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Quantitation of a Simian Virus 40 Nonhomologous Recombination Pathway

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We describe an infectious-center in situ plaque hybridization procedure which quantitates simian virus 40 (SV40) nonhomologous recombination in terms of the number of recombinant-producing cells in the DNA transfected cell population. Using this assay to measure the efficiency of recombination with SV40 DNA in permissive monkey BSC-1 cells, we found that: (i) over a range of DNA concentrations, polyomavirus DNA (which is partially homologous to SV40 DNA) cannot be distinguished from nonhomologous ϕX174 RF1 DNA with respect to its ability to recombine with SV40 DNA; (ii) at defined DNA concentrations, polyomavirus and ϕX174 RF1 DNA compete with each other for recombination with SV40 DNA; (iii) virtually all segments of the ϕX174 genome recombine, apparently at random, with SV40 DNA; (iv) the frequency of recombinant-producing cells, among the successfully transfected (virion-producing) cells, depends upon the input SV40 DNA concentration in the transfection solution; and (v) replication-defective SV40 mutant DNAs compete with wild-type SV40 DNA for recombination with ϕX174 RF1 DNA. From these observations, we conclude that the efficiency of recombination with SV40, in the system under study, is unaffected by nucleotide sequence homology and that a limiting stage in the recombination pathway occurs before SV40 DNA replication. Comparison of the dependency of recombination on initial SV40 DNA concentration with the dependency on initial ϕX174 RF1 DNA concentration indicates that SV40 DNA sequences are a controlling factor in the nonhomologous recombination pathway.

A major obstacle to the understanding of nonhomologous recombination pathways in animal cells has been the dearth of experimental systems amenable to both cellular and molecular analyses. Simian virus 40 (SV40) provides an encouraging approach to this problem in that the viral genome is highly characterized at the genetic and molecular levels and is clearly able to exploit cellular nonhomologous recombination activities for integration, for the incorporation of host DNA into the viral genome, and for a variety of extrachromosomal recombination events (reviewed in reference 23). Furthermore, the development by Villarreal and Berg (24) of an in situ plaque hybridization procedure for identifying SV40 recombinant plaques has provided the basic methodology for analyzing the cellular recombination pathways mobilized during the viral infection.

We previously reported that cotransfection of monkey BSC-1 cells with SV40 DNA and bacterial virus ϕX174 RF1 DNA (or other unrelated DNAs) gives rise to recombinant genomes encased within SV40 capsid protein which replicate in the presence of wild-type SV40 (28). The amount of recombinant virus in stocks harvested from the cotransfected cultures was quantitated by an infectious-center modification of the in situ plaque hybridization procedure. We have now devised an improved version of the infectious-center procedure which differs from that described previously (28) in that it measures recombination in terms of the number of recombinant-producing cells in the initial transfected (or infected) cell population. Using this assay, we find that the frequency of recombination with SV40 DNA is unaffected by nucleotide sequence homology, that a limiting step in the recombination process occurs before SV40 DNA replication, and that SV40 DNA sequences are a controlling factor in the recombination process. In the accompanying communication (29), we describe the structure of SV40-ϕX174 recombinant genomes derived from single recombinant-producing cells.

MATERIALS AND METHODS

Transfection. The following is a modification of the method of Milman and Herzberg (13). One day before transfection, confluent monkey BSC-1 cells were re-

plated with a twofold decrease in cell density. After overnight incubation, the cells were suspended in trypsin-EDTA (0.5 g of trypsin per liter, 100,000 U of penicillin per liter, 0.1 g of streptomycin per liter, 6 mM dextrose, 5 mM $NaHCO_3$, 5 mM KCl, 140 mM NaCl, 5 mM disodium-EDTA), collected by centrifugation, and dispersed in TD buffer (140 mM NaCl, 5 mM KCl, 0.7 mM K_2HPO_4, 25 mM Tris-hydrochloride [pH 7.4]). The cell concentration was determined by using hemacytometer counts, and appropriately sized samples were distributed into 50-ml polypropylene tubes. The cells were pelleted by centrifugation and suspended in the transfection solution at 1 ml per 4×10^6 cells. Transfection solutions contained 300 μg of DEAE-dextran per ml (molecular weight, 2×10^6; Pharmacia Fine Chemicals, Inc.) and the indicated DNA concentrations in TD buffer. After 20 min at room temperature, 5 ml of medium (Dulbecco modified Eagle medium containing 10% calf serum) was added per ml of the transfection mixture. The cell concentrations were rechecked by hemacytometer counts, and the transfected cells were plated for the infectious-center assay described below.

Infectious-center in situ plaque hybridization. The main steps in the plaque hybridization procedure are outlined in Fig. 1A. Replicate samples of the transfected cell population (500 to 1,000 cells for hybridization with SV40 [^{32}P]DNA and 5×10^4 to 1×10^5 cells for hybridization with φX174 RF1 [^{32}P]DNA or other cosubstrate DNA) were mixed with 3×10^6 uninfected BSC-1 cells and plated for monolayer formation in 9-cm-diameter dishes. After overnight incubation, the monolayers were overlaid with nutrient agar (0.9% agar and 10% calf serum in Dulbecco modified Eagle medium) and incubated until 6 days posttransfection to allow plaque formation. The agar overlay was removed, the cell monolayer was transferred quantitatively to a nitrocellulose filter (Schleicher & and Schuell Co., 0.45 μm pore diameter, 8 cm diameter), and the DNA content of the cells was immobilized on the filter as described previously (28).

Hybridization at 68°C in Denhardt buffer (4) with 6× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was carried out as described previously (28), except that the concentration of SV40 [^{32}P]DNA was 1×10^5 cpm/ml (specific activity, 5×10^7 to 1×10^8 cpm/μg) and the concentration of cosubstrate [^{32}P]DNA was 3×10^5 cpm/ml (specific activity as described above). Autoradiograms of the hybridized and washed filters were made with Agfa Curix X-ray film in conjunction with intensifying screens at −70°C for 12 h (SV40 [^{32}P]DNA probe) or 5 days (cosubstrate [^{32}P]DNA probe). The radioactive probes were prepared by nick translation as described previously (16).

DNA preparation and analysis. φX174 RF1 DNA was prepared according to Eisenberg et al. (5), and SV40 and polyomavirus DNA I were prepared according to Oren et al. (14). Bacterial plasmid DNA was prepared from *Escherichia coli* HB101 cells (grown to saturation in LB medium containing the appropriate antibiotic: 100 μg of ampicillin or 100 μg of kanamycin per ml) by a cleared lysate procedure (3) and equilibrium sedimentation in ethidium bromide-containing cesium chloride density gradients. All plasmid DNAs and DNA restriction fragments were purified by separation on agarose gels and eluted from the gel by the glass powder adsorption technique (25). Restriction endonucleases were used according to the manufacturer's (New England Biolabs) recommendations; agarose gel electrophoresis was performed as previously described, and blot hybridization was performed according to Smith and Summers (18). DNA was stored in TE buffer (10 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA) at −20°C; concentrations were determined by the absorbance at 260 nM and confirmed by gel electrophoresis staining intensity in the presence of ethidium bromide.

Source of bacterial plasmids. pMK16 plasmids (kanamycin resistant) carrying inserts of SV40 origin deletion (*ori*$^-$) mutant DNAs (6-1, 3-20, 8-11 [6]) were a gift of Y. Gluzman. Variants of pBR322 either containing or lacking the *Chi* sequence (19) were the gift of G. Smith.

Cloning of φX174 RF1 DNA fragments. Various restriction fragments of φX174 RF1 DNA (see Fig. 3B) were cloned in the *Eco*RI site of pML2 (11), using *Eco*RI linkers (New England Biolabs). The *Hpa*I A fragment (nucleotides 1,294 to 5,024) was cleaved with *Bst*NI generating fragments 1A (nucleotides 1,294 to 3,499) and 1B (nucleotides 3,499 to 5,024). The *Bst*NI ends were blunted by filling in with dATP and dTTP, using *E. coli* DNA polymerase I (8). The fragments noted above, the *Hpa*I C fragment (5,024→5,386/0→30), the *Hae*III A fragment (1,776 to 3,129), and the *Hae*III D fragment (1,173 to 1,776) were ligated to phosphorylated *Eco*RI linkers. The DNAs were then recut with *Eco*RI and religated to *Eco*RI-linearized pML2 plasmid DNA treated with calf intestinal phosphatase (12). Calcium chloride-treated HB101 *E. coli* bacteria were transformed (2) with the ligation products and selected on ampicillin plates. Individual colonies were grown to ministocks (in 2 ml of LB containing ampicillin). Plasmid DNAs prepared from the ministocks were analyzed for the presence and size of the φX174 DNA insert by *Eco*RI digestion and gel electrophoresis. The identity of the inserts was confirmed by preparing radioactive probes from each plasmid DNA isolate; the probes were hybridized to DNA blots (18) of appropriate restriction digests of authentic φX174 RF1 DNA. All recombinant DNA manipulations were carried out in accordance with local biohazard safety guidelines.

RESULTS

Quantitative assay for recombinant-producing cells. The assay for recombination used in these studies is described in detail above and is illustrated in Fig. 1A. The protocol is based upon an infectious-center modification of the in situ plaque hybridization procedure devised by Villarreal and Berg (24). Suspensions of BSC-1 monkey cells were cotransfected with SV40 DNA and a cosubstrate DNA (φX174 RF1 DNA or as designated). To determine the number of cells productively infected with SV40 (frequency of virion-producing cells; f_{vpc}), 500 to 1,000 cotransfected cells were mixed with 3×10^6 uninfected cells, plated to form a monolayer, overlaid with agar-nutrient mix, and processed for in situ plaque hybridization with SV40 [^{32}P]DNA. To determine the frequency of

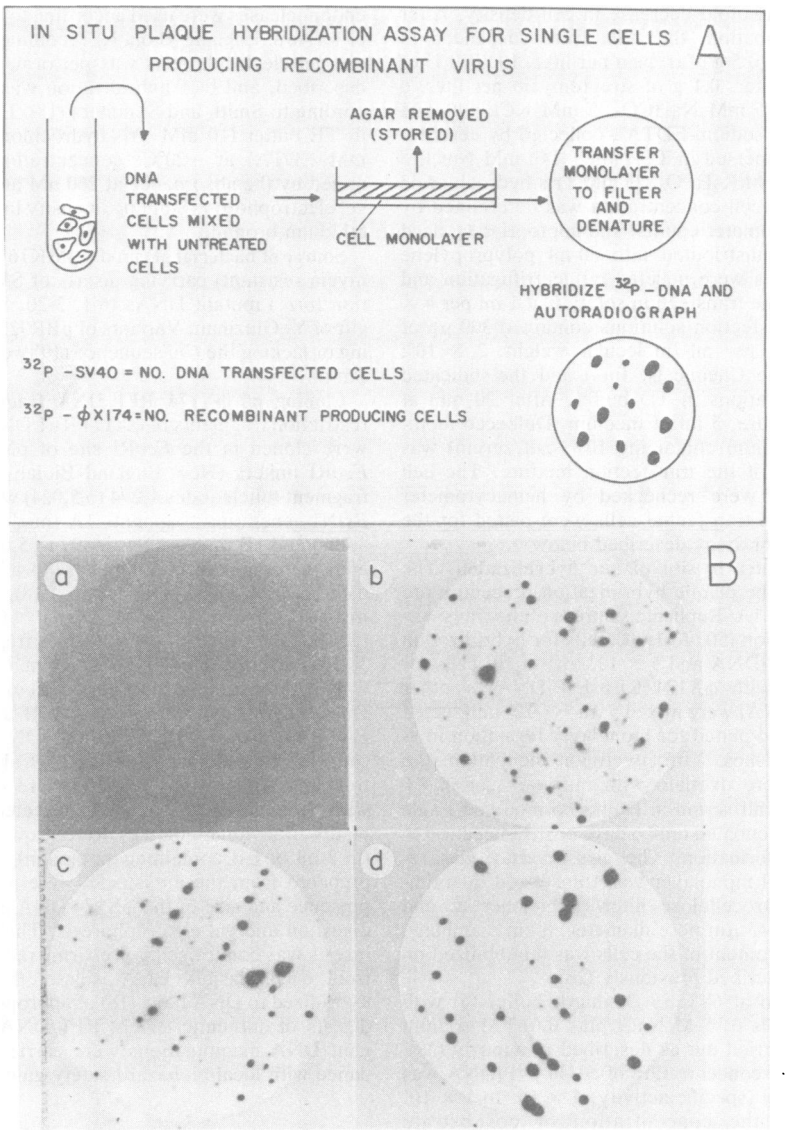

FIG. 1. The infectious-center assay for recombinant-producing cells. Panel A: Diagram of the main steps in the assay (see the text for details). Panel B: (b and c) autoradiograms with recombinant plaques visualized by hybridization with φX174 [^{32}P]DNA (5-day exposure at −70°C) which arose from plating 1×10^5 BSC-1 cells cotransfected with SV40 and φX174 RF1 DNAs (1 μg/ml each) and 3×10^6 uninfected cells; (d) plaques visualized by hybridization with SV40 [^{32}P]DNA (15-h exposure at −70°C) which arose from plating 1,000 cotransfected cells as described above; (a) the lack of hybridization response with φX174 [^{32}P]DNA (6-day exposure at −70°C) when 1×10^5 cells transfected with φX174 RF1 DNA alone (in the absence of SV40 DNA) were plated as described above. Hybridization and other conditions are described in the text.

recombinant-producing cells (f_{rpc}), 50,000 to 100,000 cotransfected cells were treated as described above, except that the hybridization probe was directed against the cosubstrate DNA (φX174 RF1 [^{32}P]DNA or as designated). The efficiency of recombination is expressed as the fraction of virion-producing cells which generated recombinants (f_{rpc}/f_{vpc}). It should be emphasized that the assay measures the number of cells giving rise to recombinant virions, rather than the amount of recombinant virus produced per cell. The assay depends on the use of a cosubstrate DNA that will not replicate significantly in monkey cells unless it recombines with the SV40 genome (controls in which SV40 DNA was omitted from the transfection mixture were used to confirm the lack of cosubstrate DNA replication in monkey cells). The replication and

cell-to-cell spread (plaque formation) of the recombinant genomes depend upon the presence of an SV40 origin of replication, encapsidation restrictions, and the availability of wild-type SV40 for helper functions.

Figure 1B shows examples of the autoradiographic signals obtained by using the infectious-center in situ plaque hybridization assay for recombination. No foci that reacted against φX174 RF1 [^{32}P]DNA were evident when the cells were exposed to φX174 RF1 DNA in the absence of SV40 DNA (Fig. 1B, panel a). The SV40-φX174 recombinant plaques (Fig. 1B, panels b and c) were more heterogeneous in size than the plaques detected with SV40 [^{32}P]DNA (Fig. 1B, panel d), possibly because of differing rates of replication among the recombinants. The transfection protocol has routinely resulted in 5 to 10% of the treated cells producing SV40 virions (SV40 DNA at 1 μg/ml), with 1 to 2% of those cells generating recombinants (cosubstrate DNA at 1 μg/ml). Although the recombination values (f_{rpc}/f_{vpc}) varied twofold from experiment to experiment, the relative levels of recombination between different groups within a given experiment were remarkably constant, with standard deviations of less than 25%. That the autoradiographic plaques shown in Fig. 1B, panels b and c, did in fact arise from the replication of SV40-φX recombinant genomes has been confirmed by isolating the hybrid virus from a portion of the agar overlay corresponding to the autoradiographic signal (24) and mapping its DNA by restriction endonuclease and heteroduplex procedures, as described in the accompanying publication (29).

Recombination with SV40 is independent of gross nucleotide sequence homology. The ability of different DNA species to recombine with SV40 was evaluated by comparing the frequency of recombinant-producing cells generated at various concentrations of different cosubstrate DNA species in the transfection reaction (SV40 DNA at a constant concentration). Figure 2 shows the comparison of polyomavirus and φX174 RF1 DNAs. Both are closed circular molecules similar in size and configuration to SV40 DNA. The f_{rpc}/f_{vpc} value was found to be dependent on the cosubstrate DNA concentration, and φX174 RF1 and polyomavirus DNAs produced the same recombination values as each other at all DNA concentrations tested. The DNAs of the papovaviruses polyoma and SV40 exhibit a certain degree of scattered nucleotide sequence homology (20). In contrast, computer scans of the SV40 DNA sequence and of the bacterial virus φX174 reveal no regions of homology comparable to those described for SV40 and polyomavirus (J. Sussman, personal communication). It appears, therefore, that neither the limited sequence homology to SV40 present in the polyoma genome nor the source (mammalian virus versus bacterial virus) of the cosubstrate DNA has an effect on the efficiency of recombination in this system. Results leading to a similar conclusion have been obtained by comparing recombination between SV40 and polyomavirus DNAs to recombination between SV40 and various bacterial plasmid DNAs (data not shown).

Polyomavirus and φX174 RF1 DNAs compete with each other for recombination with SV40 DNA. We considered the possibility that polyomavirus and φX174 DNAs recombined with SV40 DNA via different pathways which coincidentally resulted in the same recombination frequencies. If two cosubstrates recombine with SV40 via the same pathway, then they should compete with each other when they are present in the same transfection mixture at concentrations close to those which give maximum recombination. Under these conditions, the total number of recombinant-producing cells arising from a triple transfection (SV40, polyomavirus, and φX174 DNA) should be about the same as the number arising from separate cotransfections (SV40 and polyomavirus; SV40 and φX174). If, on the other hand, φX174 and polyomavirus DNAs recombine with SV40 via different pathways, then we would expect the number of recombinant-producing cells arising from the triple transfection to be the sum of those arising from the separate cotransfection reactions. The result of an experiment designed to answer this question is shown in Table 1. The total number of recombinant-producing cells arising from transfection reactions containing SV40 and φX174 DNAs, SV40 and polyomavirus DNAs, or SV40 and φX174 and polyomavirus DNAs, was about the same in each case. Hence, polyomavirus and φX174 DNAs clearly compete with each other for recombination with SV40, indicating that they recombine via the same pathway. Each of the three transfection reactions described in Table 1 generated the same number of virion-producing cells (number of foci reacting with SV40 [^{32}P]DNA). Indeed, cosubstrate DNA concentrations up to 3 μg/ml per transfection reaction have been used without decreasing the frequency of SV40 virion-producing cells. Since the entry of SV40 DNA into the cell was not affected by the increased cosubstrate concentration, the observed competition between polyomavirus and φX174 DNAs cannot have occurred at the level of DNA entry.

The results of reaction 3 in Table 1 also show that most of the triple transfected cells gave rise to recombinant plaques that hybridized only against one probe—either φX174 [^{32}P]DNA or polyomavirus [^{32}P]DNA. A small fraction of the

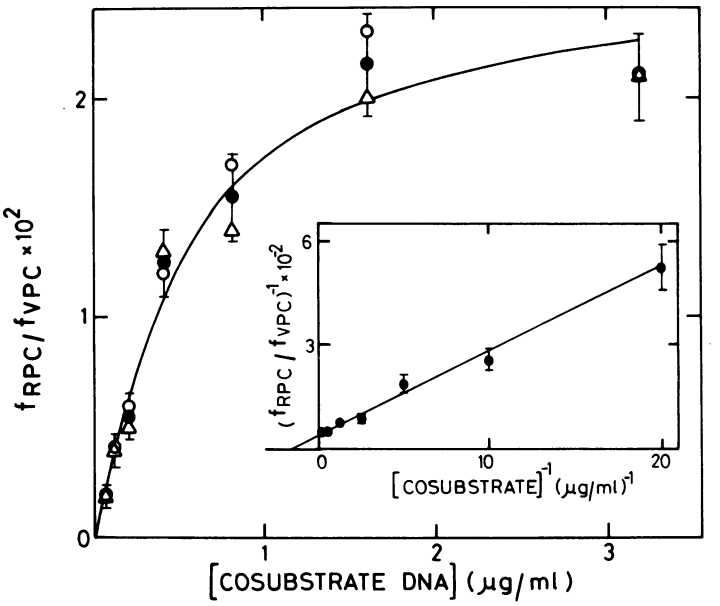

FIG. 2. Comparison of φX174 RF1 and polyomavirus DNAs in recombination with SV40 DNA. The plots show the dependence of the frequency of recombinant-producing cells (f_{rpc}/f_{vpc}; see the text) on the concentration of either cosubstrate polyomavirus DNA (△) or cosubstrate φX174 RF1 DNA (○) in the transfection mixture (SV40 DNA concentration constant at 1 μg/ml); ●, average of the values for φX174 RF1 and polyomavirus DNAs. The curve was generated from the reciprocal plot (inset), using the average of the values for φX174 RF1 and polyomavirus DNAs. Standard deviations in the plaque counts were calculated (assuming a Poisson distribution) by using standard error propagation methods, and the line of the reciprocal plot was determined by least-squares analysis (correlation = 0.97).

recombinant-producing cells (6.3×10^{-4} of 1.7×10^{-2}), however, gave rise to mixed recombinant plaques that hybridized against both probes (see Table 1 footnote *a* for the procedure used to identify these plaques). The significance of the fraction of mixed recombinant plaques will be discussed later.

No particular segment of φX174 RF1 DNA preferentially recombines with SV40. As described above, intact polyomavirus DNA and intact φX174 RF1 DNA could not be distinguished from each other with respect to their efficiencies of recombination with SV40 DNA. Both polyomavirus and φX174 are circular genomes slightly larger than 5 kilobases in size, and it is conceivable that were smaller segments of DNA examined, differences in recombination efficiency might become apparent. To test this possibility, we cloned a series of overlapping and nonoverlapping restriction fragments of φX174 RF1 DNA, encompassing 80% of the intact genome and ranging in size from 392 to

TABLE 1. Polyomavirus and φX174 RF1 DNAs compete with each other for recombination with SV40 DNA

Reaction no.	Transfection (1 μg of each DNA per ml)	f_{rpc}/f_{vpc}[a]		
		SV40-φX	SV40-Py	SV40-φX-Py
1	SV40 + φX174	1.7×10^{-2}		
2	SV40 + polyomavirus		1.6×10^{-2}	
3	SV40 + φX174 + polyomavirus	1.1×10^{-2}	0.6×10^{-2}	6.3×10^{-4}

[a] SV40-φX refers to the fraction of transfected cells (f_{rpc}/f_{vpc}) which gave rise to recombinant plaques detected by hybridization with φX174 [^{32}P]DNA; SV40-Py refers to the fraction detected by hybridization with polyomavirus [^{32}P]DNA; SV40-φX-Py refers to the fraction of transfected cells which gave rise to recombinant plaques that hybridized with both probes. To determine this latter fraction, filters that had been hybridized with φX174 [^{32}P]DNA were, after autoradiography, allowed to decay for 6 weeks and then were rehybridized with polyomavirus [^{32}P]DNA and re-autoradiographed. The autoradiograms of the two sequential hybridization reactions were oriented (24) such that individual plaques which had reacted against both probes could be identified. As a control for the fortuitous overlap of autoradiographic plaques, the autoradiograms resulting from reactions 1 and 2 were superimposed; no fortuitous overlap was observed.

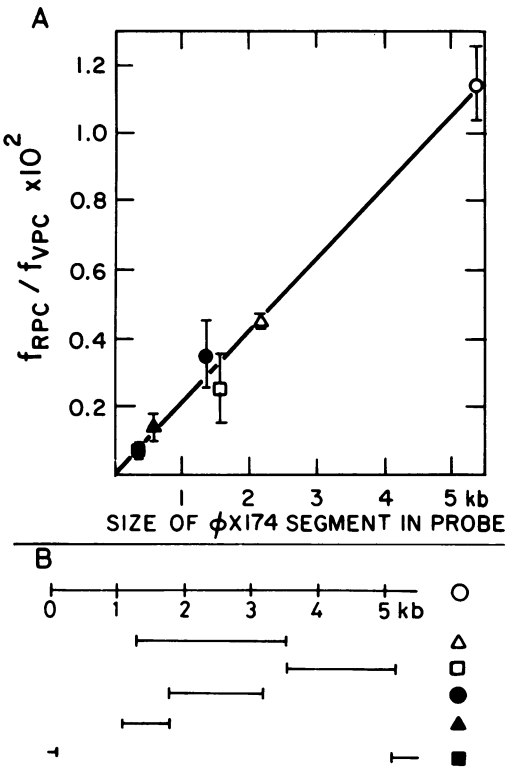

FIG. 3. Frequency of SV40-φX174 recombinant plaques measured with hybridization probes directed against cloned segments of φX174 RF1 DNA. Cells were cotransfected with SV40 DNA and intact φX174 RF1 DNA (1 μg of each per ml) and plated for infectious centers. The fraction of transfected cells giving rise to recombinant plaques was determined by using the following ^{32}P-labeled hybridization probes: ○, intact φX174 RF1 DNA; △, □, ●, ▲, ■, plasmid DNAs containing cloned segments of φX174 RF1 DNA. Panel A shows the linear relationship between f_{rpc}/f_{vpc} and the size (in kilobases) of the φX174 DNA segment represented in each hybridization probe. Panel B shows the map positions of the different cloned segments relative to the map of intact φX174 DNA, linearized at the *PstI* site (nucleotide position zero). Cloning details are given in the text.

2,205 base pairs (bp) (Fig. 3B). The five different plasmid–φX174 constructs and intact φX174 RF1 DNA were used as hybridization probes to monitor the number of recombinant plaques resulting from cotransfection with SV40 and intact φX174 RF1 DNAs. The frequency of recombinant plaques detected by each of the probes was found to be dependent, in a linear fashion, on the size of the φX174 DNA segment represented in the probe and not on the particular sequence present (Fig. 3A). Hence, no segment of the φX174 genome displayed a particular advantage or disadvantage in recombination with SV40.

The result described above was extended by cotransfecting cells with SV40 DNA and the plasmid containing the largest φX174 insert (pφX1A). Probes prepared from intact φX174 DNA, pφX1A DNA, and the plasmid vector DNA were used to detect the recombinant plaques (Table 2). Again, the number of plaques that hybridized to a particular probe was found to be proportional to the fraction of the cosubstrate DNA homologous to the probe DNA. Thus, not only did most (if not all) regions of the φX174 genome appear with equal efficiency in recombinant SV40 genomes, but both the plasmid and φX174 sectors of a plasmid–φX174 construct displayed similar recombination efficiencies with SV40 DNA.

The frequency of recombinant-producing cells depends upon the initial SV40 DNA concentration. The dissection of the relationship between the frequency of recombinant-producing cells and the SV40 DNA concentration in the transfection solution was complicated by the fact that the number of cells productively infected with SV40 was also dependent upon the SV40 DNA concentration (Fig. 4A). When the frequency of recombinant-producing cells was normalized to the frequency of virion-producing cells, however, it became apparent that at low SV40 DNA concentrations, the frequency of recombinant-producing cells was dependent upon the initial input SV40 DNA concentration (Fig. 4B). The probability that a given virion-producing cell generated a recombinant increased with the SV40 DNA concentration used to transfect the cell. Furthermore, the dependence of the recombination frequency on input SV40 DNA concentration suggested that the first recombination-related events occur before SV40 DNA

TABLE 2. Recombination between SV40 DNA and plasmid DNA containing a segment of the φX174 genome[a]

Hybridization probe	% of pφX1A represented in probe[b]	f_{rpc}/f_{vpc}	% of pφX1A f_{rpc}/f_{vpc}
pφX1A	100	1.7×10^{-2}	100
pML2	58	1.1×10^{-2}	65
φX174	42	0.7×10^{-2}	41

[a] Cells were transfected with (per ml) 1 μg each of SV40 DNA and a 5.2-kb plasmid-construct DNA (pφX1A) containing the 2.2-kb segment of φX174 RF1 DNA designated by △ in Fig. 3B. The fraction of virion-producing cells giving rise to recombinant plaques was measured by using ^{32}P-labeled hybridization probes prepared from intact pφX1A DNA, intact pML2 DNA (the plasmid vector used to construct pφX1A), and intact φX174 RF1 DNA.

[b] Calculated from the size of the φX174 DNA insert in pφX1A.

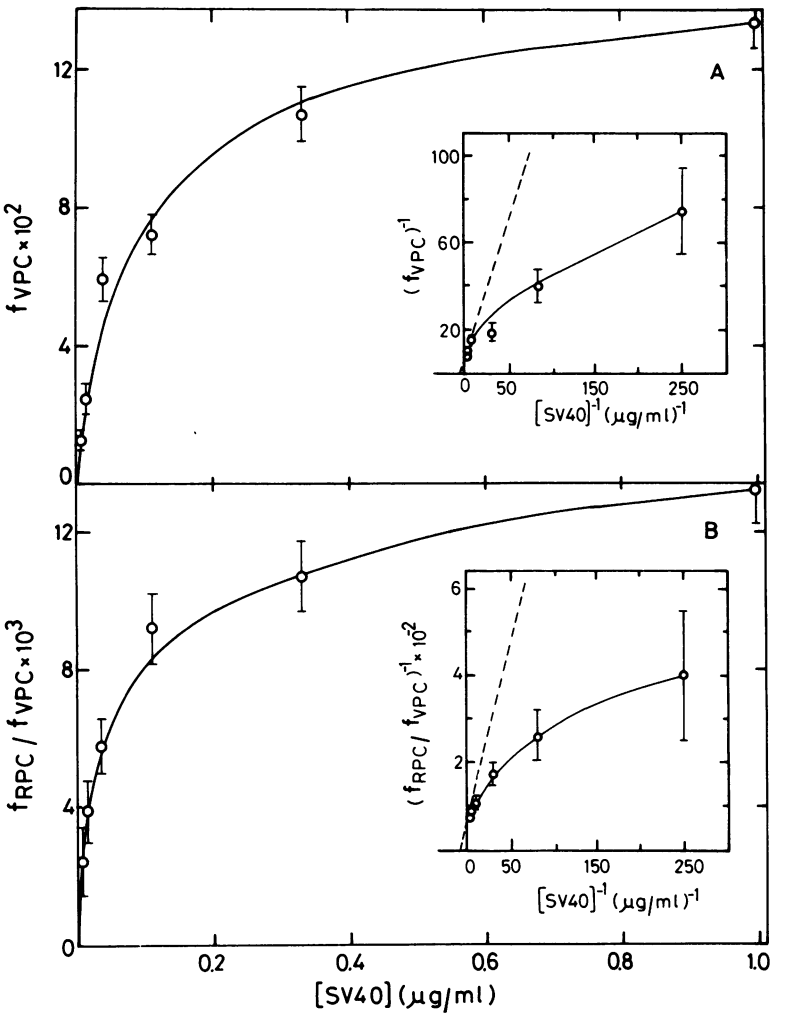

FIG. 4. Dependence of the frequency of virion-producing cells (f_{vpc}) (A) and the fraction of the virion-producing cells giving rise to SV40-ϕX174 recombinant plaques (f_{rpc}/f_{vpc}) (B) on the SV40 DNA concentration in the transfection solution. The concentration of ϕX174 RF1 DNA in the transfection solution was constant at 1 μg/ml. The insets show the reciprocal plots, calculated as in Fig. 2.

replication. Evidence in support of this hypothesis will be described later.

When the dependencies of both the f_{vpc} and f_{rpc}/f_{vpc} on the initial SV40 DNA concentration were plotted in reciprocal form, both were found to be biphasic (Fig. 4, insets). The biphasic nature of these plots cannot be related to the actual process of DNA entry into the cells, since the reciprocal plot for the dependence of f_{rpc}/f_{vpc} on the cosubstrate DNA concentration was not biphasic (Fig. 2, inset).

The biphasic character of the f_{vpc} curve (Fig. 4A) is most easily explained by the hypothesis that there is a subpopulation of the transfectable cells which requires less SV40 DNA for an infection to be initiated. It should be mentioned in this respect that the frequency of virion-producing cells was found to be dependent on the SV40 DNA concentration and relatively independent of the cell concentration in the transfection suspension; only slight increases in the frequency of virion-producing cells were observed when the cell concentration was raised from 3×10^6 per ml to 12×10^6 per ml (SV40 DNA constant at 1 μg/ml; data not shown). This indicates that it is the DNA concentration rather than the DNA-to-cell ratio that is the predominant factor, thereby ruling out a decrease in the size of the target cell population (with increasing percent infection) as a possible explanation for the biphasic dependence of f_{vpc} on SV40 DNA concentration. The biphasic nature of the dependence of the frequency of recombinant-producing cells on the SV40 DNA concentration

(f_{rpc}/f_{vpc}, Fig. 4B) is not due to the fact that the f_{vpc} dependency is itself biphasic, since the values for f_{rpc} have been normalized to the values for f_{vpc}. Possible explanations for the biphasic dependence of the recombination frequency on the initial SV40 DNA concentration include (i) the presence of more than one recombination pathway or (ii) differences in recombination proficiency among the successfully transfected cells, or both. Whatever the explanation, it should be re-emphasized that the biphasic nature of the recombination dose-response curve was observed in the reciprocal plots only when the SV40 DNA concentration varied, not when the cosubstrate DNA concentration varied.

Replication-defective SV40 mutants compete with wild-type SV40 DNA for recombination with ϕX174 DNA. The availability of the replication-defective *ori*$^-$ mutants of SV40 (6) provided the means to test the hypotheses that the first recombination-related events occur before SV40 DNA replication and that SV40 DNA sequences are specifically involved in these events. By choosing an SV40 DNA concentration that approaches saturation (0.25 μg/ml) and a cosubstrate ϕX174 DNA concentration (0.5 μg/ml) well below saturation (to minimize the effects of the competition between cosubstrates described above), one can test whether a given DNA added to the transfection reaction will compete significantly with SV40 DNA for recombination with ϕX174 DNA. If the added DNA lacks a viable origin of replication, any recombinants between it and ϕX174 DNA will not give rise to plaques that hybridize to ϕX174 [^{32}P]DNA; hence, competition will result in a reduction in the number of SV40-ϕX174 recombinant plaques. Three SV40 *ori*$^-$ mutant DNAs (6-1, 3-20, 8-11; see Table 3 and reference 6) were separated from their plasmid vectors by restriction with *Bam*HI endonuclease followed by gel electrophoresis purification and compared with linear polyomavirus and linear wild-type SV40 DNAs (both prepared by *Bam*HI digestion and gel electrophoresis) for their ability to affect the frequency of SV40-ϕX174 recombinant-producing cells, under the conditions described above. The addition of linear polyomavirus DNA had no effect on the frequency of SV40-ϕX174 plaques, linear wild-type SV40 DNA produced a slight stimulation, and all three of the *ori*$^-$ mutant DNAs inhibited the frequency of SV40-ϕX174 plaques by 50% (Table 3). The 241-bp deletion in mutant 8-11 encompasses the early promoter and most of the 72-bp transcription-enhancer repeat units (Table 3). Because this mutant produced the same inhibitory effect as the 6-1 and 3-20 *ori*$^-$ mutants, the inhibition is unlikely to be due to the production of an inhibitory product. The capacity of the replication-defective mutants to compete with wild-type SV40 DNA (for recombination with ϕX174 DNA) implies that they must represent a sizeable proportion of the viral DNA molecules present when a limiting step in recombination occurs. We argue below that this step most probably occurs before viral DNA replication.

TABLE 3. SV40 *ori*$^-$ mutant DNA competes with wild-type SV40 DNA for recombination with ϕX174 RF1 DNA

Transfection supplements[a]		f_{rpc}/f_{vpc} (% of control)[b]
None		100
Polyomavirus WT		101 ± 17
SV40 WT		138 ± 18
SV40 6-1[c]	(5,236 ——→5,243)	52 ± 7
SV40 3-20[c]	(5,186→5,243/0→ 5)	45 ± 6
SV40 8-11[c]	(5,221→5,243/0→ 220)	56 ± 7

[a] Cells were transfected with (per ml) 0.25 μg of wild-type (WT) SV40 DNA (form I) and 0.5 μg of ϕX174 RF1 DNA supplemented with 0.5 μg of the gel-purified *Bam*HI-linearized DNAs indicated.

[b] The fraction of transfected cells giving rise to recombinant plaques, as determined by hybridization with ϕX174 [^{32}P]DNA. The results are expressed as the percentage of the control value obtained with the nonsupplemented transfection reaction and are the average of three to five independent experiments; indicated error values are standard deviations.

[c] SV40 *ori*$^-$ mutants constructed by Gluzman et al. (6). Numbers in parentheses denote the extent of the deletions (BBB nucleotide numbering system [1]).

DISCUSSION

The infectious-center in situ plaque hybridization protocol described in this paper measures the frequency of recombination in terms of number of recombinant-producing cells in the initial DNA-transfected cell population. An important advantage of this method is that the recombinant genomes from individual recombinant-producing cells can be readily isolated for structural analysis (29). The main limitation of the assay is that the recombinant DNA structures must be capable of being replicated and encapsidated with the aid of the wild-type helper virus. For helper-mediated replication of the recombinant DNA, the only SV40 function that needs to be preserved is a functional replication origin. The requirements for recombinant genome encapsidation (necessary for cell-to-cell spread and plaque formation) are less clear but are known to include size restriction (±15% of the wild-type genome).

The cell-mediated joining of unrelated DNAs studied by us and others (15, 17, 27) exploits

DNA transfection procedures. Conceivably, this mode of entry into the cell favors nonhomologous recombination because large numbers of mixed DNA molecules are held in intimate association with each other by the transfection facilitator, DEAE-dextran or complexes of calcium phosphate. Nonhomologous SV40 hybrid genomes, however, do not arise solely from DNA transfection manipulations: SV40/adeno hybrid viruses have been isolated from the yields of monkey cells accidentally coinfected with SV40 and adenoviruses (7); SV40 host-substituted variants, containing various inserts of monkey DNA, have appeared in serially passaged viral stocks (9); and, most recently, recombinant-producing cells arising from the coinfection of BSC-1 monkey cells with SV40 virus and adeno-associated virus (AAV) have been detected, using the same infectious-center in situ plaque hybridization procedure described here, at the same frequency as those arising from DNA transfection (Z. Grossman, K. Berns, and E. Winocour, manuscript in preparation).

The question of whether the presence of limited regions of nucleotide sequence homology increases the frequency of recombination with SV40 was studied by comparing the partially homologous polyomavirus DNA with ϕX174 RF1 DNA which contains no comparable regions homologous to SV40. Over a range of cosubstrate DNA concentrations, ϕX174 RF1 DNA was indistinguishable from polyomavirus DNA in its ability to recombine with SV40. Other experiments, not reported here, show that various bacterial plasmid DNAs cannot be distinguished from polyomavirus DNA with respect to their ability to recombine with SV40 DNA. Hence, the SV40 homologous regions in the polyomavirus genome (scattered stretches of approximately 50 nucleotides [20]) had no measurable effect on the recombination frequency. We also considered the possibility that short DNA sequence-encoded signals, comparable to the 8-bp *Chi* sequence (21), impart a recombination advantage to certain segments of the DNA cotransfected with SV40. However, the observation that no particular segment of ϕX174 DNA displayed a recombination advantage with SV40 (Fig. 3) argues against this possibility, since it is unlikely that short signal sequences would be distributed over the entire ϕX174 genome. The authentic procaryotic *Chi* sequence itself does not effect the recombination frequency with SV40; pBR322 variants containing the *Chi* sequence (19) recombined with SV40 with the same frequency as pBR322 DNA lacking *Chi* (data not shown). Taken together, these observations indicate that the SV40 recombination pathway under study is unaffected by nucleotide sequence homology or the presence of short recombination signals encoded in the cotransfected DNA. In agreement with this, DNA sequence analysis of the recombinant joints between cellular and integrated SV40 DNAs (22) and of the junctions between SV40 and adenovirus DNAs in hybrid viral genomes (10, 26, 30) has not revealed any significant homology (>5 bp) at the recombination sites.

The frequency of recombinant-producing cells increased with increasing concentrations of cosubstrate DNA until a plateau level was reached (Fig. 2). At the plateau level, 1 to 2% of the virion-producing cells generated recombinants. The level of SV40 DNA transfection (frequency of virion-producing cells) was 5 to 10% of the total cell population exposed to DNA. Thus, in terms of the total cell population, 1 cell in 500 to 1 in 1,000 produced recombinants. In coinfections of monkey BSC-1 cells with SV40 and AAV virions, under conditions in which at least 50% of the cells were infected, the plateau frequency of SV40-AAV recombinant-producing cells was still only 1 in 1,000 relative to the total cell population, and this same frequency was obtained when the cells were cotransfected with saturating concentrations of SV40 and AAV DNAs under conditions in which 5 to 10% of the cells were infected (Grossman et al., manuscript in preparation). These observations indicate that the SV40 nonhomologous recombination events occur in a small, discrete subpopulation of cells. Whether this subpopulation is wholly contained within the subpopulation of DNA transfectable cells, and the factors (genetic or physiological or both) which determine the size of the recombinant-producing cell subpopulation, are now being studied.

One may next ask whether all members of the subpopulation of recombinant-producing cells have the same probability of supporting SV40 nonhomologous recombination events. The results of the triple transfection reaction in Table 1 (reaction 3) show that most of the cells produced only one distinguishable class of recombinants—either SV40-ϕX174 hybrid genomes or SV40-polyomavirus hybrid genomes. It should also be noted here that most of the recombinant-producing cells gave rise to only one amplifiable recombinant structure, as judged by the mapping studies reported in the accompanying paper (29). Only a small fraction of the recombinant-producing cells (6.3×10^{-4} compared with 1.7×10^{-2}; Table 1) gave rise to mixed recombinant plaques which hybridized against both SV40 [^{32}P]DNA and ϕX174 [^{32}P]DNA. Double recombination events, giving rise to mixed recombinant plaques, could have occurred purely by chance, and the low frequency observed (7 of 200 recombinant plaques) precludes the use of a statistical test to distinguish between a random

and a nonrandom event. However, the observation that the normalized recombination frequency displays a biphasic dependence on the input SV40 DNA concentration (Fig. 4B) provides an indication for the existence of recombination-proficient cells. The biphasic dependence implies that the minimum input SV40 DNA concentration required to initiate the recombination pathway is lower for some cells than for others, and the simplest explanation to account for this observation assumes the occurrence of recombination-proficient cells among the virion-producing cells.

The competition between the replication-defective *ori*$^-$ SV40 DNA mutants and wild-type SV40 DNA for recombination with φX174 RF1 DNA (Table 3) argues that a limiting stage in the recombination pathway occurs before SV40 DNA replication. If this crucial stage were to occur after the onset of SV40 DNA replication, the intracellular concentration of the replication-defective DNA would be insignificant compared with that of replicating SV40 DNA, and effective competition between the mutant and wild-type SV40 DNA molecules would be unlikely under such conditions. Moreover, the competition experiment establishes that a functional SV40 replication origin is not required for entry into the recombination pathway. Additional, supporting evidence derives from the observation that the frequency of recombinant-producing cells (among the virion-producing cells) depends upon the input SV40 DNA concentration in the transfection solution. This dependency would not be expected if recombination occurred after the onset of SV40 DNA replication.

The inability of polyomavirus DNA to compete with SV40 DNA for recombination with φX174 RF1 DNA in monkey cells (Table 3) is of particular interest since polyomavirus DNA recombines well with φX174 RF1 DNA in mouse cells (Z. Grossman, personal communication). It is possible, therefore, that the recombination pathway in monkey cells depends upon a particular SV40 DNA sequence. Other observations reported here are consistent with the hypothesis that elements encoded in the SV40 genome regulate recombination in monkey cells. First, as the amount of SV40 DNA required to achieve half the maximum frequency (~0.1 μg/ml at φX174 DNA = 1 μg/ml; Fig. 4B) is considerably less than the concentration of cosubstrate DNA required (~0.75 μg/ml at SV40 DNA = 1 μg/ml; Fig. 2), it appears that the utilization of SV40 DNA as a recombination substrate is more efficient than the utilization of the different cosubstrate DNAs used in these experiments (φX174 RF1, polyomavirus, and bacterial plasmid DNAs). Secondly, recombination displays a biphasic dependence on the SV40 DNA concentration (Fig. 4B, inset) and a monophasic dependence on the cosubstrate DNA concentration (Fig. 2, inset). Assuming that the biphasic character of the dose-response dependency results from differences in recombination proficiency among cells, the fact that these differences were detected only when SV40 DNA concentration varied is fully consistent with the hypothesis that SV40 DNA sequences are a controlling factor in the recombination process. We are currently in the process of identifying the specific SV40 DNA sequences involved.

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