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Structure and Formation of Circular Dimers of Simian Virus 40 DNA

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Most of the viral DNA extracted from simian virus 40 (SV40)-infected African green monkey kidney cells consists of circular molecules about 5.3 kilobases in contour length. However, about 1% of the viral DNA was found to occur as closed circular dimers that appeared to be formed, preferentially, late in infection. The monomeric units of dimers were organized in a head-to-tail, tandem arrangement; moreover, the monomeric units were not defective; i.e., they lacked deletions or other rearrangements. After infections with dimer DNA, nondefective monomers were formed. These findings suggest that dimers are not intermediates in the production of defective SV40 genomes. The majority of the dimers formed in mixed infections with two mutants were homodimers, but about 5% of the circular dimers were heterodimers and must have arisen by intermolecular recombination.

Several forms of simian virus 40 (SV40) DNA occur in cells multiplying the virus. Late in infection the majority of the viral DNA exists as covalently closed (SV40 I) and nicked (SV40 II) circular DNA of about 5.3 kilobases in contour length. Some of the viral DNA also occurs as circular dimers and higher oligomers (9, 12, 15) of the 5.3-kilobase monomer. Recently, it was noted (P. W. J. Rigby and P. Berg, manuscript in preparation) that an appreciable portion of the total viral DNA late in the infection occurs as long linear oligomers of SV40 DNA. The origin, function, and fate of these oligomeric forms are unknown. A goal of this work was to determine whether covalently closed circular dimers of SV40 DNA arise during replication or by recombinational processes. Another objective was to learn whether circular dimers are precursors of recombinant or defective viral genomes.

Using electron microscopy to analyze heteroduplex DNA structures formed from the dimers and mutant DNAs having identifiable alterations in their structure, we have shown that virtually all of the SV40 circular dimer DNA isolated late in infection consists of unaltered, monomeric units organized tandemly in a head-to-tail arrangement. Moreover, viral progeny genomes produced after infection of monkey cell cultures with the dimer DNA are principally (>98%) monomeric, circular SV40 DNA indistinguishable from the genomes used to generate the dimers. Therefore, it seems that the formation of SV40 dimer DNA molecules and regeneration of circular monomer DNA are not accompanied by an appreciable production of defective or reorganized genomes. Quite likely these processes involve homologous recombinational events.

When cells are infected with two distinguishable SV40 monomeric genomes, about 95% of the resultant dimer DNA molecules are homodimers, indicating that most of the circular dimers of SV40 DNA arise by an intramolecular recombination, probably occurring during replication; they could also arise by recombinational excision from longer oligomeric structures generated by replication (Rigby and Berg, in preparation). But about 5% of the dimer DNA molecules have one each of the infecting genomes, organized in a head-to-tail tandem arrangement, suggesting that these are produced by an intermolecular homologous recombinational event. Thus, dimers can act as intermediates in recombination between SV40 genomes (5).

MATERIALS AND METHODS

Our strain of SV40, SV-S (WT830), was obtained from K. K. Takemoto and grown as described by Mertz and Berg (13). Mutants *dl*-801 and *dl*-814 have already been reported (13, 14). Primary African green monkey kidney (AGMK) cells (Flow Laboratories) were grown in Dulbecco's minimal Eagle medium with 10% fetal bovine serum and passaged twice after 10-fold dilution before use. Plaque assays of virus and viral DNA were performed as previously described (13).

SV40 DNA was selectively extracted by Hirt's procedure (8) from AGMK cells that had been infected with 10 PFU/cell and labeled with 100 μCi of

[^{3}H]TdR at various times after infection. For analytical purposes with sucrose gradients, the viral DNA was recovered from the Hirt supernatant by two extractions with 2 volumes of water-saturated phenol and alcohol precipitation. The preparation and use of alkaline sucrose gradients for separating covalently closed circular DNA from nicked circular and linear DNA have already been described (1). To prepare purified dimer DNA, the Hirt supernatant was centrifuged (32,000 rpm, 15°C, 72 h in an SW50.1 rotor) in CsCl (ρ = 1.567 g/cm^3) containing 250 μg of ethidium bromide per ml. The covalently closed DNA obtained from the lower band was freed of cesium chloride and ethidium bromide with Dowex AG50 (X8) and precipitation with ethanol. Native dimer DNA was recovered from the covalently circular DNA fraction (10 μg) by centrifugation in a 5 to 30% sucrose gradient (12 ml) containing 10 mM Tris, 1 mM EDTA, and 1 M NaCl, pH 7.5 (TENN), at 4°C for 11 h at 38,000 rpm in the SW40 rotor. After 20-drop fractions were collected, the SV40 dimer DNA peak was precipitated with ethanol, dissolved, applied to a second 5 to 20% sucrose gradient (4 ml) in TENN, and centrifuged at 4°C for 200 min at 55,000 rpm. Fractions (6 drops) were collected, and the dimer DNA-containing fractions were pooled and precipitated as mentioned above.

Electron microscopy of heteroduplex DNA was performed according to Davis et al. (3). Restriction endonuclease digestions were carried out as previously described (13). The *Eco*RI endonuclease was provided by M. Thomas, and the *Hpa* II endonuclease was prepared and used according to Sharp et al. (16). For agarose gel electrophoresis, 0.5 μg of DNA was applied to gels (0.7 by 15 cm) of 1.4% agarose in 0.09 M Tris-borate-2.5 mM EDTA, pH 8.3, and electrophoresed at 40 V for 16 h. The gels were stained with 0.5 μg of ethidium bromide per ml and examined with UV light.

RESULTS

Kinetics of SV40 monomer and dimer DNA synthesis. AGMK cell cultures were infected with SV40 virus (10 PFU/cell), and viral DNA replication was followed by adding 100 μCi of [^{3}H]thymidine at various times. After 4 h of labeling, the viral DNA was selectively extracted according to Hirt (8). Sedimentation through an alkaline sucrose gradient permitted an estimation of the amount of ^{3}H label incorporated into monomer and dimer SV40 form I DNA (Fig. 1). There was little or no synthesis of SV40 DNA before 14 h postinfection, but monomer DNA was made at almost maximal rate by 20 h after infection, followed by a decline in the rate between 36 and 72 h. Dimer DNA lagged behind in its appearance, and its maximum rate of synthesis occurred at about 45 h after infection and then declined. Whereas dimer DNA synthesis was only about 1 to 2% that of the monomer 30 h after infection, nearly

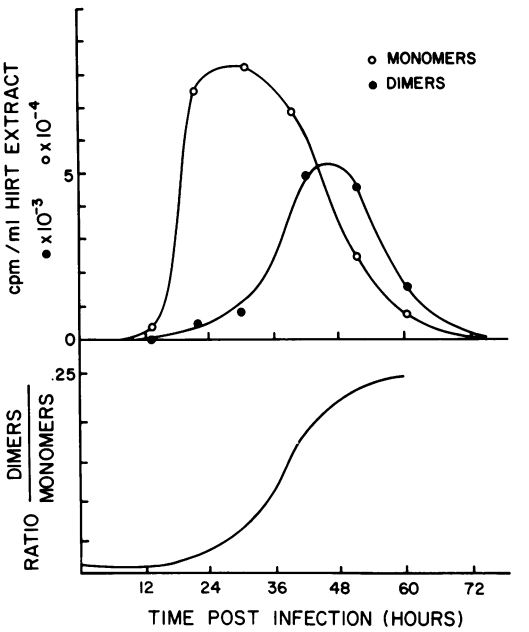

FIG. 1. *Kinetics of synthesis of SV40 monomeric and dimeric DNA species during infection of AGMK cells. AGMK cells, infected at high multiplicity with nondefective SV40, were labeled at various times with 100 μCi of [^{3}H]thymidine. After a 4-h labeling period, the viral DNA was extracted at the indicated times and sedimented through alkaline sucrose gradients as described in the text to determine the amount of labeled monomer and dimer DNA. The total ^{3}H label in the monomer and dimer DNA species is recorded as a point for each labeling period. (○ and ●) ^{3}H label in monomer and dimer DNA, respectively. The scale for the dimer DNA points is 10-fold higher than for the monomer DNA points.*

20% of the viral DNA being made at 60 h was dimer molecules. These results are in agreement with those of other studies (12).

Isolation of SV40 dimer DNA. To permit the structural analysis of SV40 dimer DNA, the DNA was purified free of cellular and other viral DNAs. Infected cells were labeled from 40 to 60 h after the infection, and DNA was extracted at the end of this labeling period. The dimer DNA was isolated from the Hirt extracts by equilibrium centrifugation followed by two successive velocity centrifugations in neutral sucrose gradients (see Materials and Methods). After the first velocity centrifugation (Fig. 2A), the pooled dimer DNA fraction was about 80% dimer circles relative to monomers, as judged by electron microscopic examination; after the second centrifugation (Fig. 2B), the fast-sedimenting peak contained 90 to 95% dimer circles.

Circular dimers have head-to-tail, tandemly

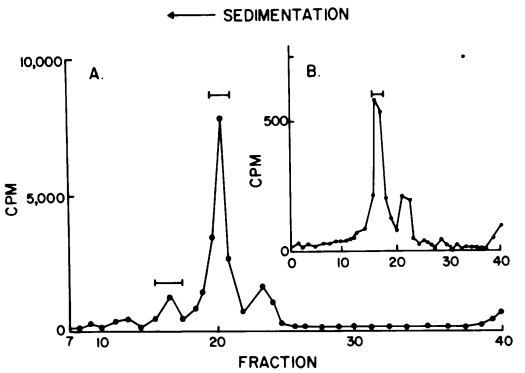

FIG. 2. *Separation of circular monomer and dimer DNA by sucrose gradient centrifugation. ^{3}H-labeled supercoiled DNA obtained from SV40-infected AGMK cells (see text) was sedimented in a 5 to 30% sucrose gradient (A); the DNA in fractions 14 to 16 was precipitated with alcohol and recentrifuged in a 5 to 20% sucrose gradient (B). The DNA recovered from fractions 16 to 17 was greater than 90% dimers as judged by electron microscopy.*

repeated monomers. The arrangement and structure of the monomeric units in the dimer DNA can be analyzed most readily by examining heteroduplexes in the electron microscope. DNA from an SV40 mutant, *dl*-814, contains a deletion of the segment between map coordinates 0.73 to 0.97 and a duplication of the segment bounded by map positions 0.62 to 0.71. Heteroduplex molecules, formed between *Eco*RI endonuclease-generated single strands of mutant *dl*-814 and single-stranded linear or circular wild-type DNA, were characteristic and easily detected by electron microscopy (Fig. 3). The wild-type strand had a deletion loop of 0.24 SV40 length, the mutant strand had a "movable" duplication loop (2) of 0.09 SV40 length, and the relative order of the single-strand loops defined the directionality of the SV40 DNA.

Heteroduplex molecules formed with the circular dimer DNA and *Eco*RI endonuclease-generated linear *dl*-814 DNA had characteristic structures with two sets of deletion and duplication loops (Fig. 4). Among 200 such heteroduplex structures examined, all had the alternating arrangement of small and large loops shown in Fig. 4, establishing the head-to-tail arrangement of the two monomer units. The only variable was the distance between adjacent small and large loops, a property inherent in tandem duplications (2).

Dimers contain principally nondefective monomers. One of several possible paths to the formation of dimers is by recombination between sister arms of a replicative intermediate followed by continued replication and normal segregation (Fig. 5A). If the recombination oc-

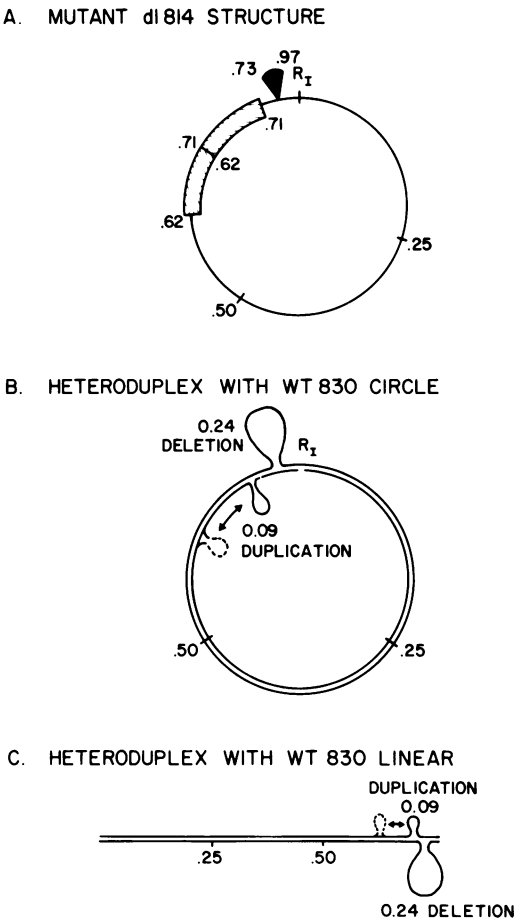

FIG. 3. *(A) Genome structure of mutant dl-814. The mutant contains a tandem duplication of the map region from 0.62 to 0.71 and a deletion of the region from 0.73 to 0.97, and therefore is 0.85 SV40 fractional length. It lacks the Hpa II endonuclease cleavage site and contains two copies of the origin of DNA replication. (B) Expected structure of a circular heteroduplex between dl-814 and WT830. Linear dl-814 DNA, prepared by cleavage with EcoRI restriction endonuclease, was denatured and renatured in the presence of nicked circles of WT830 DNA. The heteroduplexes have a single-strand loop 0.24 SV40 unit in length at the site of the deletion and another loop, 0.09 unit in length, resulting from the duplication at a somewhat variable location (see text). The order of the smaller and larger loops corresponds to the clockwise direction on the SV40 genetic map. (C) Expected structure of a linear heteroduplex between EcoRI endonuclease-generated linear DNA molecules of dl-814 and WT 830. The deletion and duplication loops, and their significance with respect to map order, are as discussed (B).*

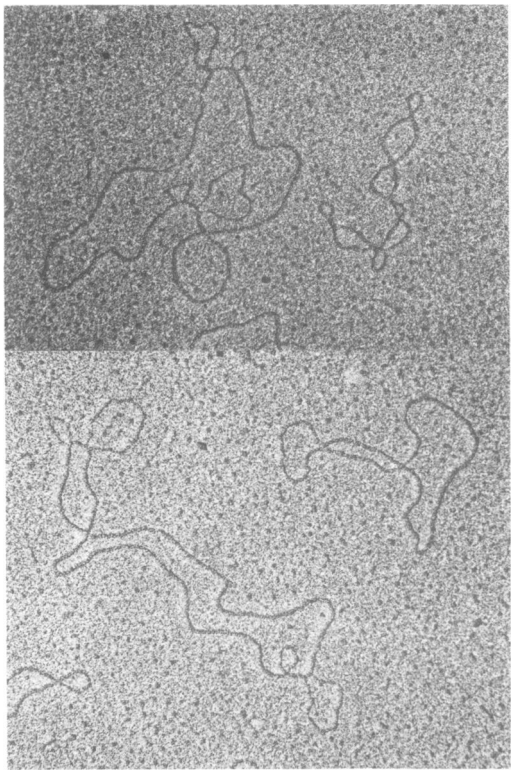

FIG. 4. *Electron micrographs of heteroduplexes between a WT830 dimer DNA circle and a monomer linear DNA of dl-814. The circular dimer DNA was obtained as described in the text, nicked, denatured, and annealed to denatured linear dl-814 DNA made with EcoRI endonuclease. Each of the heteroduplexes shown has two linear dl-814 DNA strands annealed in tandem to the dimer circle. The order of the smaller duplication and longer deletion loops verifies the head-to-tail arrangement of the monomer units.*

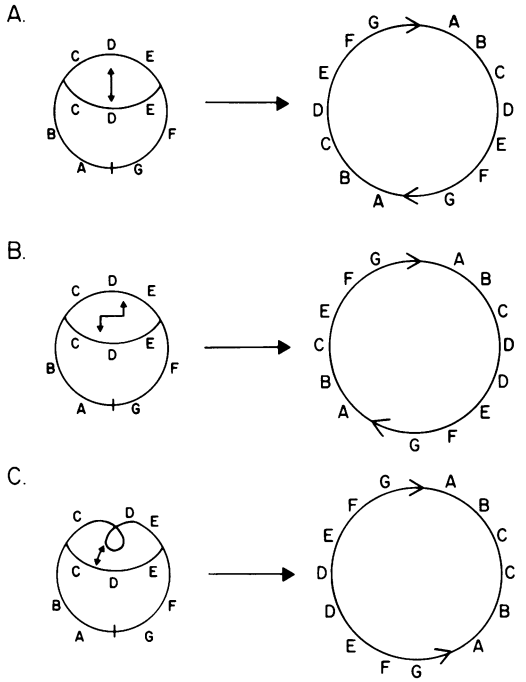

FIG. 5. *Hypothetical crossover events between newly synthesized DNA strands of replicative intermediates (theta forms). Continued replication of intermediates that undergo such intramolecular recombination leads to the formation of dimer DNA progeny. (A) Homologous crossover event leads to a perfect head-to-tail tandem repeat of the entire genome. (B) Nonhomologous crossover leads to a dimer of identical length, but one segment of the dimer contains a duplicated region and the other segment has the same region deleted. (C) Inverted, nonhomologous crossover leads to inversions of parts of the genome in the circular dimer DNA.*

curs between homologous regions, the product is a dimer with two identical monomeric units in head-to-tail arrangement (Fig. 5A). A nonhomologous recombination between sister arms would also yield a dimer-length molecule, but one monomeric unit would have a deletion and the other a corresponding duplication (Fig. 5B). More complicated types of dimer molecules, e.g., inverted, repeated segments, could result from a nonhomologous inverted recombination between the sister arms of the replicative form (Fig. 5C).

Aberrant structures of the type produced in Fig. 5B would easily be detected as heteroduplexes of dimer DNA circles and *dl*-814, since they would contain additional loops or loops of different size. Among the 200 heteroduplexes examined, there were two molecules containing a defective monomer; this is the same frequency with which defectives occurred in the monomer population. There were also no inverted repeat segments as would occur by the mechanism in Fig. 5C, since no hairpin loops with double-strand stems were observed (0/200) when single-strand dimer circles were examined under conditions known to produce "snapback" structures from inverted repeats (17).

SV40 dimer DNA is infectious and yields normal monomer progeny. The specific infectivity (PFU/mole of DNA) of SV40 monomer and dimer DNA is not distinguishable within the error of the plaque assay (1.2×10^6 PFU/μg of monomer, 5×10^5 PFU/μg of dimer). Since the progeny virion DNA and the intranuclear viral DNA produced by infection with dimer DNA are principally monomer length (>98%), the generation of monomer-length molecules

from dimers must occur quite readily in most infected cells (10). But does this process produce substantial amounts of defective monomers? Apparently not, since (i) the progeny in each of 78 different plaques produced by infection of monolayers with dimer DNA gave the same number of plaques upon reinfection in the presence or absence of *tsA*30 or *tsB*4 helper virus; (ii) viral DNAs isolated from monolayers infected with monomer or dimer DNA (after 8 to 9 days) were indistinguishable in their electrophoretic mobility in agarose gels and sedimentation properties in alkaline sucrose gradients; and (iii) heteroduplexes formed between linear wild-type DNA and monomer DNA produced by infections with dimer DNA had fewer than 2% nonhomologous structures, the value usually found after infection with monomer DNA (data not shown). Whatever the mechanism for generating monomers from dimers, it does not appear to produce a detectable number of defective monomers.

Homodimers and heterodimers are formed after infection with two distinguishable viral genomes. Other workers were unable to test for the production of heterodimers, since the genetic analysis of individual dimer DNA molecules was impossible (4). To explore the origin of the SV40 dimer DNA, cells were infected with two strains of SV40 virus, WT830 and *dl*-801 (14), whose DNAs can be distinguished by their sensitivity to *Hpa* II endonuclease. If the dimers are formed only from either one of the two parental genomes, they will be either completely resistant to cleavage by *Hpa* II endonuclease or cleaved twice to produce monomer-length linear DNAs. If heterodimers occur, they will be cleaved only once to yield dimer-length linear DNA.

AGMK cell cultures were infected with approximately equal numbers of WT830 and *dl*-801 (total multiplicity of infection, 10 PFU/cell), and after 60 h the monomer and dimer DNA was isolated as described in Materials and Methods. Limit digests of the monomer DNA with *Hpa* II endonuclease converted 32% of the DNA to linear molecules, leaving the remainder as circular DNA (Table 1); thus, both genomes replicated nearly equally well. When the same *Hpa* II endonuclease digestion was carried out with dimer DNA as substrate, 61.3% of the dimers were resistant to any cleavage, 31.3% were cleaved twice, and 7.4% were cleaved only once (Table 1).

Because the number of putative heterodimers was low, it was important to be certain that they did indeed contain one monomer from the wild-type parent and the other from *dl*-801. The singly cleaved dimer DNA might be incompletely digested molecules (although >99% of WT830 monomers are cleaved to linear structures under the same conditions) or homodimers of *dl*-801 that were inadvertently converted to dimer-length linear DNA.

Consequently, a second infection of AGMK cells, but with mutant *dl*-814 and *tsA*30 virus, was performed as before, and the monomer and dimer DNA was isolated 60 h after infection as

TABLE 1. *Proportion of monomer and dimer DNA recovered from AGMK cells mixedly infected with SV-S (WT830) and dl-801*[a]

DNA species	No./total no. of monomers	%	No./total no. of dimers	%		
				Exptl	Rec[b]	Rep[c]
SV-S monomers	157/492	32				
dl-801 monomers	335/492	68				
SV-S/SV-S homodimers			115/367	31.3	10	32
SV-S/*dl*-801 heterodimers			27/367	7.4	44	0
dl-801/*dl*-801 homodimers			225/367	61.3	46	68

[a] Monomer and dimer DNAs were isolated as described in the text. The DNAs were cleaved with *Hpa* II restriction endonuclease and mounted for electron microscopy. The various monomer and dimer species were scored as follows: the ratio of SV-S to *dl*-801 monomers was determined from the ratio of monomer-length linear to circular molecules, respectively; among the dimers, the number of SV-S/SV-S homodimers was equal to half the number of monomer-length linears, the number of SV-S/*dl*-801 heterodimers equaled the number of linear dimers, and the number of *dl*-801/*dl*-801 homodimers equaled the number of dimer-length circles.

[b] Predicted assuming that intermolecular recombination occurs at random between parental genomes. Percentages shown are the products of the percentages of the monomers that must recombine to yield that dimer species.

[c] Predicted assuming that dimers are formed only by intramolecular events during replication. In this case the percentages shown for the homodimers are equal to the percentages of the monomers that form them.

already described. Digestion of the monomer DNA with *Hpa* II endonuclease converted 15% of the DNA to linear molecules, indicating that the ratio of *dl*-814 to WT830 in the monomer pool was 5.7. Similar digestions of the dimer DNA yielded doubly resistant, doubly cleaved, and singly cleaved molecules as before (Table 2).

In this instance, however, it could be shown directly by heteroduplex analysis that the dimer-length DNA contained the two parental genomes. *Eco*RI endonuclease-cleaved WT830 DNA and the *Hpa* II endonuclease-cleaved dimer DNA were mixed, denatured, and renatured, and the heteroduplexes were mounted for electron microscopy. Two types of heteroduplexes were found with full-length *Hpa* II endonuclease-cleaved dimers and *Eco*RI endonuclease-cleaved WT830 monomer DNAs. In one type, the WT830 monomer strand annealed to the middle of the dimer strand to produce a linear heteroduplex with single-stranded "tails" (Fig. 6). These molecules contained the expected deletion and duplication loops generated by the *dl*-814 mutant sequence. Thus, an interior segment of the dimer strand originated from the infecting *dl*-814 genome.

In the other type of heteroduplex, the WT830 monomer DNA strand annealed to the dimer and formed a circular heteroduplex (Fig. 6). As in the first type of heteroduplex, the deletion and duplication loops characteristic of *dl*-814 were readily observed; here, too, the segment of the dimer that was not cleaved by *Hpa* II endonuclease must have originated from the mutant *dl*-814 parent. Measurements of the lengths of DNA from the deletion loop to the ends of the dimer (lengths A and B in Fig. 6) confirmed these conclusions. In both types of heteroduplexes, the predicted lengths of A and B are 1.00 and 0.76 SV40 fractional length, respectively; the observed values (nine molecules of the first type and five molecules of the second type) were 1.04 ± 0.021 and 0.74 ± 0.026 (Fig. 7). We conclude that the single cleavage of the dimers occurred at the *Hpa* II endonuclease cleavage site and that this portion of the dimer must have originated from the *tsA*30 parental genome. Therefore, the singly cleaved dimer DNA molecules are true heterodimers formed by intermolecular recombination between the two infecting parental molecules. Thus, although most of the dimers seemed to arise by events occurring during replication, e.g., homologous crossover between sister strands of replicative intermediates, dimers also could arise by a reciprocal intermolecular recombinational event. Our estimate of the frequency of intermolecular recombination would, in fact, be too low if the progeny of the two different DNAs used to infect the cells were confined to secluded pools within the cell nucleus. Our results, however, are not incompatible with the observed frequency of genetic recombination between temperature-sensitive mutants (5).

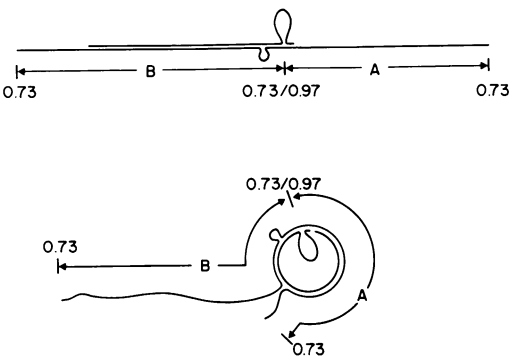

FIG. 6. *Predicted structures of heteroduplexes between EcoRI endonuclease-generated linear WT830 DNA and Hpa II endonuclease-cleaved linear heterodimer DNA (see Results). The map coordinates of several points on the molecule are shown. If cleavage of the dimer circular DNA occurs at the Hpa II endonuclease cleavage site (0.73 on the SV40 DNA map), then lengths A and B are 0.76 and 1.00 SV40 length, respectively.*

TABLE 2. *Proportion of monomer and dimer DNA fractions recovered from AGMK cells mixedly infected with tsA30 and dl-814*[a]

DNA species	No./total no. of monomers	%	No./total no. of dimers	%		
				Exptl	Rec	Rep
dl-814 monomers	249/294	85				
*tsA*30 monomers	45/294	15				
*tsA*30/*tsA*30 homodimers			30/237	13	2	15
dl-814/*tsA*30 heterodimers			12/237	5	26	0
dl-814/*dl*-814 homodimers			195/237	82	72	85

[a] See Table 1 for details.

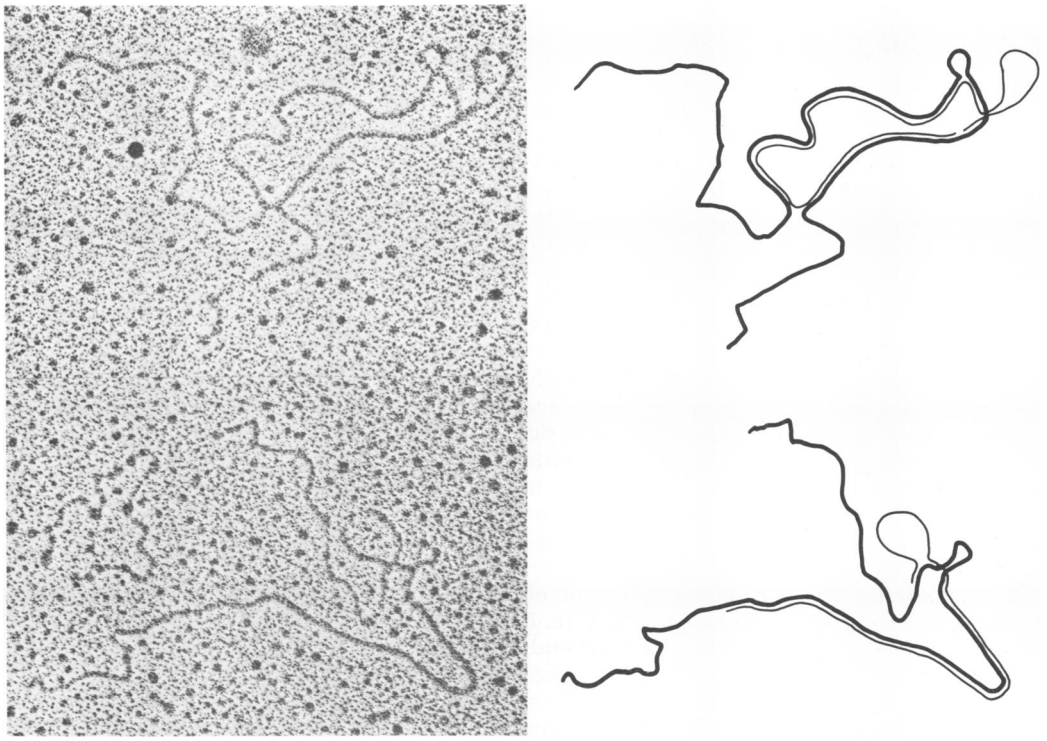

FIG. 7. *Electron micrograph and diagram of heteroduplexes between EcoRI endonuclease-generated linear WT830 DNA and Hpa II endonuclease-cleaved linear heterodimer DNA. The dimer DNA was isolated from cells infected with tsA30 and dl-814 virus. The dimer DNA was cleaved with Hpa II endonuclease, denatured, and renatured with linear WT830 DNA generated by EcoRI endonuclease. In the heteroduplex molecules shown, the WT830 linear DNA has annealed to the dl-814 portion of the dimer. The lengths of the single-stranded tails were measured as described in the text (see Fig. 6 and Results).*

DISCUSSION

Neither the formation of SV40 circular dimer DNA nor the conversion of dimers to circular monomers generated frequent or substantial alterations of the viral genome structure. Virtually all of the dimer DNA had a tandem head-to-tail arrangement of the monomer units, and fewer than 1% of the dimers contained deletions, insertions, inversions, or substitutions, a figure similar to that found in monomer DNA. If nonhomologous recombination occurs during the monomer-dimer interconversions, it happens at a frequency too low to detect by examining the fine structure of these molecules with heteroduplex analysis. Quite likely the emergence of defective DNA molecules follows one or more relatively rare events and selective growth (i.e., during replication or encapsidation) of these altered genomes.

What is the origin of circular dimer and even higher oligomeric SV40 DNA (4, 9, 12, 15)? One possibility is intermolecular recombination between monomers to form dimers, between dimers and monomers or dimers to produce trimers and tetramers, etc. If all the circular viral DNA in the pool is equally available for intermolecular recombination (there is no information for or against this assumption), then cells infected with different viral genomes should produce heterodimers and heterooligomers. Our results prove that intermolecular recombination does occur, but at only about one-seventh the frequency predicted from the abundance of the two parental genomes.

The bulk of the SV40 circular dimers are homodimers and could arise by intramolecular recombination or by an alternate path of replication. For example, recombination between homologous regions of the newly replicated sister strands in replicative intermediates (see Fig. 5A), or a failure to segregate the two daughter DNA molecules properly at the termination of replication, could result in the produc-

tion of homodimers. Aberrant replication via a "rolling-circle" model (7) could also generate oligomeric and circular molecules. Of particular interest in this regard are Rigby and Berg's findings (in preparation) that about 30% of the total SV40 DNA present late in the lytic infection occurs as long linear DNA molecules containing more than 20 tandem head-to-tail repeats of the SV40 genome; here, too, mixed infections with two distinguishable viral genomes yielded predominantly homopolymers. Conceivably, circular dimers and oligomers arise by recombinational excision between homologous regions of the linear SV40 DNA. Such a mechanism has already been invoked to account for the frequent production of monomeric SV40 DNA after infection of monkey cells with the adenovirus 2-SV40 hybrid ($Ad2^{+}HEY$), a hybrid viral genome that contains two or more tandem head-to-tail repeats of the SV40 genome (11).

The apparent disproportionate replication of SV40 dimer relative to monomer DNA that occurs late in infection may also have several explanations. Having two origins of replication per molecule could permit preferential replication of these structures, as is the case for many of the defective genomes having duplications of this region. In this regard, the conversion of "minicircular" SV40 genomes into oligomers, and the accumulation of the longer oligomeric species (6), would be similar to the formation and accumulation of SV40 dimers. Alternatively, replication of dimer DNA might increase at late times because the dimers, unlike the monomers, cannot be encapsidated and, therefore, have an increasing probability of entering the replicating pool. Since the long linear oligomeric DNA accumulates late in the infection, the preferential labeling of dimers could reflect the relatively rapid replication of the oligomers and excision of the dimers.

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