Reassortment of Simian Virus 40 DNA During Serial Undiluted Passage

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Alterations occur in the supercoiled form of viral DNA after the serial undiluted passaging of simian virus (SV) 40. We have identified a portion of the viral genome which is amplified during this process. These SV40 DNA sequences represent about 30% of the viral genetic information and are present in a reiterated form in twisted circular molecules prepared from purified virions. In addition, reiterated and unique green monkey DNA sequences are incorporated into supercoiled viral DNA. The cellular DNA appears to be inserted at numerous locations in the DNA I molecules.

Infection of African green monkey cells with simian virus 40 (SV40) results in the sequential appearance of virus-specific antigens, newly replicated viral DNA, capsid proteins, and, finally, progeny virions. The success of the lytic interaction of SV40 and mammalian cells clearly requires the precise modulation of both nucleic acid and protein synthesis as well as the mechanism(s) needed for the assembly of virion components. Several recent reports have suggested that the conditions of infection can profoundly modify the resultant progeny.

Yoshiike, for example, demonstrated that the multiplicity of infection (MOI) used greatly affected the types of particles formed during the infection of monkey cells by SV40 (15, 16). He was able to produce two classes of defective virions by successive undiluted passage of virus. One type of defective virus (type 1) was prepared from a large-plaque variant of SV40 and could be isolated as a discrete low-density band after equilibrium density centrifugation in CsCl. Virions and the extracted supercoiled DNA from such "light" particles were noninfectious and failed to induce either tumor or viral antigens in susceptible cells (16). The DNA from this first type of defective virion was about 12% smaller than the DNA prepared from infectious particles and appeared to be homogeneous with respect to size. The second class of defective particles (type 2) was derived from the

small-plaque variant of SV40 and did not form a discrete band in CsCl (15). These virions formed a broad band after isopycnic centrifugation in CsCl with a mean density slightly lower than that of infectious particles. These defective forms exhibited progressively lower infectivities that were associated with only a modest reduction in the ability to induce SV40 tumor antigen (15). The DNA isolated from virions on the light side of the main virus band was in the supercoiled configuration and was somewhat smaller than that purified from infectious particles.

Shortly thereafter, Aloni et al. (1) reported the preparation of SV40 which yielded supercoiled DNA molecules containing both viral and green monkey polynucleotide sequences. This observation was subsequently shown to be related to the MOI and the type of virus stock used to infect monkey cells (7). The undiluted, serial passaging of SV40 in BSC-1 cells resulted in the appearance of viral DNA which readily hybridized to both SV40 and monkey cellular DNAs. Heteroduplexes, prepared from single-nicked forms of these DNA molecules, contained both deletion and substitution loops presumably related, in some fashion, to the acquisition of host DNA sequences (13).

The emergence of these major changes in SV40 DNA composition occurring during serial undiluted passage of this virus led us to examine some of the properties of the altered viral genome. Alterations were detected by using the kinetics of DNA reassociation. As early as the third undiluted passage, a fraction of this serially passaged SV40 DNA, comprising about 20%

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of the total, was found to reassociate five to seven times more rapidly than did standard small-plaque SV40 DNA. This fraction consisted entirely of SV40 DNA sequences which had been conserved (or preferentially amplified, or both) relative to other regions of the viral genome. The newly acquired monkey DNA appears to consist of both reiterated and unique sequences which are distributed throughout closed circular SV40 DNA molecules.

MATERIALS AND METHODS

Cell culture. Vero and CV-1 lines of African green monkey kidney cells were grown in Eagle II medium supplemented with 10% fetal calf serum. Primary African green monkey kidney cells were cultured in Earle basal salt solution medium supplemented with 0.5% lactalbumin hydrolysate and 10% fetal calf serum.

Virus. Plaque-purified large-, small-, and minute-plaque morphology variants of SV40 were generously supplied by Kenneth Takemoto. Standard small-plaque SV40 stocks were prepared by infecting primary monkey cells at an MOI of 0.1. In experiments involving serial undiluted passage of virus, monkey cells were initially infected at an MOI of 100 with plaque-purified virus. Infected cells and media were harvested at the time of maximal cytopathic effect, frozen and thawed three times, and added, undiluted, to monolayer cultures of green monkey cells. During the course of these studies, virus was successively passaged in this fashion six times.

Preparation of viral DNA. Virus was prepared from both the medium and infected cells. Cells were sonicated and treated with 1.3% sodium deoxycholate for 20 min at 37 C in the presence of 0.025% trypsin. Virus in the medium and cell lysate was purified by sedimentation onto a CsCl cushion and followed by isopycnic centrifugation in CsCl as previously described (15, 5). Supercoiled viral DNA was prepared by treating purified virus with 1% sodium dodecyl sulfate at 50 C for 30 min followed by isopycnic centrifugation in the presence of ethidium bromide in CsCl as previously reported (5, 10, 14). ^{32}P -labeled virus was prepared from infected cells growing in phosphate-free Eagle II medium in the presence of carrier-free ^{32}P -orthophosphate (100 $\mu\text{Ci}/\text{ml}$) (5). Labeled and unlabeled viral DNAs were mechanically sheared at 50,000 lb/in² in a Ribi cell fractionator (Ivan Sorvall) to a molecular size of 3.1×10^5 daltons (5).

Preparation and fractionation of cellular DNA. Green monkey DNA was prepared from the nuclei of liver cells as previously outlined (5). Salmon sperm DNA was purchased from Mann Research Laboratories (New York, N.Y.). Cellular DNA was mechanically fragmented to a size of 3.1×10^5 daltons as described above.

Reiterated and unique fractions of monkey DNA were prepared by allowing sheared, denatured, cellular DNA fragments (5 mg/ml) to reanneal for 2.5 h in 1.05 M NaCl at 68 C. This is equivalent to a C_0t (2) of 880 in 0.12 phosphate buffer (PB) when a correction is

made for salt concentration (3). C_0t is defined as the product of the nucleic acid concentration (C_0) and the time (t) of the reassociation reaction expressed as moles of nucleotide $\times$ seconds per liter (2). The reaction mixture was diluted fivefold with distilled water and applied to a hydroxyapatite column equilibrated with 0.14 M PB at 60 C. The unique fraction was collected in the 0.14 M PB wash; reiterated monkey DNA was recovered by eluting the hydroxyapatite with 0.4 M PB. Each fraction was extensively dialyzed against 0.025 M EDTA and 0.05 M Tris-hydrochloride, pH 8.0, precipitated with 2 vol of cold ethanol, and dialyzed against 0.1 M NaCl before use.

DNA-DNA hybridization using DNA immobilized on nitrocellulose filters. DNA-DNA hybridization was carried out by using the procedure outlined by Denhardt (4). Each reaction mixture contained three 7-mm nitrocellulose filters containing standard SV40, green monkey liver, or no DNA, $4 \times$ standard saline citrate (SSC), Denhardt preincubation mixture (4), and labeled DNA in a final volume of 0.25 ml. The DNA filters were extensively washed in $4 \times$ SSC at 68 C as previously described (5), dried, and counted in a liquid scintillation spectrometer.

DNA-DNA reassociation in solution. ^{32}P -labeled viral DNA was allowed to reassociate in the presence or absence of unlabeled cellular or viral DNAs in reaction mixtures containing 2.5 mM EDTA and the indicated amounts of NaCl or PB, or both. Samples were removed at various times and analyzed for single- and double-stranded DNA on hydroxyapatite as previously described (5). The percentage of ^{32}P -labeled viral DNA reannealed at each point in time was plotted as a function of the C_0t (2).

DNA reassociation was also evaluated with the single-strand specific nuclease isolated from *Aspergillus oryzae* (12). Samples (0.05 ml) of ^{32}P -labeled reassociating DNA were removed and added to a 2.0-ml mixture containing 0.03 M sodium acetate, pH 4.0, 0.1 M NaCl, 10^{-5} M ZnSO_4 , and 60 μg of salmon sperm DNA. The reaction mixture was divided into two equal portions. An excess of the S_1 enzyme was added to one of the samples; both were incubated at 45 C for 1 h. The fraction of DNA resistant to the S_1 enzyme was determined from the nuclease-resistant radioactivity compared with the untreated sample.

Thermal denaturation of reassociated DNA. Fragmented ^{32}P -labeled supercoiled DNA, isolated from standard small-plaque, serially passaged small-plaque, or serially passaged minute-plaque variants of SV40 were heat-denatured and allowed to reassociate to 240 times their respective $C_0t_{0.5}$ in 0.14 M PB. The reassociated DNAs were separately applied to columns (1 by 1 cm) of hydroxyapatite equilibrated with 0.14 M PB at 60 C. Each column was washed with 75 ml of 0.14 M PB at 60 C. Then 0.14 M PB was pumped through the column at a rate of 2.6 ml/min as the temperature was raised. Samples were collected over the temperature range 60 to 99.9 C, and the radioactive DNA was precipitated with 5% trichloroacetic acid in the presence of yeast RNA (10 $\mu\text{g}/\text{ml}$). The proportion of the total DNA eluted was plotted as a function of the temperature.

Specific fragmentation of defective SV40 DNA.

Escherichia coli RY-13 was provided by C. Mulder and the R_1 restriction endonuclease was purified from late log-phase cells of this strain as described by Mulder and Delius (9). The purified R_1 restriction endonuclease was assayed by measuring the conversion of ^{32}P -labeled SV40 DNA I ($1-4 \times 10^5$ counts per min per μg) to DNA III. The reaction mixture (0.2 ml) consisted of 0.1 M Tris-hydrochloride, pH 7.5, 0.005 M MgCl_2 , 10 to 30 ng of DNA I, and 0.1 to 0.5 U of R_1 restriction enzyme. The reactions were incubated at 37 C for 30 min and stopped by chilling to 0 C and adding 0.01 ml of 1% Sarkosyl and 0.01 ml of 0.2 M EDTA (pH 8.0). Conversion of DNA I to DNA III was rapidly assessed by brief heat denaturation of the samples and subsequent batch elution from hydroxypapate (Fareed et al., manuscript in preparation). One unit of R_1 nuclease activity is defined as the amount needed to convert 0.1 μg of SV40 DNA I to DNA III under the assay conditions described above. Dilutions of the enzyme were made in 0.1 M Na_2HPO_4 (pH 7.5), 0.007 M β -mercaptoethanol, 0.3 M NaCl, and 0.001 M EDTA. The purified enzyme had a specific activity of 2×10^4 U/mg of protein.

^{32}P -labeled SV40 DNA from progeny virions of the sixth serially undiluted passage was cleaved with the R_1 restriction enzyme. The nuclease reaction mixtures contained 25 ng of ^{32}P -labeled DNA I (1.6×10^5 counts per min per μg), 49 ng of ^3H -labeled SV40 DNA I and II (9.6×10^4 counts per min per μg , from plaque-purified SV40 propagated at a low input MOI), and either 2 U of R_1 nuclease or no enzyme addition (control). After a 30-min incubation period at 37 C, the reactions were terminated as above. After the addition of 0.4 μg of ^{14}C -DNA I and II (3.3×10^3 counts per min per μg) to each sample, the DNAs were analyzed by sedimentation through linear sucrose gradients at pH 7.5, 0.1 M NaCl, and 0.001 M EDTA. Centrifugation was carried out for 8 h at 40,000 rpm in the SW 41 rotor (Spinco) at 10 C.

RESULTS

Our laboratory has previously examined supercoiled forms of SV40 DNA for the presence of green monkey DNA sequences (5). At that time we were unable to detect significant polynucleotide sequence homology between viral and monkey DNAs. Viral DNA prepared from virions propagated in primary and several different continuous green monkey cell lines failed to hybridize with host DNA immobilized on nitrocellulose filters. This viral DNA was thus ideally suitable as a probe for the detection of the small amounts of viral genetic information present in SV40-transformed cell lines. Our inability to verify the findings of Aloni et al. (1) was undoubtedly due to two factors: (i) virus was passaged at relatively low multiplicities and (ii) plaque-purified virus was used in all studies.

After the report by Lavi and Winocour (7), which showed that acquisition of green monkey DNA sequences by SV40 DNA required serial

undiluted passage of virus, we attempted to repeat their findings and ascertain the mechanism of host sequence incorporation. We also wanted to determine the relationship of cell type and SV40 plaque morphology variants to this phenomenon. The small-plaque variant of SV40 was passaged undiluted three times in primary African green monkey, CV-1, and Vero cells; large-plaque and minute-plaque variants were serially passed in primary monkey cells. In all cases a *single* band of virus was present as determined by isopycnic centrifugation in CsCl . The supercoiled form of viral DNA, isolated from purified virus after serial high-multiplicity passage, sedimented more broadly than did the standard SV40 DNA I. This labeled SV40 DNA was mechanically sheared to fragments of 3×10^5 daltons and incubated with nitrocellulose filters containing immobilized SV40 or green monkey DNA. The results of this experiment (Table 1) indicated that detectable polynucleotide sequence homology existed between these viral and monkey DNAs. The supercoiled DNAs from all plaque variants propagated in primary monkey cells contained host sequences; small-plaque SV40 serially passaged in Vero cells, however, did not incorporate detectable amounts of monkey DNA into its genome by the third passage.

Since it was apparent that SV40 DNA I isolated from serially passaged virus had been altered, we decided to examine its reassociation kinetics in order to further assess the changes that had occurred. The DNAs purified from the small- and minute-plaque variants grown in primary monkey cells and used in the experiments shown in Table 1 were allowed to reas-

TABLE 1. Reassociation of ^{32}P -labeled SV40 DNA prepared from virions serially passaged three times with immobilized viral or cellular DNAs^a

Cell type used to propagate virus	SV40 plaque variant used	^{32}P -DNA (%) reacting with filters containing		
		Standard SV40 DNA	Monkey liver DNA	No DNA
Primary AGMK ^b	Minute	64.9	3.0	0.03
Primary AGMK	Small	45.7	2.4	0.05
Primary AGMK	Large	57.2	2.4	0.05
CV-1	Small	51.1	1.2	0.06
Vero	Small	57.0	0.14	0.03
Primary AGMK	Small (low multiplicity)	66.7	0.04	0.05

^a ^{32}P -labeled, fragmented, SV40 DNA (6×10^{-3} μg) was incubated with filters containing approximately 1 or 18 μg of immobilized standard SV40 or green monkey DNA, respectively, as described in Materials and Methods.

^b AGMK, African green monkey kidney.

sociate in 0.14 M PB at 60 C . Standard SV40 (passaged at low multiplicity) DNA was also allowed to reassociate under similar conditions. Both of the serially passaged DNAs reassociated at a faster rate than did the standard SV40 DNA (Fig. 1). However, two aspects of these reassociation reactions deserve comment. First, the slope of the reassociation curve is considerably shallower than one would expect for a theoretical second-order reaction, suggesting that a population of DNA molecules with varying reiteration frequencies was present. Second, a portion of each curve (between C_{ot} 10^{-5} and 2×10^{-4}) was abnormally flat, indicating the possible existence of a discrete fraction of reassociating DNA.

The homogeneity of the serially passaged DNA was further assessed by examining the thermal stabilities of reassociated DNA preparations. The DNA isolated from undiluted serially passaged small- and minute-plaque SV40 variants was allowed to reanneal to approximately 240 times their $C_{ot}_{1/2}$ and applied to hydroxyapatite equilibrated with 0.14 M PB at 60 C . Thermal elution profiles (Fig. 2) were obtained as described in Materials and

FIG. 1. Reassociation kinetics of ^{32}P -labeled SV40 DNA after three serial undiluted passages. Reaction mixtures containing 7.3×10^{-5} or 2.2×10^{-3} optical density units (ODU)/ml of unfractionated, fragmented, serially passaged ^{32}P -labeled SV40 DNA, 2.5 mM EDTA , and 0.14 M PB were heat-denatured and incubated at 60 C . Samples were removed at various times, the percentage of SV40 DNA reassociated was determined on hydroxyapatite, and the results were plotted as a function of ^{32}P -labeled SV40 DNA C_{ot} (O). The ^{32}P -labeled, rapidly reassociating DNA components (●), isolated as described in the text, were allowed to reassociate at a concentration of 1.16×10^{-4} ODU/ml at 60 C in the reaction mixture described above. Standard ^{32}P -labeled SV40 DNA (Δ) (2.9×10^6 counts per min per μg), prepared from purified virions passaged at low multiplicities, was heat-denatured and allowed to reassociate at a concentration of 2.2×10^{-3} ODU/ml in the reaction mixture described above. A, Serially passaged, small-plaque SV40 DNA (1.16×10^6 counts per min per μg); B, serially passaged, minute-plaque SV40 DNA (1.44×10^6 counts per min per μg).

FIG. 2. Thermal denaturation of reassociated SV40 DNAs. Fragmented, unfractionated, serially passaged small- or minute-plaque ^{32}P -SV40 DNAs or standard small-plaque ^{32}P -SV40 DNA were heat denatured and allowed to reassociate to a C_{ot} of 7.6×10^{-2} as described in Materials and Methods. The reassociated DNA preparations were applied to hydroxyapatite columns, and samples were collected over the temperature range 60.0 to 99.9 C as outlined in Materials and Methods. The proportion of the total DNA eluted from the hydroxyapatite column with 0.14 M PB was plotted as a function of the temperature.

Methods. A sample of reassociated standard small-plaque SV40 DNA was similarly analyzed and had a T_m of 91.8 C ; reannealed DNA from serially passaged small- and minute-plaque variants of SV40 had T_m values of 90.7 and 89.7 C , respectively, suggesting a small degree of mismatching of bases and indicating some heterogeneity of the viral DNA.

In order to isolate rapidly reassociating components, the serially passaged DNAs prepared from the small- and minute-plaque variants were heat-denatured and allowed to reassociate in 0.14 M PB to a C_{ot} of 10^{-4} (rapidly reassociating component), while the remaining 80% (slowly reassociating component) was collected in the initial 0.14 M PB eluate. The rapidly reassociating components were heat-denatured and allowed to reassociate in 0.14 M PB (left curve, Fig. 1A and B). This fraction of serially passaged SV40 DNA reassociated five to six times faster than did standard small-plaque DNA, indicating that its genetic complexity was significantly less than that of viral DNA (3×10^6 daltons).

In order to be certain that the rapidly reassociating component was unique to serially passaged SV40 and was not an artifact of the

fractionation procedure, standard small-plaque SV40 DNA was denatured and allowed to reassociate to a C_{ot} of 10^{-4} . Less than 3% of the standard SV40 DNA reannealed under these conditions. Since the rapidly reassociating component represented the 20% portion of serially passaged SV40 DNA which reannealed first, standard SV40 DNA was then fractionated by incubation to a C_{ot} of 6×10^{-4} , and the 20% of the DNA which had reassociated was collected. This reannealed portion of standard SV40 DNA was denatured and allowed to reassociate in 0.14 M PB (Fig. 3). The kinetics of its reassociation were indistinguishable from unfractionated standard SV40 DNA. This is the result expected for a homogeneous DNA preparation.

The appearance of a relatively homogeneous fraction of DNA with a genetic complexity significantly less than that of standard SV40 DNA prompted us to propagate additional virus

by further serial undiluted passage. We observed the previously reported (7) fall of SV40 infectivity and an increase in the amount of monkey DNA sequences incorporated into supercoiled forms of viral DNA with successive passage (Table 2). This DNA reassociated as a collection of molecules with varying reiteration frequencies (Fig. 4). A rapidly reassociating component was readily purified which reannealed about six times more rapidly than did standard SV40 DNA (left curve, Fig. 4). This fraction was similar to the rapidly reassociating DNA components isolated from purified virions which had been serially passaged three times.

The major portion (80%) of serially propagated SV40 DNA (slowly reassociating component), purified from virions after three or six successive undiluted passages, reassociated with $C_{ot}_{1/2}$'s ranging from 1.8×10^{-3} to 2.3×10^{-3} . These values were very similar to those observed with standard SV40 DNA (2.0×10^{-3}) under these conditions.

Since serial undiluted passage of SV40 results in the incorporation of green monkey sequences into the supercoiled form of viral DNA (7), we decided to examine both the rapidly and slowly reassociating fractions to determine where the mammalian DNA was located. Because the rapidly reassociating component represented about 20% of serially passaged DNA, it seemed quite likely that it contained the host cell sequences. In order to ascertain the nature of the polynucleotide sequences present in the rapidly reannealing DNA fraction, we followed its reassociation in the presence of unlabeled standard viral or cellular DNAs. We have previously shown a direct relationship between the

FIG. 3. Reassociation of fractionated, standard, small-plaque SV40 DNA isolated from virus propagated at low MOIs. Standard, ^{32}P -labeled, small-plaque SV40 DNA fragments were heat-denatured and allowed to reassociate at a concentration of 2×10^{-3} optical density units (ODU)/ml in 0.14 M PB to a C_{ot} of 6×10^{-4} . The reaction mixture was then applied to a hydroxyapatite column and the portion (19.3%) that had reassociated was eluted with 0.14 M PB at 100 C as previously described. The fractionated standard SV40 DNA (○) was then denatured and reassociated at a concentration of 4.0×10^{-4} ODU/ml in the reaction mixture described for Fig. 1. Unfractionated standard SV40 DNA (●) was heat-denatured and allowed to reassociate at a concentration of 4×10^{-3} ODU/ml in 0.14 M PB. The percentage of SV40 DNA reassociated was determined after exposure to the S_1 single-strand specific nuclease as described in Materials and Methods.

TABLE 2. Reassociation of ^{32}P -labeled SV40 DNA prepared from purified serially passaged virus with SV40 or cellular DNAs^a

Passage no.	Infectivity (PFU/ml)	³² P-DNA (%) Reacting with		
		SV40 DNA	Monkey liver DNA	No DNA
0	2.0×10^8	75.1	0.2	0.08
1	6.5×10^7	60.3	0.6	0.06
2	1.1×10^7	61.4	1.7	0.07
3	5.0×10^6	52.3	2.5	0.07
4	1.6×10^6	57.0	4.3	0.05
5	1.6×10^5	59.6	5.2	0.05
6	3.3×10^3	54.4	5.6	0.08

^a ^{32}P -labeled, fragmented SV40 DNA ($1-8 \times 10^{-3}$ μg) was incubated with filters containing approximately 1 or 18 μg of immobilized standard SV40 or green monkey DNA, respectively, as described in Materials and Methods.

FIG. 4. Reassociation kinetics of ^{32}P -labeled SV40 DNA after six serial, undiluted passages. Reaction mixtures containing 6.9×10^{-5} or 2.1×10^{-3} optical density units (ODU)/ml of unfractionated, fragmented, ^{32}P -labeled SV40 DNA (O) isolated from virions passaged six times at high MOIs were heat-denatured and allowed to reassociate as described in Fig. 1. The ^{32}P -labeled, rapidly reassociating DNA component was isolated from unfractionated, serially passed DNA after incubation of heat-denatured DNA to a C_0t of 10^{-4} as previously described. The rapidly reassociating fraction (●) was then heat-denatured and allowed to reassociate at a concentration of 3.5×10^{-4} ODU/ml in 0.14 M PB. Standard SV40 DNA (Δ) was reassociated in 0.14 M PB at a concentration of 1.1×10^{-3} ODU/ml. DNA reassociation was monitored on hydroxyapatite columns as described in Materials and Methods.

amount of unlabeled standard SV40 DNA present in a reaction mixture and the rate of reassociation of a labeled SV40 DNA probe (5). The experiment described in Fig. 5 (right panel) shows that green monkey DNA does not effectively alter the reassociation of the rapidly reannealing DNA component. This result indicates that no cellular DNA sequences are present in this fraction. A 12.7-fold excess of unlabeled standard SV40 DNA, however, accelerated the reassociation of the labeled, rapidly reannealing DNA fraction (Fig. 5, left panel), indicating the presence of viral DNA sequences. The reassociation of the rapidly reannealing fraction was increased by only a factor of 4.1. This result suggests that the rapidly reannealing component contains only a portion (32%) of the viral DNA sequences present in standard

SV40 DNA which has been conserved (or amplified) by serial undiluted passage. The reassociation of rapidly reannealing DNA components, isolated from other serially passaged virus preparations, was affected to similar extents by unlabeled standard SV40 DNA (Table 3).

A similar experiment was carried out with the ^{32}P -labeled, slowly reassociating component to characterize the nature of its polynucleotide sequences. In the presence of an excess of unlabeled cellular DNA, the reassociation of the slowly reannealing component was accelerated 2.85-fold (Fig. 6, right panel). This effect was more than 16 times greater than that observed when labeled standard SV40 DNA was allowed to reassociate in the presence of a similar amount of unlabeled monkey DNA (5). This result indicates that the major fraction (slowly reassociating DNA component) of serially passaged DNA is greatly enriched for green monkey sequences. A surprising feature of this experiment is that the *entire* reassociation curve was shifted by the cellular DNA. This was unexpected in view of the apparently small amounts of monkey DNA present in the unfractionated, serially passaged, viral DNA. The reassociation of the slowly reannealing fraction was accelera-

FIG. 5. Reassociation of ^{32}P -labeled, rapidly reannealing DNA component in the presence of unlabeled standard SV40 or green monkey DNAs. Left panel: the ^{32}P -labeled, rapidly reannealing DNA fraction from the sixth undiluted serial passage was heat-denatured and allowed to reassociate in 0.14 M PB at a concentration of 3.2×10^{-5} optical density units (ODU)/ml in the presence of 4.0×10^{-4} ODU/ml of fragmented unlabeled SV40 (O) or salmon sperm (●) DNAs. Right panel: The ^{32}P -labeled, rapidly reannealing DNA component (3.2×10^{-5} ODU/ml) was allowed to reassociate in 0.14 M PB in the presence of 8.1 ODU/ml of fragmented, unlabeled green monkey (O) or salmon sperm (●) DNAs. The proportion of ^{32}P -labeled DNA reassociated was determined on hydroxyapatite.

TABLE 3. Reassociation of rapidly reassociating ^{32}P -DNA component in the presence of unlabeled SV40 DNA

Labeled SV40 DNA	Unlabeled standard SV40 DNA/serially passaged ^{32}P -SV40 DNA	Increase rate factor	Increase rate factor/Expected rate factor
Serially passaged five times	22.5	7.2	.32
Serially passaged five times	1077	352	.33
Serially passaged six times	12.7	4.1	.32
Standard SV40 passaged at low multiplicity	5.2	5.0	.96

FIG. 6. Reassociation of ^{32}P -labeled, slowly reannealing DNA component in the presence of unlabeled standard SV40 or green monkey DNAs. Left panel: the ^{32}P -labeled, slowly reannealing DNA fraction from the sixth undiluted serial passage was heat-denatured and allowed to reassociate in 0.14 M PB at a concentration of 2.0×10^{-4} optical density units (ODU)/ml in the presence of 3.8×10^{-3} ODU/ml of fragmented, unlabeled SV40 (○) or salmon sperm (●) DNAs. Right panel: ^{32}P -labeled, slowly reannealing DNA component (2.0×10^{-4} ODU/ml) was allowed to reassociate in 0.14 M PB in the presence of 61 ODU/ml of fragmented, unlabeled green monkey (○) or salmon sperm (●) DNAs. Samples were analyzed on hydroxyapatite.

ted by unlabeled standard SV40 DNA, although the effect was 87% of that expected for an 18.5-fold excess of viral DNA (Fig. 6, left panel).

In order to characterize the cellular sequences incorporated into serially passaged viral DNA, the experiment described in Fig. 7 was carried out. Unlabeled green monkey DNA fragments were denatured and incubated to a C_0t of 880. The preparation was applied to a hydroxyapatite column and the reiterated and unique DNA

fractions were collected as described in Methods. Under the conditions used, approximately 10% of the true "single-copy" DNA sequences reannealed and should have been present in the reiterated DNA fraction. ^{32}P -labeled, slowly reassociating DNA, prepared from serially passaged virus, was allowed to reassociate in the presence of equal amounts of either the reiterated or unique green monkey DNA fractions. Whereas both fractions of cellular DNA accelerated the reassociation of the DNA probe, the reiterated monkey sequences had a greater effect. If one assumes that the incorporated cellular sequences are representative of the green monkey genome, this observation probably reflects the fact that the cellular

FIG. 7. Reassociation of ^{32}P -labeled, slowly reannealing DNA component in the presence of unlabeled reiterated or unique green monkey DNA fractions. The ^{32}P -labeled, slowly reannealing DNA fraction from the sixth undiluted serial passage was heat-denatured and allowed to reassociate in 0.14 M PB at a concentration of 1.97×10^{-4} optical density units (ODU)/ml in the presence of 14.1 ODU/ml of fragmented, unlabeled reiterated (▲) or unique (■) green monkey or salmon sperm (●) DNAs. Samples were analyzed on hydroxyapatite.

DNAs reassociated to a C_0t of 815 during their incubation with the labeled probe. This is sufficient to allow nearly all of the repetitious DNA sequences to react with complementary sequences in the labeled probe. However, no more than 20% of the unique cellular DNA

sequences react under these conditions, and this could explain the apparent lower efficiency of the non-reiterated fraction in accelerating the reassociation of the labeled DNA probe.

The R_1 restriction endonuclease of *E. coli* cleaves SV40 DNA I at a specific site, converting it to unique linear DNA molecules (DNA III) (8, 9). It has been previously reported that a large fraction of viral DNA prepared from virions that have been serially passaged at high multiplicities are resistant to the R_1 restriction enzyme (9). The experiment shown in Fig. 8 indicates that approximately 60% of the supercoiled DNA isolated from serially passaged virus is not cleaved by the enzyme. In addition, a discrete cleavage product, sedimenting at 9S in neutral sucrose, appeared along with DNA III. This 9S product, which represented about 10% of the labeled DNA, was purified from preparative neutral sucrose gradients and subsequently analyzed by sedimentation through neutral sucrose (Fig. 8B). The purified 9S product sedimented as a relatively homogeneous population of molecules with a peak S value of 9.3. This is equivalent to DNA molecules approximately 7.5×10^5 daltons in size (11). A small amount (16%) of contaminating DNA III was also present in this 9S DNA preparation.

The purified ^{32}P -labeled 9S component was heat-denatured and allowed to reassociate in 0.14 M PB. Fragmented DNA I, isolated from standard SV40, as well as the rapidly reannealing DNA component, were similarly denatured and the kinetics of DNA reassociation were examined. The results of this experiment (Fig. 9) indicate that the 9S cleavage product reanneals about 3.2 times more rapidly than does standard SV40 DNA. The DNA reassociation curve for the 9S DNA, in contrast to the one obtained for the rapidly reannealing component, has a slope identical to that for standard SV40 DNA, reflecting the homogeneity of the cleavage product. The genetic complexity of 9S DNA is approximately 31% of standard SV40 DNA. This value is very similar to the one obtained from experiments in which the ^{32}P -labeled, rapidly reassociating component was allowed to reassociate in the presence of unlabeled standard SV40 DNA (Fig. 5 and Table 3).

DISCUSSION

The serially passaged SV40 we have described in this report shares many properties with the type 2 defective particles isolated by Yoshiike which were also propagated by passaging small-plaque SV40 in primary green monkey cells. In all cases only a single band of virus appeared,

FIG. 8. Specific fragmentation of serially passaged SV40 DNA by the R_1 restriction endonuclease. A: ^{32}P -labeled DNA I, isolated from purified virions serially passaged six times, was incubated with the R_1 restriction enzyme as described in Materials and Methods. (^{14}C -labeled SV40 DNA I, isolated from standard virus propagated at low multiplicity, was also present in the reaction.) The reaction mixture was layered onto the surface of a 5 to 30% (wt/vol) neutral sucrose gradient and sedimented at 40,000 rpm in the SW41 rotor for 9 h at 10 C. The locations of ^{14}C -labeled SV40 DNA I and II, added to the sample before centrifugation, are indicated by the arrows. B: In another reaction mixture, 500 ng of ^{32}P -labeled DNA I was incubated with 5 U of the R_1 restriction enzyme. The 9S cleavage product was obtained from a preparative gradient similar to that shown in panel A and concentrated by pressure dialysis against 0.01 M Tris-hydrochloride, pH 7.5, 0.01 M NaCl, and 0.001 M EDTA. A 100- μ liter sample of the ^{32}P -9S product was mixed with 40 ng of ^{14}C -labeled SV40 DNA I and II and analyzed by sedimentation through neutral sucrose as described above.

FIG. 9. Reassociation of ^{32}P -labeled 9S DNA cleavage product. 9S ^{32}P -labeled DNA was produced by digesting the supercoiled DNA, obtained from purified virions after six undiluted serial passages, with R_1 restriction endonuclease as described in Materials and Methods and Fig. 8. The purified 9S DNA component (Fig. 8B) was heat-denatured and allowed to reassociate in 0.14 M PB at a concentration of 6.2×10^{-4} optical density units (ODU)/ml ($\bullet$). The rapidly reannealing SV40 DNA fraction from the sixth undiluted passage ($\blacktriangle$) and standard SV40 DNA ($\circ$), both labeled with ^{32}P , were heat-denatured and allowed to reassociate at concentrations of 5.4×10^{-4} and 1.9×10^{-3} ODU/ml, respectively. All samples were incubated at 60 C and analyzed on hydroxyapatite.

and the isolated SV40 DNA sedimented as a broad band. This kind of defective SV40 is different from type 1, which forms a discrete "light" virus band (16) in addition to a fully infectious and apparently normal virus band after isopycnic centrifugation in CsCl. The DNA isolated from type 1 "light" particles is homogeneous in size, is missing specific SV40 DNA sequences, and contains no host cell DNA (Martin et al., manuscript in preparation).

Our experiments characterizing serially passaged viral DNA are in agreement with those of Lavi and Winocour (7), who reported an incorporation of monkey DNA sequences into the supercoiled form of SV40 DNA. The closed circular DNA molecules isolated from purified virions propagated in such a manner sedimented in a broader band than did standard viral DNA, and about two-thirds lacked the R_1 restriction endonuclease site. Both of these observations have been noted previously (9, 15).

As pointed out above, the kinetics of serially passaged viral DNA reassociation differ from

those observed for standard SV40 DNA and do not fit the theoretical curve for a second-order reaction. This is undoubtedly due to the presence, in the population of supercoiled DNA molecules, of DNA sequences with varying reiteration frequencies. The slightly lower thermal stability of reassociated, serially passaged viral DNA is further evidence of its heterogeneity. Previous studies have concentrated on the insertion of host cell DNA sequences into the closed circular form of viral DNA. Our results indicate that the alterations of SV40 DNA involve both viral and mammalian DNA sequences. The presence of a rapidly reassociating DNA component, unique to serially passaged virus and composed entirely of SV40 DNA sequences, contributes greatly to the observed heterogeneity of unfractionated DNA. This component reassociates five to seven times faster than does standard viral DNA, suggesting that its genetic complexity is considerably less than that of SV40 DNA. Since the rapidly reassociating fraction isolated on hydroxyapatite is not completely homogeneous, we cannot determine what proportion of the SV40 genome it represents. When it is allowed to reassociate in the presence of unlabeled viral DNA, this component behaves as if it contained 30% of the information present in SV40 DNA (Fig. 5 and Table 3). The 9S component, however, prepared by digesting DNA I isolated from serially passaged virus, reassociates like a homogeneous DNA preparation and appears to have a genetic complexity equivalent to 31% of SV40 DNA (Fig. 9). Since the bulk of the DNA (slowly reannealing fraction), isolated from virus passaged at high multiplicity, reassociated with a $C_0t_{0.5}$ very similar to standard SV40, it seems most likely that the 9S product represents a homogeneous form of the rapidly reassociating component.

The 9S component arose after treatment of serially passaged viral DNA I with the R_1 restriction endonuclease. Its appearance suggests that a portion of the supercoiled DNA contains three to four R_1 sites. In this regard we have observed DNA I molecules containing three symmetrically placed R_1 sites in preparations of an SV40-like virus obtained from a patient with progressive multifocal leukoencephalopathy (Fareed and Martin, manuscript in preparation). Thus, the alteration of viral DNA sequences we have described (rapidly reassociating component and 9S product) may be distinct from the process involved in the incorporation of cellular DNA sequences. A specific region (about 30%) of the viral genome appears to be reiterated in supercoiled DNA molecules containing three to four R_1 sites.

The monkey DNA sequences incorporated into serially passaged viral DNA are associated with the DNA component that has reassociation kinetics similar to those of standard SV40 DNA. Our studies indicate that *both* reiterated and unique mammalian DNA sequences become incorporated into viral DNA (Fig. 6). Another aspect of the acquired monkey DNA sequences deserves comment. When the slowly reannealing DNA component was reassociated in the presence of cellular DNA, the *entire* reaction was accelerated (Fig. 6 and 7). This result was unexpected in view of the small amounts of green monkey DNA found in the unfractionated viral DNA by filter hybridization assays (Tables 1 and 2). One explanation for the shift of the entire reassociation curve is that the cellular DNA may be inserted at numerous locations in the closed circular viral DNA molecules. The labeled, slowly reassociating DNA fragments used in these studies consist predominantly of SV40 DNA sequences (Fig. 6). The small portion consisting of monkey DNA must be present in a majority of the 3×10^5 dalton DNA fragments since the reassociation curve is symmetrically displaced by the unlabeled cellular DNA. It is known that hydroxyapatite does not discriminate between partially and completely duplexed nucleic acid structures (6). In this case, the portion of each labeled DNA fragment containing cellular DNA sequences is apparently able to form a stable duplex with the unlabeled monkey DNA. The entire molecule then behaves like double-stranded DNA on hydroxyapatite columns even though it is predominantly single stranded.

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LITERATURE CITED

1. Aloni, Y., E. Winocour, L. Sachs, and J. Torten. 1969. Hybridization between SV40 DNA and cellular DNAs. *J. Mol. Biol.* **44**:333-345.
2. Britten, R. J., and D. E. Kohne. 1968. Repeated sequences in DNA. *Science* **161**:529-540.
3. Britten, R. J., and J. Smith. 1970. In *Yearb. Carnegie Inst.* **68**:385.
4. Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* **23**:641-646.
5. Gelb, L. D., D. E. Kohne, and M. A. Martin. 1971. Quantitation of simian virus 40 sequences in African green monkey, mouse and virus-transformed cell genomes. *J. Mol. Biol.* **57**:129-145.
6. Khoury, G. and M. A. Martin. 1972. Comparison of SV40 DNA transcription in vivo and in vitro. *Nature N. Biol.* **238**:4-6.
7. Lavi, S., and E. Winocour. 1972. Acquisition of sequences homologous to host deoxyribonucleic acid by closed circular simian virus 40 deoxyribonucleic acid. *J. Virol.* **9**:309-316.
8. Morrow, J. F., and P. Berg. 1972. Cleavage of simian virus 40 at a unique site by a bacterial restriction enzyme. *Proc. Nat. Acad. Sci. U.S.A.* **69**:3365-3369.
9. Mulder, C., and H. Delius. 1972. Specificity of the break produced by restricting endonuclease R₁ in simian virus 40 DNA as revealed by partial denaturation mapping. *Proc. Nat. Acad. Sci. U.S.A.* **69**:3215-3219.
10. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in Hela cells. *Proc. Nat. Acad. Sci. U.S.A.* **57**:1514-1521.
11. Studier, F. W. 1965. Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* **11**:373-390.
12. Sutton, W. D. 1971. A crude nuclease preparation suitable for use in DNA reassociation experiments. *Biochim. Biophys. Acta* **240**:522-531.
13. Tai, H. T., C. A. Smith, P. A. Sharp, and J. Vinograd. 1972. Sequence heterogeneity in closed simian virus 40 deoxyribonucleic acid. *J. Virol.* **9**:317-325.
14. Trilling, D. M., and Axelrod, D. 1970. Encapsulation of free host DNA by simian virus 40: a simian virus 40 pseudovirus. *Science* **168**:268-271.
15. Yoshiike, K. 1968. Studies on DNA from low-density particles of SV40. I. Heterogeneous defective virions produced by successive undiluted passages. *Virology* **34**:391-401.
16. Yoshiike, K. 1968. Studies on DNA from low density particles of SV40. II. Noninfectious virions associated with a large plaque variant. *Virology* **34**:402-409.