

Isolation and propagation of a segment of the simian virus 40 genome containing the origin of DNA replication

(origin of simian virus 40 DNA replication/S1 endonuclease cleavage of heteroduplexes/repeated simian virus 40 DNA segments)

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ABSTRACT Heteroduplex DNA molecules formed from two DNAs that differ from each other by a deletion can be cleaved at the mismatched region (a deletion loop) with the single-strand-specific S1 endonuclease. A heteroduplex DNA molecule, constructed from the DNA of a simian virus 40 (SV40) mutant with a deletion of the map region 0.54-0.55 and the DNA of a second SV40 mutant having a deletion of the map segment 0.70-0.73, is cleaved twice with S1 endonuclease. One of the products is a DNA fragment of about 0.13 the length of SV40 DNA which contains the origin of SV40 DNA replication (0.67 on the SV40 DNA map).

Infection of cultured CV-1 monkey kidney cells with the fragment and intact SV40 DNA yields, in addition to the expected full-length wild-type circular DNA molecules, a population of discrete size circular DNAs whose lengths are very nearly integral multiples of the infecting fragment. Restriction endonuclease digestion patterns and heteroduplex analysis indicate that the small circular DNAs are oligomers of the infecting fragment, organized in "head-to-tail" and, less frequently, "head-to-head" arrangement.

Heteroduplex DNA molecules formed from DNAs that differ from one another by a deleted, added, or substituted sequence can be cleaved at the mismatched region by the single-strand-specific S1 endonuclease. This fact has already been used to map the location of such alterations in the simian virus 40 (SV40) genome (1, 2). We suggested earlier (1) that a segment of DNA between two deletion sites could be isolated by an adaptation of this procedure.

This supposition has been examined using a heteroduplex molecule having two deletion loops. It was formed from an SV40 mutant having a deletion of the region 0.54-0.55 on the SV40 map and another mutant with a deletion of the region 0.70-0.73. After treatment of the heteroduplex structure with S1 endonuclease, a small fragment corresponding in length to the distance between the two deletions was produced. Since the segment between the two deleted regions contains the origin of SV40 DNA replication, it was possible to propagate the segment in CV-1P cells.

MATERIALS AND METHODS

Cells and Viruses. The source and the procedures for growing CV-1P monkey cells have been described (3). The wild-type SV40, which served as parent for the deletion mutants, was a plaque-purified derivative of the SVS strain (4). The deletion mutants *dl* 883 and *dl* 894 have already been described (2); their deletions extend from 0.54 to 0.55 and 0.70 to 0.73 on the SV40 map, respectively.

DNA and Enzymes. SV40 DNA was extracted (5) from

CV-1P cells when >90% of the cells showed cytopathic effect. Covalently closed viral DNA [SV40(I)] was obtained by equilibrium centrifugation in a CsCl (1.56 g/cm³)-ethidium bromide (200 µg/ml) gradient followed by removal of the ethidium bromide with Dowex 50 (6).

*Eco*RI-, *Hpa* II-, and *Hind* III endonucleases, as well as S1 endonuclease, were prepared and used according to published protocols (*Eco*RI (7, 8), *Hpa* II (9), *Hind* III (10, 11), and S1 (1, 12)). One unit of S1 endonuclease releases 1 nmol of nucleotides per min at 37°, from sonicated, denatured salmon sperm DNA at pH 4.4 in the presence of 0.5 mM Zn⁺⁺ and 280 mM Na⁺.

Preparation of Heteroduplex DNA and Its Cleavage with S1 Endonuclease. Equal amounts of *Eco*RI endonuclease-cleaved *dl* 883 and *dl* 894 DNAs (5 µg/ml of each) were denatured in 0.1 M-NaOH. After 10 min at room temperature the solution was titrated to pH 7-8 with HCl, the Na⁺ concentration was raised to 300 mM, and the DNA was annealed at 68° for 3 min. The reannealed DNA was treated with S1 endonuclease (1400 units/ml) at room temperature in the presence of Zn⁺⁺ (4.5 mM), Na⁺ (280 mM), and CH₃COO⁻ (30 mM) at pH 4.4. The reaction was terminated after 30 min by adding 0.05 volume of Tris base (2 M) and increasing the Na⁺ concentration to 500 mM. To reduce the volume and lower the Na⁺ concentration prior to electrophoresis, we precipitated the DNA at -20° after the addition of yeast RNA (20 µg/ml) and 2 volumes of ethanol.

Gel Electrophoresis. Agarose gels (1.2%, 6 × 200 mm) were prepared in Tris-borate buffer (89 mM Tris-OH, 89 mM boric acid, 2.5 mM EDTA, pH 8.2) (7). Samples were applied in 50 µl of Tris-borate buffer containing sucrose (20% wt/vol). After electrophoresis, DNA bands were stained with ethidium bromide and visualized under a short wavelength ultraviolet light; the fluorescent bands were photographed using a Vivitar orange (02) filter and Polaroid type 105 film.

Infection of Monkey Kidney Cells with DNA. Monolayers of CV-1P cells (10⁶ cells) were infected with either SV40(I) DNA (5 × 10⁻³ µg) or the fragment of the SV40 genome (2.5 × 10⁻³ µg) in the presence of DEAE-dextran, as previously described (3).

RESULTS

Short DNA fragments containing the SV40 origin of DNA replication can be isolated

S1 endonuclease can cleave heteroduplex DNAs at the site of a single-stranded loop (1). Logically, DNA heteroduplex molecules formed from two DNAs, each with a different and nonoverlapping deletion, should contain two single-stranded loops and, therefore, be cleaved twice by S1 endonuclease (Fig. 1a). The availability of a collection of viable deletion mutants that bracket the origin of SV40 DNA replication (Orep) pro-

Abbreviations: SV40, simian virus 40; Orep, origin of SV40 DNA replication; SV40(I) DNA, covalently closed SV40 DNA.

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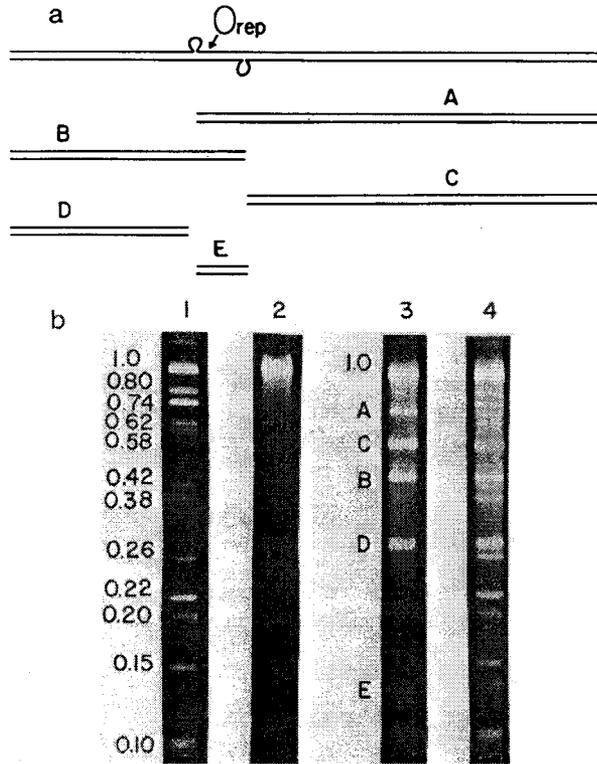


FIG. 1. Isolation of a segment of the SV40 genome containing the origin of DNA replication using S1 endonuclease. (a) Diagram of the expected cleavage products of a heteroduplex molecule prepared from two mutant linear DNAs whose deletions bracket the SV40 origin of DNA replication (Orep). (b) Cleavage products generated by S1 endonuclease digestion of *EcoRI* endonuclease-cleaved mutant DNAs. Samples of 0.2–0.4 μ g of DNA were applied to each agarose gel; electrophoresis was for seven hours at 60 V. Gel 1: marker fragments. These include *EcoRI* endonuclease-generated SV40 linear DNA, fragments obtained by sequential cleavage of SV40 DNA with *Hpa* II and *EcoRI* endonucleases, fragments obtained by partial cleavage of SV40 DNA with *Hpa* I endonuclease, and fragments obtained by cleavage of SV40 DNA with the *Hind* II + III endonucleases. Gel 2: S1 endonuclease-treated homoduplexes. Gel 3: S1 endonuclease-treated heteroduplexes formed between *dl* 883 and *dl* 894 DNA. Gel 4: same as gel 3 plus marker fragments. The letter designations of the DNA bands in gel 3 are those used in Fig. 1a.

vided an opportunity to test that supposition and to isolate that small segment of the viral genome (Fig. 1a).

Accordingly, heteroduplex DNA was prepared using *EcoRI* endonuclease-generated linear molecules from *dl* 883, an SV40 deletion mutant lacking the region 0.54–0.55 on the SV40 map, and *dl* 894, another deletion mutant lacking the region 0.70–0.73. After cleavage of the heteroduplex DNA with S1 endonuclease, the expected five fragments (Fig. 1a) were readily detected by agarose gel electrophoresis (Fig. 1b, gels 3 and 4). Two sets of fragments were produced by cleavages at only one of the two deletion loops (fragments A and D or fragments B and C in Fig. 1a and b), and one additional fragment, which presumably contained the SV40 Orep (fragment E in Fig. 1a and b), was produced by cleavages at both deletion loops. Table 1 shows that the observed fragment lengths (expressed in SV40 fractional length) agree well with the expected values based on prior mapping data (2).

We cannot account for the production of two small fragments (0.12 and 0.13 SV40 fractional length) in the S1 endonuclease digestion (Fig. 1b, gel 3 and 4). This result was obtained in di-

Table 1. Fragments generated by S1 endonuclease cleavage of *dl* 883 \times *dl* 894 heteroduplex DNA

DNA fragment*	Fragment size (SV40 fractional length)	
	Predicted†	Found‡
A	0.69	0.69
B	0.54	0.55
C	0.43	0.45
D	0.28	0.29
E	0.15	0.13, 0.12

* The letter designations of the fragments are those assigned in Fig. 1.

† The predicted lengths were calculated taking into account that the single-stranded loops of the heteroduplexes are digested; a correction was made for the expected shortening of fragments due to "nibbling" by S1 endonuclease (1).

‡ Determined from the fragments' electrophoretic mobilities using SV40 DNA fragments of known lengths as standards; the lengths have been corrected for "nibbling" by S1 endonuclease (1).

gests of two separately prepared heteroduplex preparations from the mutant DNAs. Perhaps one of the mutant DNAs contains two closely spaced deletions (e.g., *dl* 894 in the region 0.70–0.73). In that case heteroduplex structures with three single-stranded loops would be produced and random cleavage at two of the three sensitive sites would produce two small fragments differing in length by the distance between the two closest deletion sites.

The fragment containing the SV40 origin of DNA replication can be propagated

The smallest fragment (fragment E, Fig. 1a and b) should contain the SV40 Orep. We determined if that fragment could replicate *in vivo* by recovering it from the agarose gel (the 0.12 and 0.13 SV40 fractional length fragments were pooled) and using it to infect CV-1P monolayers. Cells were infected with SV40(I) DNA alone, with the fragment alone, or with a mixture of SV40(I) DNA and the fragment. Extracts (5) from cells infected with SV40(I) DNA alone contained a single DNA species with an electrophoretic mobility characteristic of SV40(I) DNA (Fig. 2a, gel 1). There was no detectable small circular DNA from the comparable extract of cells infected with the fragment alone, but the production of very small amounts (<0.5 μ g per 10^7 cells) would have gone undetected. Extracts from cells infected with the fragment plus SV40(I) DNA contained a population of small, circular DNAs in addition to SV40(I) DNA (Fig. 2a, gel 2). Virus stocks were prepared both from cells infected with SV40(I) DNA alone and from cells infected with the fragment plus SV40(I) DNA. These stocks were used to infect a second set of CV-1P cells; here, too, only SV40(I) DNA was found in the cells receiving virus obtained from the original infection with SV40(I) DNA alone (Fig. 2a, gel 3), and both SV40(I) DNA and small circular DNAs were found after infection with virus obtained from the mixedly infected cells (Fig. 2a, gel 4).

The small, closed-circular DNAs are oligomers of a segment containing the SV40 origin of DNA replication

EcoRI endonuclease cleaves the SV40(I) DNA in each of the DNA preparations to full-length linear structures, and these can be removed from the uncut circular DNA by centrifugation in a CsCl-ethidium bromide gradient. The uncut circular DNA consists of several different size molecules after the first in-

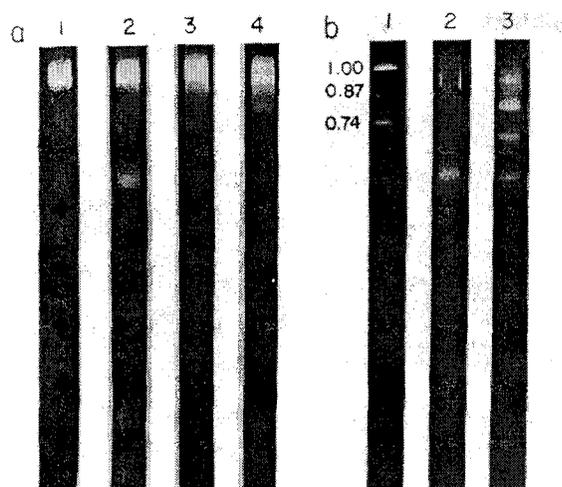


FIG. 2. Analysis of viral DNAs produced in cells infected with SV40(I) DNA and fragments of SV40 DNA containing the origin of DNA replication. (a) Electrophoretic analysis of total closed-circular DNAs isolated from infected CV-1P cells. Cells were infected and viral DNAs were extracted and purified as described in *Materials and Methods*. Samples of 1.0 μ g of DNA were applied to each gel and electrophoresed for 12 hr at 50 V. Gel 1: closed-circular DNA from cells infected with SV40(I) DNA alone. Gel 2: closed-circular DNA from cells infected with SV40(I) DNA plus the fragment. Gel 3: closed-circular DNA from cells infected with virus obtained from the original infection with SV40(I) DNA alone. Gel 4: closed-circular DNA from cells infected with virus obtained from the original infection with SV40(I) DNA plus the fragment. (b) Electrophoretic analysis of *Eco*RI endonuclease-resistant DNAs from infected CV-1P cells. The same DNA preparations described in Fig. 2a were cleaved with *Eco*RI endonuclease, and the uncut, closed-circular DNA was isolated by equilibrium centrifugation in a CsCl-ethidium bromide gradient (*Materials and Methods*). Samples of 0.2 μ g of DNA were applied to each gel and electrophoresed for 12 hr at 50 V. Gel 1: closed-circular marker DNAs. These include full-length SV40(I) DNA and several deletion mutant DNAs of 0.87 and 0.74 SV40 fractional length. Gel 2: *Eco*RI endonuclease-resistant, closed-circular DNA derived from the DNA shown in gel 2 of Fig. 2a. Gel 3: *Eco*RI endonuclease-resistant, closed-circular DNA derived from the DNA shown in gel 4 of Fig. 2a.

fection (Fig. 2b, gel 2), and there is clearly an increase in the size of these molecules after the second passage (Fig. 2b, gel 3). Although there are many minor species in the second passage DNA, the majority of the *Eco*RI endonuclease-resistant DNA migrated in four broad bands of approximately 0.56, 0.70, 0.83, and 0.96 SV40 fractional length. A reasonable inference from this result is that these DNA molecules contain segments of about 0.12–0.14 SV40 fractional length that are repeated four to seven times.

If the *Eco*RI endonuclease-resistant DNAs obtained from these mixed infections have multiple repeats of the segment containing the SV40 Orep, they probably also contain multiple repeats of the *Hind* III endonuclease cleavage site that occurs at 0.655 on the SV40 DNA map (13). Consequently, digestion of the *Eco*RI endonuclease-resistant DNA with *Hind* III endonuclease should generate discrete, small, linear DNA fragments. Fig. 3, gel 2, shows that *Hind* III endonuclease digestion of the DNA yields predominantly fragments of 0.13 and 0.08 SV40 fractional length, though others are also evident. Thus, the *Eco*RI endonuclease-resistant closed-circular DNA contains molecules with repeated segments 0.13 and 0.08 SV40 fractional length.

What is the orientation of the repeating segments in the multimeric circles? Are they arranged "head-to-tail" or do both

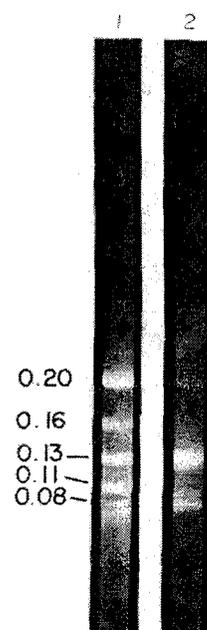


FIG. 3. *Hind* III endonuclease cleavage of *Eco*RI endonuclease-resistant DNAs produced in infections of CV-1P cells with SV40(I) DNA plus the fragment containing the origin of DNA replication. The DNA preparation shown in gel 3 of Fig. 2b was digested with *Hind* III endonuclease. A sample of 0.2 μ g of DNA was electrophoresed on an agarose gel for 12 hr at 50 V. Gel 1: *Eco*RI endonuclease-cleaved SV40 DNA as length markers. Gel 2: *Hind* III endonuclease cleavage products derived from the *Eco*RI endonuclease-resistant DNA shown in gel 3 of Fig. 2b.

"head-to-tail" and "head-to-head" configurations occur? *Eco*RI endonuclease-resistant circular DNA that contained a single-strand break was denatured and mounted for examination by electron microscopy without renaturation. If the repeating segments are arranged in a "head-to-tail" configuration only, then single-stranded open circular and linear molecules would be seen; if they contain any "head-to-head" joints, the molecules will "snap back" spontaneously and form structures that have single- and double-stranded regions. Both types of molecules were observed in large numbers: relaxed, single-stranded circles ("head-to-tail" arrangement) and circles containing a variety of "snap back" structures because of segments arranged in both "head-to-tail" and "head-to-head" configurations (Fig. 4).

Direct observation of heteroduplexes formed from linear SV40 DNA and the *Hind* III endonuclease-generated fragments establishes that the fragments are derived from the region of the SV40 genome containing the Orep (Fig. 5). When *Eco*RI endonuclease-cleaved linear molecules of SV40 DNA are annealed to the *Hind* III endonuclease-generated fragments, heteroduplexes with a small double-stranded loop (about 0.10–0.15 SV40 fractional length) are seen about one-third of an SV40 DNA length from one end (Fig. 5a and c). Heteroduplexes formed with *Hpa* II endonuclease-generated linear SV40 DNA and the *Hind* III endonuclease-produced small fragments have about the same size double-stranded loop very near one end (Fig. 5b and d).

The structure of these heteroduplexes can be rationalized if the *Eco*RI endonuclease-resistant DNA that is generated after infection with the fragment containing the SV40 Orep is an oligomer of that fragment. If we designate its structure as ABCDE, then the oligomers are (ABCDEABCDE)_n [and some (ABCDEEDCBA)_n]. Since the *Hind* III endonuclease cleavage

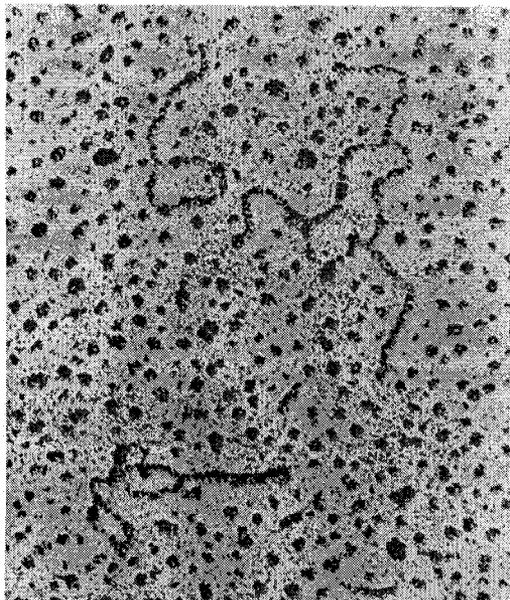


FIG. 4. "Snap back" molecules present in the single-stranded DNA derived from the *Eco*RI endonuclease-resistant DNA from cells infected with SV40(I) DNA plus the fragment containing the origin of DNA replication. The *Eco*RI endonuclease-resistant preparation shown in Fig. 2b, gel 3 was exposed to ultraviolet light to introduce on the average one single-stranded cleavage per molecule. The DNA was denatured in NaOH (0.1 M) for 10 min at room temperature. Then the pH was adjusted to 8.5 with Tris-HCl (2 M), and the DNA was spread immediately for electron microscopy by the formamide method of Davis *et al.* (20) and examined in a Philips EM300.

site is within the repeated sequence, the fragments produced by *Hind* III endonuclease digestion will have a rearranged sequence, DEABC. Heteroduplexes formed with long linear molecules that have the sequence ABCDE will necessarily be circular in the homologous portion and have single-stranded tails. *Hind* III endonuclease-generated fragments produced from "head-to-head" arrangements will "snap back" and therefore not be available for heteroduplex formation.

DISCUSSION

This report describes the isolation and propagation of a segment of the SV40 genome that contains the origin of DNA replication (Orep). The procedure used to isolate the DNA segment involves cleavage between two deletion loops in a heteroduplex molecule using S1 endonuclease. In principle, S1 endonuclease could be used to isolate any region of a genome that can be bounded by deletions or additions. The small linear DNA fragments can be used without additional modifications or prior circularization to infect CV-1P cells; apparently circularization occurs after infection, though how it occurs is a mystery. One possibility is that a cellular exonuclease activity produces single-stranded termini that promote circularization according to the pathway suggested by Carbon *et al.* (14). Alternatively, blunt-ended molecules may be joined by ligation (15) or by an illegitimate recombination reaction.

Though not described in this report, we have performed similar experiments using two other mutants with deletions at 0.54 to 0.58 and 0.70 to 0.72. The fragment containing the Orep (0.10 SV40 fractional length) also gave rise to oligomeric circular structures after co-infection with SV40(I) DNA. However, the size distribution of the oligomers was more complex than

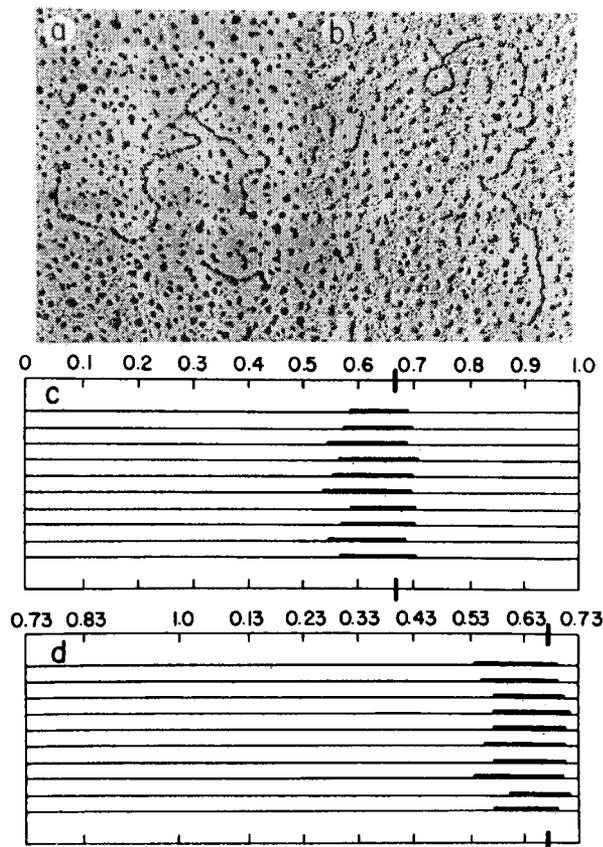


FIG. 5. Heteroduplex DNAs prepared between linear SV40 DNA and fragments derived from the *Eco*RI endonuclease-resistant DNA by cleavage with *Hind* III endonuclease. The *Hind* III endonuclease fragments are those shown in Fig. 3, gel 2 [the second passage in a series initiated by infection with SV40(I) DNA plus the fragment containing the origin of DNA replication]. Either *Eco*RI (a, c) or *Hpa* II endonuclease-generated (b, d) linear SV40 DNA was mixed with the *Hind* III endonuclease-generated fragments (the ratio of full-length linear DNA to fragments was 1 to 10). The mixture was denatured and partially reannealed. The DNA was mounted for electron microscopy by the formamide technique of Davis *et al.* (20) and examined in a Philips EM300. Panels a and b show electron micrographs of representative heteroduplexes; panels c and d show the results of length measurements of 10 heteroduplex molecules in each experiment. The thick bar represents the position and length of the double-stranded circular portion; the thin bar represents the lengths of the single-stranded tails. The actual length measurements of double-stranded regions were normalized (multiplied by 0.9) to correspond to the measurements of single-stranded segments.

simple multimers of 0.10 SV40 fractional length, and the fragments produced by *Hind* III endonuclease cleavage of these molecules were a complex mixture of discrete sized small fragments that was not readily interpretable. At present we do not know if the different behavior of the two DNA fragments reflects some unknown feature of the DNAs or whether the progeny molecules depend on some chance occurrence and selective conditions subsequent to infection. Perhaps the infecting DNA fragments can be trimmed or cleaved further and the shortened fragments containing the Orep can also replicate. Alternatively, portions of the fragment could be lost or only partially duplicated at some stage during the recombination events that duplicated, triplicated, etc. the monomers.

The mechanism by which these complex oligomeric structures (which include both "head-to-tail" and "head-to-head"

repeats) are formed is not clear. However, it is clear that there is a strong selection for oligomerization because the efficiency of encapsidation is probably very sensitive to the size of the genome. As a result, molecules below half SV40 fractional length are probably not encapsidated and may be excluded from successive cycles of infection.

Variant genomes such as those described here, and also naturally occurring variants (16) which contain tandem repeats of the origin of DNA replication, should prove useful in biochemical and physiological analyses of the region containing *cis*-acting functions for replication and packaging. These oligomers of the SV40 Orep may also be a useful reagent for the construction and propagation of hybrid DNA molecules, e.g., DNA molecules joined to a segment containing the Orep could be cloned and amplified, as is now being done with plasmids (17, 18) and phage genomes (19). Such hybrid molecules would, however, require the use of wild-type SV40 as a helper to supply the required replication function. Hybrid molecules of the proper size could be encapsidated by virion coat proteins supplied by the helper virus genome. These "pseudovirus" particles could be useful for transducing a variety of DNAs into animal cells.

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