 1981. Characterization of a temperature-sensitive, DNA-positive, nontransforming mutant of simian virus 40. *Virology* 112:605-624.
8. Fiers, W., R. Contreras, G. Haegeman, R. Rogiers, A. Van de Voorde, H. Van Heuverswyn, J. Van Herreweghe, G. Volckaert, and M. Ysebaert. 1978. Complete nucleotide sequence of SV40. *Nature (London)* 273:113-120.
9. Flint, S. J., and G. A. Beltz. 1979. Expression of transforming viral genes in semipermissive cells transformed by SV40 or adenovirus type 2 or type 5. *Cold Spring Harbor Symp. Quant. Biol.* 44:89-102.
10. Gerard, R. D., and Y. Gluzman. 1985. New host cell system for regulated simian virus 40 DNA replication. *Mol. Cell. Biol.* 5:3231-3240.
11. Girardi, A. J., and F. C. Jensen. 1966. Transformation of human cells by oncogenic virus SV40, p. 126-135. *In* W. H. Kirsten (ed.), *Recent results of cancer research*, vol. 6. Springer-Verlag, New York.
12. Girardi, A. J., F. C. Jensen, and H. Koprowski. 1965. SV40-induced transformation of human diploid cells: crisis and recovery. *J. Cell. Comp. Physiol.* 65:69-84.
13. Gluzman, Y. 1981. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* 23:175-182.
14. Gluzman, Y., and B. Ahrens. 1982. SV40 early mutants that are defective for viral DNA synthesis but competent for transformation of cultured rat and simian cells. *Virology* 123:78-92.
15. Gluzman, Y., R. J. Frisque, and J. Sambrook. 1979. Origin-defective mutants of SV40. *Cold Spring Harbor Symp. Quant. Biol.* 44:293-300.
16. Graham, F. L., and A. J. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456-467.
17. Griffin, J. D., G. Spangler, and D. M. Livingston. 1979. Enzymatic activities associated with the SV40 large T antigen. *Cold Spring Harbor Symp. Quant. Biol.* 44:113-122.
18. Gruss, C., E. Baumann, and R. Knippers. 1984. DNA-binding properties of a mutant T antigen from the simian virus 40-transformed human cell line simian virus 80. *J. Virol.* 50:943-946.
19. Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557-580.
20. Hanahan, D., and M. Meselson. 1980. Plasmid screening at high colony density. *Gene* 10:63-67.
21. Henderson, C. I., and D. M. Livingston. 1974. Partial purification and characterization of the SV40 T antigen. *Cell* 3:65-70.
22. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365-369.
23. Kimura, G., and R. Dulbecco. 1972. Isolation and characterization of temperature-sensitive mutants of simian virus 40. *Virology* 49:394-403.
24. Kimura, G., and R. Dulbecco. 1973. A temperature-sensitive mutant of simian virus 40 affecting transforming ability. *Virology* 52:529-534.
25. Kriegler, M. P., J. D. Griffin, and D. M. Livingston. 1978. Phenotypic complementation of the SV40 *tsA* mutant defect in viral DNA synthesis following microinjection of SV40 T antigen. *Cell* 14:983-994.
26. Kucherlapati, R., S. P. Hwang, N. Shimizu, J. K. McDougall, and M. R. Botchan. 1978. Another chromosomal assignment for a simian virus 40 integration site in human cells. *Proc. Natl. Acad. Sci. USA* 75:4460-4464.
27. Lai, C.-J., and D. Nathans. 1974. Mapping the genes of simian virus 40. *Cold Spring Harbor Symp. Quant. Biol.* 39:53-60.
28. Lai, C.-J., and D. Nathans. 1974. Mapping temperature-sensitive mutants of simian virus 40: rescue of mutants by fragments of viral DNA. *Virology* 60:466-475.
29. Lusky, M., and M. Botchan. 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. *Nature (London)* 293:79-81.
30. MacPherson, I. 1969. Agar suspension culture for quantitation of transformed cells, p. 214-219. *In* K. Habel and N. P. Salzman (ed.), *Fundamental techniques in virology*. Academic Press, Inc., New York.
31. Manos, M. M., and Y. Gluzman. 1984. Simian virus 40 large

- T-antigen point mutants that are defective in viral DNA replication but competent in oncogenic transformation. *Mol. Cell. Biol.* **4**:1125–1133.
32. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499–560.
 33. McCutchan, J. S., and J. S. Pagano. 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Natl. Cancer Inst.* **41**:351–357.
 34. Miller, J., P. Bullock, and M. Botchan. 1984. Simian virus 40 T antigen is required for viral excision from chromosomes. *Proc. Natl. Acad. Sci. USA* **81**:7534–7538.
 35. Murkami, Y., C. R. Wobbe, L. Weissbach, F. Dean, and J. Hurwitz. 1986. Role of DNA polymerase α and DNA primase in simian virus 40 DNA replication *in vitro*. *Proc. Natl. Acad. Sci. USA* **83**:2869–2873.
 36. Myers, R. M., D. C. Rio, A. K. Robins, and R. Tjian. 1981. SV40 gene expression is modulated by the cooperative binding of the T antigen to DNA. *Cell* **25**:373–384.
 37. Myers, R., and R. Tjian. 1980. Construction and analysis of SV40 origins defective in T antigen binding and DNA replication. *Proc. Natl. Acad. Sci. USA* **77**:6491–6495.
 38. Myers, R., R. Williams, and R. Tjian. 1981. Oligomeric structure of a simian virus 40 T antigen in free form and bound to DNA. *J. Mol. Biol.* **148**:347–353.
 39. Paucha, E., D. Kalderon, R. W. Harvey, and A. E. Smith. 1986. Simian virus 40 origin DNA-binding domain on large T antigen. *J. Virol.* **57**:50–64.
 40. Pintel, D., N. Bouck, G. di Mayorca, B. Thimmappaya, B. Swardlow, and T. Shenk. 1979. SV40 mutant *tsA1499* is heat-sensitive for lytic growth but generates cold-sensitive rat cell transformants. *Cold Spring Harbor Symp. Quant. Biol.* **44**:305–309.
 41. Risser, R., and R. Pollack. 1974. A nonselective analysis of SV40 transformation of mouse 3T3 cells. *Virology* **59**:477–489.
 42. Smale, S. T., and R. Tjian. 1986. T-antigen-DNA polymerase α complex implicated in simian virus 40 DNA replication. *Mol. Cell. Biol.* **6**:4077–4087.
 43. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**:503–517.
 44. Spangler, G. J., J. D. Griffin, H. Rubin, and D. M. Livingston. 1980. Identification and initial characterization of a new low-molecular-weight virus-encoded T antigen in a line of simian virus 40-transformed cells. *J. Virol.* **36**:488–498.
 45. Stringer, J. R. 1982. Mutant of simian virus 40 large T-antigen that is defective for viral DNA synthesis, but competent for transformation of cultured rat cells. *J. Virol.* **42**:854–864.
 46. Tooze, J. (ed.). 1982. DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 47. Tornow, J., M. Polvino-Bodnar, G. Santangelo, and C. N. Cole. 1985. Two separable functional domains of simian virus 40 large T antigen: carboxyl-terminal region of simian virus 40 large T antigen is required for efficient capsid protein synthesis. *J. Virol.* **53**:415–424.
 48. Weiss, B., A. Jacquemin-Sablon, T. R. Live, G. C. Fareed, and C. C. Richardson. 1968. Enzymatic breakage and joining of deoxyribonucleic acid. VI. Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4. *J. Biol. Chem.* **243**:4543–4555.
 49. Zouzas, D., K. K. Jha, C. Mulder, C. Basilico, and H. L. Ozer. 1980. Human fibroblasts transformed by the early region of SV40 DNA: analysis of “free” viral DNA sequences. *Virology* **104**:439–453.