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Circular and Linear Simian Virus 40 DNAs Differ in Recombination

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Linear forms of simian virus 40 (SV40) DNA, when added to transfection mixtures containing circular SV40 and φX174 RFI DNAs, enhanced the frequency of SV40/φX174 recombination, as measured by infectious center in situ plaque hybridization in monkey BSC-1 cells. The sequences required for the enhancement of recombination by linear DNA reside within the SV40 replication origin/regulatory region (nucleotides 5,171 to 5,243/0 to 128). Linearization of φX174 RFI DNA did not increase the recombination frequency. The SV40/φX174 recombinant structures arising from transfections supplemented with linear forms of origin-containing SV40 DNA contained φX174 DNA sequences interspersed within tandem head-to-tail repeats derived from the recombination-enhancing linear DNA. Evidence is presented that the tandem repeats are not formed by homologous recombination and that linear forms of SV40 DNA must compete with circular SV40 DNA for the available T antigen to enhance recombination. We propose that the enhancement of recombination by linear SV40 DNA results from the entry of that DNA into a rolling circle type of replication pathway which generates highly recombinogenic intermediates.

The pathways and underlying mechanisms responsible for recombination in animal cells are poorly understood. Integrative recombination by DNA tumor viruses such as simian virus 40 (SV40), polyoma virus, and adenovirus does not generally involve specific sites in the viral or cellular genome, and extensive base sequence homology is not required (reviewed in reference 69), although a possible role for very short regions of homology has not been excluded (11). Because the molecular analysis of viral integration sites in transformed cells is feasible only many cell generations after the initial cell-virus recombination event, we and others have devised short-term extrachromosomal recombination systems, based either upon the cell-mediated excision of infectious SV40 DNA from transfected plasmid constructs (65, 73, 74) or upon intermolecular recombination between cotransfected SV40 and foreign DNAs (1, 24, 31, 75, 76). Some of these studies have led to the realization that, in contrast to lower eucaryotes, nonhomologous recombination in higher eucaryotes is prevalent.

Previously, we have shown that DEAE-dextran-mediated cotransfection of monkey BSC-1 cells with circular SV40 and φX174 RFI DNAs generates recombinant virus which replicates in the presence of wild-type SV40 (76). The frequency of recombination was measured, in terms of the proportion of SV40-replicating cells which generate SV40/φX174 hybrid virions, by an infectious center in situ plaque hybridization procedure (24). Only recombinants which retain a functional SV40 origin of replication and are of a size which can be encapsidated into SV40 virions are scored by this assay. The number of recombinant-producing cells (10^{-3} with respect to the total cell population and 10^{-2} with respect to the successfully transfected subpopulation) is unaffected by the level of gross nucleotide sequence homology between SV40 and the cotransfected DNA (24). The illegitimate recombination events observed are not artifacts of the DEAE-dextran-mediated transfection process since in a study of recombination between SV40 and adeno-associated virus (AAV), the same maximum number of SV40/AAV recombinant-producing cells was recorded irrespective of the way in which the DNAs were introduced into the cells, either by transfection or by covirion infection (31).

Several reports indicate that linear DNA is more recombinogenic than circular DNA in animal cells. When plasmid DNA containing a selectable marker gene is introduced into mouse cells by microinjection, at low input DNA concentrations, linear molecules lead to higher transformation frequencies than circular molecules, and the ends of the linear molecules are involved in recombination with the mouse chromosomal DNA (27). Intramolecular end-to-end ligation has been observed after the introduction of linear DNA molecules into cells by microinjection (38) and transfection (14, 38, 74). Intermolecular ligation has been reported after calcium phosphate-mediated transfection (46, 53) and, at high DNA concentrations, after microinjection of DNA (27). Linearization of SV40 DNA has been shown, at low DNA concentrations, to increase the frequency of neoplastic transformation of animal cells in culture (Z. Grossman and E. Winocour, unpublished data), and linearization of polyoma virus DNA enhances its oncogenic potential in animals of a susceptible species (9, 35). Taken together, these reports indicate that linearization of DNA before its introduction into animal cells enhances the recombinogenic potential of that DNA at both the chromosomal and extrachromosomal levels. To explain this phenomenon, some have argued that linearization of DNA by increasing the concentration of free ends enhances the frequency of end-to-end ligation (74), whereas others (12, 39, 57) have suggested that damage to DNA (including double-strand breaks) may stimulate the production of cellular enzymes involved in the recombination in a manner analogous to the stimulation of SOS proteins by damaged DNA in bacteria (40). The results described in the present report provide arguments for an additional hypothesis, namely, that linear forms of SV40 DNA have a propensity for an unusual replication pathway (probably of the rolling circle type) which generates highly recombinogenic intermediates.

MATERIALS AND METHODS

Transfection. Suspensions of BSC-1 monkey kidney cells were transfected with the indicated concentrations of SV40

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DNA I, φX174 RFI DNA, and various linear forms of SV40 DNA, using DEAE-dextran (molecular weight, 2×10^6; Pharmacia Fine Chemicals) as previously described (24, 27).

Recombination frequency. After DNA cotransfection, the number of SV40 replicating cells and the number of recombinant-producing cells were determined by an infectious center in situ plaque hybridization procedure described fully elsewhere (24). The frequency of recombination (*R*) is defined as the proportion of recombinant-producing cells normalized to the proportion of SV40-replicating cells (24).

Preparation of virion stocks containing recombinant genomes. (i) Stocks containing a variety of recombinant genomes produced from many individual cells (mass lysate stocks) were prepared by incubating the transfected cell population until full cytopathic effect (CPE) had occurred (8 to 10 days posttransfection); the lysate was then collected, freeze-thawed three times, and used to infect fresh cell monolayers for progeny DNA preparation (described below). (ii) Distribution of transfected cells in microwell plates was used to isolate virion stocks containing recombinant genomes produced originally from a single transfected cell (77, 78); the transfected cells were mixed with uninfected cells and plated in microwells such that each well received on the average 2×10^4 uninfected cells and 10 transfected cells. After the appearance of full CPE in some of the wells (12 to 14 days), the microwell plates were freeze-thawed three times, and a sample of each well was removed for dot-blot hybridization (77, 78) with the appropriate DNA probe. Aliquots from the hybridization-positive wells were then used to infect fresh BSC-1 cell cultures; upon development of full CPE, the lysates were collected, freeze-thawed three times, and used to infect fresh BSC-1 cells for progeny DNA preparation. (iii) Virion stocks containing SV40/φX174 recombinant genomes (produced from a single transfected cell) were isolated from the agar overlays of the infectious center assay (77, 78). Autoradiograms of filters hybridized to φX174 [^{32}P]DNA were aligned with the agar overlays as previously described (70, 77, 78), and plugs of agar corresponding to the autoradiographic signals were picked, freeze-thawed, and used to infect fresh monolayers in microwells; upon the occurrence of full CPE, the microwell supernatants were assayed for the presence of φX174 sequences by dot-blot hybridization. The yields of the positive wells were passaged by infection of fresh BSC-1 cells, and after the occurrence of full CPE, the supernatants were collected and used as the inoculum for progeny DNA preparation.

Plasmid constructs. The plasmid containing the *dl*1135 mutant of SV40 (pSV*dl*1135) was a gift from K. Peden and D. Nathans. The pML2K plasmid was prepared (by C. Kahana) from the pML2 (43) derivative of pBR322 by insertion of *Kpn*I linkers (New England Biolabs) into the unique *Bam*HI site. SV40 DNA (strain 777) was cloned via the *Kpn*I site in the *Kpn*I site of pML2K, using procedures described previously (24). The orientation in which the direction of SV40 early gene transcription is the same as the direction of the plasmid ampicillin gene transcription was designated pSVK1 (Fig. 1). *Sph*I digestion of pSVK1 followed by religation was used to generate pSVK1S; pSVK1H was produced by *Hin*dIII digestion of pSVK1 and religation. *Stu*I and *Eco*RI digestion of pSVK1H followed by filling in the *Eco*RI end (with dATP and dTTP, using the Klenow fragment of *E. coli* DNA polymerase I [44]) and religation resulted in pSVK1St (Fig. 1). The p306 plasmid (see below) was produced from pSVK1H by *Eco*RI and *Hin*dIII digestion and repair of the nonflush ends with deoxynucleotides with Klenow polymerase followed by religation.

DNA preparation and analysis. Wild-type SV40 DNA (strain 777), DNA of the *dl*1019, *dl*1020, *dl*1021, and *dl*1083 mutants, and DNA of the various SV40 recombinant viral stocks were extracted from virion-infected BSC-1 cells 48 h postinfection by the method of Hirt (34) and further purified by equilibrium sedimentation in ethidium bromide-containing CsCl gradients (51). Deletion mutants of SV40 were a gift from D. Nathans. The supercoiled replicative form of φX174 DNA (φX174 RFI DNA) was prepared by the method of Eisenberg et al. (25). Bacterial plasmid DNA was extracted from *Escherichia coli* HB101 cultures grown to saturation in LB medium containing 100 μg of ampicillin by a cleared-lysate procedure (17) and equilibrium sedimentation in ethidium bromide-containing CsCl gradients. DNA to be used in transfection solutions was further purified by adsorption to and elution from RPC5 resin (4). Plaskon powder and Adogen 464 used in the preparation of RPC5 was the gift of K. B. Jacobson and G. D. Novelli. DNA restriction fragments were purified by separation on agarose gels and elution from the gels by the glass powder adsorption technique (71). 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.

Restriction enzymes were used according to the recommendations of the manufacturer (New England Biolabs). Agarose gel electrophoresis was performed as previously described (52), and transfer of DNA to nitrocellulose sheets was performed as described by Smith and Summers (64). The resulting blots were hybridized to [^{32}P]DNA probes (200,000 cpm/ml, prepared by nick translation [59]) for 18 h at 68°C in a solution (150 ml per blot) containing Denhardt buffer (21), 6× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 100 μg of denatured salmon sperm DNA per ml. After several washes at room temperature in 2× SSC and one wash in 0.1× SSC at 68°C for 1 h, the blots were dried, and autoradiograms were made with Agfa Curix X-ray film in conjunction with intensifying screens at −70°C.

RESULTS

The infectious center in situ plaque hybridization assay, which we use to compare the recombinogenic potentials of linear and circular SV40 DNAs, quantitates SV40/φX174 recombination in terms of the number of recombinant-producing cells normalized to the number of SV40 virion-producing cells (24). Replication of the SV40/φX174 hybrid virus, and consequently the development of recombinant plaques, depends upon the retention of a functional SV40 replication origin, encapsidation restrictions, and a sufficiency of wild-type SV40 (helper virus) to provide functions missing in the recombinant genome. Because the specific infectivity of linear SV40 DNA is only 1 to 5% that of circular SV40 DNA, transfection with φX174 RFI DNA and the linear form of SV40 DNA may give rise to recombinant-producing cells which are not detected because of an insufficiency of helper virus for infectious center plaque formation (unpublished data). Accordingly, we devised protocols in which the linear SV40 DNA was added as a supplement to standard transfection reactions containing circular SV40 DNA (at a concentration close to the saturation level for plaque formation) and φX174 RFI DNA. The number of recombinant plaques arising from the supplemented transfection reaction, compared with the number arising from the standard transfection, was taken as a measure of the recombination-enhancing properties of the linear DNA.

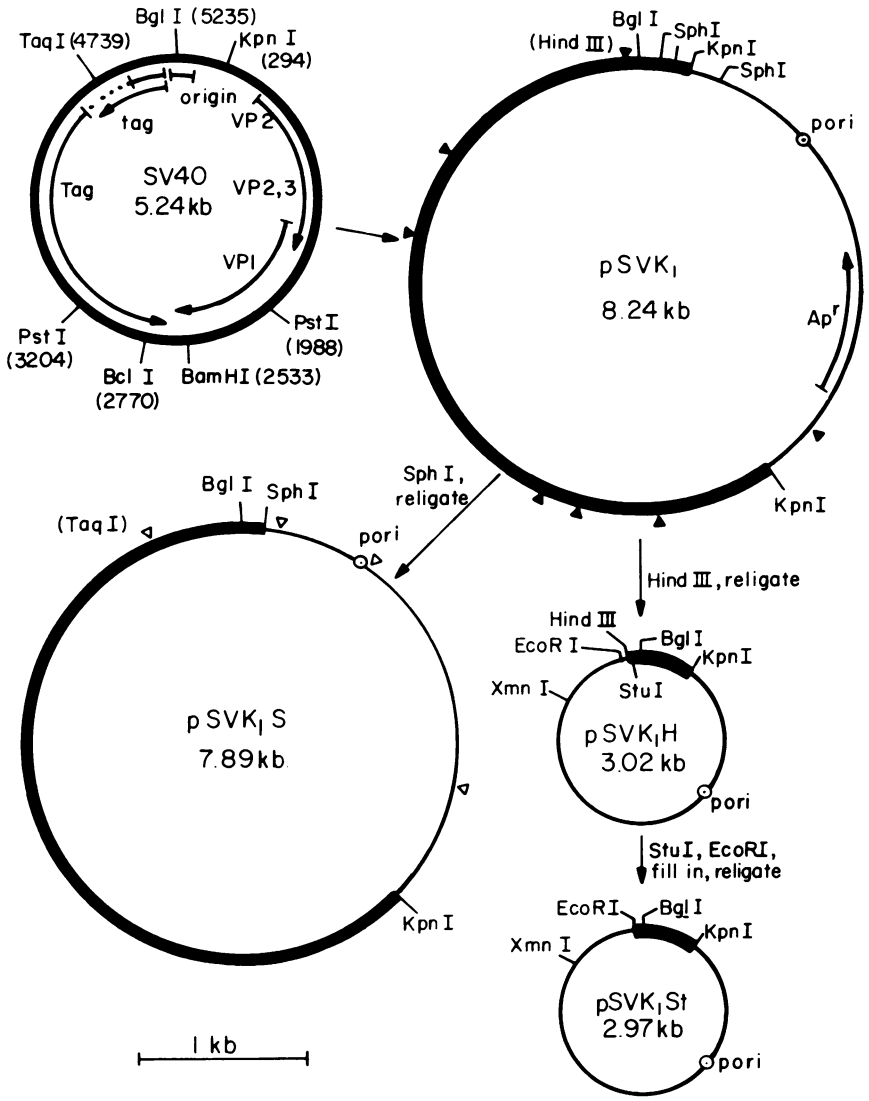

FIG. 1. Construction of SV40 plasmids (see the text). SV40 nucleotide numbering is according to the BBB system (10). Thick lines represent SV40 sequences, and thin lines represent plasmid sequences.

Presence of linear SV40 DNA increases frequency of recombination. To examine the recombinogenic potential of linear SV40 DNA, monkey BSC-1 cells were transfected with a mixture containing 0.1 μg of supercoiled SV40 DNA (SV40 DNA I) and 1.0 μg of φX174 RFI DNA per ml supplemented with either 1.0 μg of intact linear SV40 DNA per ml (produced by *Kpn*I digestion of SV40 DNA I) or, as a control, an additional 1.0 μg of SV40 DNA I per ml. The frequency of SV40/φX174 recombinant foci was determined by the infectious center in situ plaque hybridization procedure (φX174 DNA probe) described above. Table 1 shows that the addition of 1.0 μg of *Kpn*I-linearized SV40 DNA per ml led to a threefold increase in the number of recombinant-producing cells. In contrast, the addition of the same amount of SV40 DNA I produced little change in the recombination frequency.

The viable SV40 mutant DNA *dl*1083 (22) in the supercoiled configuration exhibited the same recombination values as did wild-type SV40 DNA I (Table 1). However, in striking contrast to *Kpn*I-linearized wild-type SV40 DNA, the addition of *Kpn*I-linearized mutant DNA did not increase the frequency of recombinant-producing cells. Other properties of the SV40 *dl*1083 mutant DNA are described in more detail below.

Conversion of φX174 RFI DNA to a linear form does not influence recombination frequency. We next examined the

TABLE 1. SV40/φX174 recombination is enhanced in the presence of linear SV40 DNA[a]

Supplement	*R*	
	SV40 DNA I	*dl*1083 DNA I
None	0.4×10^{-2}	0.5×10^{-2}
SV40 DNA I	0.5	0.5
*Kpn*I-linearized SV40 DNA	1.4	4.2
*Kpn*I-linearized *dl*1083 DNA	0.3	2.0

[a] Monkey BSC-1 cells were transfected with mixtures containing 1.0 μg of supercoiled φX174 RFI DNA per ml and 0.1 μg of supercoiled SV40 DNA I (or supercoiled mutant *dl*1083 DNA I) per ml. These mixtures were supplemented with 1.0 μg of either SV40 DNA I or *Kpn*I-linearized wild-type or mutant *dl*1083 SV40 DNA per ml as indicated. Recombination (*R*) is the proportion of virion-producing cells that also produce SV40/φX174 recombinants as measured by the infectious center in situ plaque hybridization assay, using a φX174 [^{32}P]DNA probe (see the text).

TABLE 2. Linearization of φX174 RFI DNA does not enhance SV40/φX174 recombination[a]

SV40 DNA (μg/ml)	Supplement	R	
		φX174 RFI supercoiled DNA	φX174 RFI linear DNA
0.05	−	0.4×10^{-2}	0.3×10^{-2}
1.0	−	0.6	0.5
0.05	+	2.3	2.6

[a] BSC-1 cells were transfected with SV40 DNA I, 1.0 μg of supercoiled φX174 RF I DNA or φX174 RFI DNA per ml linearized at the single *Pst*I site, and supplemented (where indicated) with 0.5 μg of the *Kpn*I-*Taq*I B fragment of SV40 DNA per ml. See Table 1, footnote *a*, and text for a description of the measurement of recombination (*R*).

possibility that the enhanced recombinogenic potential of linear SV40 DNA results from an increased frequency of random intermolecular end-to-end ligation events. If this were the case, we would expect that linearization of the φX174 RFI DNA should result in a similar increase in the recombination frequency. However, substitution of *Pst*I-linearized φX174 RFI DNA for supercoiled φX174 RFI DNA did not alter the frequency of recombinant-producing cells (Table 2). Even when the SV40/φX174 recombination level was enhanced by the addition of the linear *Kpn*I-*Taq*I origin-containing fragment of SV40 DNA (see below), the recombination frequencies with linear and supercoiled forms of φX174 RFI DNA remained the same. Similar results were obtained in recombination frequency measurements between SV40 DNA I and supercoiled or linear forms of pBR322 DNA (data not shown). These observations, as well as others reported below, do not support the notion that the enhanced recombinogenic potential of linear SV40 DNA can be explained solely on the basis of an increase in random end-to-end ligation events.

Identification of sequences required for enhanced recombinogenic potential of linear SV40 DNA. To identify the region of the SV40 genome required for the higher recombinogenic potential of the linearized form, standard transfection reactions containing 0.05 or 0.25 μg of SV40 DNA per ml and 0.5 μg of φX174 RFI DNA per ml were supplemented with SV40 DNA restriction fragments (Table 3; the map positions of these fragments are shown in Fig. 1). The SV40/φX174 recombination values in Table 3 are presented as proportional values (Δ*R*); that is, the proportional increase in the SV40/φX174 recombination frequency which results from the addition of a given SV40 DNA restriction fragment to the standard transfection reaction. The Δ*R* values are adjusted to the concentration of linear DNA in the transfection reaction, both in terms of weight (micrograms per milliliter) and molarity (nanomolar) so that SV40 DNA restriction fragments of different sizes may be compared.

Table 3 shows that the addition of SV40 DNA restriction fragments which lack the replication origin and regulatory region (for example, the *Kpn*I-*Taq*I A fragment, the *Kpn*I-*Bam*HI B fragment, and the *Taq*I-*Bam*HI B fragment) did not significantly enhance the frequency of SV40/φX174 recombinant-producing cells. In contrast, the origin-containing (*ori*$^+$) fragments and full-length linear DNA, prepared from wild-type SV40, increased the frequency of recombinant-producing cells to various extents. Table 3 also shows that the *Kpn*I-*Bam*HI A, *Kpn*I-*Pst*I A, and *Kpn*I-*Taq*I B *ori*$^+$ fragments of SV40 DNA, which contain decreasing portions of early gene sequences, were as effective as each other on a weight basis, and therefore their molar efficiencies decreased proportionally with their length. This observation provides further evidence that the higher recombinogenic potential of linear SV40 DNA fragments cannot be explained solely on the basis of an increased frequency of end-to-end ligation events since there are more free ends per unit weight in small fragments than in larger fragments.

When the *Kpn*I-*Taq*I B fragment of SV40 DNA (nucleotides 4,739 to 5,243/0 to 294) was further reduced in size to produce either the *Sph*I-*Taq*I B fragment (nucleotides 4,739 to 5,243/0 to 128) or the *Kpn*I-*Hin*dIII fragment (nucleotides 5,171 to 5,243/0 to 294) the recombination-enhancing activity was decreased on both a molar and weight basis (Table 4). This loss of efficiency, however, was due to size. Extension of *Sph*I-*Taq*I B fragment with plasmid DNA sequences (the *Taq*I C fragment of the pSVK1S construct shown in Fig. 1) fully restored the recombination-enhancing activity; similarly, extension of the *Kpn*I-*Hin*dIII fragment with plasmid

TABLE 3. Only origin-containing SV40 DNA fragments enhance SV40/φX174 recombination[a]

SV40 DNA[b] supplement	Size (kilobases)[c]	Origin[d]	Proportional recombination increase[e] at SV40 DNA I concn:			
			0.25 μg/ml		0.05 μg/ml	
			Δ*R* (μg/ml)	Δ*R* (nM)	Δ*R* (μg/ml)	Δ*R* (nM)
Intact						
*Kpn*I linearized	5.24	+	1.0 ± 0.1	3.3 ± 0.4	7.0	23.1
*Bam*HI linearized	5.24	+	0.8 ± 0.1	2.6 ± 0.3	2.2	6.6
Fragments						
*Taq*I-*Bam*HI A	3.04	+	1.1 ± 0.2	2.1 ± 0.4	12.0 ± 1.7	23.6 ± 3.3
*Kpn*I-*Bam*HI A	3.00	+	5.0 ± 0.9	9.8 ± 1.7	20.0 ± 1.6	39.5 ± 3.2
*Kpn*I-*Pst*I A	2.33	+	5.0 ± 0.6	7.6 ± 0.9		
*Kpn*I-*Taq*I B	0.80	+	4.0 ± 0.6	2.1 ± 0.3	16.0 ± 3.2	8.3 ± 1.6
*Kpn*I-*Taq*I A	4.44	−	0.1 ± 0.1			
*Kpn*I-*Bam*HI B	2.24	−	0.3 ± 0.3			
*Taq*I-*Bam*HI B	2.20	−	0.0 ± 0.04			

[a] Transfections contained 0.5 μg of φX174 RFI DNA per ml, 0.25 or 0.05 μg of SV40 DNA I per ml, and 0.5 μg of the indicated SV40 linear DNA supplements per ml.

[b] Fragments are denoted by the restriction enzymes used to digest SV40 DNA I and a capital letter to indicate the particular fragment, where A represents the largest product and so forth (restriction maps are shown in Fig. 1).

[c] Calculated by using restriction maps of SV40.

[d] +, Presence of an SV40 origin of replication; −, absence.

[e] Δ*R* = (*R* in the presence of supplement − *R* in the absence of supplement)/(*R* in the absence of supplement). These values have been normalized to fragment concentration in terms of both weight and molarity. Values are the result of 2 to 10 independent transfections; error values are standard deviations.

DNA sequences (the *Kpn*I-*Xmn*I B fragment of the pSVK1H construct) restored efficiency (Table 4). These results indicate that the SV40 sequences required for expression of the recombination-enhancing activity of linear DNA are located between the *Hin*dIII site at nucleotide 5,171 and the *Sph*I site at nucleotide 128, a 200-base-pair (bp) region that contains the replication origin and the three T antigen-binding sites.

To pinpoint the essential sequence more precisely, *Kpn*I-*Taq*I B fragments from a number of viable deletion mutants (containing various deletions around the *Hin*dIII site at nucleotide position 5,171) were compared for their recombination-enhancing activities (Table 5). The *Kpn*I-*Taq*I B fragment of *dl*1135 (Δ = 5,082 to 5,144 [54]) was as active as the wild-type fragment. In contrast, *Kpn*I-*Taq*I B fragments derived from *dl*1019 (Δ = 5,170 to 5,199 [50]), *dl*1020 (Δ = 5,164 to 5,180 [50]), and *dl*1021 (Δ = 5,171 to 5,182 [50]) were only 30 to 50% as efficient as the wild-type fragment. These latter deletions affect A+T-rich sequences that form part of a 10-bp palindrome, TTTTGCAAAA. Most striking of all was the virtual total loss of recombination-enhancing activity exhibited by the *Kpn*I-*Taq*I B fragment of *dl*1083 (Δ = 5,177 to 5,197 [22]), in which the deletion affects both the A+T-rich stretch and two of the three pentamer contact sequences constituting T antigen-binding site I (20).

The *dl*1083 mutant replicates as well as does wild-type SV40 at 37°C (22). Supercoiled *dl*1083, in the absence of linear forms of SV40 DNA, recombined as well as did wild-type SV40 (Table 1). However, in agreement with the results obtained with the *dl*1083 *Kpn*I-*Taq*I B fragment (Table 4), the intact *Kpn*I-linearized form of *dl*1083 exhibited no recombination-enhancing activity when it was added to transfections containing wild-type SV40 DNA I as the helper (Table 1). Hence, the sequences deleted in *dl*1083 influence the production of recombinants from linear but not circular SV40 DNA (see below).

Recombination enhancement depends upon input ratio of circular to linear DNA. The enhancement of SV40/ϕX174 recombination, by the addition of SV40 *ori*$^+$ restriction fragments to the transfection mix, was more effective at an SV40 DNA I concentration of 0.05 compared with 0.25 μg/ml (Table 3). To examine this concentration effect in more detail, a dose-response experiment (Fig. 3A) was undertaken. Increasing the SV40 DNA I concentration in the presence of constant amounts of ϕX174 RFI DNA and SV40 *ori*$^+$ fragment DNA led to a decrease in the frequency of SV40/ϕX174 recombinant foci. Indeed, the recombination-enhancing activity of these SV40 DNA fragments virtually disappeared at SV40 DNA I concentrations above 1.0 μg/ml. Increasing the fragment DNA concentration overcame the inhibitory effects of higher SV40 DNA I levels (Fig. 3B).

The inhibitory effects of higher SV40 DNA I concentrations on the recombination enhancement phenomenon were not due to a decrease in the efficiency of the transfection process. Thus, when the concentration of ϕX174 RFI DNA was increased in the presence of constant amounts of SV40 DNA I and *ori*$^+$ fragment DNA, proportional increases in the recombination frequency were observed (Fig. 3C). We conclude, therefore, that the extent to which the SV40 *ori*$^+$ fragments enhance SV40/ϕX174 recombination is specifically related to the SV40 DNA I/SV40 fragment DNA ratio in the transfection mixture.

Recombination-enhancing linear SV40 DNA gives rise to a characteristic class of recombinant structures. To gain insight into the mechanism by which linear SV40 *ori*$^+$ DNA enhances recombination, we examined the structure of recombinants arising in cells transfected with SV40 DNA I, ϕX174 RFI DNA, and the *Kpn*I-*Xmn*I B fragment of pSVK1H (the recombination-enhancing DNA fragment containing the SV40 origin and flanking sequences, nucleotides 5171-5243/0-294, linked to 429 bp of plasmid DNA; Fig. 1 and 2). The structure of the recombinants was determined by using two hybridization probes: a plasmid DNA probe to monitor the participation of the recombination-enhancing linear DNA itself, and a ϕX174 DNA probe to identify sequences derived from ϕX174 RFI DNA. The evidence summarized below establishes (i) that the recombinant structures contain tandem (head-to-tail) repeats of sequences derived from the recombination-enhancing DNA fragment and that these repeats are linked to DNA derived from wild-type SV40 DNA I; (ii) that the head-to-tail repeats are not formed by homologous recombination; and (iii) that ϕX174 DNA sequences

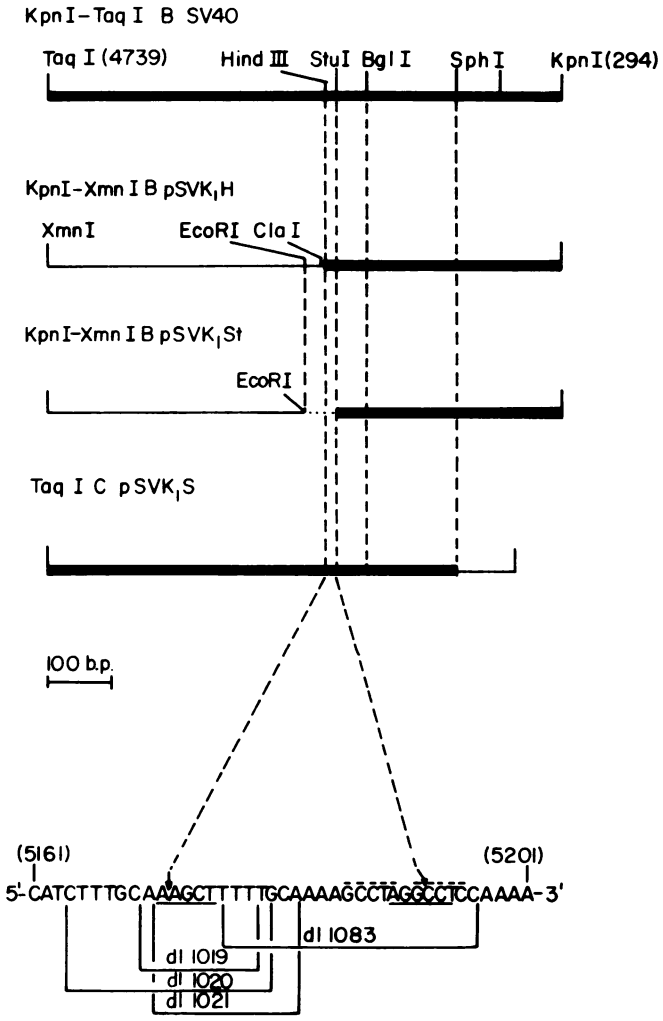

FIG. 2. SV40 origin-containing DNA fragments and SV40 deletion mutants. Heavy lines indicate SV40 sequences and thin lines indicate plasmid sequences. Dotted line (*Kpn*I-*Xmn*I B fragment of pSVK1St) indicates the sequences deleted in the construction of pSVK1St from pSVK1H. Vertical dashed lines indicate the position of restriction sites. The bottom portion shows the nucleotide sequence (early strand) deleted in the SV40 mutants *dl*1019, *dl*1020, *dl*1021, and *dl*1083 (22, 50). The *Hin*dIII and *Stu*I recognition sequences are underlined, and the nucleotides constituting part of the contact pentamers of T antigen-binding site I (20) are overlined (dashed).

TABLE 4. SV40 region required for enhanced recombinogenic potential of linear DNA[a]

SV40 DNA fragment[b]	Size (kilobases)	SV40 nucleotides	Proportional recombination increase[c]	
			ΔR (μg/ml)	ΔR (nM)
*Kpn*I-*Taq*I B	0.83	4,739–5,243/0–294	16.0 ± 3.2	8.3 ± 1.6
*Sph*I-*Taq*I B	0.63	4,739–5,243/0–128	7.4	3.0
*Kpn*I-*Hin*dIII F	0.37	5,171–5,243/0–294	4.7 ± 0.5	1.1 ± 0.1
*Taq*I C pSVK1S	0.72	4,739–5,243/0–128	18.0	8.4
*Kpn*I-*Xmn*I B pSVK1H	0.80	5,171–5,243/0–294	16.2 ± 1.6	8.4 ± 0.8

[a] Transfections contained 0.05 μg of SV40 DNA I, 0.5 μg of φX174 RFI DNA, and 0.5 μg of the indicated SV40 DNA fragment per ml.
[b] Maps are shown in Fig. 2. The *Taq*I C fragment of the pSVK1S construct and the *Kpn*I-*Xmn*I B fragment of the pSVK1H construct contain the SV40 DNA sequences linked to 90 and 400 bp, respectively, of plasmid DNA sequences.
[c] Proportional increase in recombination frequency per unit fragment was calculated as described in Table 3, footnote *e*. Values are the averages of 2 to 10 independent transfections. Standard deviations were calculated when three or more tansfections were performed.

are interspersed within the tandem repeats in a subset of the recombinant population.

(i) Tandem repeat structure. To analyze a variety of recombinant structures, a population of BSC-1 cells transfected as described above was incubated until CPEs were prominent; the virus-containing lysate was harvested and used to infect fresh BSC-1 cell cultures from which supercoiled DNA was prepared by Hirt extraction and by equilibrium centrifugation in CsCl gradients. The population of supercoiled DNA molecules (wild-type SV40 and recombinants) was then analyzed by restriction digestion and gel blot hybridization (Fig. 4). The supercoiled recombinant DNA molecules detected by the plasmid DNA probe (lane 1) were either equal to or smaller than wild-type SV40 DNA. After digestion with *Bgl*I (which cuts intact SV40 DNA or the *Kpn*I-*Xmn*I B fragment DNA once but which does not cut φX174 DNA), more than 90% of the plasmid DNA sequences were detected in small fragments (700 to 800 bp; lane 2). An identical pattern was obtained after *Cla*I digestion (lane 3). *Cla*I cuts the *Kpn*I-*Xmn*I B fragment once in the plasmid DNA sector (70 bp distal from the SV40 *Bgl*I site; Fig. 2) but does not cut authentic SV40 or φX174 RFI DNA. These results indicate, therefore, that the majority of recombinant structures contain head-to-tail repeats of DNA derived from the *Kpn*I-*Xmn*I B fragment DNA. That a transfected monomeric SV40 *ori*$^+$ fragment can give rise to molecules containing head-to-tail repeats has been reported previously by others (62).

The head-to-tail repeat structure was also observed in recombinant DNA populations derived from the progenies of single transfected BSC-1 cells. Single-cell yields were obtained by seeding small numbers of transfected cells (mixed with a large excess of untreated cells) in multiwell plates such that each well received on the average only 10 cells that had been exposed to the DNA transfection solution. A total of 60% of the wells exhibited SV40 CPEs, and

TABLE 5. Requirement for T antigen-binding site I and adjoining sequences for full recombinogenic potential of linear SV40 DNA[a]

*Kpn*I-*Taq*I B fragment from:	Deletion[b]	Proportional recombination increase (ΔR [μg/ml])[c]
Wild-type SV40		16.0 ± 3.2
*dl*1135	5,082–5,144	21.5
*dl*1019	5,170–5,179	5.0
*dl*1020	5,164–5,180	7.0
*dl*1021	5,171–5,182	5.0
*dl*1083	5,177–5,197	1.0

[a] Transfections contained 0.05 μg of SV40 DNA I, 0.5 μg of φX174 RFI DNA, and 0.5 μg of the *Kpn*I-*Taq*I B fragment derived from wild-type SV40 or the SV40 mutants per ml as indicated.
[b] Deletion mutants were a gift from D. Nathans. The nucleotides deleted are as described by Pipas et al. (54) and Nathans (50). Maps of *dl*1019, *dl*1020, *dl*1021, and *dl*1083 are shown in Fig. 2.
[c] Determined as described in Table 3, footnote *e*. Values are the result of 1 to 10 transfections (standard deviations were calculated when three or more transfections were performed).

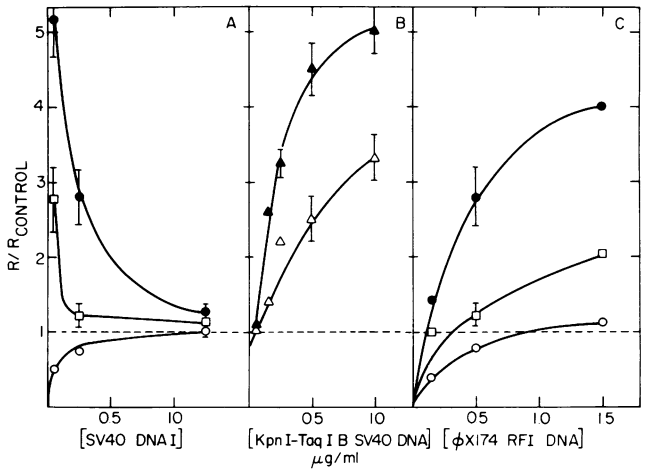

FIG. 3. Extent to which linear SV40 DNA enhances recombination depends on the ratio of linear to circular SV40 DNA in the transfection mixture. (A) Dependence on the concentration of SV40 DNA I. Cells were transfected with mixtures containing 0.5 μg of φX174 RFI DNA per ml, the indicated concentrations of SV40 DNA I, and no linear SV40 DNA supplement (○), 0.5 μg of SV40 *Kpn*I-*Bam*HI A fragment DNA per ml (●), or 0.5 μg of SV40 TaqI-BamI A fragment DNA per ml (□). *R* control is the recombination frequency obtained after transfection with 0.5 μg of φX174 RFI DNA and 1.25 μg of SV40 DNA I per ml (the highest SV40 DNA I concentration tested). The recombination values obtained from both supplemented and nonsupplemented transfections are presented relative to *R* control (*R*/*R* control). The recombination frequency measurements are derived from two to five independent transfection experiments; error values are standard deviations calculated when three or more experiments were performed. (B) Dependence on the concentration of SV40 *Kpn*I-*Taq*I B fragment DNA. Transfection mixtures contained 0.5 μg of φX174 RFI DNA per ml, 0.05 (▲) or 0.25 (△) μg of SV40 DNA I per ml, and the indicated concentrations of fragment DNA. Recombination values are derived from one to five independent transfections and are expressed as described for A above. (C) Influence of φX174 RFI DNA concentration. Transfection mixtures contained 0.25 μg of SV40 DNA I per ml, 0.5 μg of SV40 *Kpn*I-*Bam*HI A fragment DNA per ml (●), 0.5 μg of SV40 *Taq*I-*Bam*HI A fragment DNA per ml (□), or no fragment DNA (○) and the indicated concentrations of φX174 RFI DNA. Recombination values (presented as described for A above) are derived from one to five independent experiments.

of these, 10% produced recombinant virus genomes containing sequences derived from *Kpn*I-*Xmn*I B fragment DNA as detected by dot-blot hybridization of the well lysates with a plasmid DNA probe. The recombinants were then amplified and purified as were those derived from the mass lysates described above. The analyses of 2 of 12 independent isolates (each derived from a different recombinant-producing cell) are shown in Fig. 5. Like those derived from the mass lysates, the recombinants derived from single-cell yields were of various sizes (lanes 1 and 5) and contained tandem repeats of sequences from *Kpn*I-*Xmn*I B fragment DNA as judged by the digestion patterns with *Cla*I (lanes 4 and 8) and *Bgl*I (lanes 3 and 7). The recombinant structures were sensitive to *Bcl*I digestion (lanes 2 and 6). Since *Bcl*I cuts authentic SV40 DNA once (Fig. 1) and does not cut either the *Kpn*I-*Xmn*I B fragment DNA or authentic φX174 RFI DNA, the tandem repeats must be linked to sequences derived from wild-type SV40 DNA. The majority of the recombinants present in the mass lysates were also found to be sensitive to *Bcl*I digestion (data not shown).

(ii) Tandem repeats are not formed by homologous recombination. To determine whether the tandem repeats are formed by homologous recombination, we constructed a derivative (p306) of pSVK1H in which the *Cla*I site of the plasmid DNA section 70 bp distal from the SV40 *Bgl*I site was deleted (see above). BSC-1 cells were then transfected with SV40 DNA I, φX174 RFI DNA, and either a 1:1 mixture of p306 and pSVK1H *Kpn*I-*Xmn*I B fragments or (as a control) the p306 *Kpn*I-*Xmn*I B fragment alone. Mass lysate DNA populations were prepared and subjected to restriction enzyme and blot hybridization analyses as described above. If the tandem repeats form by homologous recombination, individual recombinant structures arising from the transfection with the mixture of *Kpn*I-*Xmn*I B fragments (mixture of *Cla*I$^+$ and *Cla*I$^-$ fragments) should contain a mixture of repeat units (both *Cla*I$^+$ and *Cla*I$^-$); consequently, *Cla*I digestion of this recombinant DNA population should generate a series of products, which hybridize to the plasmid DNA probe, ranging in size from ca. 800 bp to that of full-length SV40 DNA. If, however, the tandem repeats are formed by a replication-related mechanism, individual recombinant structures would be expected to contain repeat units of the same type (either *Cla*I$^+$ or *Cla*I$^-$), and digestion of the recombinant DNA should yield only two classes of products containing plasmid DNA sequences: fragments of about 800 bp (structures containing repeats that are all *Cla*I$^+$) and molecules insensitive to *Cla*I digestion (structures containing repeats uniformly *Cla*I$^-$). The results (Fig. 4A, lanes 4 to 9) indicated that most of the repeat units are not generated by homologous recombination. After *Bgl*I digestion of the control group (recombinant structures arising from transfections containing only the *Cla*I$^-$ p306 *Kpn*I-*Xmn*I B fragment), the expected pattern of small products containing plasmid DNA sequences was observed (lane 5), and as expected, all the recombinant DNA molecules were insensitive to *Cla*I digestion (lane 6). The recombinant

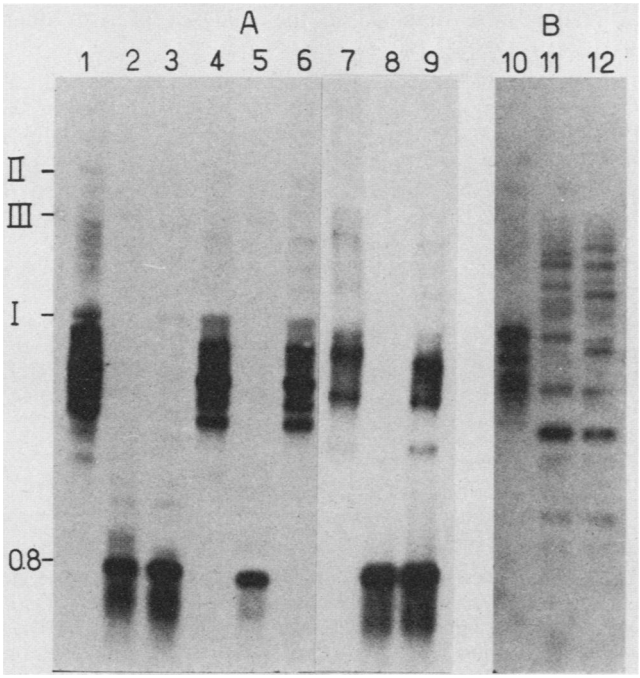

FIG. 4. Blot hybridization analyses of recombinant genomes isolated from a mass lysate of transfected cells. (A) Recombinant genomes were isolated (see text) from cells transfected with 0.25 μg of SV40 DNA I per ml, 1.0 μg of φX174 RFI DNA per ml, and 1.0 μg of *Kpn*I-*Xmn*I B fragment DNA from pSVK1H (*Cla*I$^+$) (lanes 1 to 3), 1.0 μg of *Kpn*I-*Xmn*I B fragment DNA from p306 (*Cla*I$^-$) (lanes 4 to 6), or 1.0 μg of a 1:1 mixture of the p306 (*Cla*I$^-$) and pSVK1H (*Cla*I$^+$) fragments (lanes 7 to 9) per ml. Lanes 1, 4, and 7, uncut; lanes 2, 5, and 8, *Bgl*I digest; lanes 3, 6, and 9, *Cla*I digest. Blot was hybridized with pML2K [^{32}P]DNA and autoradiographed (−70°C with intensifying screen) for 24 h. Each slot contained 1 μg of DNA. I, II, and III indicate the supercoiled, relaxed, and linear forms of SV40 DNA, respectively; other size markers are expressed in kilobase pairs. (B) Duplicate blot of the above hybridized with φX174 [^{32}P]DNA. Lanes 10 to 12 are the same as lanes 1 to 3 in A. Autoradiographic exposure was for 1.5 weeks at −70°C with an intensifying screen.

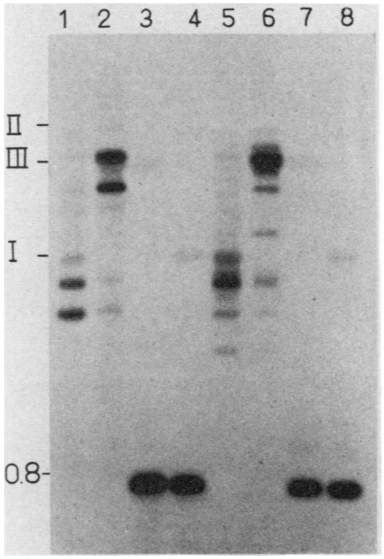

FIG. 5. Blot hybridization of recombinant genomes isolated from transfected cells partitioned in microwells. Cells were transfected with 0.25 μg of SV40 DNA I and 1.0 μg of the *Kpn*I-*Xmn*I B fragment DNA from pSVK1H per ml. The transfected cells were partitioned in microwells, and the recombinant progeny were isolated as described in the text. Lanes: 1 to 4, isolate I; 5 to 8, isolate II; 1 and 5, uncut; 2 and 6, *Bcl*I digest; 3 and 7, *Bgl*I digest; 4 and 8, *Cla*I digest. Each slot contained 1 μg of DNA. Hybridization was with pML2K [^{32}P]DNA; autoradiographic exposure was for 24 h at −70°C with an intensifying screen. Size markers are as described in the legend to Fig. 4.

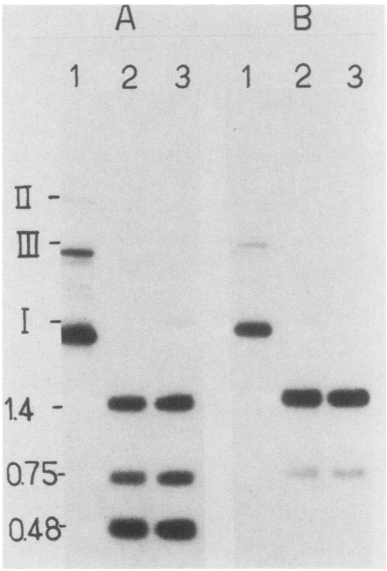

FIG. 6. Blot hybridization of recombinant genomes isolated from infectious center plaques. Cells were transfected with 0.25 μg of SV40 DNA I, 1.0 μg of φX174 RFI DNA, and 1.0 μg of the *Kpn*I-*Xmn*I B fragment DNA from pSVK1H per ml. Recombinants were isolated from the agar overlay as described in the text. (A) Blot hybridized with pML2K [^{32}P]DNA. Lanes: 1, uncut; 2, *Bgl*I digest; 3, *Cla*I digest. Exposure was for 24 h at −70°C with an intensifying screen. Size markers are as described for Fig. 4. (B) Duplicate blot hybridized with φX174 [^{32}P]DNA; lanes 1 to 3 are as in A above. Autoradiographic exposure was for 12 h at −70°C with an intensifying screen. Each slot contained 1 μg of DNA.

structures which arose in cells transfected with the 1:1 mixture of *Cla*I$^-$ p306 *Kpn*I-*Xmn*I B fragment and *Cla*I$^+$ pSVK1H *Kpn*I-*Xmn*I B fragment DNAs displayed the expected pattern after *Bgl*I digestion (lane 8); after *Cla*I digestion, however, most of the plasmid sequences were detected in either small fragments (comigrating with those generated by *Bgl*I digestion) or in DNA structures insensitive to *Cla*I digestion (lane 9). Thus, in the majority of recombinant structures, the sequences derived from p306 *Kpn*I-*Xmn*I DNA and those derived from pSVK1H *Kpn*I-*Xmn*I DNA were present in separate molecules. These results were confirmed in a repeat experiment in which recombinant structures, amplified from single-cell yields, were examined (data not shown). We conclude, therefore, that the tandem repeats of sequences derived from the recombination-enhancing linear DNA are not formed by homologous recombination. As discussed below, we propose a replication-related process, such as the rolling circle mode of DNA amplification (29), as the most likely mechanism for the formation of polymeric head-to-tail repeated DNA.

(iii) φX174 DNA sequences are interspersed within tandem repeats in a subset of the recombinant population. The technique used to transfer the recombinant DNA restriction products from the agarose gel to nitrocellulose paper provided duplicate blots from each gel (64). One of the duplicate blots was hybridized with the plasmid DNA probe as described above; the twin blot was hybridized with a φX174 DNA probe to identify the presence and position of inserts derived from φX174 RFI DNA. From the analysis of the recombinant DNA structures in mass lysates (Fig. 4B), it is evident that most of the structures containing φX174 DNA sequences were sensitive to *Cla*I digestion (lane 12) and *Bgl*I digestion (lane 11). Since there are no *Cla*I or *Bgl*I restriction sites in authentic φX174 RFI DNA, and no *Cla*I site in authentic SV40 DNA, it follows that the φX174 DNA inserts must be present in molecules that also contain sequences derived from the pSVK1H *Kpn*I-*Xmn*I B fragment DNA. Furthermore, the similarity of the *Bgl*I and *Cla*I digestion patterns suggests that the φX174 DNA sequences are contained within the tandem repeats.

To establish at the level of individual structures (rather than at the level of mass lysates) the presence of φX174 DNA within the tandem repeats, we next examined the progeny derived from single transfected cells. φX174 DNA-containing populations were isolated by sampling the agar overlay at points corresponding to recombinant plaques identified by infectious center in situ plaque hybridization with φX174 [^{32}P]DNA (77). The analysis of one of three isolates derived by this procedure is shown in Fig. 6. *Bgl*I digestion generated three small products, two of which hybridized to both plasmid DNA and φX174 DNA probes (lanes A2 and B2). Furthermore, *Cla*I digestion generated identically sized products with the same hybridization pattern (lanes A3 and B3). Therefore, φX174 DNA sequences are present within two of the three tandemly arranged segments derived from *Kpn*I-*Xmn*I B fragment DNA. Similar results were obtained in the analyses of two other

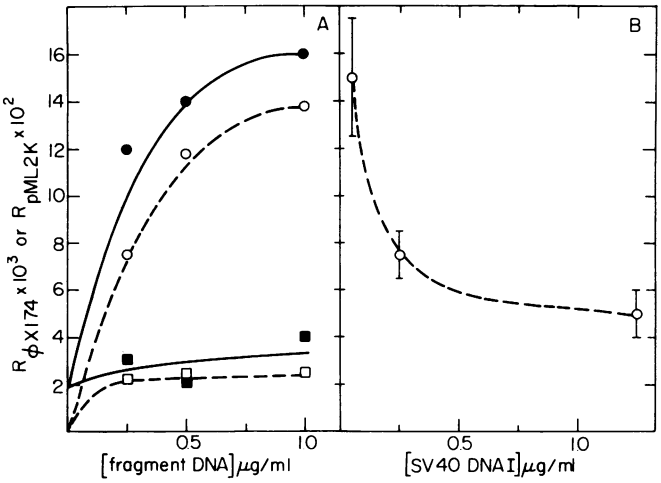

FIG. 7. φX174 recombinants are a subset of recombinants containing tandem repeats. (A) Recombination-enhancing activity of DNA fragments containing (*Kpn*I-*Xmn*I B fragment of pSVK1H) or lacking (*Kpn*I-*Xmn*I B fragment of pSVK1St) T antigen-binding site I. Cells were transfected with 0.05 μg of SV40 DNA I per ml, 0.5 μg of φX174 RFI DNA, and the indicated concentrations of the *Kpn*I-*Xmn*I B fragment DNA of either pSVK1H or pSVK1St (see the text). $R_{\phi X174}$ is the frequency of recombinant plaques detected with φX174 [^{32}P]DNA normalized to the frequency of virion-producing cells; R_{pML2K} is the frequency of recombinant plaques detected with pML2K [^{32}P]DNA normalized as above. Note 10-fold difference in the $R_{\phi X174}$ and R_{pML2K} scales. $R_{\phi X174}$ was determined in the presence of the *Kpn*I-*Xmn*I B fragment DNA from pSVK1H (●) and from pSVK1St (■). Similarly, R_{pML2K} was determined in the presence of the fragment from pSVK1H (○) and from pSVK1ST (□). (B) Recombination between pSVK1H *Kpn*I-*Xmn*I B fragment DNA and SV40 DNA is inhibited at higher SV40 DNA I concentrations. Cells were transfected with 0.5 μg of pSVK1H *Kpn*I-*Xmn*I B fragment DNA and the indicated concentrations of SV40 DNA I. R_{pML2K} was determined as in A above. Recombination values are derived from three independent experiments; error values are standard deviations.

independent agar overlay recombinant isolates (data not shown).

The φX174 [^{32}P]DNA hybridization data (Fig. 4B and 6B) do not address the question of whether φX174 DNA sequences are present in all the recombinant structures containing tandemly repeated SV40 *ori*$^+$/plasmid DNA. To clarify this issue, cells were transfected with SV40 DNA I, φX174 RFI DNA, and *Kpn*I-*Xmn*I B fragment DNA as described above; then the frequency of recombinant-producing cells was determined by in situ plaque hybridization with plasmid pML2K [^{32}P]DNA and, separately, with φX174 [^{32}P]DNA. Figure 7A shows that the number of recombinant foci detected with the plasmid DNA probe was 10-fold higher than the number detected with φX174 [^{32}P]DNA, indicating that only a minor fraction of the recombinants contain φX174 DNA. The question then arose of whether the generation of the recombinants which lack φX174 DNA depends upon the same requirements described for the φX174 DNA-containing subpopulation. To resolve this question, we isolated the *Kpn*I-*Xmn*I B fragment of pSVK1St which, in contrast to the same fragment from pSVK1H, contains a deletion affecting the A+T-rich sequences and adjoining T antigen-binding pentamers (Δ = 5,172 to 5,197) and is thus analogous to the *Kpn*I-*Taq*I B fragment of the *dl*1083 mutant (Fig. 2). Figure 7A shows that the recombination activity of the deleted pSVK1St fragment was considerably lower than that of the wild-type pSVK1H fragment with respect to both recombinant populations: those lacking φX174 DNA as well as those with φX174 DNA. Furthermore, the generation of the larger fraction of recombinants detected with the plasmid DNA probe was influenced by the ratio of circular SV40 DNA to linear fragment DNA in the transfection reaction (Fig. 7B) in a manner essentially identical to that observed with the φX174 DNA-containing recombinants (Fig. 3A). Hence, the φX174 DNA-containing recombinants constitute a subset of a larger recombinant population containing head-to-tail repeats derived from the SV40 *ori*$^+$/plasmid linear DNA fragment.

DISCUSSION

We have shown that the addition of linear SV40 *ori*$^+$ DNA to transfection mixtures containing circular SV40 and φX174 RFI DNAs enhances the frequency of SV40/φX174 recombination. The key feature of the recombinant structures arising from such transfections is the presence of tandem head-to-tail repeats derived specifically from the added SV40 *ori*$^+$ linear DNA. To explain the findings described in this report, we propose that linear forms of SV40 DNA containing an active origin of replication have a tendency to enter an aberrant nontheta replication pathway (rolling circle replication) that is associated with a high frequency of recombination. The arguments for this hypothesis are discussed below.

Input linear DNA is not by itself the recombinogenic species. We considered initially the possibility that linear SV40 DNA is more recombinogenic than circular forms because of an increased availability of free ends for end-to-end ligation. Indeed, several reports have indicated that free ends of DNA molecules, introduced into animal cells, undergo ligation and are involved in some recombination pathways (14, 27, 38, 56, 74). Our results, however, imply that the availability of free ends is not, by itself, a limiting factor. First, only linear molecules containing the SV40 replication origin were more recombinogenic than circular molecules. Furthermore, linearization of the cosubstrate φX174 RFI DNA did not alter the frequency of recombinant-producing cells. Second, the recombinogenic potentials of linear SV40 *ori*$^+$ DNA fragments ranging in size from 700 to 3,000 bp were equivalent on a weight basis; on a molar basis, their recombinogenic potential decreased proportionally with length. Because the smaller DNA fragments contain more free ends per mass unit, this latter observation implies that the total concentration of free DNA ends in the transfection mixture has little or no influence on the frequency of recombinant-producing cells. These considerations suggested that linearization of SV40 DNA enhances recombination by other mechanisms.

Requirement for T antigen-binding site I. The sequences required for linear SV40 DNA to display a high recombinogenic potential are contained within a 200-bp region (nucleotides 5,171 to 5,243/0 to 128) which comprises the origin of replication (3, 22, 49), the auxilary origin sequences (3), and T antigen-binding sites I, II, and III (20, 61, 67, 68). Deletion of nucleotides 5,171 to 5,197 (*dl*1083 mutant) or nucleotides 5,171 to 5,191 (pSVK1St construct), which eliminates T antigen-binding site I, was found to substantially reduce the recombinogenic potential of linear SV40 DNA. Furthermore, since mutant *dl*1083 DNA in the circular configuration replicates (22) and recombines as well as does the wild-type genome (Table 1), the deletion of T antigen-binding site I affects only a recombination pathway exploited by linear DNA. The function of T antigen-binding site I (the strongest binding site) is known to be involved in the autoregulation of T antigen synthesis (23, 33, 60). Mutants like *dl*1083 overproduce T antigen (23) because of the absence of the autoregulatory signal. We assume that binding of T antigen to the origin region, which is required for normal SV40 DNA replication (63, 66), is also required for the aberrant replication process which generates tandem repeats. Hence, entry of linear DNA into replication will depend upon its ability to compete with circular SV40 DNA for the available T antigen molecules.

Given these considerations, the dependency of the recombination frequency on the ratio of circular DNA to linear DNA in the transfection mixture (Fig. 3A and 7B) can be explained on the basis that with increasing concentrations of input circular SV40 DNA, the linear DNA can no longer compete effectively for the available amount of parental T antigen. Linear DNA which lacks T antigen-binding site I is at a further disadvantage since it lacks the strongest of the three binding sites. The poor recombination-enhancing properties of linear forms of *dl*1083 DNA (or those derived from the pSVK1St construct) can be ascribed to an inability to compete effectively with circular DNA for the available pool of T antigen molecules, which in turn decreases the probability that the linear DNA will give rise to recombinogenic replication intermediates.

When supercoiled *dl*1083 DNA was used instead of supercoiled wild-type SV40 DNA as the helper genome in transfections containing linear SV40 DNA and φX174 RFI DNA, higher SV40/φX174 recombination values were observed (Table 1). In line with the argument developed above, we assume that because circular *dl*1083 DNA overproduces T antigen (and binds less itself), it provides the linear DNA with a more abundant pool of T antigen molecules. Significantly, the recombination-enhancing ability of *Kpn*I-linearized *dl*1083 DNA was partly rescued by using circular *dl*1083 rather than wild-type SV40 DNA as the helper genome (Table 1). Therefore, the data available at present suggest a rather indirect role for T antigen-binding site I, i.e., indirect in the sense that it influences the ability of linear DNA to compete with circular DNA for the level of T antigen

required for entry into the replication pathway that generates recombinogenic intermediates.

Recombinant structures. SV40/φX174 recombinant genomes arising from the cotransfection of circular DNAs in the absence of linear DNA generally possess noncomplex structures characterized by single inserts of φX174 DNA within SV40 DNA which outside the region of the substitution is indistinguishable from the wild-type SV40 genome (77; D. Dorsett and A. Roman, unpublished data). In contrast, the SV40/φX174 recombinant structures arising from the cotransfection of circular DNA molecules supplemented with SV40 *ori*$^+$ fragments as described herein exhibited a complex array of head-to-tail repeats derived from the recombination-enhancing *ori*$^+$ DNA fragments (Fig. 4, lanes 1 to 3). Using appropriate restriction markers, we showed that the tandem repeats are not formed by homologous recombination (Fig. 4, lanes 7 to 9) and that the tandemly repeated structure is linked to wild-type SV40 DNA sequences (Fig. 5, lanes 2 and 6). A subset of the recombinant population contained, additionally, φX174 DNA sequences interspersed within the tandem repeats (Fig. 6). Preliminary data (not shown) indicate that all of the φX174 DNA sequences in a given recombinant structure are derived from the same region of the authentic φX174 genome. In considering how such φX174 DNA-containing recombinant structures might arise, two recombination events have to be accounted for: the event responsible for the presence of φX174 DNA in some of the tandem repeats, and the recombination between the tandemly repeated DNA and wild-type SV40 DNA.

The rolling circle replication process, which we propose is responsible for the formation of the tandem repeats, gives rise to an intermediate structure comprising a circular domain (the rolling template) and a tail of newly synthesized tandemly repeated DNA (29). The circular portion of the intermediate is not covalently closed. The simplest explanation for the observed SV40/φX174 recombinant structures assumes recombination between φX174 RFI DNA and the circular template portion of the rolling circle intermediate such that continuation of the replication process generates tandem repeats containing both φX174 DNA and SV40 *ori*$^+$ DNA. Consistent with this proposal are our preliminary data suggesting that the interspersed φX174 DNA sequences in a given structure are of the same type. Alternative explanations assuming recombination with φX174 RFI DNA after the formation of the tandem repeats appear less likely. For example, recombination between φX174 DNA and the tail of the rolling circle intermediate would require that the generation of a single interspersed recombinant structure be the consequence of multiple recombination events with the same region of the φX174 genome. Recombination between φX174 DNA and the SV40 *ori*$^+$ DNA fragment before rolling circle replication, although possible, would not be consistent with our data that the availability of free ends has little or no influence on the frequency of recombination.

The generation of the observed recombinant structures must also involve a recombination step which links the tandemly repeated *ori*$^+$ DNA to SV40 DNA sequences not present in the *ori*$^+$ recombination-enhancing fragment DNA. This step occurs at a relatively high frequency since the number of tandemly repeated recombinant structures lacking φX174 DNA is 10-fold greater than the number containing interspersed φX174 DNA (Fig. 7A). The higher frequency may be due to a greater efficiency of a homologous recombination process or to the fact that the tandemly repeated DNA, in contrast to φX174 RFI DNA, is a replicating recombination substrate. We have no data to date which directly bear upon this question.

Rolling circle DNA replication and recombination in the papovavirus life cycle. Although in the normal lytic cycle SV40 and polyoma virus DNA replications proceed bidirectionally from unique origins around the circular DNA template (yielding theta-type intermediates [69]), a fraction of the DNA synthesized late in the replication cycle has been observed to be tandemly repeated (28, 58). Electron microscopic visualization of this DNA has revealed structures reminiscent of rolling circle intermediates (6). Such tandemly repeated structures continue to be synthesized when the more usual theta-type replication is suppressed by an inhibitor of reinitiation (6). Recently, we have obtained evidence that linear SV40 *ori*$^+$ fragments exhibit a propensity for rolling circle replication (I. Deichaite, D. Dorsett, and E. Winocour, unpublished data). We have observed that transfection of SV40-transformed COS monkey cells which produce T antigen constitutively (30) with an SV40 *ori*$^+$ DNA fragment (the *Kpn*I-*Xmn*I B fragment of pSVK1H [Fig. 2], which enhances SV40/φX174 recombination) leads to the synthesis of variously sized DNA molecules made up of head-to-tail repeats of the input DNA. Electron microscopy of this DNA revealed the presence of circles with tails characteristic of rolling circle intermediates. Circularization of the *Kpn*I-*Xmn*I B fragment DNA by in vitro ligation before transfection led to higher levels of replication, primarily by a theta mode generating covalently closed monomeric circular DNA molecules. The question of why the linear forms of SV40 DNA exhibit a propensity for rolling circle replication is currently under investigation.

The rolling circle replication pathway that occurs in papovavirus lytic infection may also promote the formation of host DNA-substituted (26, 32, 36, 45, 52, 55, 72) and other rearranged viral genomes (18, 26, 36, 41, 42) characterized by head-to-tail repeats of the origin region. Because this mode of replication usually occurs late in the lytic cycle (28, 58), its effect on recombination between transfected circular SV40 and φX174 RFI DNAs may be minimal because degradation of the nonreplicating φX174 RFI DNA takes place before significant amounts of rolling circle intermediates have formed. Because linear SV40 DNA exhibits a preference for rolling circle replication, the addition of linear *ori*$^+$ SV40 DNA to the transfection will result in the postulated recombinogenic intermediates appearing early in the lytic cycle at a time when the cosubstrate φX174 RFI DNA is still available for recombination.

SV40 rolling circle replication has also been noted in nonpermissive mouse cells (15). This may explain the frequent occurrence of head-to-tail repeats in the integrated viral genomes (8, 13, 37, 48). Head-to-tail repeats in integrated polyoma virus genomes have been reported as well (2, 5, 16, 19). Cells transformed by polyoma virus DNA capable of producing active T antigen contained inserts with tandem duplication, whereas those transformed by DNA incapable of producing active T antigen contained nonduplicated viral inserts (19). Similar results have been reported in transformation studies with polyoma virus DNA bearing deletions in the origin of replication (16). These observations have led others to suggest that rolling circle replication of the papovavirus DNA may generate intermediates with a higher probability of integrating into the host genome (5, 7, 13, 16, 19). Interestingly, it has also been reported that linear polyoma virus DNA is more tumorigenic than circular DNA when directly injected into animals of a susceptible species (9, 35).

The infectious center in situ plaque hybridization assay (24) which we used to compare the recombination pathways pursued by circular and linear forms of SV40 DNA detects only those recombinant structures which retain an active origin of replication and which are of a size commensurate with SV40 encapsidation restrictions. Therefore we cannot exclude the possibility that linear SV40 DNA may, in addition, recombine by other mechanisms (including end-to-end ligation), generating products that would not be detected under our assay conditions. Nevertheless, the parallels between the observations on integrative recombination noted above and the extrachromosomal recombinations reported in the present study are striking and suggest that the postulated recombinogenic intermediates of rolling circle replication are responsible for a variety of recombination events in animal cells infected with papovaviruses.

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LITERATURE CITED

1. **Bandopadhyay, P. K., S. Watanabe, and H. M. Temin.** 1984. Recombination of transfected DNAs in vertebrate cells in culture. Proc. Natl. Acad. Sci. U.S.A. **81**:3476–3480.
2. **Basilico, C., S. Gattoni, D. Zouzia, and G. Della Valle.** 1979. Loss of integrated viral DNA sequences in polyoma transformed cells is associated with an active viral A function. Cell **17**:645–659.
3. **Bergsma, D. J., D. M. Olive, S. W. Hartzell, and K. N. Subramanian.** 1982. Territorial limits and functional anatomy of the simian virus 40 replication origin. Proc. Natl. Acad. Sci. U.S.A. **79**:381–385.
4. **Best, A. N., D. P. Alison, and G. D. Novelli.** 1981. Purification of supercoiled DNA of plasmid ColE1 by RPC-5 chromatography. Anal. Biochem. **114**:235–243.
5. **Birg, F., R. Dulbecco, M. Fried, and R. Kamen.** 1979. State and organization of polyoma virus DNA sequences in transformed rat cell lines. J. Virol. **29**:633–648.
6. **Björsell, G.** 1978. Effects of 2′-deoxy-2′-azido-cytidine on polyomavirus replication: evidence for rolling circle-type mechanism. J. Virol. **26**:136–142.
7. **Botchan, M., J. Stringer, T. Mitchison, and J. Sambrook.** 1980. Integration and excision of SV40 DNA from the chromosome of a transformed cell. Cell **20**:143–152.
8. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in the DNA of transformed cells. Cell **9**:269–287.
9. **Bouchard, L., C. Gelinas, C. Asselin, and M. Bastin.** 1984. Tumorigenic activity of polyoma virus and SV40 DNAs in newborn rodents. Virology **135**:53–64.
10. **Buchman, A. R., L. Burnett, and P. Berg.** 1980. The SV40 nucleotide sequence, p. 799–829. *In* J. Tooze (ed.), The molecular biology of tumor viruses, part 2, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Bullock, P., W. Forrester, and M. Botchan.** 1984. DNA sequence studies of simian virus 40 chromosomal excision and integration in rat cells. J. Mol. Biol. **174**:55–84.
12. **Calos, M. P., J. S. Lebkowski, and M. R. Botchan.** 1983. High mutation frequency in DNA transfected into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **80**:3015–3019.
13. **Campo, M. S., I. R. Cameron, and M. E. Rogers.** 1978. Tandem integration of complete and defective SV40 genomes in mouse-human somatic cell hybrids. Cell **15**:1411–1426.
14. **Carbon, J., T. Shenk, and P. Berg.** 1975. A biochemical procedure for the production of small deletions in simian virus 40 DNA. Proc. Natl. Acad. Sci. U.S.A. **72**:1392–1396.
15. **Chia, W., and P. W. J. Rigby.** 1981. Fate of viral DNA in nonpermissive cells infected with simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **78**:6638–6642.
16. **Dailey, L., S. Pellegrini, and C. Basilico.** 1984. Deletion of the origin of replication impairs the ability of polyoma virus DNA to transform cells and to form tandem repeats. J. Virol. **49**:984–987.
17. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 116–117. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Davoli, D., D. Ganem, A. L. Nussbaum, G. C. Fareed, P. M. Howley, G. Khoury, and M. A. Martin.** 1977. Genome structures of reiteration mutants of simian virus 40. Virology **77**:110–124.
19. **Della Valle, G., R. G. Fenton, and C. Basilico.** 1981. Polyoma large T antigen regulates the integration of viral DNA sequences into the genome of transformed cells. Cell **23**:347–355.
20. **DeLucia, A. L., B. A. Lewton, R. Tjian, and P. Tegtmeyer.** 1983. Topography of simian virus 40 A protein-DNA complexes: arrangement of pentanucleotide interaction sites at the origin of replication. J. Virol. **46**:143–150.
21. **Denhardt, D.** 1966. A membrane filter technique for detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
22. **DiMaio, D., and D. Nathans.** 1980. Cold sensitive regulatory mutants of simian virus 40. J. Mol. Biol. **140**:129–142.
23. **DiMaio, D., and D. Nathans.** 1982. Regulatory mutants of simian virus 40, effect of mutations at a T antigen binding site on DNA replication and expression of viral genes. J. Mol. Biol. **156**:531–548.
24. **Dorsett, D. L., I. Keshet, and E. Winocour.** 1983. Quantitation of a simian virus 40 nonhomologous recombination pathway. J. Virol. **48**:218–228.
25. **Eisenberg, S., B. Harbers, C. Hours, and D. T. Denhardt.** 1975. The mechanism of replication of ϕX174 DNA. XII. Nonrandom locations of gaps in nascent ϕX174 RFII DNA. J. Mol. Biol. **99**:107–123.
26. **Fareed, G. C., and D. Davoli.** 1977. Molecular biology of papovaviruses. Annu. Rev. Biochem. **46**:471–522.
27. **Folger, K. R., E. A. Wong, G. Wahl, and M. R. Capecchi.** 1982. Patterns of integration of DNA microinjected into cultured mammalian cells: evidence for homologous recombination between injected plasmid DNA molecules. Mol. Cell. Biol. **2**:1372–1387.
28. **Ganz, P. R., and R. Sheinen.** 1983. Synthesis of multimeric polyoma virus DNA in mouse L-cells: role of the tsA159 gene product. J. Virol. **46**:768–777.
29. **Gilbert, W., and D. Dressler.** 1968. DNA replication: the rolling circle. Cold Spring Harbor Symp. Quant. Biol. **33**:473–484.
30. **Gluzman, Y.** 1981. SV40-transformed simian cells support the replication of early SV40 mutants. Cell **23**:175–182.
31. **Grossman, Z., E. Winocour, and K. I. Berns.** 1984. Recombination between simian virus 40 and adeno-associated virus: virus coinfection compared to DNA cotransfection. Virology **134**:125–137.
32. **Gutai, M. W., and D. Nathans.** 1978. Cellular DNA sequences and sequences at the recombinant joints of substituted variants. J. Mol. Biol. **126**:275–288.
33. **Hansen, U., D. G. Tenen, D. M. Livingston, and P. A. Sharp.** 1981. T antigen repression of SV40 early transcription from two promoters. Cell **27**:603–612.
34. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
35. **Israel, M. A., D. T. Simmons, S. L. Hourihan, W. P. Rowe, and M. A. Martin.** 1979. Interrupting the early region of polyomavirus DNA enhances tumorigenicity. Proc. Natl. Acad. Sci. U.S.A. **76**:3713–3716.
36. **Kelly, T. J., Jr., and D. Nathans.** 1977. The genome of simian virus 40. Adv. Virus Res. **21**:85–173.
37. **Ketner, G., and T. Kelly, Jr.** 1976. Integrated simian virus 40 sequences in transformed cell DNA: analysis using restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. **73**:1102–1106.

38. **Kopchick, J. J., and D. W. Stacey.** 1984. Differences in intracellular DNA ligation after microinjection and transfection. Mol. Cell. Biol. **4**:240–246.
39. **Lin, F.-L., K. Sperle, and N. Sternberg.** 1984. Models for homologous recombination during transfer of DNA into mouse cells: role for DNA ends in the recombination process. Mol. Cell. Biol. **4**:1020–1034.
40. **Little, J. W., and D. W. Mount.** 1982. The SOS regulatory system of *Escherichia coli*. Cell **29**:11–22.
41. **Lund, E., M. Fried, and B. E. Griffin.** 1977. Polyoma virus defective DNAs. I. Physical map of a related set of defective molecules (D76, D91, D92). J. Mol. Biol. **117**:473–495.
42. **Lund, E., B. E. Griffin, and M. Fried.** 1977. Polyoma virus defective DNAs. II. Physical map of a molecule with rearranged and reiterated sequences (D74). J. Mol. Biol. **117**:497–513.
43. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 sequences. Nature (London) **293**:79–81.
44. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning (a laboratory manual), p. 113–114. Cold Spring Harbor Laboratory, Cold Spring Habor, N.Y.
45. **McCutchan, T., M. Singer, and M. Rosenberg.** 1979. Structure of SV40 recombinants that contain both host and viral DNA sequences. II. The structure of variant 1103 and its comparison to variant CVP8/1/P2 (*Eco*RI res.). J. Biol. Chem. **254**:3592–3597.
46. **Miller, C. K., and H. M. Temin.** 1983. High efficiency ligation and recombination of DNA fragments by vertebrate cells. Science **200**:606–609.
47. **Milman, G., and M. Herzberg.** 1981. Efficient DNA transfection and rapid assay for thymidine kinase activity and viral antigenic determinants. Somatic Cell Genet. **7**:161–170.
48. **Mougneau, E., F. Birg, M. Rassoulzadegan, and F. Cuzin.** 1980. Integration sites and sequence arrangement of SV40 DNA in a homogenous series of transformed rat fibroblast lines. Cell **22**:917–927.
49. **Myers, R. M., and R. Tjian.** 1980. Construction and analysis of simian virus 40 origins defective in tumor antigen binding and DNA replication. Proc. Natl. Acad. Sci. U.S.A. **77**:6491–6495.
50. **Nathans, D.** 1981. Mutational analysis of regulatory elements of simian virus 40. Juselius Found. Symp. **8**:57–65.
51. **Oren, M., E. L. Kuff, and E. Winocour.** 1976. The presence of common host sequences in different populations of substituted SV40 DNA. Virology **73**:419–430.
52. **Oren, M., S. Lavi, and E. Winocour.** 1978. The structure of a cloned substituted SV40 genome. Virology **85**:404–421.
53. **Perucho, M., D. Hanahan, and M. Wigler.** 1980. Genetic and physical linkage of exogenous sequences in transformed cells. Cell **22**:309–317.
54. **Pipas, J. M., K. W. C. Peden, and D. Nathans.** 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T-antigen gene. Mol. Cell. Biol. **3**:203–213.
55. **Rao, G. R. K., and M. F. Singer.** 1977. Studies on defective variant of simian virus 40 that is substituted with DNA sequences derived from monkey. II. Structures of DNA. J. Biol. Chem. **252**:5124–5134.
56. **Razzaque, A., S. Chakrabarti, S. Joffee, and M. Seidman.** 1984. Mutagenesis of a shuttle vector plasmid in mammalian cells. Mol. Cell. Biol. **4**:435–441.
57. **Razzaque, A., H. Mizusawa, and M. M. Seidman.** 1983. Rearrangement and mutagenesis of a shuttle vector plasmid after passage in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **80**:3010–3014.
58. **Rigby, P. W. J., and P. Berg.** 1978. Does simian virus 40 DNA integrate into cellular DNA during productive infection? J. Virol. **28**:475–489.
59. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
60. **Rio, D., A. Robbins, R. Myers, and R. Tjian.** 1980. Regulation of simian virus 40 early transcription *in vitro* by a purified tumor antigen. Proc. Natl. Acad. Sci. U.S.A. **77**:5706–5710.
61. **Shalloway, D., T. Kleinberger, and D. M. Livingston.** 1980. Mapping of SV40 DNA replication origin region binding sites for the SV40 T antigen by protection against exonuclease III digestion. Cell **20**:411–422.
62. **Shenk, T., and P. Berg.** 1976. Isolation and propagation of a segment of the simian virus genome containing the origin of DNA replication. Proc. Natl. Acad. Sci. U.S.A. **73**:1513–1517.
63. **Shortle, D., R. F. Margolskee, and D. Nathans.** 1979. Mutational analysis of the simian virus 40 replicon: pseudorevertants of mutants with a defective replication origin. Proc. Natl. Acad. Sci. U.S.A. **76**:6128–6131.
64. **Smith, G. E., and M. D. Summers.** 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem. **109**:123–129.
65. **Subramani, S., and P. Berg.** 1983. Homologous and nonhomologous recombination in monkey cells. Mol. Cell. Biol. **3**:1040–1052.
66. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591–598.
67. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen-related protein. Cell **13**:165–179.
68. **Tjian, R.** 1978. Protein-DNA interactions at the origin of simian virus 40 DNA replication. Cold Spring Harbor Symp. Quant. Biol. **43**:655–662.
69. **Tooze, J. (ed).** 1980. Molecular biology of tumor viruses, part 2, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
70. **Villareal, L. P., and P. Berg.** 1977. Hybridization *in situ* of SV40 plaques: detection of recombinant SV40 virus carrying specific sequences of nonviral DNA. Science **196**:183–185.
71. **Vogelstein, B., and D. Gillespie.** 1979. Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. U.S.A. **76**:615–619.
72. **Wakamiya, T., T. McCutchan, M. Rosenberg, and M. Singer.** 1979. Structure of simian virus 40 recombinants that contain both host and viral DNA sequences. I. The structure of variant CVP8/1/P2 (*Eco*RI res). J. Biol. Chem. **254**:3584–3591.
73. **Wake, C. T., T. Gudewicz, T. Porter, A. White, and J. H. Wilson.** 1984. How damaged is the biologically active subpopulation of transfected DNA? Mol. Cell. Biol. **4**:387–398.
74. **Wilson, J. H., P. B. Berget, and J. M. Pipas.** 1982. Somatic cells efficiently join unrelated DNA segments end-to-end. Mol. Cell. Biol. **2**:1258–1269.
75. **Winocour, E., D. Dorsett, Z. Grossman, and D. Vernade.** 1984. Recombination promoted by papovaviruses: model systems for a nonhomologous recombination pathway, p. 75–89. *In* C. Chagas (ed.), Modern biological experimentation. Proceedings of the Pontifical Academy of Sciences, Vatican City, Rome.
76. **Winocour, E., and I. Keshet.** 1980. Indiscriminate recombination in simian virus 40-infected monkey cells. Proc. Natl. Acad. Sci. U.S.A. **77**:4861–4865.
77. **Winocour, E., V. Lavie, and I. Keshet.** 1983. Structure of simian virus 40-ϕX174 recombinant genomes isolated from single cells. J. Virol. **48**:229–238.
78. **Winocour, E., M. Singer, and E. Kuff.** 1980. Rapid detection, isolation and amplification of host-substituted SV40 variants. Cold Spring Harbor Symp. Quant. Biol. **44**:621–628.