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Fate of viral DNA in nonpermissive cells infected with simian virus 40

(DNA replication/polymeric DNA/transformation/integration/tandem duplications)

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ABSTRACT **Mouse cells are nonpermissive for simian virus 40 (SV40): replication of viral DNA is undetectable and progeny virions are not produced. Infection leads instead to the establishment of stably transformed cell lines in which viral DNA is covalently integrated into cellular DNA. We have followed the fate of SV40 DNA in infected mouse cells to define steps in viral DNA metabolism that precede integration. A novel high molecular weight form of SV40 DNA is synthesized shortly after infection by a process sensitive to the inhibition of DNA replication. This DNA represents polymers in which viral genomes are organized as tandem "head-to-tail" arrays. Recombination can be demonstrated with mutant viruses, but the recombination frequency is not high enough to account for the synthesis of polymers by recombination between infecting genomes. We conclude that polymers are synthesized by DNA replication and that they then recombine with one another. We believe that the polymers also recombine with cellular DNA and are thus the precursor to integrated viral DNA. Such a model accounts directly for the high frequency of tandemly duplicated viral insertions in transformed cells and also leads to experimentally testable predictions.**

Mouse cells are nonpermissive for simian virus 40 (SV40). Viral DNA replication involving circular, θ-form, replicative intermediates is not detectable (1), and consequently no progeny virions are produced and most infected cells survive. Viral early gene products are synthesized, inducing transformation in a proportion of the cells (2). Stably transformed cells contain viral DNA covalently integrated into chromosomal DNA (3) and the continued expression of large tumor antigen from such integrated templates is required for maintenance of the transformed phenotype (2). Integration is nonspecific and in many viral DNA insertions all or part of the genome is tandemly duplicated (4–13).

Although our knowledge of the structure of the integrated viral DNA in transformed cells is now quite detailed, we know little about the processes that occur between infection and the establishment of a transformed cell line. We have therefore used transfer hybridization to follow directly the fate of SV40 DNA in cultures of infected BALB/c 3T3 cells. These experiments should allow us to define steps in viral DNA metabolism that precede integration and to detect and characterize intermediates in the integration process.

MATERIALS AND METHODS

Cells and Viruses. African green monkey kidney cell lines were grown as described (14). BALB/c 3T3 clone A31 mouse cells (obtained from M. Fried, Imperial Cancer Research Fund, London) were grown similarly but with 10% fetal calf serum. High-titer stocks of SV40 strains *wt*830, *dl(pm)*861, *dlF*884 (refs. 16–20) and *ts*A58 (ref. 21) (obtained from P. Berg, Stanford University Medical Center) were prepared by published procedures (15).

DNAs and Enzymes. Covalently closed circular SV40 DNA [SV40(I) DNA] was purified from infected CV-1 cells (18). The methods for isolation of total DNA from infected cells and for purification of high molecular weight DNA by sucrose density gradient centrifugation have been published (15). Restriction endonucleases were purified by standard procedures (22).

Gel Electrophoresis and Transfer Hybridization. Agarose gels were cast and run in 89 mM Tris/89 mM H_3BO_3/2.5 mM EDTA (pH 8.2); electrophoresis was at 1 V·cm^{-1} in horizontal slab gels. The molecular lengths of DNA fragments were determined relative to SV40, λ, and pBR322 DNA standards. DNA was transferred (23) from gels to nitrocellulose filters, which were preincubated, hybridized, and washed as described by Jeffreys and Flavell (24) and autoradiographed at −70°C, using Fuji RX x-ray film and Mach 2 intensifying screens (25). Hybridization probes, prepared by nick translation (26), had specific activities of between 3×10^7 and 2×10^8 cpm/μg of SV40 DNA. For quantitative experiments ethidium bromide-stained gels were irradiated with short-wave UV light for 5 min so that all fragments were transferred with approximately equal efficiency. The samples included standards of known amounts of linear SV40 DNA. Autoradiography was performed such that the resultant bands were of an intensity within the linear response range of the film. Films were scanned with a Joyce–Loebl microdensitometer and the amount of DNA in each band was quantitated by cutting out the peaks from the resultant tracings and weighing them. We find, in agreement with Lis *et al.* (27), that the intensity of the autoradiographic response is directly proportional to the amount of DNA. Thus the amount of SV40 DNA in a particular band in an experimental track can be read off from a calibration curve constructed from the standards.

RESULTS

Infected Nonpermissive Cells Accumulate a High Molecular Weight Form of SV40 DNA. Confluent monolayers of BALB/c 3T3 cells were infected with *wt*830 virions, and 72 hr postinfection (p.i.) total DNA was isolated and analyzed; in a parallel series of cultures arabinosylcytosine was present in the medium from the time of infection. Fig. 1*A* shows that cells infected in the absence of inhibitor contain a high molecular weight form of viral DNA; in the presence of arabinosylcytosine

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Abbreviations: SV40, simian virus 40; SV40(I) DNA, covalently closed circular SV40 DNA; SV40(II) DNA, relaxed circular SV40 DNA; moi, multiplicity of infection; p.i., postinfection; kb, 1000 base pairs.

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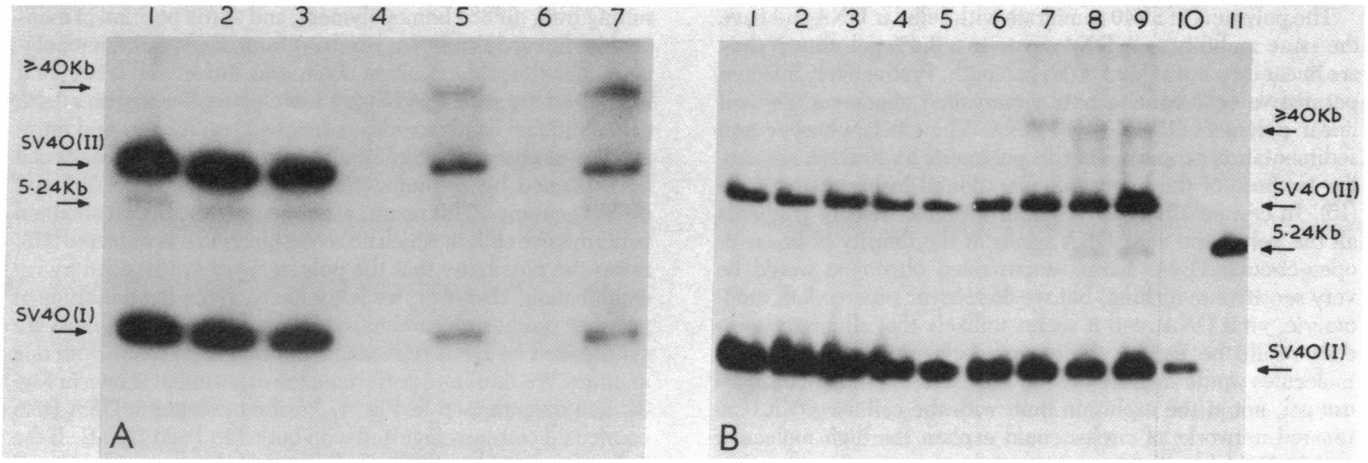

FIG. 1. SV40-infected mouse cells contain a high molecular weight form of viral DNA. (*A*) Inhibition by arabinosylcytosine. Total DNA was isolated 3 days p.i. from *wt*830-infected cells incubated in medium containing 10% fetal calf serum and analyzed by electrophoresis (0.7% gel) and transfer hybridization. The conditions of infection were: track 1, multiplicity of infection (moi) = 300, plus arabinosylcytosine at 20 μg/ml; track 2, moi = 300, plus arabinosylcytosine at 5 μg/ml; track 3, moi = 300, plus arabinosylcytosine at 1 μg/ml (arabinosylcytosine was added at the time of infection); track 4, mock infection; track 5, moi = 25; track 6, moi = 2.5; track 7, moi = 100. (*B*) Time of appearance of high molecular weight SV40 DNA. Confluent monolayers infected with *wt*830 virions (moi = 100) were incubated in medium containing 2% fetal calf serum; total DNA was isolated and analyzed by electrophoresis (0.7% gel) and transfer hybridization. The samples are: track 1, 7 hr p.i.; track 2, 20 hr p.i.; track 3, 32 hr p.i.; track 4, 45 hr p.i.; track 5, 56 hr p.i.; track 6, 72 hr p.i.; track 7, 122 hr p.i.; track 8, 190 hr p.i.; track 9, 242 hr p.i.; track 10, SV40(I) DNA and relaxed circular SV40 DNA [SV40(II) DNA]; track 11, linear SV40 DNA [5243 base pairs (5.24 kb)].

the high molecular weight DNA does not appear. In cells infected at a moi of 100 and held in medium containing 2% fetal calf serum the high molecular weight viral DNA is first detectable 122 hr p.i. (Fig. 1*B*). Incubation in medium containing 10% serum leads to its appearance 64 hr p.i. (data not shown). The high molecular weight DNA is synthesized under conditions that lead to transformation, because cultures in which it was detectable several days after infection gave rise to foci of morphologically transformed cells two weeks later.

Structure of the High Molecular Weight SV40 DNA. The high molecular weight viral sequences could be covalently integrated into cellular DNA or they could represent a polymeric form of SV40 DNA similar to that we described in productively infected permissive cells (15). Total DNA from infected cells was fractionated by sucrose density gradient centrifugation to separate the high molecular weight form from monomeric viral DNA. Fig. 2*A* shows that digestion of the purified DNA with restriction enzymes that cleave cellular DNA but not SV40 DNA does not affect the mobility of the high molecular weight viral sequences. Digestion with enzymes that cleave SV40 DNA once results in the loss of the high molecular weight band and the appearance of a single band comigrating with monomeric, linear viral DNA (Fig. 2*B*). Thus the cells contain polymers of SV40 DNA organized as tandem, "head-to-tail" arrays. These data cannot exclude the covalent integration of large tandem arrays, for example decamers; however, the results of the recombination experiments discussed below argue against this possibility.

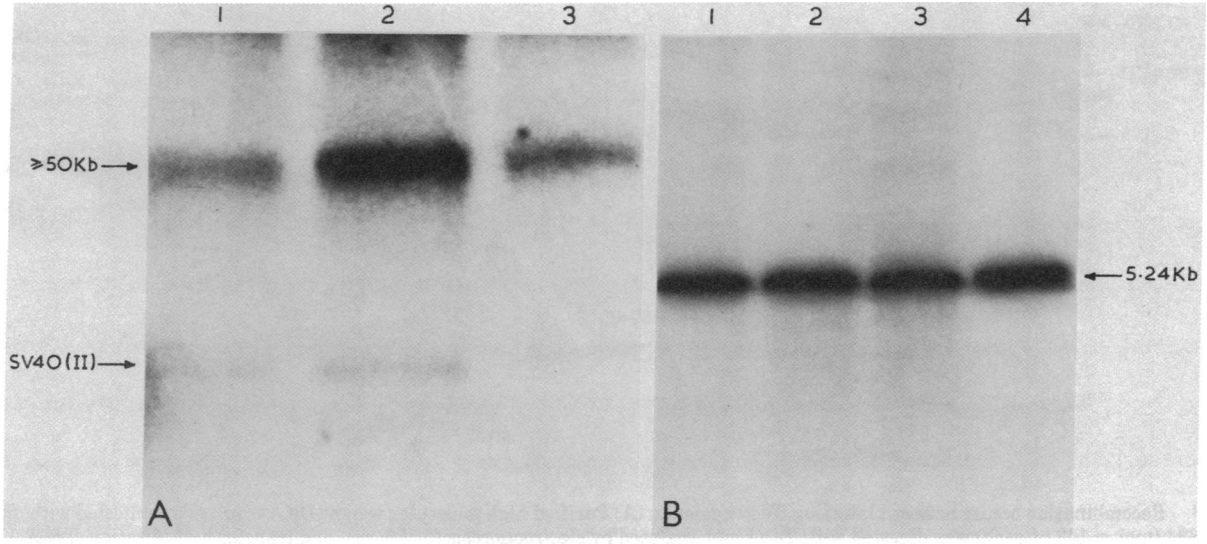

FIG. 2. SV40 genomes in high molecular weight viral DNA are organized in tandem, "head-to-tail" arrays. Purified high molecular weight DNA from *wt*830-infected cells (moi = 300, 10% fetal calf serum, 3 days p.i.) was digested with restriction enzymes and analyzed by electrophoresis (0.6% gel) and transfer hybridization. (*A*) Track 1, 0.3 μg of DNA, *Sma* I-digested; track 2, 0.3 μg of DNA, *Sst* I-digested; track 3, 0.3 μg of DNA, undigested. (*B*) Track 1, 0.3 μg of DNA, *Taq* I-digested; track 2, 0.3 μg of DNA, *Kpn* I-digested; track 3, 0.3 μg of DNA, *Eco*RI-digested; track 4, 0.1 ng of linear SV40 DNA (5.24 kb).

The polymers of SV40 comigrate with cellular DNA and have the same mobility as λ DNA, even in a 0.4% gel; thus if they are linear they are at least 50 kb in length. Productively infected permissive cells contain both supercoiled oligomers (28) and linear polymers (15) of SV40 DNA. The electrophoretic and sedimentation properties of this polymeric SV40 DNA are similar to those of the linear polymers found in permissive cells (15). In cesium chloride/ethidium bromide density gradients all the polymeric viral DNA bands at the density of linear or open-circular DNA. Large, supercoiled oligomers would be very sensitive to nicking, but we do recover supercoiled, monomeric, viral DNA, and it seems unlikely that all large supercoils would be nicked. Moreover, large open-circular DNA molecules would migrate behind the exclusion limit of an agarose gel, not at the exclusion limit with the cellular DNA. Catenated networks of circles could explain the high molecular weight DNA but could not account for the recombination data presented below. In particular, restriction enzyme digestion of catenates would not give rise to linear oligomers. Infected permissive cells contain catenated dimers but these molecules are supercoiled (29, 30). We therefore believe that the polymers are predominantly linear.

Synthesis of the Polymeric Viral DNA. Polymeric SV40 DNA could be synthesized either by DNA replication or by recombination between infecting DNA molecules. We have assessed the role of recombination by infecting cells with viruses with physically distinguishable genomes. *dl(pm)*861 has a deletion of approximately 30 base pairs including the single *Hpa* II site at map position 0.73; *dlF*884 has a deletion of 247 base pairs including the single *Taq* I site at map position 0.57. Both mutants induce the synthesis of polymeric DNA at the same level as *wt*830 (data not shown).

Fig. 3*A* shows an analysis of purified polymeric DNA from cells coinfected with equal multiplicities of the two mutants. *Taq* I digestion gives bands not only at the exclusion limit, resulting from *dlF*884 homopolymers, and at the position of monomeric, linear SV40 DNA, resulting from *dl(pm)*861 homopolymers, but also at the position of dimeric, linear viral DNA (10.2 kb). When the same *Taq* I digest was electrophoresed on a 0.4% gel, additional bands corresponding to linear trimers and tetramers were observed (Fig. 3*B*). The presence of such bands can be explained by recombination between the *dl(pm)*861 and *dlF*884 genomes. This result, which contrasts with that obtained in permissive cells in which no recombination was detected (15), raises the possibility that the polymers are synthesized by recombination. However, we felt it likely, given the inhibition of polymer synthesis by arabinosylcytosine, that the polymers are synthesized by DNA replication and then recombine with one another. We therefore performed the experiment shown in Fig. 3*C* and diagrammed in Fig. 4. Purified polymeric DNA from coinfected cells was digested with both *Taq* I and *Hpa* II. If the polymers recombine in the longer interval between the *Hpa* II and *Taq* I sites, digestion of the recombinant polymer will generate the two linear parental genomes of 5.21 kb [*dl(pm)*861] and 5.0 kb (*dlF*884), plus two novel fragments of 5.82 and 4.39 kb. Similarly, recombination in the shorter interval will lead to the presence of novel fragments of 9.36 and 0.85 kb. Fig. 3*C* shows that the recombinant fragments of 9.36, 5.82, and 4.39 kb are detected in addition to the two parental genomes.

Transfer hybridization experiments were also used to quantitate the polymeric viral DNA and to determine the frequency of recombination. Fig. 5*A* shows total DNA and purified polymeric DNA from *wt*830-infected cells digested with *Eco*RI. On the same gel known amounts of monomeric, linear SV40 DNA were run as standards. The polymeric form (track 6) accounts for approximately 12% of the total intracellular viral DNA (track 3); this is equivalent to 250 viral genome equivalents per cell—i.e., 25 molecules per cell of a linear polymer of 10 genomes (52.4 kb in length). Fig. 5*B* shows a similar experiment in which DNA from cells coinfected with *dl(pm)*861 and *dlF*884

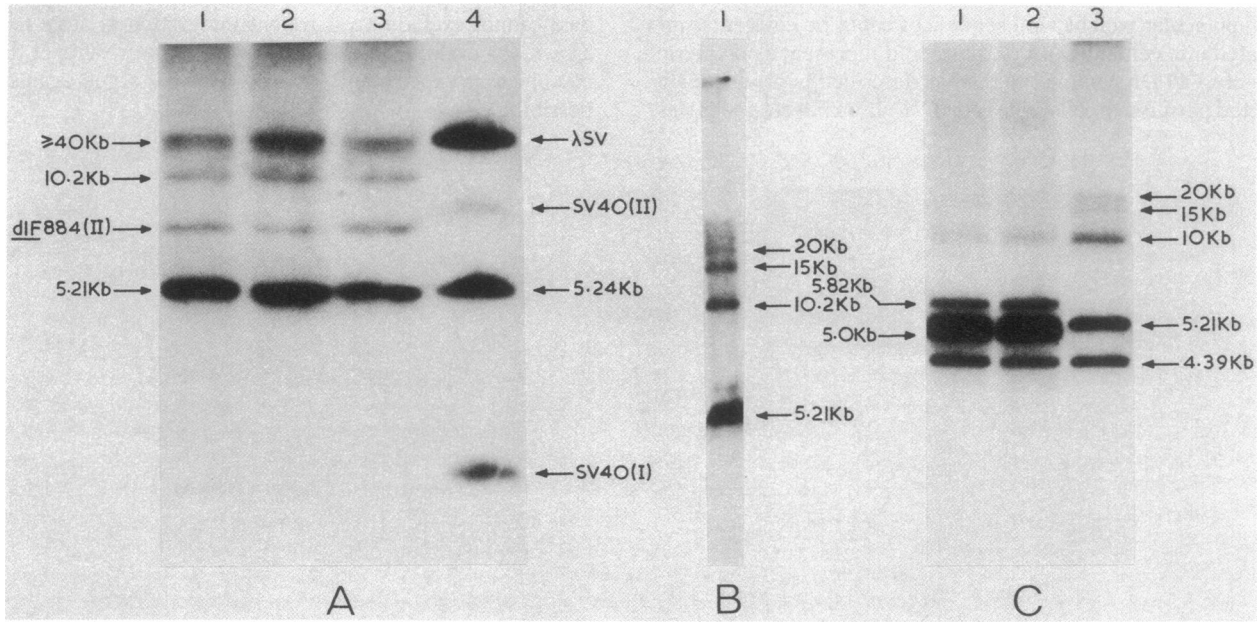

FIG. 3. Recombination occurs between infecting SV40 genomes. (*A*) Purified high molecular weight DNA from cells coinfected with *dl(pm)*861 and *dlF*884 (moi = 100 of each) was digested with *Taq* I and analyzed by electrophoresis (0.75% gel) and transfer hybridization. Track 1, 1 μg of DNA, 66 hr p.i.; track 2, 1 μg of DNA, 69 hr p.i.; track 3, 0.5 μg of DNA, 73 hr p.i.; track 4, markers. λSV is the DNA of a recombinant phage containing SV40 DNA cloned in λ. (*B*) The same DNA sample as in track 2 of *A*, analyzed by electrophoresis in a 0.4% gel and transfer hybridization. (*C*) DNA prepared as in *A* above was digested with restriction enzymes and analyzed by electrophoresis (0.75% gel) and transfer hybridization. Track 1, 0.5 μg of DNA, 69 hr p.i., digested with *Taq* I and *Hpa* II; track 2, 0.5 μg of DNA, 73 hr p.i., digested with *Taq* I and *Hpa* II; track 3, 0.5 μg of DNA, 77 hr p.i., digested with *Taq* I plus 30 pg of SV40 DNA digested with *Taq* I and *Hpa* II. The 0.83-kb *Taq* I/*Hpa* II fragment of SV40 DNA has been run off this gel.

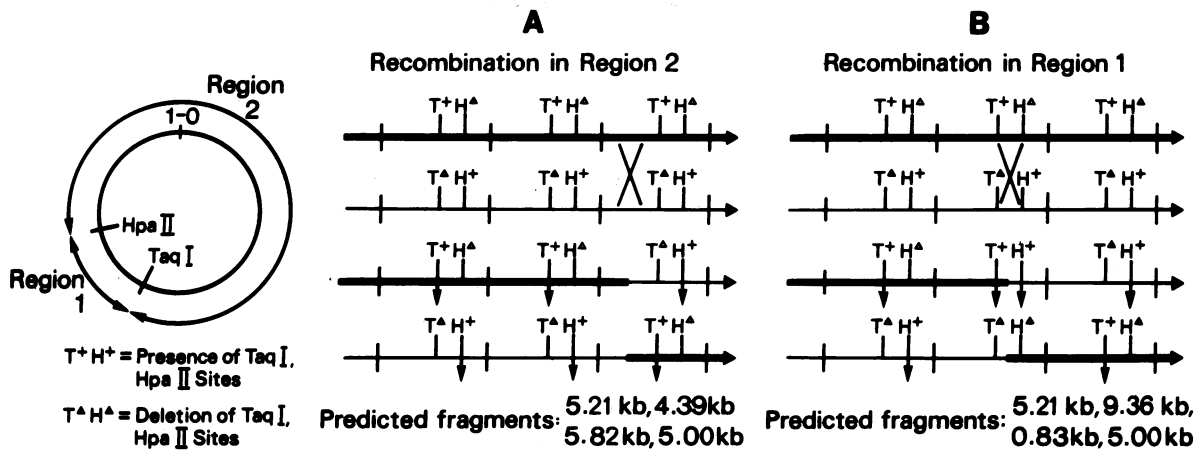

FIG. 4. Schematic representation of recombination between polymeric SV40 DNAs. The map at the left shows the location of the *Hpa* II and *Taq* I sites relative to the *Eco*RI site (position 1–0) on the SV40 DNA map. In *A* and *B* thick lines indicate *dl(pm)*861 homopolymers and sequences derived from them, while thin lines indicate *dlF*884 homopolymers and sequences derived from them. Vertical arrows indicate the *Taq* I and *Hpa* II cleavages that produce the predicted fragments. *dl(pm)*861 DNA is 5.21 kb, *dlF*884 DNA is 5.0 kb.

was digested with *Eco*RI or with *Hpa* II and *Taq* I. Microdensitometer scanning allows determination of the total amount of polymeric DNA (*Eco*RI digestion, tracks 5 and 7) and of the proportion of the polymeric DNA found in the recombinant fragments (*Taq* I plus *Hpa* II digestion, track 4) and thus calculation of the recombination frequency. The relative intensities of the recombinant fragments (track 4) are those expected from the sizes of regions 1 and 2 (Fig. 4), indicating that there is no preferred site for recombination. Twenty percent of the polymeric DNA is in the 5.82- and 4.39-kb recombinant fragments (tracks 4 and 5). Given the sizes of regions 1 and 2, these bands should represent 84% of the total recombination and thus the recombination frequency is 24%. If the polymers were synthesized by recombination then, assuming that the two mutants are not sequestered in separate pools, total reassortment of the markers would occur, giving a recombination frequency of 50%.

We have attempted to determine whether polymer synthesis depends upon the activity of the viral large tumor antigen. Confluent monolayers were infected with *tsA*58 virions at a moi of 500 and then incubated at 39.5°C; total DNA was isolated 76 hr p.i. and analyzed. In three separate experiments we observed that polymeric viral DNA is synthesized under these conditions (data not shown), although our results would not allow us to accurately detect a decrease in the efficiency of polymer synthesis.

DISCUSSION

We have shown that large linear polymers of viral DNA, organized as tandem head-to-tail arrays, are synthesized in SV40-

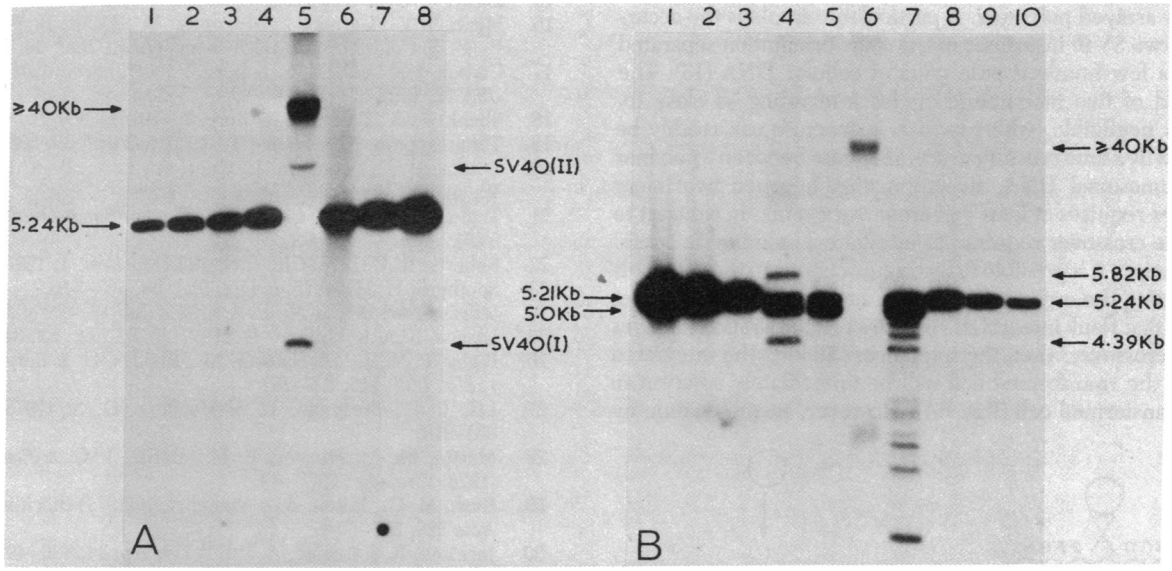

FIG. 5. Quantitative transfer hybridization experiments. The DNAs were analyzed by electrophoresis in 0.7% gels. (*A*) Track 1, 5 pg of SV40 DNA; track 2, 10 pg of SV40 DNA; track 3, 6.25 ng of *Eco*RI-digested total DNA from *wt*830-infected cells (moi = 100, 10% fetal calf serum, 72 hr p.i.); track 4, 20 pg of SV40 DNA; track 5, 0.1 μg of purified high molecular weight DNA from *wt*830-infected cells (moi = 100, 10% fetal calf serum, 72 hr p.i.); track 6, 0.1 μg of same DNA as track 5 but *Eco*RI-digested; track 7, 40 pg of SV40 DNA; track 8, 80 pg of SV40 DNA. (*B*) Track 1, 0.2 ng of SV40 DNA; track 2, 0.1 ng of SV40 DNA; track 3, 50 pg of SV40 DNA; track 4, 0.125 μg of purified high molecular weight DNA from cells coinfected with *dl(pm)*861 and *dlF*884 (moi = 100 of each, 10% fetal calf serum, 72 hr p.i.) digested with *Taq* I and *Hpa* II; track 5, 0.125 μg of same DNA as track 4, undigested; track 7, 12.5 ng of total DNA from coinfected cells (same infection as track 4) digested with *Eco*RI; track 8, 25 pg of SV40 DNA; track 9, 12.5 pg of SV40 DNA; track 10, 6.25 pg of SV40 DNA. The lower molecular weight bands in track 7 result from a low level of *Eco*RI* activity in this particular digestion. The autoradiograms shown here are heavily overexposed and are therefore not in the linear response range of the film. The autoradiograms that were used for microdensitometer scanning were very lightly exposed.

infected mouse cells under culture conditions that lead to stable transformation. The role of viral gene products in polymer synthesis is unclear because the polymeric DNA is synthesized in cells incubated at 39.5°C after infection with *tsA*58, a mutant defective for the initiation of θ-form DNA replication in permissive cells incubated at 41°C. Our "nonpermissive" conditions, a moi of 500 and a temperature of 39.5°C, may allow sufficient replication to produce 25 decameric polymers per cell. Moreover, in permissive cells infected at 41°C with *tsA* virus, limited replication occurs, initiating at sites other than the origin (31). Equally, polymer synthesis may be independent of viral functions or it may depend on the function of tumor antigen required for the induction of cell DNA synthesis, which is inactivated only at 41.5°C (32).

Our data cannot rigorously eliminate the possibility that the polymers are covalently integrated into chromosomal DNA, although the production of such integrated polymers is problematical. They are unlikely to arise by the integration of one monomeric circular viral DNA molecule followed by a series of homologous recombination events because the retransformation of revertant cells that already contain SV40 sequences leads to integration at a second cellular site (33, 34). *In situ* amplification requires that the initial insertion is already tandemly duplicated (35). A final argument against the polymers being integrated is provided by our recombination experiments; such high frequency recombination is unlikely to occur between integrated sequences.

We believe that the polymers are synthesized by DNA replication and then recombine with one another. One would expect them to also recombine, albeit at a much lower frequency, with chromosomal DNA, and we therefore suggest that the polymers are the immediate precursor to integrated viral DNA. This directly explains the high frequency with which tandemly duplicated viral insertions are found in transformed cells. Moreover, SV40-transformed mouse cells contain other types of structure most easily explained by integration occurring from tandemly arrayed polymers. A particular example is the occurrence of two SV40 insertions in the same orientation separated by only a few hundred base pairs of cellular DNA (13). The likelihood of two monomeric circles integrating so close together is negligible, whereas such a structure can readily be produced by a four-crossover recombination between a polymer and chromosomal DNA. Recombination between two linear molecules requires at least two crossover events, in contrast to the single crossover required to integrate a monomeric circle. Consequently it is possible to distinguish between these models by analyzing the organization in untransformed cells of the sequences that flank integrated viral DNA. If integration involves a single crossover, then the sequences flanking the integrated DNA in the transformed cell will be immediately adjacent in the untransformed cell (Fig. 6*A*). However, recombination involving two crossovers will cause deletion of chromosomal DNA at the site of insertion and thus the flanking sequences will be noncontiguous in untransformed cell DNA (Fig. 6*B*). Recent evidence (refs. 38 and 39; unpublished data) has revealed that SV40 integration patterns are not stable; therefore any deletions that are detected could have occurred during the growth of the cells and not as a result of the primary integration event. To verify the prediction of our model it will be necessary to characterize the cellular DNA sequences that flank viral DNA immediately after integration.

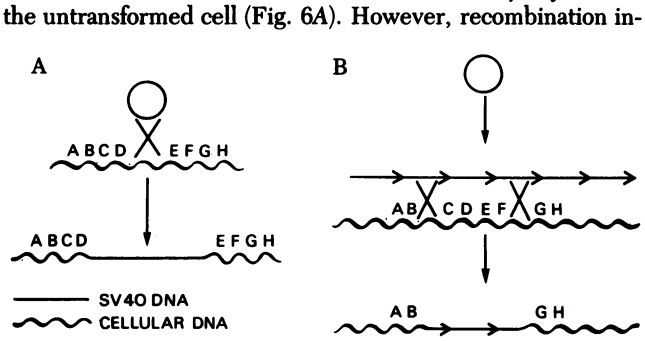

FIG. 6. Single-crossover (*A*) and double-crossover (*B*) models for the integration of SV40 DNA into cellular DNA.

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