JOURNAL OF VIROLOGY, Oct. 1983, p. 229–238
0022-538X/83/100229-10$02.00/0

Vol. 48, No. 1

Structure of Simian Virus 40-φX174 Recombinant Genomes Isolated from Single Cells

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Received 1 April 1983/Accepted 29 June 1983

Three simian virus (SV40)-φX174 recombinant genomes were isolated from single BSC-1 monkey cells cotransfected with SV40 and φX174 RF1 DNAs. The individual cell progenies were amplified, cloned, and mapped by a combination of restriction endonuclease and heteroduplex analyses. In each case, the 600 to 1,000 base pairs of φX174 DNA (derived from different regions of the φX174 genome) were present as single inserts, located in either the early or late SV40 regions; the deletion of SV40 DNA was greater than the size of the insert; and the remaining portions of the hybrid genome were indistinguishable from wild-type SV40 DNA, as judged by both mapping and biological tests. Hence, apart from the deletion which accommodates the φX174 DNA insert, no other rearrangements of SV40 DNA were detected. The restriction map of a SV40-φX174 recombinant DNA isolate before molecular cloning was indistinguishable from those of two separate cloned derivatives of that isolate, indicating that the species cloned was the major amplifiable recombinant structure generated by a single recombinant-producing cell. The relative simplicity of the SV40-φX174 recombinant DNA examined is consistent with the notion that most recombinant-producing BSC-1 cells support single recombination events generating only one amplifiable recombinant structure.

Recombination occurs between simian virus 40 (SV40) and bacterial virus φX174 RF1 DNAs cotransfected into monkey BSC-1 cells (14). The frequency of this nonhomologous recombination occurrence has been measured in terms of the quantity of hybrid virus in the yield of a cotransfected culture of cells (14) and in terms of the number of recombinant-producing cells in the initial cotransfected cell population (4). Evidence has been reported that SV40 nonhomologous recombination events occur in a discrete subpopulation of cells: 1 to 2% of the successfully transfected fraction of cells or 1 in 500 to 1 in 1,000 of the total cell population (4). An analysis of the SV40-φX174 recombinant genomes in the total yield of a cotransfected cell population indicated that a variety of recombinant structures had arisen (14). This variety may result from the presence of a few recombination-proficient cells supporting multiple recombination events; alternatively, cells may support only single recombination events, each cell giving rise to a different recombinant structure. We report here an analysis of three cloned SV40-φX174 recombinant genomes, each of which arose from a single recombinant-producing cell. The results indicate that most recombinant-producing cells, in fact, support single recombination events generating only one amplifiable recombinant structure.

MATERIALS AND METHODS

DNA and cells. The procedures used for the preparation of wild-type SV40 DNA I and φX174 RF1 DNA, and for the culture of monkey BSC-1 cells, were as described elsewhere (4).

Isolation and amplification of SV40-φX174 recombinant progeny from individual transfected cells. Suspensions of BSC-1 cells were cotransfected with SV40 and φX174 RF1 DNAs (1 μg of each DNA per 4×10^6 cells per ml), using DEAE-dextran as the facilitator as described previously (4). The recombinant yields of individual cotransfected cells were isolated either by cell partition in microwell plates (15) or from the agar overlay of infectious-center in situ plaque hybridization foci (4). In the microwell method, the transfected cells were mixed with a 20-fold excess of uninfected BSC-1 cells and distributed into microwell plates (Microtest II, Falcon Plastics) such that each well received 1,000 cotransfected cells and 20,000 uninfected cells in 0.1 ml of medium. When all wells showed cytopathic effects (after 9 days of incubation), the plates were subjected to three cycles of freeze-thawing, and a 40-μl sample of each well lysate was analyzed for φX174 DNA by using a dot hybridization procedure (15). Strong autoradiographic signals, after hybridization with φX174 RF1 [^{32}P]DNA, were detected in 12 of 192 well lysates. Control wells seeded with

cells transfected with φX174 RF1 DNA alone (or with SV40 DNA alone) gave rise to no autoradiographic signals after hybridization with φX174 [^{32}P]DNA. To amplify the recombinant isolates, 3-cm BSC-1 cell cultures were inoculated with 50-μl samples of the positive well lysates, and progeny virus was harvested when full cytopathic effects became apparent (usually after 5 days of incubation). These viral yields were then used to infect 15-cm BSC-1 cell plates from which supercoiled progeny DNA was prepared (at 50 h postinfection) by the Hirt extraction procedure followed by equilibrium centrifugation in cesium chloride-ethidium bromide density gradients (7). Isolates 9 and 32 (see below) were purified and amplified by the procedure described above.

To isolate progeny from recombinant infectious-center plaques, the contransfected cells (mixed with a 40-fold excess of uninfected cells) were plated for infectious centers, overlaid with agar, transferred to nitrocellulose membrane filters, and titrated for the production of SV40-φX174 recombinant plaques as described previously (4). After hybridization with φX174 [^{32}P]DNA and autoradiography (4), well-separated "hybridization plaques" appearing on the autoradiogram were aligned with the agar overlay by means of the system of orientation marks described by Villarreal and Berg (12). A plug of agar was withdrawn from the region of the overlay corresponding to the autoradiographic plaque, suspended in 1 ml of medium, and used to inoculate 3-cm cultures of BSC-1 cells. After the appearance of full cytopathic effects (9 days post-inoculation), progeny virus was harvested and used to infect 15-cm BSC-1 cell plates from which supercoiled viral progeny DNA was isolated as described above. Isolate 2 (see below) was prepared and amplified by this procedure.

Restriction digestion, gel electrophoresis, and blot hybridization. DNA was digested with restriction endonucleases under the conditions specified by the supplier (New England Biolabs), and the products were separated by electrophoresis on 1% agarose–Tris-acetate gels as described previously (4). Gels were stained with 0.5 μg of ethidium bromide per ml and photographed under UV light. In early experiments, the DNA was transferred to nitrocellulose sheets (Schleicher & Schuell Co., BA 85) by the Southern procedure (11); in later experiments, the transfer procedure of Smith and Summers (10) was utilized. Hybridization with in vitro-labeled [^{32}P]DNA probes (9) was as described elsewhere (14). Probe concentration and autoradiography conditions are noted elsewhere in the text.

Molecular cloning. The closed-circular DNA yields of individual recombinant-producing cells (amplified as described above) were digested with *Eco*RI, ligated to *Eco*RI-cleaved DNA arms of the λ-Charon phage 21A, and packaged in vitro by using previously described procedures (16). Phage plaques were screened by hybridization with SV40 [^{32}P]DNA and with λφX174 RF1 [^{32}P]DNA. Those which reacted with φX174 RF1 [^{32}P]DNA (0.1% of those which reacted with SV40 [^{32}P]DNA) were isolated, plaque purified by two consecutive cycles of replating (at which point 100% of the plaques hybridized with both probes), and grown to stocks. DNA from CsCl-purified phage was digested with *Eco*RI, and the recovered SV40-φX174 DNA was then subcloned in the *Eco*RI site of pBR322. All cloning procedures were carried out under the containment conditions specified by the local biohazard safety committee.

Heteroduplex analysis and electron microscopy. Wild-type SV40 DNA and SV40-φX174 recombinant DNA were excised from their pBR322 cloning vectors by *Eco*RI digestion, purified on 1% agarose gels, and extracted from the gel by glass powder adsorption (13). For heteroduplex formation between wild-type SV40 DNA and SV40-φX174 recombinant DNA, or SV40-φX174 recombinant DNA and wild-type φX174 RF1 DNA, the DNA samples (final concentration of each DNA, 2.6 μg/ml) were heated at 70°C for 10 min, followed by 60 min at 57°C, in the presence of 62.6% formamide, 412 mM NaCl, 83 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid)-NaOH (pH 7.8), 16 mM Tris-hydrochloride, and 3 mM EDTA (1). Samples were prepared for electron microscopy (Phillips EM400) by the formamide-cytochrome *c* monolayer spreading procedure (3). The conversion factor (length measurements to base pair [bp] units) for double-stranded DNA regions was calculated by using homoduplexes derived from linearized wild-type SV40 DNA (5,243 bp) or φX174 RF1 DNA (5,386 bp); that for single-stranded DNA regions was derived from single-stranded φX174 DNA (virion plus strand DNA, 5,386 nucleotides) added at the end of heteroduplex reactions.

RESULTS

Isolation and preliminary characterization of SV40-φX174 recombinants from individual transfected cells. The yields of single recombinant-producing transfected cells were isolated by the infectious-center protocol, or by partition of transfected cells in microwells, and were amplified as described above. The supercoiled DNA of these amplified isolates consisted of 99.9% wild-type SV40 DNA and 0.1% recombinant DNA (see below). Despite the overwhelming background of wild-type SV40 DNA, it was possible to determine the loss or retention of SV40 restriction sites in the SV40-φX174 recombinant genomes by digesting the DNA with restriction enzymes which cut wild-type SV40 DNA but not φX174 DNA, and by using φX174 RF1 [^{32}P]DNA as a hybridization probe. Conveniently, the SV40 single-cut enzymes *Eco*RI, *Bgl*I, *Bam*HI, *Kpn*I, and the SV40 multi-cut enzyme *Hin*dIII have no recognition sites on the φX174 genome (Fig. 1). The digestion products were separated by agarose gel electrophoresis and transferred to nitrocellulose paper (11), and those containing φX174 DNA sequences were identified by hybridization with φX174 RF1 [^{32}P]DNA. All five independent SV40-φX174 recombinant isolates (each from a different recombinant-producing cell) examined by this procedure contained single SV40 *Eco*RI and *Bgl*I restriction sites, as judged by the conversion of form I or from II to unit-length linear (form III) DNA. The analysis of two of the five isolates is shown in Fig. 2. In addition to the

retention of single *Eco*RI and *Bgl*I sites, the SV40-φX174 recombinant DNA in isolate 2 retained single sites for *Bam*HI and *Kpn*I (lanes 7 and 9); moreover, all of the φX174 DNA sequences in this recombinant DNA isolate were present within one *Hin*dIII digestion product which also contained the single *Kpn*I site (lanes 10 and 11). This preliminary characterization of the SV40-φX174 recombinant DNAs derived from single cotransfected cells was primarily intended to identify an appropriate restriction site for molecular cloning. In addition, the data obtained with isolate 2 suggested that all of the φX174 DNA sequences were located in one region adjacent to the single SV40 *Kpn*I restriction site.

Molecular cloning and structure of three SV40-φX174 recombinant genomes. The SV40-φX174 DNAs in three single cell yields (isolates 2, 9, and 32) were cloned by insertion into the *Eco*RI site of the λ-Charon phage 21A and were subcloned in the *Eco*RI site of pBR322 as described above. The proportion of first-cycle λ-Charon 21A plaques (see above) which reacted against both the SV40 [^{32}P]DNA and φX174 RF1 [^{32}P]DNA probes was 0.1% of the number which reacted with the SV40 [^{32}P]DNA probe alone, indicating that the ratio of recombinant to wild-type SV40 DNA molecules in the three isolates was 1:1,000. The cloned SV40-φX174 recombinant DNAs were mapped by a combination of restriction endonuclease digestion and heteroduplex analyses. Figure 3 shows the maps that were constructed on the basis of the data from these two procedures. The essential features of the recombinant structures are as follows. (i) The φX174 DNA sequences (600 to 1,000 bp) were present as single inserts, located within either the early or the late SV40 regions. (ii) The deletion of SV40 DNA sequences was greater than the size of the φX174 DNA insert, resulting in a shortening (by 6 to 10%) of the recombinant genome relative to wild-type SV40. (iii) Other than the deletion which accommodated the φX174 DNA insert, the remainder of the SV40 DNA in the recombinant structure was retained in an unaltered, intact form. We summarize below the evidence for these conclusions and for the maps shown in Fig. 3.

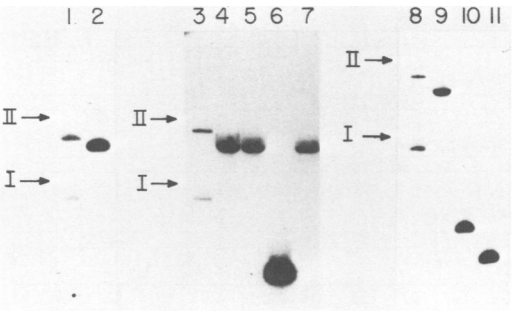

FIG. 2. Restriction mapping, before cloning, of SV40-φX174 recombinant DNA in isolates derived from single recombinant-producing cells. DNA of isolates 2 and 32 (see the text), either undigested or digested with restriction enzymes which do not cut authentic φX174 RF1 DNA, was fractionated on 1% agarose gels, transferred to nitrocellulose paper, and hybridized with φX174 RF1 [^{32}P]DNA. Lanes 1 and 2, isolate 32 DNA undigested or digested with *Eco*RI, respectively; lanes 3 to 7, isolate 2 DNA undigested or digested with *Bgl*I, *Eco*RI, *Hin*dIII, and *Bam*HI, respectively; lanes 8 to 11, isolate 2 DNA undigested or digested with *Kpn*I, *Hin*dIII, and *Kpn*I-*Hin*dIII, respectively. The arrows point to the positions of wild-type SV40 DNA, form I and form II, deduced from the ethidium bromide stain of the gel before blot hybridization. Autoradiograms were exposed at −70°C for 40 to 45 h. Although the DNAs of isolates 2 and 32 were subjected to cesium chloride-ethidium bromide density gradient centrifugation to purify form I, partial conversion to form II occurred during storage at −20°C.

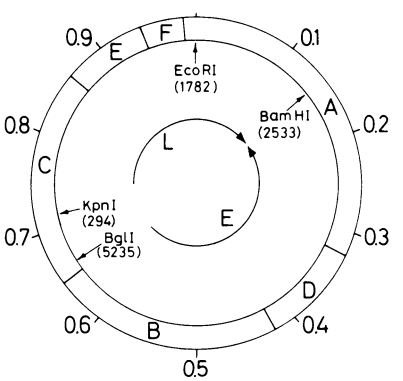

FIG. 1. Wild-type SV40 genome, showing the sites of restriction endonucleases used to map the SV40-φX174 recombinant DNAs. The outer circle shows the positions of the six *Hin*dIII fragments labeled A to F in descending order of size. The bracketed figures under the single-cut enzymes are the SV nucleotide numbers of these restriction sites (2). *Bgl*I cuts within the replication origin sequence. The letters L and E denote, respectively, the approximate coding regions for late and early functions. None of the restriction endonucleases noted above cut φX174 RF1 DNA.

(i) Restriction endonuclease digestion and blot hybridization. The pBR322 plasmids carrying the recombinant genomes (or wild-type SV40 DNA as control) cloned in the *Eco*RI site were digested with *Eco*RI, *Eco*RI-*Bgl*I, *Eco*RI-*Bam*HI, or *Eco*RI-*Hin*dIII. As noted above, none of these enzymes cut φX174 DNA. The products were separated on a 1% agarose gel, stained with ethidium bromide, photographed under UV light, and transferred to nitrocellulose paper by the method of Smith and Summers

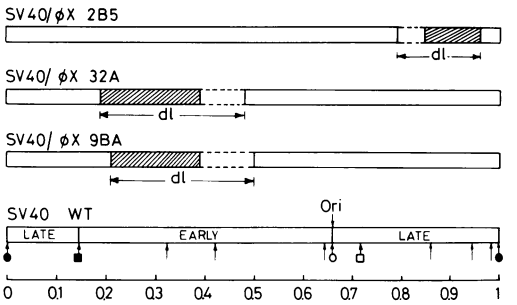

FIG. 3. Structure of the SV40-φX174 recombinant genomes. The maps of the recombinant DNAs are drawn relative to that of the wild-type (WT) SV40 genome and are based upon data from heteroduplex measurements and restriction cleavage-blot hybridization (see the text). The cross-hatched boxes denote the φX174 DNA inserts; the open boxes denote SV40 DNA sequences. dl refers to the deletion of SV40 DNA sequences. This deletion is greater than the φX174 DNA insert; as a consequence, the recombinant genomes are shorter than the SV40 WT genome by an amount corresponding to the dashed lines (the size order is WT > 2B5 > 32A > 9BA). The WT SV40 map, at the bottom, also shows the sites (in map units) of the restriction enzymes used to analyze the structure of the recombinant genomes (see also the circular map in Fig. 1). Symbols: ↥, *Eco*RI; ↥, *Bam*HI; ↑, *Hin*dIII; ↥, *Bgl*I; ↥, *Kpn*I. Ori denotes origin of replication.

(10), which provided two identical blots from each gel. One blot was hybridized with SV40 [^{32}P]DNA (5,000 cpm/ml); the twin blot was hybridized with φX174 RF1 [^{32}P]DNA (50,000 cpm/ml). The *Eco*RI digestion patterns (Fig. 4) showed that each of the intact, linearized SV40-φX174 recombinant genomes reacted, as expected, with both probes and was slightly smaller than wild-type SV40 DNA (the order of size is wild-type SV40 DNA > 2B5 DNA > 32A DNA > 9BA DNA).

*Eco*RI-*Bgl*I digestion of wild-type SV40 DNA divides the genome into two sectors (Fig. 1): a 3,453-bp fragment (0 to 0.66 map unit) which comprises all of the early region and a part of the late region spanning the *Eco*RI-*Bam*HI sites; and a 1,790-bp fragment (0.66 to 1.0 map unit) which is composed of late region and origin-flanking sequences. *Eco*RI-*Bgl*I digestion of 2B5 recombinant DNA shows that the φX174 DNA sequences are located in the SV40 late region; one of the two products was found to comigrate with the wild-type SV40 3,453-bp early DNA fragment and contained only SV40 sequences (Fig. 5; Table 1). The second digestion product contained both SV40 and φX174 DNA sequences and migrated faster than the authentic 1,790-bp SV40 late DNA fragment. As judged by gel electrophoresis, the size of the product containing both SV40 and φX174 DNA sequences is 1,450 bp, 340 bp shorter than the wild-type *Eco*RI-*Bgl*I product.

In contrast to 2B5 recombinant DNA, the φX174 DNA inserts in recombinant DNAs 32A and 9BA are located within shortened forms of the SV40 early region. Of the two products generated by *Eco*RI-*Bgl*I digestion, the smaller fragment comigrated with the wild-type 1,790-bp SV40 late segment and contained only SV40 sequences; the larger digestion product contained both SV40 and φX174 DNA sequences and migrated faster than the wild-type 3,453-bp SV40 early region fragment (Fig. 5; Table 1). The estimated sizes of the larger products are 3,000 bp for recombinant 32A (453 bp smaller than the comparable wild-type product) and 2,900 bp for recombinant 9BA (553 bp shorter than the wild-type fragment). A part of the late region (the 751-bp *Eco*RI-*Bam*HI segment) is contained within the larger *Eco*RI-*Bgl*I product (Fig. 1). However, *Eco*RI-*Bam*HI digestion of 9BA and 32A DNA showed that the wild-type 751-bp *Eco*RI-*Bam*HI fragment was retained in both recombinants and did not contain φX174 DNA sequences (Fig. 5; Table 1). The inserts in 9BA and 32A must therefore be located within the *Bam*HI-*Bgl*I early region of SV40.

The 2B5 SV40-φX174 recombinant retains an intact authentic SV40 early region (the φX174 DNA sequences being located in a truncated

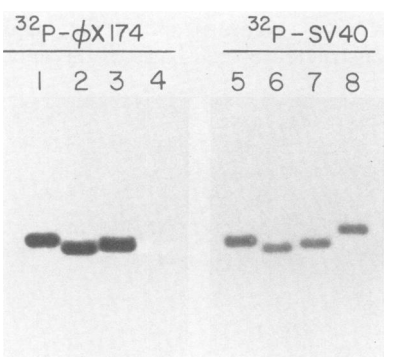

FIG. 4. *Eco*RI digestion of cloned SV40-φX174 recombinants. DNA of plasmids containing wild-type SV40 (lanes 4 and 8) or the recombinants 2B5 (lanes 1 and 5), 9BA (lanes 2 and 6), and 32A (lanes 3 and 7) was digested with *Eco*RI. The products were electrophoresed on 1% agarose gels and transferred to nitrocellulose papers by the method of Smith and Summers (10), which provided two replicate blots from each gel. One blot was hybridized with 50,000 cpm of φX174 RF1 [^{32}P]DNA per ml (lanes 1 to 4); the twin blot was hybridized with 5,000 cpm of SV40 [^{32}P]DNA per ml (lanes 5 to 8). The bands on the autoradiograms, resulting from hybridization with either probe, coincided exactly with bands (other than pBR322 DNA fragments) visualized by ethidium bromide staining of the gel before transfer and hybridization.

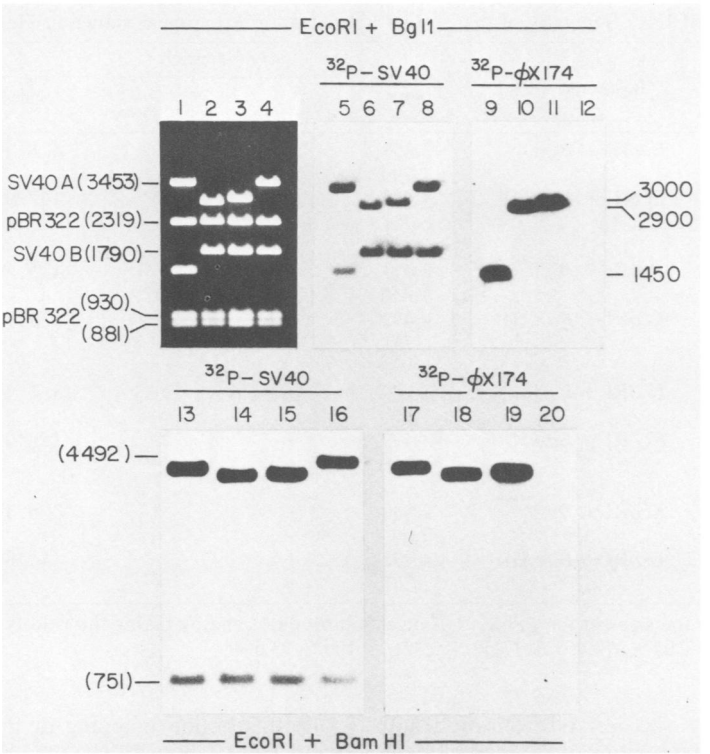

FIG. 5. *Eco*RI-*Bgl*I digestion (upper panel) and *Eco*RI-*Bam*HI digestion (lower panel) of cloned SV40-φX174 recombinants. Procedural details are as noted in the legend to Fig. 4. Upper panel: recombinant 2B5, lanes 1, 5, 9; 9BA, lanes 2, 6, 10; 32A, lanes 3, 7, 11; wild-type SV40 DNA, lanes 4, 8, 12. Lanes 1 to 4 show the electrophoretic gel patterns, stained with ethidium bromide, before transfer and hybridization; lanes 5 to 8, autoradiograms after hybridization with SV40 [^{32}P]DNA; lanes 9 to 12, autoradiograms after hybridization with φX174 RF1 [^{32}P]DNA. The lower panel shows the products of 2B5 (lanes 13 and 17), 9BA (lanes 14 and 18), 32A (lanes 15 and 19), and wild-type SV40 DNA (lanes 16 and 20) after digestion with *Eco*RI-*Bam*HI and hybridization with SV40 [^{32}P]DNA (lanes 13 to 16) or φX174 RF1 [^{32}P]DNA (lanes 17 to 20). The figures in parentheses (left side) are the sizes (in bp) of the known pBR322 and wild-type SV40 DNA digestion products which provided the size markers for the gel. The unbracketed figures on the right side are the sizes (in bp) of the recombinant DNA fragments containing φX174 sequences, as estimated from an electrophoretic mobility plot of the stained gel. Table 1 summarizes these data.

form of the late region segment), whereas the 9BA and 32A recombinants retain intact authentic SV40 late region segments (the φX174 DNA sequences being located in truncated forms of the early region segment) (Table 1). Additional evidence for this conclusion was provided by gel blot hybridization patterns with *Eco*RI-*Hin*dIII (data not shown). 2B5 DNA gave rise to four products, three of which comigrated with the wild-type early SV40 fragments and contained solely SV40 DNA sequences (the *Hin*dIII restriction sites at 0.860 and 0.945 map unit in the late SV40 region are missing; Fig. 1 and 3). *Eco*RI-*Hin*dIII digestion of 9BA and 32A DNA generated products which comigrated with the wild-type *Hin*dIII C, E, and F fragments (spanning the SV40 replication origin and late region sector; Fig. 1 and 3) and contained solely SV40 sequences.

(ii) Heteroduplex analysis. SV40-φX174 recombinant DNA strands (*Eco*RI-linearized) were annealed with wild-type SV40 DNA strands (*Eco*RI-linearized) or with φX174 RF1 DNA strands (*Pst*I-linearized) and were examined by electron microscopy as described in the legend to Fig. 6. Figure 6 depicts the heteroduplex segments that were measured, and Table 2 shows the measurements in summary form.

The single-strand arms of the loop-out apparent in each recombinant–wild-type SV40 heteroduplex are of unequal length. Because the restriction mapping data indicated that the deletion of SV40 DNA sequences is greater than the size of the φX174 DNA insert (Table 1), the size estimates of the φX174 DNA inserts are derived from the length of the shorter loop-out arm (Table 2, column a). As an independent check, the values can be compared with the

TABLE 1. Summary of the gel blot hybridization experiment shown in Fig. 5

DNA	Restricted with	Products (bp)	Hybridization		Fig. 5, lanes	Relative size of genome (%)[a]
			SV40 [^{32}P]DNA	φX174 RF1 [^{32}P]DNA		
Wild-type SV40	*Eco*RI + *Bgl*I	3,453	+	−	4, 8, 12	100
		1,790	+	−		
	*Eco*RI + *Bam*HI	4,492	+	−	16, 20	
		751	+	−		
Recombinant 2B5	*Eco*RI + *Bgl*I	3,453	+	−	1, 5, 9	93.5
		1,450	+	+		
	*Eco*RI + *Bam*HI	<4,492	+	+	13, 17	
		751	+	−		
Recombinant 32A	*Eco*RI + *Bgl*I	3,000	+	+	3, 7, 11	91.2
		1,790	+	−		
	*Eco*RI + *Bam*HI	<4,492	+	+	15, 19	
		751	+	−		
Recombinant 9BA	*Eco*RI + *Bgl*I	2,900	+	+	2, 6, 10	89.4
		1,790	+	−		
	*Eco*RI + *Bam*HI	<4,492	+	+	14, 18	
		751	+	−		

[a] Calculated from the sizes of the *Eco*RI-*Bgl*I digestion products and by taking the wild-type genome size as 5,243 bp (thus, e.g., 2B5 = [(3,453 + 1,450)/(5,243)] × 100 = 93.5%.

estimates of insert size derived from the length of the double-strand regions in the recombinant-φX174 RF1 heteroduplexes. The agreement between the two sets of measurements is excellent (Table 2, column a). Estimates of the size of the SV40 deletion which accommodates the insert can be derived from the lengths of the longer loop-out arm in the recombinant–wild-type SV40 heteroduplex (Table 2, column b) or by subtracting the sum of the double-strand regions (c and d) from the wild-type SV40 DNA length of 5,243 bp. By both calculations, the relative order of recombinant genome size (wild type > 2B5 > 32A > 9BA) is the same as that established by restriction mapping (Fig. 4; Table 1).

Examination of 130 recombinant–wild-type SV40 heteroduplex structures also established (i) that the φX174 DNA sequences are present as single inserts in each case and (ii) that other than the deletion which accommodates the insert, the remainder of the SV40 genome is retained intact. The structures of the 2B5, 32A, and 9BA recombinant genomes are thus relatively simple in that they do not exhibit rearrangements outside the region of the substitution.

The distance of the loop-out from either end of the *Eco*RI-linearized recombinant–wild-type SV40 heteroduplex structure (Table 2, columns c and d) provides two alternative positions for the φX174 DNA insert relative to the *Eco*RI site on the circular SV40 DNA map. The ambiguity is resolved by combining the heteroduplex measurements with the restriction mapping data as follows.

(i) Restriction mapping of the 2B5 recombinant DNA shows that the φX174 DNA sequences are located in the SV40 late region, somewhere between 0.67 and 1.0 map unit. The loop-out in the 2B5–wild-type SV40 DNA heteroduplex is 183 ± 34 bp from one *Eco*RI-cleaved end and 4,108 ± 196 bp from the other end. Clearly, to be consistent with the restriction mapping, the insert must be located 4,108 ± 196 bp clockwise from the *Eco*RI site (~0.79 map unit) and extend to a point 183 ± 34 bp distant from the *Eco*RI site on the circular map (~0.96 map unit).

(ii) Restriction analysis of the 32A DNA establishes that the φX174 DNA insert is present in the early SV40 region, within the *Bam*HI-*Bgl*I segment (0.143 to 0.659 map unit). The heteroduplex loop-out is 1,000 ± 89 bp from one *Eco*RI end and 2,922 ± 160 bp from the other end. A distance of 2,922 ± 160 bp clockwise from the *Eco*RI site on the circular SV40 map would position the φX174 DNA between 0.56 and 0.80 map unit, a possibility which is clearly excluded by the restriction mapping data (and which is theoretically impossible since it would involve a deletion of the SV40 origin sequences). The insert in 32A must therefore be located 1,000 ± 89 bp clockwise from the *Eco*RI site, replacing the wild-type segment from 0.19 to 0.48 map unit. This determination is also consistent with the *Hin*dIII restriction mapping data which indicate loss of sites at 0.324 and 0.423 map unit. The heteroduplex and restriction analysis of recombinant 9BA is similar to that of 32A; using

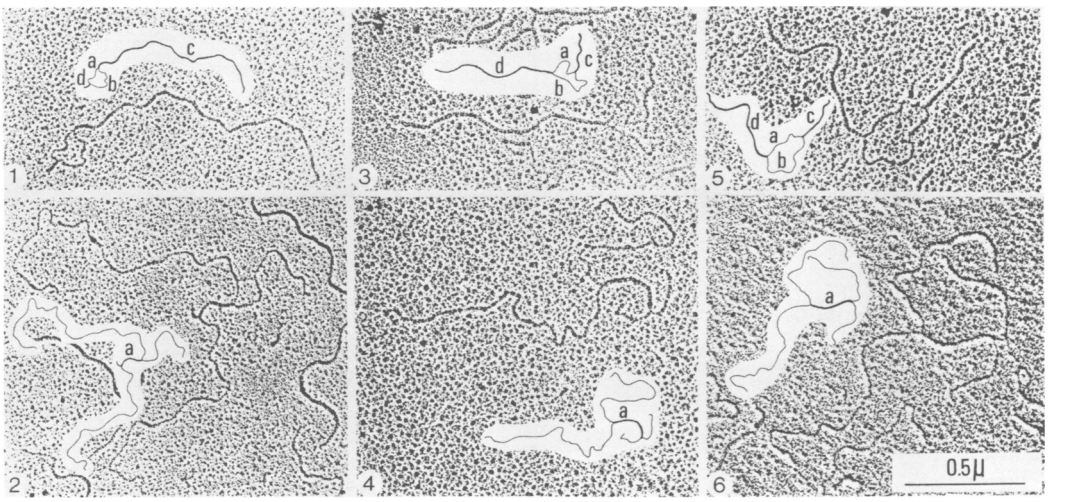

FIG. 6. Electron micrographs showing heteroduplex structures formed between SV40-φX174 recombinant DNAs and wild-type SV40 DNA or wild-type φX174 RF1 DNA. SV40-φX174 recombinant DNAs 2B5 (panels 1 and 2), 32A (panels 3 and 4), and 9BA (panels 5 and 6) were excised from their pBR322 vectors by *Eco*RI digestion, denatured, and annealed with *Eco*RI-linearized wild-type SV40 DNA (panels 1, 3, 5) or *Pst*I-linearized wild-type φX174 RF1 DNA (panels 2, 4, 6). The annealing conditions and the preparation for electron microscopy are described in the text. The superimposed inset drawings show the heteroduplex segments that were measured, as described in Table 2.

the same arguments, the position of the φX174 DNA insert in 9BA is estimated to replace the wild-type region, 0.21 to 0.50 map unit.

The φX174 DNA sequences in the recombinant genomes. To determine the regions of the φX174 genome from which the inserts in 2B5, 32A, and 9BA were derived, *Hae*III and *Hpa*I fragments of wild-type φX174 RF1 DNA were separated by gel electrophoresis and hybridized with ^{32}P-labeled SV40-φX174 recombinant DNAs (Fig. 7). ^{32}P-labeled 2B5 and 9BA DNAs hybridized solely with the contiguous *Hae*III fragments designated 1 and 4 in Fig. 7, indicating that the inserts in 2B5 and 9BA were derived from a region which straddles the *Hae*III site at nucleotide position 1,776 (within the capsid-coding region of the φX174 genome). In contrast, the insert in the 32A recombinant was derived from a portion of the φX174 genome closer to the 3′ end, spanning *Hae*III fragments 2 and 6a/6b (within the coding region for replication functions). Although *Hae*III fragments 5 and 6a/6b of φX174 RF1 DNA were poorly resolved by gel electrophoresis, the 32A insert cannot contain

TABLE 2. Heteroduplex analysis of three cloned SV40-φX174 recombinants[a]

Heteroduplex	*n*	Heteroduplex segment (bp)			
		a	b	c	d
2B5–WT-SV40	39	594 ± 66	860 ± 73	183 ± 34	4,108 ± 196
2B5–φX174 RF1	19	630 ± 75			
32A–WT-SV40	46	1,010 ± 141	1,562 ± 178	2,922 ± 160	1,000 ± 89
32A–φX174 RF1	38	894 ± 71			
9BA–WT-SV40	45	949 ± 124	1,653 ± 176	2,683 ± 179	1,097 ± 103
9BA–φX174 RF1	28	927 ± 120			

[a] Recombinants 2B5, 32A, and 9BA DNAs (*Eco*RI cut) were denatured and annealed with denatured wild-type (WT) SV40 DNA (*Eco*RI cut) or with denatured φX174 RF1 DNA (*Pst*I cut) as described in the legend to Fig. 6 and in the text. *n*, Number of heteroduplex structures analyzed. The a to d heteroduplex segments measured are depicted in Fig. 6. In the heteroduplex structures formed between the recombinant and wild-type SV40 DNAs, segment a is the shorter arm of the single-strand loop-out (the φX174 DNA insert); segment b is the larger arm of the single-strand loop-out (the deletion of SV40 DNA); c and d are the double-stranded portions of the structure (distance of the loop-out from either end). In the heteroduplex formed between the recombinant and wild-type φX174 RF1 DNAs, segment a is the double-strand region (the φX174 DNA insert in the recombinant genome). The conversion factor (length measurement to base pairs) for double-stranded DNA was derived from linear measurements of wild-type SV40 homoduplexes (5,243 bp); that for single-stranded DNA was derived from linear measurements of the φX174 virion plus strand (5,386 nucleotides) added before electron microscopy.

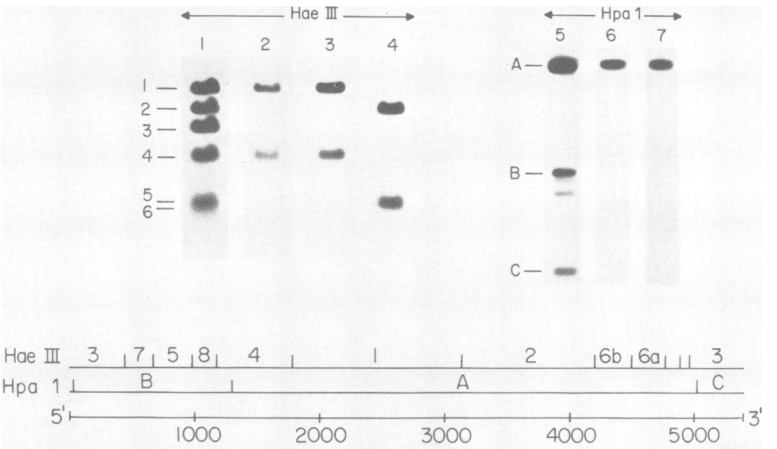

FIG. 7. The origin of the ϕX174 DNA inserts in recombinants 2B5, 9BA, and 32A. Wild-type ϕX174 RF1 DNA was digested with *Hpa*I or with *Hae*III. The products were separated on 1% agarose and transferred to nitrocellulose paper. Replicate nitrocellulose strips were hybridized with ϕX174 RF1 [^{32}P]DNA (lanes 1 and 5) or with ^{32}P-label DNAs of the cloned SV40-ϕX174 recombinants 2B5 (lanes 2 and 6), 9BA (lane 3), and 32A (lanes 4 and 7). The lower panel shows the *Hae*III and *Hpa*I restriction maps of ϕX174 DNA, linearized at the *Pst*I site (which is the zero point for both map units and nucleotide numbers).

sequences derived from *Hae*III fragment 5 because 32A [^{32}P]DNA did not hybridize with the overlapping *Hpa*I B fragment (Fig. 7). Other types of experiments (4) show that all segments of the ϕ174 genome recombine equally well with SV40 DNA; indeed, no single portion of the ϕX174 genome appears to possess a recombination advantage.

Comparison of recombinant genomes before and after molecular cloning. Comparison of the endonuclease restriction patterns of the recombinant structure before and after molecular cloning provides information on (i) possible alterations that might have occurred during cloning and (ii) whether the structure cloned is representative of the predominant type present in the amplified yield of a given recombinant-producing cell. We therefore compared the restriction patterns of the recombinant DNA yield from a single recombinant-producing cell (isolate 2) with those of two independent molecularly cloned recombinant genomes (2B5a and 2B5b) derived from that yield; 2B5a and 2B5b were isolated from two separate λ-Charon plaques. The *Eco*RI, *Eco*RI-*Hin*dIII, and *Eco*RI-*Kpn*I digestion patterns (Fig. 8) indicate that the recombinants in the single cell yield and the two independently cloned derivatives of that yield all contain ϕX174 DNA sequences within restriction fragments of identical electrophoretic mobility. The use of three different restriction endonuclease combinations in this experiment provides strong evidence that these recombinant structures must be very similar, if not identical. The results, therefore, indicate not only that no gross genomic alterations occurred during molecular cloning, but also, and more importantly, that the structures cloned represent the predominant types generated by a given recombinant-producing cell.

DISCUSSION

The SV40-ϕX174 recombinant DNA structures described in this report originated from single recombinant-producing cells. One isolate (2B5) was derived from a recombinant infectious-center plaque that arose from plating cotransfected monkey BSC-1 cells in the presence of a large excess of uninfected cells (4). The frequency of recombinant-producing cells, under the cotransfection conditions used, was about 10^{-3} with respect to the total cell population exposed to SV40 and ϕX174 RF1 DNAs (4), thus ensuring that the plaque sampled was initiated by the progeny of a single recombinant-producing cell. Isolates 9BA and 32A were obtained by distributing recombinant-producing cells in microwells (15) such that only a minor fraction of the microwells (12 of 192) gave rise to recombinant progeny. The isolation of recombinants from transfected cells partitioned in microwells averts a difficulty encountered in the isolation of recombinants from plaques identified by in situ hybridization; the successful sampling of the agar above the plaque requires rather precise alignment of autoradiogram, nitrocellulose filter, and agar overlay (12). It should be emphasized that successful isolation, by either method, also requires that the recombinant DNA structures be capable of replication and encapsidation, aided by the presence of

wild-type SV40 helper virus. These requirements for recombinant DNA amplification introduce an unavoidable degree of selection into the isolation procedures.

The proportion of recombinant to wild-type SV40 DNA molecules, in stocks raised from the microwell or plaque progenies, was 1:1,000. Application of molecular cloning procedures to remove the large excess of wild-type DNA raised the question of whether the recombinant species cloned was the major amplifiable species generated by a given recombinant-producing cell. However, comparison of the restriction maps before and after molecular cloning (isolate 2 and its cloned derivatives 2B5a and 2B5b) established that the species cloned was in fact the predominant recombinant structure in the amplified progeny of a single recombinant-producing cell. Recombinant genome mapping before molecular cloning was possible—despite the presence of a large excess of wild-type molecules—because of the availability of a relatively large number of restriction enzymes which cut SV40 but not φX174 RF1 DNA.

The structures of the three SV40-φX174 recombinant genomes examined are remarkably simple in the sense that the φX174 DNA sequences are present as single inserts and that other than the deletion of SV40 DNA which accommodates the insert, the remainder of the genome is indistinguishable from wild-type SV40 DNA, as judged by heteroduplex and restriction analyses. The retention of an intact SV40 early region in recombinant 2B5 and of intact SV40 late regions in recombinants 9BA and 32A has also been confirmed by biological tests. Independent replication of recombinant 2B5 DNA, transfected into BSC-1 cells in the absence of wild-type SV40 DNA, has been detected by in situ hybridization with φX174 [^{32}P]DNA. In contrast, no replication of 9BA or 32A DNA was detected under the same conditions. The retention of intact SV40 late regions in the 9BA and 32A recombinant genomes was confirmed by the observation that these DNAs (but not 2B5 DNA) replicated, synthesized SV40 capsid proteins, and underwent encapsidation in the COS line of monkey cells (5) whose chromosomally integrated SV40 genome expresses T antigen constitutively (data not shown). The relatively simple, unscrambled structures of the three SV40-φX174 recombinant genomes analyzed are consistent with the conclusion that most (but not all) recombinant-producing cells support only single recombination events (4). This conclusion, however, should be viewed within the context that recombinant-producing cells are themselves a rare occurrence, appearing at a frequency of 1 in 500 to 1 in 1,000 cells (4).

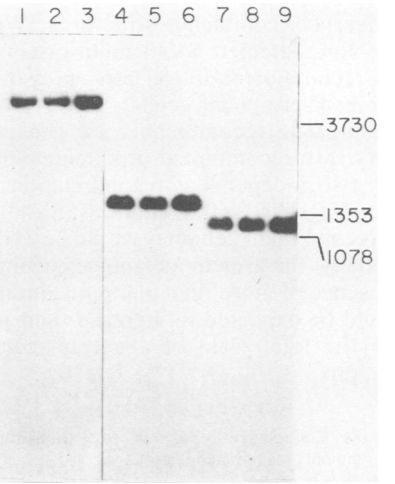

FIG. 8. Comparison of a SV40-φX174 recombinant genome before and after molecular cloning. The DNAs of the SV40-φX174 recombinants in isolate 2 and the two molecularly cloned derivatives 2B5a and 2B5b were digested with *Eco*RI (lanes 1, 2, and 3, respectively), *Eco*RI-*Hin*dIII (lanes 4, 5, and 6, respectively) and *Eco*RI-*Kpn*I (lanes 7, 8, and 9, respectively). The products were separated by electrophoresis and were blot hybridized with φX174 RF1 [^{32}P]DNA as described in Fig. 2 and 4. 2B5a and 2B5b are derived from two separated λ-Charon plaques (see the text). Size markers are the 3,730-bp *Hpa*I fragment A and the 1,353- and 1,078-bp *Hae*III products of authentic φX174 RF1 DNA.

A previous investigation (14) of SV40-φX174 recombinant DNA derived from the total viral yield of a cotransfected culture of BSC-1 cells indicated that a variety of different recombinant structures had arisen, many with multiple SV40 origins of replication reminiscent of the host substituted variants (8). In the three SV40-φX174 recombinant DNA structures described herein (each originating from a separate recombinant-producing cell), the position of the φX174 DNA insert with respect to the SV40 genome varied from structure to structure. A fourth structure, recently defined, contained φX174 DNA sequences in yet another location (data not shown). Although the number of recombinant genomes studied is too small to permit conclusions on the presence or absence of recombination "hot spots" is SV40 DNA, it is clear that each recombinant-producing cell can generate a different recombinant structure. It is therefore not surprising that the total yield of hybrid viral genomes from a mass-cotransfected culture displayed a highly complex pattern of recombinant structures (14); a single culture of 4×10^6 BSC-1 cells would be expected to contain 4,000 to 8,000 recombinant-producing cells (1 in 500 to 1 in 1,000) and many, perhaps most, of these gener-

ate different recombinant structures. We have not, so far, detected SV40 multi-origin SV40-ϕX174 recombinants in the progenies of single recombinant-producing cells. Most probably, the multi-origin recombinants are generated in the very rare recombinant-producing cells supporting two independent recombination events (4). Although the proportion of SV40 multi-origin recombinant genomes would be extremely low initially, the growth advantage ensuing from the presence of more than one replication origin (6) would be expected to increase their proportion in the total yield of a cotransfected cell population.

ACKNOWLEDGMENTS

We thank Kenneth I. Berns and Ann Roman for their critical comments on the manuscript.

This work was supported by a grant from the Leo and Julia Forchheimer Center for Molecular Genetics.

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