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

 или

THOMAS E. SHENK* AND PAUL BERG

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Contributed by Paul Berg, February 17, 1976

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 DNA molecules 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 containing the origin of DNA replication/S1 endonuclease cleavage of heteroduplexes/repeated simian virus 40 DNA segments

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 SV5 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).

EcoRI, HpaII, and Hind III endonucleases, as well as S1 endonuclease, were prepared and used according to published protocols [EcoRI (7, 8), HpaII (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 EcoRI endonuclease-cleaved dl 883 and dl 894 DNAs (5 μg/ml of each) were denatured in 0.1 M-NaOH. After 15 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 vol of Tris base (2 M) and increasing the Na+ concentration to 500 mM. To reduce the volume and lower the Na+ concentration 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, 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 × 10⁻⁵ μg) or the fragment of the SV40 genome (2.5 × 10⁻⁵ 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-
two mutant linear DNAs whose deletions bracket the expected cleavage products of a heteroduplex molecule prepared from DNA replication (Orep). (b) Cleavage products generated by these include EcoRI endonuclease-generated fragments obtained by sequential 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.

provided 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 894, 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 digest 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 μg 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 uncult circular DNA by centrifugation in a CsCl-ethidium bromide gradient. The uncult circular DNA consists of several different size molecules after the first in-

---

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

<table>
<thead>
<tr>
<th>DNA fragment*</th>
<th>Predicted†</th>
<th>Found†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.69</td>
<td>0.69</td>
</tr>
<tr>
<td>B</td>
<td>0.54</td>
<td>0.55</td>
</tr>
<tr>
<td>C</td>
<td>0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>D</td>
<td>0.28</td>
<td>0.29</td>
</tr>
<tr>
<td>E</td>
<td>0.15</td>
<td>0.13, 0.12</td>
</tr>
</tbody>
</table>

* 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).
What is the orientation of the repeating segments in the multimeric circles? Are they arranged "head-to-tail" or do they contain any "head-to-head" arrangements? The repeating segments are arranged in a "head-to-tail" configuration only.

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.

Direct observation of heteroduplexes formed from linear SV40 DNA and the Hind III endonuclease-generated fragments established that the fragments are derived from the region of the SV40 genome containing the Orep (Fig. 5). When EcoRI endonuclease-cleaved linear molecules of SV40 DNA are annealed to the Hind III endonuclease-generated fragments, heteroduplexes were formed with a small double-stranded loop (about 0.10-0.15 SV40 fractional length) that contains 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 EcoRI 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 ABCDEABCDE (ABCDEDCBA), then the other oligomers are (ABCDEABCDE)ₙ and some (ABCDEDCBA)ₙ. Since the Hind III endonuclease cleavage

...
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 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.

This work was supported in part by research grants from the U.S. Public Health Service (GM-13235-06) and the American Cancer Society (VC-25C). T. E. S. was a Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.


