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Genetics

Simian virus 40 T antigen is required for viral excision from chromosomes

(simian virus 40 excision/homologous recombination/onion skin/transfection)

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ABSTRACT **We describe experiments that show that simian virus 40 (SV40) T antigen is required for viral excision from host chromosomes at some point prior to or during the homologous recombination events that create circular wild-type virus. Two recombinant SV40-pBR322 plasmids were constructed such that homologous recombination across similar-sized but different duplications of SV40 would reconstitute wild-type viral DNA. One plasmid (pSVED) was constructed such that the duplication separates the viral early T-antigen promoter from the coding sequences; the other recombinant (pSVLD) contains a duplication of the late viral sequences and thus maintains a complete T-antigen gene. These plasmids were individually established in Rat 2 cells via cotransformation with the herpes virus *Tk* gene. Both classes of cell lines contained integrated tandem arrays of the plasmids and yielded equivalent levels of infectious virus after cell fusions with COS-7 cells; however, only the T^+ lines yielded virus after cell fusion with CV-1 cells. These results are consistent with the notion that viral excision is initiated by T-antigen-mediated in situ replication of viral DNA as proposed in the "onion skin" model. In contrast, both plasmids yielded infectious virus when transiently introduced via transfection into CV-1 cells. This latter finding is discussed in terms of the possible induction of cellular repair and recombination pathways evoked by the introduction of damaged DNA into the nucleus.**

Cells that can be infected productively by simian virus 40 (SV40), such as African green monkey cells, are thought to be permissive for the lytic cycle because they express one or more chromosomal genes that specify factor(s) that are essential for viral replication. In addition, studies of permissive cells infected with temperature-sensitive mutations in the SV40 *A* gene (3) demonstrated that SV40 large T antigen is also required for successful initiation of viral replication. In contrast, SV40 can neither replicate in nor productively infect cells lacking the permissive factor(s), although viral sequences can persist in these nonpermissive cells if they become integrated and subsequently carried in the host's genome in a proviral form (3).

Though the mechanism for SV40 integration is poorly understood, recent sequencing of parental DNAs indicates that small DNA homologies at the crossover points between virus and chromosome may play some role in this process (2, 4). Integration of SV40 into the chromosomes of nonpermissive cells generally results in a tandem duplication of the inserted proviral sequences though nontandem, single-copy, proviral inserts have been described (1, 5–7).

The association between proviral and chromosomal sequences in these SV40-transformed cells can be destroyed by inducing cell fusion to cells permissive for SV40 replication, such as those of the simian line CV-1 (3). When transformed cells containing tandem duplications of SV40 are fused to permissive cells, freely replicating, form I SV40 DNA molecules are detected in 10–50% of the resulting heterokaryons and these forms are generated via homologous recombination across the tandem duplications (8, 9).

Botchan *et al.* (8) proposed that rather than providing a specific factor for viral excision, fusion to permissive cells induced viral excision by establishing within the heterokaryons all of the components necessary for the initiation of *in situ* proviral replication. They found that an increase in high molecular weight viral DNA still associated with the chromosome preceded viral excision and they proposed that upon initiation of replication at a given proviral locus, multiple rounds of DNA synthesis occurred to form a localized "onion skin" of amplified sequences. They suggested that resolution of this aberrant, polytenized region would require the intervention of repair enzymes that either directly or indirectly lead to the excised forms. It was clear that this sort of *in situ* amplification model could also be used to explain the spontaneous induction of virus in "semi-permissive" lines as well as the apparent expansion and contraction of integrated tandem repeats (1, 10–15, 25).

A fundamental tenet of the onion skin model for viral excision is that within the heterokaryons, SV40 large T antigen is not only required for postexcisional viral replication but is essential, in *trans*, to bind to and initiate unscheduled replication at proviral origins. This aspect of the onion skin model is consistent with *in vitro* studies that have shown that T antigen specifically binds to the SV40 origin of replication and genetic evidence suggesting that a direct interaction between T antigen and the SV40 origin of replication is required for viral replication (3). Though both genetic and physical data suggested that the SV40 *A* gene is the only *trans*-acting viral protein required for this process (8, 16), it was difficult to clearly separate and distinguish a requirement for this protein in the recombination process as opposed to postexcisional replication.

To address the question of the role played by T antigen in the excision process, we have constructed two classes of Rat 2 cell lines using pBR322-SV40 chimeras pSVED (*SV40 E*arly *D*uplication) and pSVLD (*SV40 L*ate *D*uplication) (Fig. 1). Both plasmids contain similar-sized direct repeats of SV40 DNA and both were designed such that intramolecular recombinations across the direct repeats reconstitute wild-type (wt) SV40 DNA. However, a distinguishing feature of plasmid pSVLD is that the SV40 *A* gene is intact and cell lines derived via cotransformation of this plasmid with the herpes virus thymidine kinase *Tk* gene constitutively express T antigen. Cell lines derived via *Tk* cotransformation with pSVED, a plasmid which does not contain an intact *A* gene, are T-antigen negative; however, the *A* gene in these cell lines can be reformed by homologous recombinations across the direct repeats. In this report we demonstrate that upon

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Abbreviations: SV40, simian virus 40; wt, wild-type; Tk, thymidine kinase; pfu, plaque-forming unit(s).

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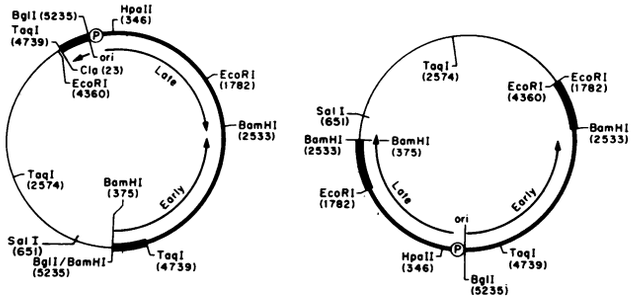

FIG. 1. Structure of plasmids pSVED and pSVLD. Both plasmids contain SV40 sequences (thick line) joined to pBR322 sequences (thin line) and both were designed such that homologous recombinations across the direct repeats of SV40 (dark rectangles) reconstitute wt SV40 DNA. SV40 residues are numbered according to the BB numbering system as found in the second printing of the second edition of Tooze (3). The numbering system used to designate pBR322 sequences is that of Sutcliffe. (*Left*) The SV40 *A* gene promoter (P) in plasmid pSVED is separated from the early coding sequences. However, the promoter can be correctly repositioned, and an intact *A* gene can be reconstituted, by homologous recombination across the 496-base-pair direct repeats. (*Right*) In contrast, the SV40 *A* gene in plasmid pSVLD, which contains 751-base-pair direct repeats, is intact. For both plasmids, the arrows represent the direction of transcription of the SV40 early and late regions, and "ori" marks the unique SV40 origin of replication.

fusion to permissive CV-1 cells, excision of wt SV40 occurs only from T-antigen-positive SVLD cell lines. However, when T antigen is provided in *trans*, by fusion to COS-7 cells, wt SV40 can be rescued from cell lines harboring either pSVLD or pSVED.

We also report that in contrast to the demonstrated requirement for SV40 T antigen during the initiation of excision of wt SV40 from nonpermissive *host chromosomes*, SV40 T antigen is not required for the homologous recombination events that generate wt SV40 upon transfection of these chimeras directly into permissive cells.

MATERIALS AND METHODS

Plasmids. Plasmid pSVED was constructed by first isolating the SV40 *Taq* I/*Bam*HI late fragment from pJY1 (a recombinant plasmid consisting of the full-length *Bam*HI linear sequence of SV40 inserted into the unique pBR322 *Bam*HI site) and ligating this fragment into the *Cla* I/*Bam*HI fragment of pBR322 creating plasmid pPD. Plasmid pPD was cleaved with *Bam*HI and a fragment containing the SV40 early region extending from the *Bgl* I site to the *Bam*HI site was inserted into the *Bam*HI site of plasmid pPD with the aid of a *Bam*HI linker ligated to the *Bgl* I site, thus generating pSVED.

Plasmid pSVLD (previously called pDAX) was constructed by ligating the 751-base-pair *Bam*HI/*Eco*RI fragment of SV40 into *Eco*RI-cleaved plasmid pJY1, which had also been partially cleaved with *Bam*HI at the *Bam*HI site proximal to the pBR322 *Eco*RI site.

Cell Culture and Transfections. For calcium phosphate transfections, Rat-2 cells were seeded ≈24 hr pretransfection at 6×10^5 cells per 100-mm plate. Calcium phosphate precipitates, carrier free, were formed as described in ref. 17. Fifty nanograms of herpes simplex virus *Tk* DNA was transfected along with 150 ng, 250 ng, 275 ng, or 500 ng of either pSVLD or pSVED. Eighteen hours posttransfection, fresh medium was added and after an additional 24 hr the medium was replaced by selective HAT (hypoxanthine, 15 μg/ml; aminopterin, 1 μg/ml; thymidine, 5 μg/ml) medium. Individual Tk^+ colonies were established as cell lines.

Cell Fusions and SV40 Plaque Assays. For the experiments shown in Fig. 3, low molecular weight DNA extractions were performed 48 hr after cell fusions as described in ref. 8. For plaque assays, virus was harvested 60 hr after fusion and stocks were titered by standard protocols on CV-1 cells.

RESULTS

Establishment of SVLD and SVED Cell Lines. The structures of the pBR322-SV40 recombinants pSVED and pSVLD are shown in Fig. 1, and their construction is described in *Materials and Methods*. Plasmids pSVED and pSVLD were cotransfected into Rat 2 cells with the herpes virus *Tk* gene (7) by using plasmid DNA concentrations intended to limit the number of proviral loci in these cell lines.

The Rat 2 cell line (18) is nonpermissive for SV40 replication and contains a mutated, nonfunctional *Tk* gene. Upon transfection, Tk^+ colonies were selected in HAT medium and individual Tk^+ colonies were cloned and expanded into cell lines. The cell lines studied in this report are presented in Table 1, the legend of which explains the nomenclature used to name the various cell lines.

Characterization of SVED and SVLD Cell Lines. Since the SV40 *A* gene is intact in plasmid pSVLD, we anticipated that Tk^+ Rat 2 cell lines cotransfected with pSVLD would express T antigen if (*i*) pSVLD integration did not disrupt the *A* gene and (*ii*) the plasmid DNA resided in genomic regions that allowed for viral expression (7). In contrast, Tk^+ Rat 2 cell lines established with plasmid pSVED were not expected to express SV40 T antigen since, in this plasmid, the cod-

Table 1. Characteristics of SVED and SLVD cell lines

Cell line	Circular DNA upon fusion		Virus yield		Large T immunofluorescence
	CV-1	COS-7	CV-1	COS-7	
SVLD 150-7	+	+	3×10^4	2×10^5	+
SVLD 375-4	+	+	6×10^4	4×10^5	+
SVLD 375-5*	+	+	2×10^4	5×10^5	+
SVLD 500-5*	+	+	5×10^4	6×10^5	+
SVED 150-14*	−	+	0	1×10^5	−
SVED 250-1	−	+	0	4×10^3	−
SVED 250-9*	−	+	1	3×10^5	−
SVED 375-8	−	+	0	1×10^5	−
SVED 375-9†	+	+	5×10^4	5×10^5	+

The first three digits used to designate the various SVLD and SVED lines represent the nanogram amounts of plasmid pSVLD and pSVED that were cotransfected with 50 ng of the herpes virus *Tk* gene into Rat 2 cells: numbers following the hyphens refer to individual Tk^+ cell lines. Titers for infectious virus are given in terms of plaque-forming units (pfu) per ml. For each experiment 10^6 transformed cells were plated with 2×10^6 CV-1 or COS-7 cells in 10 ml of medium. The number of pfu in the freeze-thawed cell lysates was determined by adding 1 ml of either undiluted or serially diluted (diluted 10^{-2} or 10^{-4} in serum-free Dulbecco's minimal essential medium) stocks of virus to nearly confluent monolayers of CV-1 cells on 60-mm plates. To estimate the level at which we cannot detect SV40 excision from the SVED lines, the following calculation is provided. If a hetero-fusion efficiency of 10% is assumed, and if, as in excision from T-antigen-positive cell lines, 10% of these viable heterokaryons are competent for excision (8), then, out of an initial population of 1×10^6 cells, 1×10^4 SVED (T-antigen negative) CV-1 heterokaryons should be competent for excision. The sensitivity of our plaque assay is such that were excision to take place in only one of the excision-competent SVED-CV-1 heterokaryons, with a burst size of ≈100 pfu, we would detect 10 pfu/ml of virus isolate as only 1 ml of medium was tested. Since no plaques are detected, SV40 T-antigen-independent excision is occurring at a frequency of $< 1 \times 10^{-4}$.

*SVED and SVLD cell lines for which fusion data are presented in Fig. 3.

†SVED line 375-9 is listed in a separate category from the other SVED lines since, as detailed in the *Results*, the proviral *A* gene was reconstituted during formation of this cell line and this line is T-antigen positive.

ing region for the viral *A* gene is split from the early promoter.

The presence of SV40 T antigen in cell lines cotransfected with either pSVED or pSVLD was detected by indirect immunofluorescent screening. As shown in Table 1, cell lines established with plasmid pSVLD were invariably T-antigen positive, whereas most cell lines established with pSVED were T-antigen negative. SVED line 375-9 is the exception since it was determined by immunofluorescent screening to be T-antigen positive. Southern blot analysis of genomic 375-9 DNA utilizing an SV40 DNA probe reveals a fragment that comigrates with linear viral DNA after cleavage with either *Taq* I, *Bam*HI, or *Eco*RI. These results together with our ability to rescue wt infecting SV40 DNA from this line (see below) show that a tandem A^+ SV40 genome reformed presumably by recombination across the repeats during establishment of this cell line.

Previous studies have demonstrated that infectious SV40, or precise copies of input recombinant DNAs containing the SV40 origin, can be rescued from those cell lines that contain tandemly duplicated sequences of the proviral sequences (7, 8, 16, 19). Thus, as diagrammed in Fig. 2, precise excision of wt SV40 from either SVED or SVLD cell lines requires that, irrespective of a need for T antigen, the SV40 direct repeats be intact at their respective proviral loci. Accordingly, we have analyzed the genomic DNAs from cell lines established with either of the plasmids for the presence of tandem repeats and for copy number. Tandems were probed by hydrolysis of SVED DNA with either *Taq* I or by *Bgl* I and *Sal* I double digestions. Lines established with pSVLD were screened for tandem duplications of SV40 by cleavage with either *Eco*RI or *Bam*HI. As expected, bands comigrating with linear SV40 indicated that tandem inserts of the SV40 genome were established within the rat genome. Furthermore, cleavage of cellular DNAs with *Sal* I enzyme, a restriction enzyme that does not hydrolyze SV40 DNA, and subsequent blot analysis showed that the copy numbers of separate inserts for either class of cell lines were equivalent, with the copy number ranging from 1 to 5 per genome equivalent (20).

SV40 T Antigen Is Required for Viral Excision. To induce excision of SV40 DNA from cell lines harboring proviral genomes individual lines were fused to either CV-1 or COS-7 cells. Viral excision was assayed by two methods. First, 48 hr after fusion, low molecular weight DNA was extracted from the heterokaryons by the method of Hirt (41). Fig. 3 shows that when representative T-antigen-positive SVLD lines are fused to CV-1 cells (lanes a and c), DNA that comigrates with form I, wt SV40 DNA is detected in the Hirt supernatants. Upon fusion to COS-7 cells, which provide T antigen in *trans*, the same result is obtained (lanes b and d). SVED (T-antigen negative) cell lines also yield SV40 wt sized DNA when fused to COS-7 cells (lanes f and h). However, upon fusion to CV-1 cells (lanes e and g) freely replicating SV40 DNA could not be detected. As expected from the immunofluorescence analysis and genomic organization of the *A* gene in SVED line 375-9, form I sized, SV40 DNA is detected when this cell line is fused to either CV-1 or COS-7 cells (lanes i and j). We have presented these physical data for two SVED lines and two SVLD lines; however, as indicated in Table 1, equivalent and consistent results were obtained with all of the lines.

To show that the extrachromosomal DNA detected was truly wt SV40 and to provide a quantitative measure of virus rescue, fusion-induced excision of SV40 was also monitored by assaying for the production of infectious SV40 virus in the heterokaryons. SV40 virus was isolated prior to the first

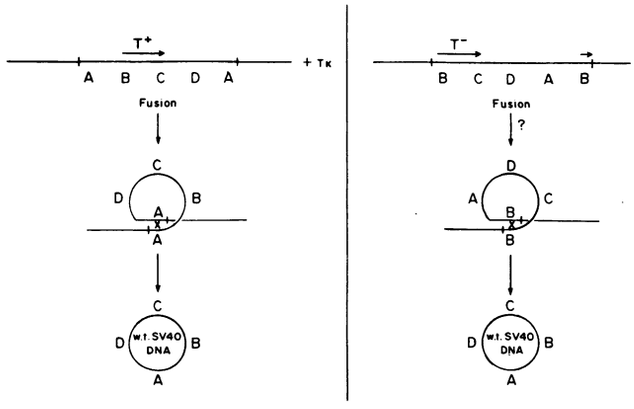

FIG. 2. Experimental design for testing the involvement of T antigen in excision. (*Left*) T-antigen-positive SVLD cell lines were constructed by cotransfecting the herpes virus *Tk* gene with plasmid pSVLD. Upon fusion to permissive cells, wt SV40 DNA should excise via homologous recombination across the direct repeats. (*Right*) T-antigen-negative SVED cell lines were created by cotransfecting plasmid pSVED with the herpes virus *Tk* gene. If T antigen is required for excision prior to the homologous recombination event, wt SV40 should not be liberated upon fusion to T-antigen-negative CV-1 cells; however, excision should occur upon fusion to T^+ COS-7 cells.

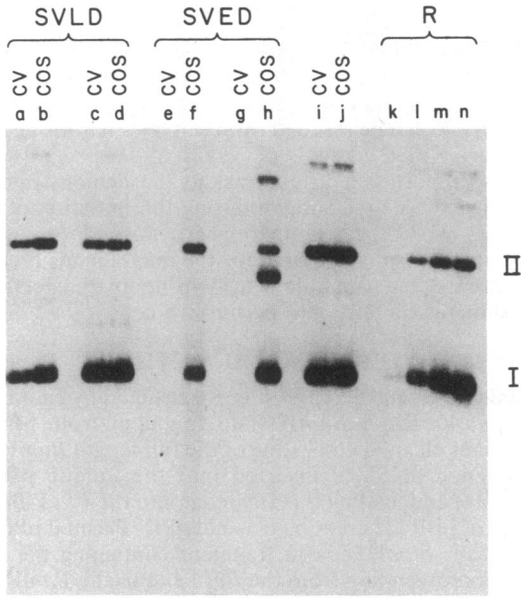

FIG. 3. Physical detection of excised SV40 in heterokaryons formed by fusion. Low molecular weight DNA in heterokaryons formed by fusing SVED or SVLD cells (1×10^6 cells per plate) to either CV-1 or COS-7 cells (2×10^6 cells per plate) was isolated by the method of Hirt (41). DNA samples, containing supercoiled form I and nicked circular form II DNA, were fractionated on a 1% agarose gel, transferred to nitrocellulose, and probed with nick-translated SV40 DNA. Low molecular weight DNA was isolated from heterokaryons formed between SVLD 375-5 and CV-1 cells (lane a), SVLD 375-5 and COS-7 cells (lane b); SVLD 500-5 and CV-1 cells (lane c), SVLD 500-5 and COS-7 (lane d); SVED 150-14 and CV-1 cells (lane e), SVED 150-14 and COS-7 cells (lane f); SVED 250-9 and CV-1 cells (lane g), SVED 250-9 and COS-7 cells (lane h); SVED 375-9 and CV-1 cells (lane i), and SVED 375-9 and COS-7 cells (lane j). Lanes marked R are reconstructions of SV40 containing 0.1 ng (lane k), 0.4 ng (lane l), 0.7 ng (lane m), and 1.0 ng (lane n). Lanes e and g were loaded with the entire Hirt isolates from 100-mm plates of the indicated heterokaryons. To prevent overloading of the gel, the following percentages of the isolates from the remaining heterokaryons (one 100-mm plate) were used: 15% (lane a), 2% (lane b), 15% (lane c), 2% (lane d), 4% (lane f), 4% (lane h), 15% (lane i), and 2% (lane j).

burst (60 hr) and viral titers were determined in CV-1 cells. The results of the viral plaque assays conducted on numerous fused cell lines (Table 1) substantiates the results presented in Fig. 3. SVLD lines, and SVED line 375-9, produce infectious virus when fused to either CV-1 or COS-7 cells. SVED lines do not yield infectious SV40 virus when fused to CV-1 cells, though these same lines produce infectious SV40 when T antigen is provided in *trans*, by fusion with COS-7 cells.

Transient Transfections of pSVED and pSVLD into CV-1 Cells. The results presented above address the role of the *A* gene in excision from chromosomes. Indirect arguments lead to the suggestion that naked DNA transiently introduced into the nucleus at high concentrations may be more recombinogenic than similar events occurring in the chromosome. For example, one of five of our Tk^+ + pSVED cotransformed lines had a $T\text{-}ag^+$ phenotype generated via homologous recombination. We do not know when this recombination occurred, but certainly prior to our first DNA extractions. Once established, however, we detect spontaneous reversion from the transformed state of this, or any of the other T^+ lines, at levels below 10^{-4} per generation. Similarly, spontaneous transformation *to* the T^+ phenotype of the T^- SVED lines is occurring at levels below 10^{-4} to 10^{-5}. Such spontaneous transformants might be expected to arise, for example, via unequal crossovers between sister chromatids. Though differences in effective DNA concentrations (transfection versus chromosomal events) may explain these contrasting results, it is conceivable that damage to incoming DNA may also play some role (see also ref. 22 and below).

We were therefore interested in measuring SV40 recombination from plasmids pSVED and pSVLD after transient introduction into permissive CV-1 cells via DEAE-dextran transfection. Recombinations yielding SV40 were scored for by plaque formation. This assay for recombination relies on the fact that these prokaryote plasmids are too large to be packaged into viral capsids. Thus, plaque formation indicates that pBR322 sequences in these chimeras have been deleted, presumably via homologous recombination across the duplications. As a control, wt SV40 DNA was transfected into CV-1 cells and produced 3.4×10^6 pfu/μg of transfected DNA. pSVLD, containing an intact *A* gene, plaqued at a relative efficiency of 10% of that of wt SV40. pSVED, although incapable of producing T antigen, plaqued with a relative efficiency of 3% of that of wt SV40.

DISCUSSION

Our results show that SV40 excision from chromosomes requires the *trans*-acting functions of the viral T antigen prior to, or during, the recombination process. Different sorts of tandem duplications of the viral DNA were established within cells, and simple recombination as illustrated in Fig. 2 would clearly generate identical wt SV40 for either construction. However, virus rescue after cell fusion was detected only in the T^+ situations. Cell lines containing duplications of viral DNA that split the viral early region were capable of yielding excised virus only if the T antigen was provided in *trans* by fusion with the COS-7 cell line. These findings virtually eliminate the possibility that SV40 T antigen is needed in the virus rescue cycle simply for the replication of free viral genomes that excised via T-antigen-independent pathways. A calculation provided in the legend to Table 1 suggests that in the absence of T antigen, homologous recombination across direct repeats takes place at a frequency of <1 in 10^4 cells. It is clear that these arguments assume that prior exposure to or the presence of T antigen within the cells does not influence the chances of an excised species to be degraded. In this regard we point out that even single copies of SV40 DNA microinjected into CV-1 cells are capable of initiating plaque formation (23). Our results are in good agreement with other studies on the stability of DNA repeats within the chromosomes of cultured cells. For example, Liskay *et al.* (24) studied the stability of tandemly duplicated copies of the herpes simplex virus *Tk* gene in the genome of mouse LTK^- cells and found that recombination across the repeats occurred infrequently ($\approx 10^{-4}$ to 10^{-5} per cell plated). Thus, excision of SV40 DNA from the host chromosome is a special type of homologous recombination event that requires SV40 T antigen. When T antigen is present in *trans*, homologous recombination across the SV40 direct repeats is stimulated 1000- to 10,000-fold.

The stability of the association between integrated polyoma DNA and the host DNA in transformed rat cells is also dependent on its *A* gene product (large T antigen). Thus, when tsA-transformed rat cell lines are passed at 40°C, they are devoid of detectable free polyoma DNA. When shifted to the permissive temperature of 33°C, a low but constant percentage of the transformed cells is induced to yield free polyoma DNA; and when returned to 40°C, the polyoma tsA-transformed rat cells lose the free viral DNA (19, 25–27). Additionally, at the permissive temperature, curing of integrated viral DNA in tsA-transformed cells occurs at a frequency that is at least 100-fold higher than at the nonpermissive temperature (28). These latter results suggest that as in SV40 excision, the requirement for a functional polyoma A protein is not limited to the replication of excised viral DNA but is essential to initiate excision. One study by Lania *et al.* (29) reached the opposite conclusion regarding the involvement of T antigen in excision from polyoma-transformed rat cell lines fused to permissive mouse cells. However, the presence of polyoma T antigen increased the rate of excision 100-fold, and there is now some evidence that, at least in certain polyoma-transformed cell lines, wt large T antigen may not be absolutely required in large amounts to initiate viral replication (30).

The exact role played by T antigens in stimulating viral excision is not known, and it must still be considered a speculation that the detected *in situ* replication (8, 26, 31) is the immediate cause of the excisional recombination. There is no evidence that SV40 T antigen has an intrinsic recombination activity. Indeed, Pellegrini *et al.* (32) have recently shown that within polyoma-transformed cells (T-antigen positive), tandem arrays lacking effective replication origins are stable; whereas, those arrays with origins can excise and amplify *in situ*. If the large T antigen were simply increasing the rate of homologous recombination across repeats, this dependence upon the replication origin should not be observed. Our study complements those of Pellegrini *et al.* (32) in that we show that T antigen is required for some step prior to, or during, recombination and their work establishes the importance of the replication origin in this process. *In situ* replication by itself may increase the chances for excisional recombination (or amplification—see refs. 14 and 15) by creating appropriate substrates; however, it is just as likely that SV40 replication induces, or utilizes, certain cellular enzymes that catalyze these recombination events (see below).

Unlike chromosomal excision, the results of our transient transfection studies indicate that SV40 T antigen in *trans* is not a prerequisite for reconstituting wt SV40 from pSVED or pSVLD. Since the SV40 *A* gene is disrupted in pSVED, this finding shows that upon direct transfection into CV-1 cells, the recombination events that regenerate wt SV40 do not depend on the prior replication of the input DNA. This same conclusion has been reached by Subramani and Berg (33) with analogous, replication-deficient pBR322-SV40 recombinants.

The mechanism for T-antigen-independent recombination upon transfection into CV-1 cells is unknown, though the finding that, in addition to point mutations, newly transfect-

ed DNA is prone to deletions, inversions, and translocations (34–37) suggests that newly transfected DNA is recombinogenic. One way to understand the differences in frequencies between transfection-mediated recombination and identical events occurring within the chromosome is to propose that a critical concentration of recombining forms is low in one situation and high in another. Although concentration of reactive substrates must always influence rates, we believe these considerations are not as relevant to our experiments in that the recombination events scored in our studies are intramolecular. We have performed our transient transfection experiments under a wide range of DNA concentrations, in the linear range of response, and at all concentrations find no dependence upon T antigen. Indeed, it is formally possible that the lesions that signal DNA damage in newly transfected DNA—e.g., nicks, gaps, non-histone covered DNA, etc.—are analogous to the signals that cause excision upon induction of unscheduled SV40 proviral replication. It is well known that DNA-damaging agents will increase the spontaneous excision of SV40 and polyoma in semi-permissive transformed cell lines (21, 25, 31, 38, 39). Direct DNA damage in the region of the integrated viral chromosome need not be invoked in the mode of action of these agents in inducing excision. It may well be that the virus uses a certain class of repair enzymes in its normally lytic replication and that these same proteins are called upon to both initiate *in situ* viral replication and excise the viral DNA from the chromosome to yield discrete replicating free forms. That damage to DNA actually induces enzymes involved in viral excision has recently been indicated by the work of Lambert *et al.* (40). These workers found that polyoma excision is enhanced by fusing normal rat fibroblasts pretreated with a DNA-damaging agent to unexposed polyoma-transformed rat fibroblasts. Since, in this experiment, the proviral-containing genomic DNA was not exposed to the DNA-damaging agent, one cannot argue that the enhanced levels of viral excision were the results of DNA damage around the provirus. Instead, this study suggests that treating nonpolyoma containing cells with DNA-damaging agents induces *trans*-acting cellular factor(s) that stimulate polyoma excision upon heterokaryon formation. In a similar manner we have also observed that pretreatment of CV-1 cells with the DNA-damaging agent mitomycin C elevates by at least 10-fold the amount of SV40 excision that results when these cells are fused to SV40 transformed Rat 1 cells (unpublished observation).

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