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Does Simian Virus 40 DNA Integrate into Cellular DNA During Productive Infection?

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Late after infection of permissive monkey cells by simian virus 40 (SV40), large amounts of SV40 DNA (30,000 to 220,000 viral genome equivalents per cell) can be isolated with the high-molecular-weight fraction of cellular DNA. Hirai and Defendi (J. Virol. **9**:705-707, 1972) and Hölzel and Sokol (J. Mol. Biol. **84**:423-444, 1974) suggested that this SV40 DNA is covalently integrated into the cellular DNA. However, our data indicate that the high-molecular-weight viral DNA is composed of tandem, "head-to-tail" repeats of SV40 DNA and that very little, if any, of this viral DNA is covalently joined to the cellular DNA. This was deduced from the following experimental findings. The size of the SV40 DNA associated with the high-molecular-weight cellular DNA fraction is greater than 45 kilobases, based on its electrophoretic mobility in agarose gels. In this form the SV40 DNA did not produce heteroduplex structures with a marker viral DNA (an SV40 genome with a characteristic deletion and duplication). After the high-molecular-weight DNA was digested with *Eco*RI or *Hpa*II endonucleases, enzymes which cleave SV40 DNA once, more than 95% of the SV40 DNA migrated as unit-length linear molecules and, after hybridization with the marker viral DNA, the expected heteroduplex structures were easily detected. Digestion of the high-molecular-weight DNA fraction with restriction endonucleases that cleave cellular, but not SV40, DNA did not alter the electrophoretic mobility of the polymeric SV40 DNA, nor did it give rise to molecules that form heteroduplex structures with the marker viral DNA. Polymeric SV40 DNA molecules produced after coinfection by two physically distinguishable SV40 genomes contain only a single type of genome, suggesting that they arise by replication rather than by recombination. The polymeric form of SV40 DNA is highly infectious for CV-1P monolayers (6.5×10^4 PFU per μg of SV40 DNA), yielding virtually exclusively normal, covalently closed circular, monomer-length DNA. Quite clearly these cells have an efficient mechanism for generating monomeric viral DNA from the SV40 DNA polymers.

Simian virus 40 (SV40) DNA persists in the chromosomal DNA of cells that have been stably transformed by the virus (2, 11). That these viral DNA sequences are covalently joined to the cellular DNA is shown by their copurification with high-molecular-weight cellular DNA even under denaturing conditions (35) and by restriction endonuclease mapping experiments (3, 22). Cells transformed by other DNA tumor viruses, e.g., polyoma virus and adenovirus type 2, also appear to contain viral DNA covalently integrated into cellular DNA (10, 46).

It is generally assumed that there is a causal relationship between the transformation of nonpermissive cells and the integration of viral genetic information. Consequently, clarifying the mechanism of viral DNA integration may be crucial to understanding viral oncogenesis. The findings by Hirai and Defendi (16), later confirmed by Hölzel and Sokol (18), that large quantities of SV40 DNA are associated with cellular DNA during the productive infection of permissive cells seemed to provide a model for examining the mechanism of integration and the organization of integrated sequences. Integration was inferred from the copurification of viral DNA sequences with cellular DNA under a variety of conditions which separate large, linear, cellular DNA from the much smaller, circular, viral DNA (16, 18). It is known that SV40 and cellular DNAs can recombine during productive infection to generate defective viral DNAs carrying substitutions of cellular DNA (24); such recombinational events could proceed through transient integration. It has also been reported

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(44) that small, linear DNA molecules containing both viral and cellular sequences occur in infected cells.

The analysis of integration in permissive cells is necessarily more complex than that in nonpermissive cells because extensive viral DNA replication occurs in the former, and, therefore, to rigorously prove integration, one must exclude fortuitous associations between replicating viral DNA and cellular DNA, as well as novel forms of viral DNA produced by the replication process. It has, for example, been shown that covalently closed circular oligomers of SV40 DNA as large as hexamers occur in considerable amounts in productively infected permissive cells and that these DNAs cosediment with cellular DNA under denaturing conditions (27).

Accordingly, cellular DNA has been purified from productively infected cells by a variety of procedures. In agreement with previous reports (16, 18), we found that this fraction contains large amounts of SV40 DNA sequences, but the behavior of these sequences in electron microscopic and restriction endonuclease mapping experiments indicates that most, if not all, of the viral DNA is not covalently joined to the cellular DNA. Rather, our experiments indicate that the viral DNA is organized into large polymeric molecules of wholly viral origin. Because the appearance of these polymers follows upon extensive replication of the viral DNA and homopolymers rather than heteropolymers are produced after coinfections with two physically distinguishable SV40 genomes, we conclude that they are synthesized by a replicational rather than a recombinational mechanism.

Although our data cannot exclude a low level of SV40 DNA integration during productive infection, it is clear that the sedimentation criteria used previously to demonstrate integration are misleading because novel forms of viral DNA, which copurify with cellular DNA, mimic integrated genomes.

MATERIALS AND METHODS

Cells and viruses. The CV-1, CV-1P, MA-134, and BSC-1 lines of African green monkey kidney cells were grown as previously described (28). Virus stocks were prepared by using MA-134 cells either as described (28) or by a modification of method 4 of Estes et al. (9). The combined supernatants obtained by extraction of the polyethylene glycol precipitate were layered over a CsCl cushion (3 ml, ρ = 1.38; 3 ml, ρ = 1.30) and centrifuged in a Beckman SW27 rotor at 25,000 rpm for 3 h at 4°C. The lower band, containing virus, was collected, dialyzed against 25 mM Tris-hydrochloride (pH 7.6)-150 mM NaCl-1 mM $MgCl_2$, sterilized with chloroform, made 10% (vol/vol) in calf serum, and frozen in liquid nitrogen. Such preparations have titers of 10^{10} to 10^{11} PFU/ml. Plaque assays of either SV40 virus or DNA were performed on monolayers of CV-1P cells according to published methods (28). The wild-type SV40 strains, *wt*800 and *wt*830, and the deletion mutants *dl*(*pm*)861 and *dl*-814 have already been described (5, 28, 30).

DNAs. Covalently closed circular viral DNA [SV40(I) DNA] was obtained from infected CV-1 cells and purified as previously described (37). DNA for nick translation reactions was purified further by sedimentation in neutral sucrose gradients.

Cellular DNA was isolated from SV40-infected cells by two methods. (i) In the first method, CV-1 cells, labeled with [^{3}H]thymidine (2 μCi/ml, 20 Ci/mmol) when subconfluent, were infected with SV40 (multiplicity of infection [MOI] = 5) when they were confluent and relabeled with [^{3}H]thymidine for 10 h before harvesting. The infected cells were collected by trypsinization and washed with Tris-saline, and nuclei were isolated by a procedure developed by F. Cuzin and M. Dieckmann (personal communication). Briefly, the cells were suspended (at 10^7 cells per ml) in cold buffer containing 0.1% (vol/vol) Triton X-100–250 mM sucrose in 10 mM Tris-hydrochloride (pH 7.7), blended in a Vortex mixer (Scientific Products Deluxe Mixer) at maximum speed for 2 min at 4°C, and centrifuged at 4,000 × *g* for 10 min. The nuclear pellet was suspended in 10 mM Tris-hydrochloride (pH 7.7)–250 mM sucrose–5 mM $MgCl_2$, washed twice with this buffer, and extracted by the Hirt procedure (17). The "Hirt pellet" was suspended in 0.1× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate), digested with pancreatic RNase (50 μg/ml) at 25°C for 1 h and then with Pronase (100 μg/ml) at 25°C for 19 h, and extracted with 4% (vol/vol) isoamyl alcohol in chloroform. The aqueous phase was dialyzed exhaustively against TNE (10 mM Tris-hydrochloride [pH 8], 10 mM NaCl, 2 mM EDTA). (ii) In the second method, CV-1 cells, labeled with [^{3}H]thymidine (1 μCi/ml, 20 Ci/mmol) when they were subconfluent, were infected with SV40 (MOI = 5) when they were confluent, and [^{3}H]thymidine (2 μCi/ml, 20 Ci/mmol) was added for about 24 h before harvesting. DNA was isolated by the procedure of Gross-Bellard et al. (15), except that extractions were with isoamyl alcohol-chloroform rather than with phenol.

λ*dvgal*120, PM2, and ColE1 DNAs were the gifts of P. C. Wensink, F. H. Schachat, and D. M. Glover, respectively.

In vitro labeling of SV40 DNA. SV40(I) DNA was labeled with ^{32}P in vitro by nick translation with *Escherichia coli* DNA polymerase I (31). The labeled DNAs had specific activities of 5×10^7 to 2×10^8 cpm/μg and an average single-strand length of about 400 nucleotides.

Synthesis of SV40 cRNA. ^{32}P-labeled RNA complementary to SV40 DNA (cRNA) was synthesized with *E. coli* RNA polymerase (45). The reaction mixture contained 50 mM Tris-hydrochloride (pH 7.9), 80 mM NaCl, 40 mM KCl, 15 mM $MgCl_2$, 1 mM dithiothreitol, 10% (vol/vol) glycerol, and 120 μg/ml of SV40(I) DNA together with ribonucleoside triphosphates. To prepare highly labeled cRNA for in situ hybridization experiments (39), 1 mM each ATP, CTP, and GTP and 7 μM [^{32}P]UTP (100 Ci/mmol) were used. For the preparation of less highly labeled cRNA,

e.g., for quantitative filter hybridization experiments, all four triphosphates were present at 1 mM together with 20 μM [^{32}P]GTP (72 Ci/mmol). At the completion of the reaction, 40 μg of yeast RNA per ml was added, the template DNA was digested with 50 μg of pancreatic DNase per ml at 25°C for 1 h, protein was then removed by phenol extraction, and the mixture was extracted with ether and passed over a column of Sephadex G50 (fine) to remove unincorporated triphosphates.

Enzymes. Restriction endonucleases were prepared as follows: *Eco*RI by a modification (M-T. Hsu, T. A. Landers, and P. Berg, unpublished data) of the method of Greene et al. (14); *Hpa*II according to Sharp et al. (36); *Hinc*II and *Hin*dIII by Bio-Gel A0.5M chromatography, ammonium sulfate fractionation, and phosphocellulose and DEAE-cellulose chromatography, as described by R. J. Roberts (personal communication); *Hae*III by the same procedure omitting the final step (33); *Sal*I by the same procedure omitting steps 2 and 4 (Roberts, personal communication); *Sst*I by the same procedure omitting step 2 and adding hydroxylapatite chromatography (S. P. Goff and A. Rambach, unpublished data); and *Hga*I by the method of Takanami (41). *Sma*I was a gift from J. Newbold; *Kpn*I and *Taq*I were gifts from A. Atkinson.

E. coli DNA polymerase I, the homogeneous preparation of Jovin et al. (20), was obtained from A. Kornberg. *E. coli* RNA polymerase, purified by an unpublished procedure, was given to us by W. Wickner, and preparations purified by the method of Burgess and Jendrisak (4) were provided by C. Yanofsky. S1 nuclease was purified by the method of Vogt (43).

DNA/DNA reassociation kinetics. The amount of SV40 DNA present in cellular DNA fractions was determined from the increase in the rate of reassociation of ^{32}P-labeled, nick-translated SV40 DNA in the presence of the test DNA (11); the results were treated as described by J. F. Morrow (Ph.D. thesis, Stanford University, Stanford, 1974).

Reassociation mixtures contained sufficient ^{32}P-labeled SV40 DNA to give 2,000 cpm/ml (10 to 100 pg/ml), sonically treated salmon sperm DNA (250 μg/ml), and the test DNA (sonically treated to an average single-strand length of 400 nucleotides) in 10 mM Tris-hydrochloride (pH 7.5)-1.5 M NaCl-2 mM EDTA. The mixtures were denatured in the absence of NaCl at 100°C for 3 min and cooled in ice water; the NaCl was added, and the samples were incubated at 68°C. Reassociation was monitored by resistance to S1 nuclease. Portions (0.5 ml) were withdrawn into 2.5 ml of S1 nuclease digestion buffer (30 mM sodium acetate [pH 4.55], 0.5 mM $ZnCl_2$), stored at −20°C, and then digested with excess S1 nuclease at 37°C for 2 h.

Gel electrophoresis. Polyacrylamide gels contained 4% (wt/vol) acrylamide and 0.2% (wt/vol) *N,N'*-methylenebisacrylamide; the electrophoresis buffer contained 89 mM Tris, 89 mM H_3BO_3, and 2.5 mM EDTA (pH 8.2) (14). The composition of agarose gels, cast in the same buffer, and the electrophoresis conditions are given in the appropriate figure legends.

Detection of SV40 sequences in agarose gels. DNA was transferred from agarose gels to nitrocellulose strips as described by Southern (39). The strips were baked under vacuum at 80°C for 2 h and then hybridized with ^{32}P-labeled SV40 cRNA at 42°C for 16 h. The hybridization solution (1 ml) contained 25 ng of SV40 cRNA per ml (4.3×10^7 cpm/μg), 100 μg of yeast RNA per ml, and 50% (vol/vol) formamide in 5× SSC. After hybridization, the filters were washed in the hybridization buffer at 42°C and in 2× SSC at 25°C, digested with pancreatic RNase (20 μg/ml) at 25°C for 1 h, and washed again in 2× SSC at 25°C. In some experiments the filters were hybridized with SV40 DNA labeled with ^{32}P by nick translation using the procedure we have described previously (31). Filters were radioautographed using Dupont Cronex 4 or Kodak X-omatic XH-1 X-ray film, in some cases at −70°C with a Kodak regular intensifying screen (23).

Electron microscopy. DNA was spread for electron microscopy, under aqueous conditions or from formamide, by the methods of Davis et al. (6). Grids were rotary shadowed with platinum-palladium and examined in a Phillips EM300 electron microscope.

RESULTS

Productively infected cells contain SV40 DNA which copurifies with cellular DNA. CV-1 cells were infected with SV40 (unless otherwise stated, all work was performed with strain *wt*830) at an MOI of 5, and nuclei isolated at various times were extracted by the Hirt procedure (17), in which circular viral DNA partitions mainly into the supernatant while high-molecular-weight cellular DNA is in the pellet (17). Cellular DNA, isolated from the pellet (see above), was further freed of traces of circular viral DNA by two steps; first, the DNA was sedimented through a neutral sucrose gradient, and the rapidly sedimenting fraction was then subjected to electrophoresis through 4% agarose gels. This latter step separates linear from circular DNA because linear DNA migrates in 4% gels at a rate essentially independent of its molecular weight, whereas circular DNA does not enter the gel (1, 5). At each stage of the purification procedure, DNA fractions were assayed for their content of SV40 DNA by DNA/DNA reassociation kinetics and for contamination with circular viral DNA by electron microscopy (Table 1).

By 47 h postinfection, but even more strikingly at 72 h, the cells contained considerable amounts of SV40 DNA associated with the cellular DNA. After the DNA was subjected to electrophoresis through 4% agarose gels, the quantity of circular forms of viral DNA was less than 5% the amount of SV40 DNA detected by reassociation kinetics. These results, obtained using a different procedure for purifying the cellular DNA, support earlier conclusions (16, 18) that cellular DNA from SV40-infected cells is associated with appreciable amounts of SV40 DNA. About the same quantity of SV40 DNA

TABLE 1. *Association of SV40 DNA with cellular DNA*

Time postinfection (h)	DNA purified by centrifugation[a]			DNA purified by electrophoresis[b]		
	SV40 genome equivalents/cell[c]	Fraction of cellular DNA as SV40[c] (%)	Contamination of cellular DNA by SV40 DNA[d] (%)	SV40 genome equivalents/cell	Fraction of cellular DNA as SV40 (%)	Contamination of cellular DNA by SV40 DNA[e] (%)
10	350	0.01		400	0.01	
24	750	0.03		300	0.01	
47	79,000	3.0	2.7	27,000	1.0	0
72	159,000	6.0	2.2	103,000	3.9	0.2

[a] DNA isolated from Hirt pellets as described in the text was sedimented through 5 to 20% neutral sucrose gradients in TNE (Beckman SW27 rotor, 27,000 rpm, 4°C, 5.5 h). Fractions containing high-molecular-weight DNA, located by counting portions of each fraction, were pooled, and the DNA was precipitated with ethanol and resuspended in TNE.

[b] DNA purified by sedimentation was subjected to electrophoresis through 4% agarose plugs as previously described (5). The DNA which passed through the plug was collected in a dialysis bag.

[c] Measured by DNA/DNA reassociation kinetics as described in the text.

[d] DNA was spread for electron microscopy, random fields were photographed, and the numbers of cellular and circular viral DNA molecules present were counted. The lengths of many cellular DNA molecules were measured relative to SV40 (II) DNA as a length standard. From these measurements the contamination by free viral DNA on a weight basis could be computed and expressed as a percentage of total DNA.

[e] The measurement was performed as described in *d* above except that open circular PM2 DNA was used as a length standard.

copurified with the high-molecular-weight cellular DNA after infections with another strain of SV40, *wt*800 (Rh 911), or with several viable SV40 mutants which carry deletions at 0.735 on the SV40 map (29). Moreover, similar amounts of high-molecular-weight viral DNA accumulate in the BSC-1 line of African green monkey kidney cells.

The kinetics of appearance of this high-molecular-weight SV40 DNA were followed by measuring the amount of viral DNA that partitioned between the Hirt supernatant and pellet fractions obtained from infected cells at various times after infection. The amount of SV40 DNA present in each fraction was measured by DNA/DNA reassociation kinetics (Fig. 1). Although some circular viral DNA can be detected by electron microscopic examination of the DNA obtained from the Hirt pellet, the level of this contamination is insufficient to account for the quantity estimated from the reassociation kinetics data.

We found that the high-molecular-weight form of the viral DNA appears mainly late in the infection, after considerable amounts of circular viral DNA have appeared in the supernatant fraction. At 6 days after infection, about one-third of all the viral DNA sequences in the cell appear with the cellular DNA in the Hirt pellet.

Cellular DNA isolated by the method described above has an average length of only about 29 kb (1 kb = 1,000 base pairs). Given the need to characterize the organization of the SV40 DNA sequences in this DNA, we sought

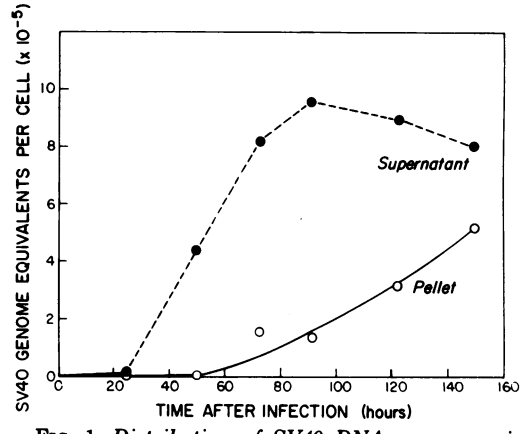

FIG. 1. *Distribution of SV40 DNA sequences in Hirt supernatant and pellet fractions of infected CV-1 cells. CV-1 cells were infected with SV40 (MOI = 15). At the indicated times the cells were extracted by the Hirt procedure (17), and the SV40 DNA content of both supernatant and pellet fractions was measured by DNA/DNA reassociation kinetics.*

to obtain cellular DNA preparations of the highest molecular weight possible. Consequently, DNA was isolated from SV40-infected CV-1 cells by the method of Gross-Bellard et al. (15) and further purified by sedimentation in neutral sucrose gradients. In this procedure the DNA was resolved into three components with different sedimentation behavior (Fig. 2). The rapidly sedimenting DNA at the bottom of the gradient was used in all of the experiments described below. Under these centrifugation conditions (relatively high DNA concentration and low

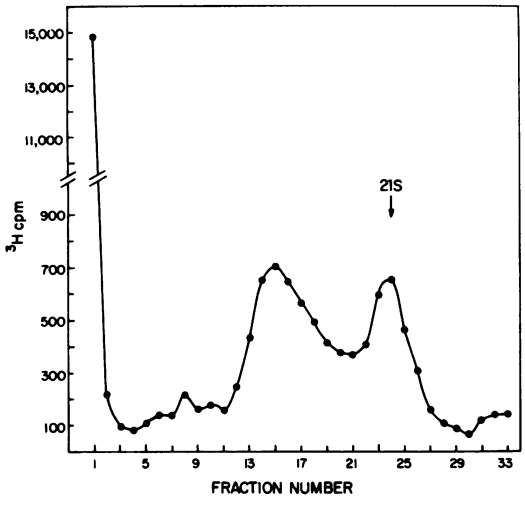

FIG. 2. *Sedimentation behavior of total DNA from SV40-infected CV-1 cells. DNA was isolated from infected cells by the second method described in the text. The second labeling was for 15 h before harvesting at 39 h postinfection. Sedimentation, from right to left, was through a 5 to 20% neutral sucrose gradient in TNE (Beckman SW40 rotor, 39,000 rpm, 4°C, 2.5 h). A parallel gradient, containing ^{32}P-labeled SV40(I) DNA, was used to obtain the position of 21S DNA.*

salt), the cellular DNA, although aggregated, is separated from the conventional forms of viral DNA. The material in the middle of the gradient is mainly partially degraded cellular DNA, although it contains some monomeric and oligomeric supercoiled viral DNA (up to at least tetramers). The partially degraded cellular DNA in this fraction presumably arises during the viral infection (32), because its amount is greatly reduced in CV-1 cells infected for only a short time (15 h) and in infected BSC-1 cells, in which virus-induced fragmentation of cellular DNA does not occur (32). The slowest sedimenting fraction is free viral DNA synthesized during the infection as it sediments at the same position as authentic SV40(I) DNA.

The high-molecular-weight DNA isolated from the bottom of the gradient (Fig. 2) had an average length of 52 kb; its electrophoretic mobility in 0.2% agarose gels was less than that of phage λ DNA (46.7 kb). It contained 66,000 SV40 genome equivalents per cell, as measured by DNA/DNA reassociation kinetics, and less than 1,600 viral length circular DNA molecules per cell, as judged by electron microscopy. When this DNA was fractionated on 0.2% agarose gels and then transferred to nitrocellulose filters (39), hybridization with ^{32}P-labeled SV40 cRNA also indicated that all of the viral DNA sequences migrate more slowly than λ DNA. The sizes of the SV40 DNA sequences were further analyzed by sedimenting samples of this DNA through 5 to 20% neutral sucrose gradients containing 1 M NaCl. The DNA in each gradient fraction was immobilized on a nitrocellulose filter, and the location of viral DNA sequences was determined by hybridization with ^{32}P-labeled SV40 cRNA. These data confirmed the electrophoretic analysis, indicating that more than 85% of the viral DNA sedimented more rapidly than λ DNA, but revealed that the SV40 DNA sequences are quite heterogeneous in size, the majority sedimenting at positions characteristic of linear DNA molecules of 45 to 100 kb in length. In a variety of experiments, high-molecular-weight DNA isolated in this way contained from 30,000 to 220,000 viral genome equivalents per cell, depending on the precise conditions of the infection. Thus, the 50 kb DNA fraction contains 60 to 80% of the total high-molecular-weight forms of SV40 DNA, defined as those viral sequences found in the Hirt pellet fraction, and has sedimentation and electrophoretic properties distinct from those of the circular oligomers studied by Martin et al. (27).

Organization of the high-molecular-weight SV40 DNA sequences. The SV40 DNA sequences associated with high-molecular-weight cellular DNA could be organized in several ways, some of which are diagramed in Fig. 3. To discriminate among these, several experimental approaches were employed.

(i) Electron microscopic mapping of heteroduplexes. Considering the very large number of viral genomes associated with the high-molecular-weight cellular DNA, electron microscopic characterization of heteroduplex structures should shed some light on the organization of the viral DNA. In principle, such experiments could establish whether the viral DNA is cova-

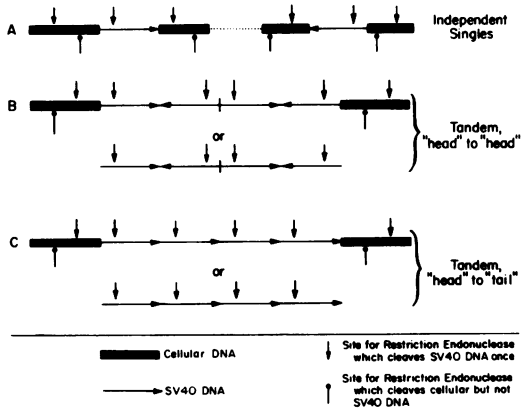

FIG. 3. *Possible organizations of high-molecular-weight SV40 DNA.*

lently integrated, how the integrated genomes are oriented, and where the integration sites are located on the viral DNA.

To detect viral DNA sequences in the high-molecular-weight DNA, a mutant of SV40, *dl*-814, was used as a probe (Fig. 4). *dl*-814 carries a deletion between map coordinates 0.73 and 0.97 and a duplication of the region 0.63 to 0.71 (J. E. Mertz and P. Berg, unpublished data). When mutant and wild-type linear DNAs, generated with *Eco*RI endonuclease, are denatured and renatured together, linear heteroduplexes having two single-stranded loops (deletion and duplication loops) are produced (Fig. 4). When the same *Eco*RI endonuclease-cleaved *dl*-814 DNA is heteroduplexed with SV40 DNA which has been opened up at any other point on the map, circular heteroduplexes with deletion and duplication loops are formed (Fig. 4). Heteroduplex molecules formed between *Eco*RI endonuclease-cleaved *dl*-814 DNA and integrated SV40 DNA could be either linear or circular, but will have cellular DNA tails (Fig. 4). The vast majority of cellular DNA strands larger than 40 kb contain clearly visible inverted duplications; therefore, linear or circular heteroduplexes with tails bearing "lollipop" structures (snapback regions corresponding to inverted duplications) should be diagnostic of covalently joined cellular and viral DNA sequences.

All attempts to produce linear or circular heteroduplex structures between *Eco*RI endonuclease-generated linear *dl*-814 DNA and the high-molecular-weight SV40 DNA have failed. However, the expected structures are readily detected if either *Eco*RI or *Hpa*II endonuclease-generated linear viral DNA is added to the cellular DNA fraction in amounts equivalent to the known SV40 sequence content of this fraction. A considerable amount of "networking" (tangling) occurs during annealing of cellular DNA of this size, and such networks could obscure the heteroduplexes. Prior digestion of the DNA with endonucleases *Sma*I or *Sst*I, which do not cleave SV40 DNA and should thus preserve the structure of any integrated genomes, considerably reduces the average molecular weight and thus the extent of networking, but still none of the predicted heteroduplexes were detected.

That such heteroduplex structures can form

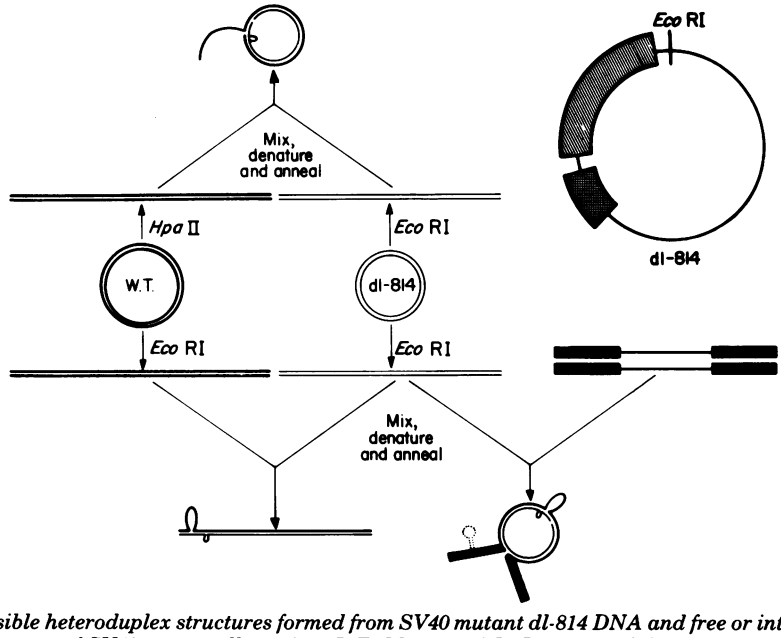

FIG. 4. *Possible heteroduplex structures formed from SV40 mutant dl-814 DNA and free or integrated SV40 DNA. The structure of SV40 mutant dl-814 (23; J. E. Mertz and P. Berg, unpublished data) is shown in the upper right-hand corner. The hatched area shows the location of a deletion of 24% of the SV40 genome (from 0.73 to 0.97 on the SV40 map); the stippled area shows the location of a tandem duplication of 8% of the genome (from 0.63 to 0.71). dl-814 DNA is sensitive to EcoRI endonuclease but resistant to HpaII endonuclease (the HpaII site is at 0.735 on the map). The structures expected when dl-814 EcoRI endonuclease-generated linear DNA is heteroduplexed to EcoRI or HpaII endonuclease-cleaved wild-type DNA are in the center of the diagram. In the bottom right-hand corner is the type of structure that would be generated if a strand of linear (EcoRI) dl-814 DNA anneals with an SV40 genome joined at its ends to cellular DNA. The dotted lollipop structure corresponds to an inverted duplication, which occurs in most cellular DNA strands of the size used.*

is demonstrated in Fig. 5A, which shows a heteroduplex generated from *Eco*RI endonuclease-cleaved *wt*830 DNA and a linear dimer of SV40 DNA that had been cleaved once by *Hpa*II endonuclease (13). The dimer mimics an integrated or oligomeric SV40 DNA, and, as expected, a double-stranded circle with two single-stranded loops and two single-stranded tails is observed (Fig. 4). *dl*-814 DNA preparations contain trace amounts of *tsA*30 DNA, derived from the helper virus used in its growth (28), and *dl*-814/*tsA*30 heteroduplexes were observed at the expected low frequency in all of these experiments, providing an internal control for the efficiency of the reannealing step.

The cellular DNA fraction contains SV40 DNA sequences capable of forming heteroduplexes because, if it is digested with either *Eco*RI or *Hpa*II endonucleases and then heteroduplexed with *Eco*RI endonuclease-cleaved *dl*-814

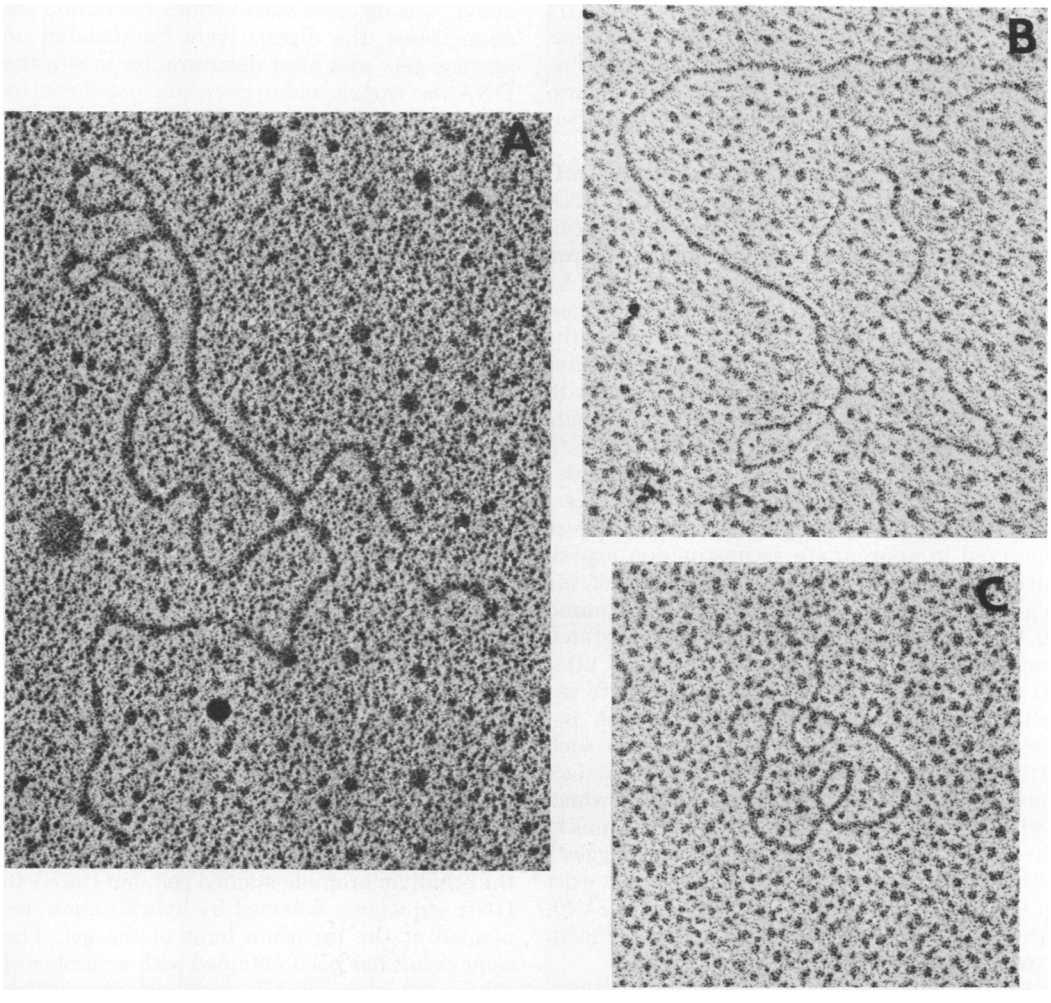

FIG. 5. *Heteroduplex DNA molecules formed between mutant dl-814 DNA and various types of SV40 DNA. (A) EcoRI endonuclease-cleaved wt830 DNA was mixed with linear dimers of SV40 DNA; the mixture was denatured, renatured, and spread for electron microscopy from 40% (vol/vol) formamide. The dimeric DNA used contained one HpaII endonuclease-sensitive (tsA30) and one HpaII endonuclease-resistant (dl-814) SV40 genome and had been converted to a linear dimer by HpaII endonuclease digestion (13). We thank Steve Goff for providing this micrograph. (B) EcoRI endonuclease-generated linear dl-814 DNA was mixed with EcoRI endonuclease-cleaved cellular DNA; the mixture was denatured, renatured, and spread from 60% (vol/vol) formamide. A representative heteroduplex molecule is shown. (C) EcoRI endonuclease-digested dl-814 DNA was mixed with HpaII endonuclease-cleaved cellular DNA; the mixture was denatured, renatured, and spread from 60% (vol/vol) formamide. A representative heteroduplex molecule is shown. The DNAs were denatured, annealed, and spread for electron microscopy by the procedures of Davis et al. (6).*

DNA, the expected heteroduplex structures are observed (Fig. 4; for example, Fig. 5B and C). After *Eco*RI endonuclease digestion of the high-molecular-weight DNA from infected cells, there is a marked increase in the frequency of linear heteroduplexes of the type shown in Fig. 5B. An average of 56 such molecules were observed per grid square, whereas with undigested DNA only one-sixth as many were seen. These are derived from the *tsA*30 DNA which contaminates the *dl*-814 DNA. More importantly, when *Hpa*II endonuclease-digested DNA was heteroduplexed to *Eco*RI endonuclease-cleaved *dl*-814 DNA, characteristic slipped-circle structures were seen, an example of which is shown in Fig. 5C. An average of 51 of these very easily identified molecules was seen per grid square, whereas with undigested DNA none was observed.

This experiment shows that *Eco*RI and *Hpa*II endonuclease digestions of the cellular DNA release linear SV40 DNA molecules indistinguishable, at the resolution level of an electron microscope, from linear molecules generated by digestion of SV40(I) DNA with these enzymes. The absence of foreign DNA tails on any of the 680 heteroduplex molecules examined indicates that the SV40 DNA sequences associated with the cellular DNA are organized in tandem, "head-to-tail" arrays (model C, Fig. 3). A comparison of the frequency at which heteroduplexes were observed after endonuclease digestion with the frequency at which they were observed in appropriate reconstruction experiments suggests that a large proportion of the high-molecular-weight SV40 DNA is organized in this way. Such arrays could be integrated (model C, Fig. 3); if so, one would expect, after *Eco*RI or *Hpa*II endonuclease digestion, to see heteroduplexes containing the junctions between viral and cellular DNAs, but no such structures were observed. The failure to detect such heteroduplexes, in an experiment in which 680 structures derived from internal positions in the long, tandem repeats were seen, suggests either that integrated viral DNA does not exist in the high-molecular-weight DNA from SV40-infected cells or that any integrated tandem arrays are greater than 50 units in length.

An alternative possibility is that the high-molecular-weight form of SV40 DNA is large polymers of viral DNA molecules organized in tandem, head-to-tail arrays. Such polymers probably would not form heteroduplexes effectively with the *dl*-814 DNA because the polymer strands would anneal with each other at a much higher rate. Even if an SV40 sequence in the polymer annealed with a *dl*-814 strand, it is likely that the small marker strand would be displaced by a complementary sequence on a polymer strand.

(ii) Restriction endonuclease mapping of SV40 sequences in high-molecular-weight DNA. A critical distinction between integrated and polymeric viral genomes can be made by examining the distribution of SV40 DNA sequences among the fragments generated by restriction endonuclease cleavage of the cellular DNA fraction.

High-molecular-weight cellular DNA, isolated from SV40-infected CV-1 cells as described above, was digested with various restriction endonucleases; the digests were fractionated on agarose gels, and after denaturation in situ the DNA was transferred to nitrocellulose sheets by the method of Southern (39). The location of SV40 DNA was determined by hybridization with ^{32}P-labeled SV40 cRNA (Fig. 6). With undigested DNA the hybridized ^{32}P-labeled SV40 cRNA coincided with the position of the high-molecular-weight cellular DNA, i.e., at the exclusion limit of the gel; no detectable hybridization occurred at any of the positions on the gel corresponding to the conventional forms of viral DNA, indicating that the SV40 DNA sequences associated with the high-molecular-weight DNA cannot be attributed to contamination. When the cellular DNA was digested with *Eco*RI endonuclease, all of the viral DNA was found at the position of full-length, linear SV40 DNA. Indeed, the band of excised linear SV40 DNA is so intense that it can be seen on the gels stained with ethidium bromide. Even if the radioautograms were grossly overexposed, the only additional bands seen were those resulting from partial digestion, e.g., linear dimers and trimers of SV40 DNA.

When the DNA was digested with *Sst*I endonuclease, an enzyme which cleaves cellular DNA but not SV40 DNA (S. P. Goff and A. Rambach, unpublished data), the cellular DNA was degraded to approximately the same extent as by *Eco*RI endonuclease digestion (as judged from the ethidium bromide-stained gel), but the SV40 DNA sequences, detected by hybridization, remained at the exclusion limit of the gel. The same result has been obtained with a number of other enzymes. *Hpa*II endonuclease, which cleaves SV40 DNA once, yielded full-length, linear SV40 DNA; *Sal*I, *Sma*I, and *Hga*I endonucleases, each of which cleaves cellular but not SV40 DNA, failed to alter the mobility of the high-molecular-weight SV40 DNA.

The models shown in Fig. 3 predict that if SV40 DNA were joined covalently to cellular DNA sequences, cleavage with *Eco*RI endonuclease would generate fragments whose sizes

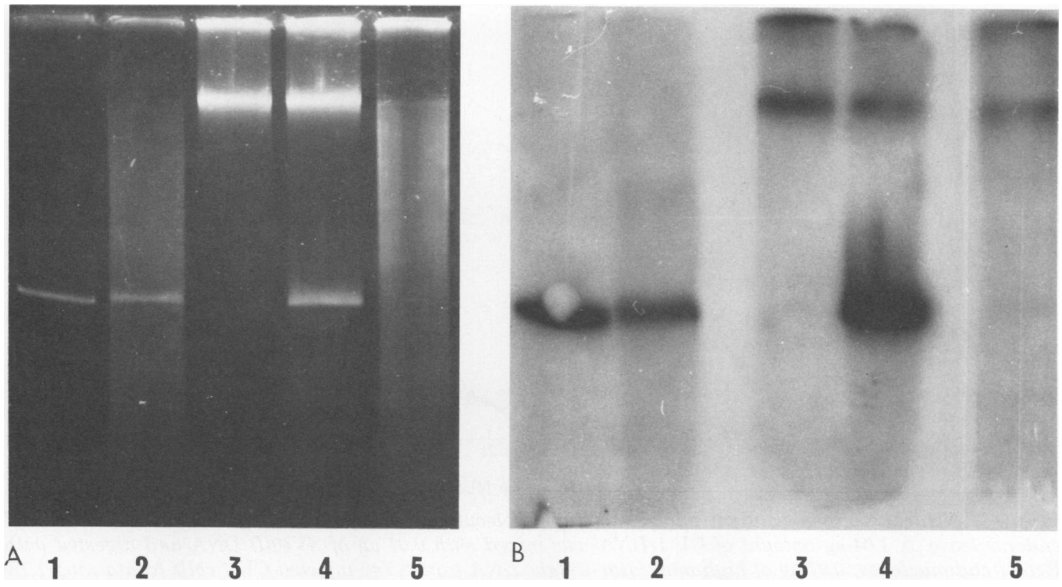

FIG. 6. *Restriction endonuclease analysis of SV40 DNA sequences associated with high-molecular-weight cellular DNA. High-molecular-weight cellular DNA, isolated from SV40-infected CV-1 cells as described in the text, was digested with various restriction endonucleases. The digests were fractionated on a 0.4% agarose gel run in a horizontal slab apparatus at 0.83 V/cm for 15 h. (A) shows the ethidium bromide-stained gel. The DNA in the gel was transferred to nitrocellulose filters as described by Southern (39); the filters were hybridized with ^{32}P-labeled SV40 cRNA, as described in the text, and then radioautographed for 3 days with Dupont Cronex 4 X-ray film. The radioautogram is shown in (B). The DNA samples were as follows: (1) 0.04 μg of EcoRI-digested SV40 DNA; (2) 0.45 μg of cellular DNA (containing 1.1% SV40 sequences) digested with EcoRI; (3) 0.45 μg of cellular DNA, undigested; (4) 0.45 μg of cellular DNA, undigested, plus 0.04 μg of EcoRI-digested SV40 DNA; (5) 0.85 μg of cellular DNA digested with SstI.*

would depend on the location of the *Eco*RI endonuclease cleavage sites in the cellular DNA; it seems unlikely that viral-length DNA would be formed after either *Eco*RI or *Hpa*II endonuclease digestion of DNA with singly integrated genomes. Moreover, the integration models predict that the electrophoretic mobility of the SV40 DNA sequences would change after *Sst*I digestion since the average size of the cellular DNA fragments is markedly reduced. Our data are more consistent with the likelihood that the bulk of the SV40 DNA sequences occur as linear, tandem, head-to-tail polymers, probably greater than 10 units in length and unlinked to cellular DNA.

It remains possible, however, that a small proportion of the viral DNA found associated with the high-molecular-weight cellular DNA is covalently integrated and that this integration is random with respect to the cellular DNA. If this were so, *Eco*RI endonuclease digestion would produce a considerable number of viral DNA-containing fragments present at the level of one copy per cell genome; these would not be detected as bands by the hybridization procedure that we have used; they would at best contribute to the general background on the autoradiogram. To test further for covalent integration, a mixture of DNA from uninfected CV-1 cells (1.04 μg) and SV40 DNA (0.01 μg) was digested with *Eco*RI endonuclease; a sample (0.97 μg) of high-molecular-weight DNA from SV40-infected CV-1 cells (containing 1.1% SV40 DNA sequences) was digested with the same enzyme. Both samples were centrifuged separately in neutral sucrose gradients, and the amount of SV40 DNA in each fraction of the gradient was assayed by DNA/DNA reassociation kinetics (Fig. 7) to determine whether SV40 DNA sequences in the second digest are associated with molecules that are larger or smaller than full-length linear SV40 DNA. The data show that the position of the SV40 DNA in the two gradients (the peak corresponds to full-length linear viral DNA) is, within the accuracy of the annealing kinetics measurements and the gradient fractionation procedure, indistinguishable. If the DNA contained several hundred covalently integrated viral genome equivalents per cell, then the amount of SV40 DNA detected in each fraction containing DNA larger than full-length viral DNA would be consistently larger than

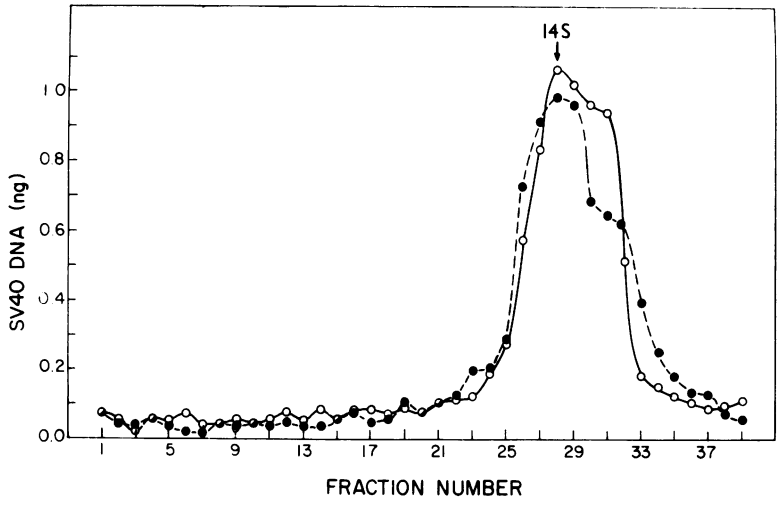

FIG. 7. *Neutral sucrose gradient analysis of high-molecular-weight cellular DNA digested with EcoRI endonuclease. A 1.04-μg amount of CV-1 DNA was mixed with 0.01 μg of SV40(I) DNA and digested with EcoRI endonuclease; 0.97 μg of high-molecular-weight DNA from SV40-infected CV-1 cells (containing 1.1% SV40 DNA sequences) was similarly digested. The samples were centrifuged through 5 to 20% neutral sucrose gradients (in 10 mM Tris-hydrochloride [pH 8]-1 M NaCl-2 mM EDTA) in a Beckman SW41 rotor (39,000 rpm, 20°C, 2.5 h). The gradients were fractionated by dripping, and the SV40 DNA content of each fraction was determined by DNA/DNA reassociation kinetics. A 15-μl amount of 5 N NaOH was added to each fraction (260 μl), and the solution was boiled for 10 min to reduce the average single-strand length of the DNA to 400 nucleotides (10). After neutralization of the solution with 15 μl of 5 N HCl, the reassociation assays were performed as described in the text except that the buffer contained 50 mM Tris-hydrochloride, pH 7.5. The position of the 14S peak was determined from a parallel gradient containing labeled linear SV40 DNA. Symbols: ●, infected-cell DNA; ○, uninfected-cell DNA plus SV40 DNA. From control experiments in which the same amount of the ^{32}P-labeled SV40 DNA probe was reassociated, in eight parallel assays, with a fixed amount of sonically treated, unlabeled SV40 DNA, we estimate the accuracy of each determination to be ±10%.*

that detected in the control gradient. We note that there is in the experimental gradient a slight excess of SV40-containing fragments smaller than full-length viral DNA. It seems unlikely that covalently integrated viral genomes would give rise only to *Eco*RI endonuclease fragments smaller than viral DNA; this discrepancy is probably due to imperfect matching of the two gradients. The data obtained from this experiment and the gel electrophoresis experiments clearly exclude high levels of covalent integration, but neither would have detected integration at the level found in transformed cells.

(iii) Infectivity of high-molecular-weight SV40 DNA. Monkey cells can rescue infectious SV40 from the tandemly integrated arrays of SV40 genomes in the adenovirus type 2-SV40 hybrids HEY and LEY (21, 25). As the SV40 DNA associated with high-molecular-weight cellular DNA in productively infected cells appears to be organized in tandem, head-to-tail arrays, its infectivity on monolayers of CV-1P cells was examined (Table 2). The specific infectivity of the DNA was unexpectedly high, 2.5% the value for SV40(I) DNA. Monkey cells apparently possess an efficient mechanism for rescuing virus from tandemly organized polymers.

Digestion of the DNA with either *Eco*RI or *Hpa*II endonucleases increased the infectivity about twofold, but if the DNA was digested with both of these enzymes, the infectivity was virtually abolished. The results of the digestions with each of the enzymes alone indicate that infectivity is not due to contamination by free viral DNA; if that were so, the digestions would have reduced the infectivity about fivefold. Furthermore, the increased infectivity is not due merely to a reduction in the size of the infecting DNA; digestion with *Sma*I or *Sst*I endonucleases, which cleave cellular but not SV40 DNA and, therefore, reduce considerably the average size of the DNA, did not increase the infectivity.

Generally, the viral DNA recovered from infections with the high-molecular-weight DNA or the endonuclease-cleaved DNAs was indistinguishable from the original infecting genomes, as judged by its *Hin*d (-II and -III) and *Hae*III endonuclease restriction patterns. Occasional plaques (3%), produced after infection with undigested DNA or with DNA that had been digested with *Sma*I or *Sst*I endonucleases, yielded virus which on further propagation contained

TABLE 2. *Infectivity of high-molecular-weight SV40 DNA*[a]

Sample	Infectivity (PFU/μg)[b]	
	Uncorrected	Corrected[c]
SV40(I) DNA	2.3×10^6	
Undigested DNA	6.5×10^4	
*Eco*RI endonuclease-digested DNA[d]	1.2×10^5	2×10^6
*Hpa*II endonuclease-digested DNA[d]	10^5	10^6
*Eco*RI/*Hpa*II endonuclease-digested DNA[d]	10^2	
*Sma*I endonuclease-digested DNA[e]	4.5×10^4	
*Sst*I endonuclease-digested DNA	5.5×10^4	

[a] Infectivities determined by plaque assays on monlayers of CV-1P cells (28). Numbers are the average of five experiments; in each experiment, about 500 plaques were counted for each sample.

[b] Calculated as PFU per microgram of SV40 DNA (the SV40 DNA content of the samples was determined by DNA/DNA reassociation kinetics).

[c] Data corrected (×4.5) for reduced infectivity of SV40 *Eco*RI endonuclease-generated linear DNA or (×6.5) for reduced infectivity of SV40 *Hpa*II endonuclease-generated linear DNA. Also corrected (×5) for competition by *Eco*RI endonuclease-digested cellular DNA or (×1.5) for competition by *Hpa*II endonuclease-digested cellular DNA. These latter correction factors were determined by performing plaque assays with appropriate mixtures of linear SV40 DNA and endonuclease-digested CV-1 DNA.

[d] λ*dvgal*120 DNA was added to the samples as an internal control for the efficiency of digestion. After digestion, the DNA was examined by electron microscopy to insure that the reaction was complete.

[e] ColE1 DNA was used to control for the efficiency of digestion as described in *d* above.

defective DNA molecules, i.e., DNA containing deletions, but because of their very low frequency we have not pursued the question of their origin.

Synthesis of the high-molecular-weight form of SV40 DNA. The kinetics of appearance and the amount of the high-molecular-weight form of SV40 DNA (Fig. 1) suggest that viral DNA replication is required for its synthesis. Consistent with this view is the observation that in cells infected wtih wild-type virus in the presence of arabinosyl-cytosine or with the temperature-sensitive mutant *tsA*58 at the nonpermissive temperature, 41°C, conditions which permit no viral DNA replication (26, 42), the high-molecular-weight form of viral DNA was not synthesized.

Possibly, the polymers are made only from progeny, rather than parental, DNA molecules. Polymers and oligomers could be synthesized by recombination between progeny molecules or directly in the replication process. To distinguish between these possibilities, CV-1 cells were coinfected with equal multiplicities of *dl*(*pm*)861, a mutant which lacks the *Hpa*II endonuclease cleavage site at map position 0.735 (5), and *wt*830; at 72 h after infection, supercoiled viral DNA was isolated from one infected plate, and high-molecular-weight cellular DNA was isolated from the remainder. The ratio of *dl*(*pm*)861 DNA to *wt*830 DNA in the supercoiled viral DNA was 37:63, as judged by the amounts of circular and linear DNAs after digestion with *Hpa*II endonuclease.

After *Eco*RI or *Taq*I endonuclease digestions of the high-molecular-weight DNA, all of the SV40 DNA was at the position of full-length, linear viral DNA (Fig. 8). However, after *Hpa*II endonuclease digestion, three bands were observed, one at the position of full-length, linear viral DNA, one at the position of undigested DNA, and the third at a position corresponding to linear trimers of SV40 DNA. All three bands remained even if the DNA was digested with a large excess of *Hpa*II endonuclease. As expected, digestion with *Sst*I endonuclease did not alter the mobility of the viral DNA sequences (Fig. 8).

If the polymeric form of SV40 DNA is produced by a replication process, for example, by a "rolling-circle" mechanism, any individual polymer molecule will contain only one type of viral genome. Consequently, *Hpa*II endonuclease digestion should produce only two bands, that at the full-length linear position and that at the position of undigested DNA. Conversely, if the polymers result from recombination between progeny molecules, the two types of viral genome should be randomly mixed in any individual polymer molecule. In this case *Hpa*II endonuclease digestion should produce a series of bands at the positions of linear monomers, dimers, trimers, tetramers, etc., of SV40 DNA.

The presence of the trimer-size band fits simply with neither model. We have occasionally detected a band at this position after digestion of the high-molecular-weight DNA with other endonucleases which cleave SV40 DNA once, for example, *Eco*RI, *Kpn*I, and *Taq*I. However, in these cases digestion with excess enzyme eliminates the band. The trimer-size band found in *Hpa*II endonuclease digests survives phenol extraction and heating to 55°C in 30% (vol/vol) formamide; it is, therefore, not an artifact caused by residual protein or by interactions between the short, complementary termini produced by *Hpa*II endonuclease. The appearance of this band is not a consequence of the mixed infection as it can be detected in *Hpa*II endonuclease

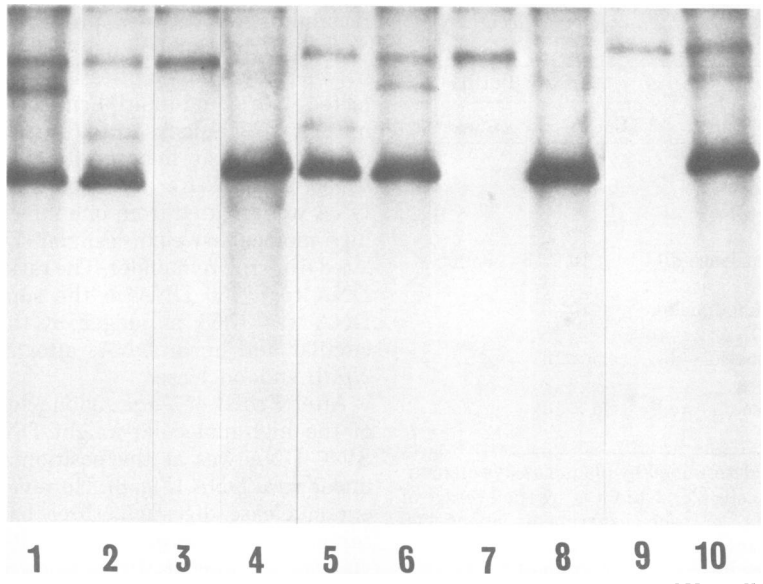

FIG. 8. *Restriction endonuclease analysis of polymeric SV40 DNA synthesized in CV-1 cells coinfected with wt830 and dl(pm)861. High-molecular-weight DNA was isolated from CV-1 cells at 72 h after infection with equal multiplicities of wt830 and dl(pm)861 and digested with various restriction endonucleases. The digests were fractionated on 0.8% agarose gels run in a horizontal slab apparatus at 0.7 V/cm for 17 h. The DNA in the gels was transferred to nitrocellulose filters (39) which were hybridized with ^{32}P-labeled SV40 DNA as described in the text. Fragments containing viral sequences were detected by radioautography for 12 h at −70°C, using presensitized Kodak XH-1 film and an intensifying screen (23). The DNA samples were as follows: each track contained 0.125 μg of high-molecular-weight DNA containing 3% SV40 sequences. (1) DNA digested with a twofold excess of HpaII; (2) undigested DNA plus 0.25 ng of HpaII-digested SV40 DNA (containing a trace of open circular SV40 DNA); (3) DNA digested with SstI; (4) DNA digested with TaqI; (5) as for (2); (6) DNA; digested with a fourfold excess of HpaII; (7) as for (3); (8) DNA digested with EcoRI; (9) undigested DNA; (10) DNA digested with a sixfold excess of HpaII.*

digests of high-molecular-weight DNA isolated from cells infected only with wild-type virus. Its appearance requires the presence of *Hpa*II endonuclease sites in the polymeric DNA as it is not found after *Hpa*II endonuclease digestion of high-molecular-weight DNA isolated from cells infected only with *dl(pm)*861. There are many possible explanations for the appearance of this band, for example, methylation of some subset of the *Hpa*II endonuclease sites in the polymeric DNA, but we have no data that can directly explain its origin.

However, given the assumption that the two types of genome replicate in the same pool, the data strongly suggest that the polymers are synthesized by a replicative rather than a recombinational mechanism. Were they produced by recombination, *Hpa*II endonuclease digestion of the polymeric SV40 DNA would have produced an easily detected ladder of bands between the positions of full-length, linear viral DNA and undigested, high-molecular-weight DNA.

DISCUSSION

After infection of permissive monkey cells, SV40 DNA sequences remain associated with cellular DNA throughout purifications which separate viral and cellular DNAs. It has been inferred from this finding (16, 18) that SV40 DNA becomes covalently integrated into the chromosomal DNA of productively infected cells. But our experiments on the nature of this association, in particular on the organization of the SV40 DNA sequences, indicate that most, if not all, of them occur in large, tandem, head-to-tail polymeric structures and not in covalent linkage with the cellular DNA.

This conclusion rests upon the following observations. (i) Heteroduplexes between the high-molecular-weight SV40 DNA and an appropriate mutant SV40 DNA probe were not formed unless the former was first digested with *Eco*RI or *Hpa*II endonucleases, enzymes which cleave SV40 DNA once. (ii) The electrophoretic mobility of the high-molecular-weight SV40 DNA is not perceptibly altered by digestion with several restriction endonucleases that cleave cellular DNA but not SV40 DNA. However, digestion with *Eco*RI, *Hpa*II, *Kpn*I, or *Taq*I endonucleases converts virtually all of the SV40 DNA into unit-length, linear molecules. (iii) The high-molecular-weight SV40 DNA is highly infectious in

the standard plaque assay, and this infectivity is increased by digestion with *Eco*RI or *Hpa*II endonucleases.

Although these experiments do not exclude the possibility that the polymeric SV40 DNA arrays are associated with cellular DNA sequences at their ends, this seems unlikely because, even though the oligomers are, on average, 10 to 20 SV40 genomes in length, characteristic heteroduplex molecules and restriction endonuclease fragments, containing the junctions of viral and cellular DNAs, should have been detected in experiments (i) and (ii).

Our data cannot eliminate the possibility that covalent integration occurs at the low level found in transformed cells, nor do they bear on whether covalently linked viral and cellular sequences exist as viral-size molecules (44). Dealing with each of these questions requires other experimental approaches. The existence of oligomeric and polymeric forms of viral DNA underscores the need for rigorous criteria for assessing integration in systems in which viral DNA replicates. The appearance in gel electropherograms of novel restriction endonuclease fragments containing viral sequences is insufficient, as they could be derived from defective viral DNA molecules. Heteroduplex analysis of the type illustrated in Fig. 4 could be helpful in establishing the existence of putative viral-cellular DNA recombinants.

Circular dimers of SV40 DNA (13, 19) and the adenovirus type 2-SV40 hybrids HEY and LEY (21, 25), each of which contains SV40 DNA sequences in tandem array, yield SV40 progeny (with monomer-length DNA) after infection of monkey cells. A similar phenomenon was found with the high-molecular-weight SV40 DNA. A plausible model to account for the infectivity in each case is a recombinational excision between homologous regions of the tandemly repeated segments yielding a circular DNA molecule. The oligomeric molecules may provide a novel substrate for a search for such recombinational excision.

The oligomers are not derived solely from parental molecules; viral DNA replication is required for their appearance. Also, the production of homopolymers, rather than heteropolymers, after infection with two distinguishable viral genomes suggests that they arise by a replicational rather than a recombinational mechanism. (This conclusion rests on the assumption that the two different viral genomes are not sequestered in noninteracting pools.) Studies of SV40 DNA replication have focused on the replication of monomer circles via "theta-form" replicative intermediates (34). Quite possibly replication can also occur by a rolling-circle mechanism (7, 12), to yield the polymers. This would be analogous to the situation with bacteriophage λ, where DNA replication via circular replicative intermediates yielding monomeric progeny molecules is followed by a rolling-circle mechanism yielding long, tandemly repeated polymeric structures (8, 38). Because of the asynchrony of SV40 infection, it would be difficult to detect a distinct switch between the two replication modes. Whether SV40 polymers play a role in the maturation and packaging of DNA into virions, as is the case for phage λ (40), is conjectural.

Martin et al. (27) have recently described circular oligomeric forms of SV40 DNA as large as hexamers, which they estimate to account for as much as 10% of the total SV40 DNA late in infection. The polymeric SV40 DNA characterized in this work differs from these circular oligomers in its sedimentation behavior in neutral sucrose gradients and electrophoretic mobility in agarose gels. Conceivably, the linear polymeric SV40 DNA was formed by cleavage of polymeric circular molecules during the isolation. We consider this unlikely as electron microscopic examination of our DNA preparations did not reveal any large, supercoiled molecules; moreover, it seems unlikely that the isolation procedure would convert all of the circular molecules to linear structures since supercoiled oligomers as large as tetramers are readily recovered by the same procedure. The sedimentation characteristics of the polymeric SV40 DNA molecules indicate that if supercoiled they would range in size from tetramers to decamers. Martin et al. (27) estimated that even late in infection the amount of circular oligomers larger than tetramers was very small, whereas the DNA that we have examined accounts for a considerable proportion of the intracellular viral DNA. Additional experiments should be done to search for circular oligomers larger than those analyzed by Martin et al. (27).

Of interest are the observations that circular dimers (13), as well as circular oligomers (27) and linear polymers, of SV40 DNA are synthesized, predominantly, late in infection, although the detailed kinetics differ somewhat. Perhaps they are produced by a common mechanism; possibly, after a period of monomer circle replication via theta-form intermediates, linear polymers are synthesized by a rolling-circle process or by some as yet unknown mechanism. Such linear oligomers could, by homologous recombination between repeat units, generate a series of smaller, circular oligomers.

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