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Acquisition of Sequences Homologous to Host DNA by Closed Circular Simian Virus 40 DNA

II. Further Studies on the Serial Passage of Virus Clones

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Three plaque isolates of SV40 strain 777 and 1 plaque isolate of strain 776 were grown to high-titer stocks and serially passaged, undiluted, in monkey BS-C-1 cells. In each case, the serial passaging procedure resulted in the accumulation of closed-circular SV40 DNA molecules containing covalently linked sequences homologous to reiterated host cell DNA (called substituted virus DNA). The relative yields, at a given passage level, of SV40 DNA with measurable homology to host DNA varied in different sets of serial passages, including passages of the same virus clone. More reproducible yields of substituted viral DNA progeny were obtained when the serial passaging procedure was initiated from earlier passages rather than from the original plaque-purified stock. Fractionation of closed-circular SV40 DNA molecules on alkaline sucrose gadients indicated that the majority of substituted virus DNA molecules are not plaque producers and are slightly smaller in size than plaque-forming DNA molecules which display no detectable homology to host DNA. Evidence that substituted SV40 DNA molecules replicate during serial undiluted passage was obtained from experiments which demonstrated (i) the presence of host sequences in replicative forms of the viral DNA and (ii) the incorporation of ^{3}H-thymidine into host sequences isolated from the mature substituted virus DNA molecule.

In previous studies from this laboratory, we reported the presence of host DNA sequences in closed-circular DNA molecules obtained from purified simian virus 40 (SV40) virions (1), and we described the special conditions of infection leading to the synthesis of virus DNA containing covalently linked host sequences (6). Lytic infection of BS-C-1 monkey kidney cells resulted in the synthesis of such DNA when the cells were infected either with serial, undiluted passages of a plaque-purified virus stock or with non-plaque-purified SV40 at high multiplicity of infection. Infection with plaque-purified virus which was not serially passaged, or with non-plaque-purified virus at low multiplicity, did not yield viral DNA with significant levels of detectable homology to host DNA under the conditions of hybridization used. These hybridization conditions detect only sequences homologous to reiterated host DNA.

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In this paper we report more extensive results on serial undiluted passage of plaque-purified SV40 on BS-C-1 cells. The data indicate that the accumulation of closed-circular viral DNA molecules containing covalently linked sequences homologous to host DNA (termed substituted virus DNA) is a general phenomenon, observable with several separately isolated plaque-purified virus preparations derived from two different virus strains. However, we have observed striking differences in the yields of viral DNA molecules with detectable homology to host DNA in different sets of serial passages. We will also report experiments showing that the substituted virus DNA is (i) somewhat smaller in size than the DNA of plaque-purified virus, (ii) defective with respect to plaque-forming ability, and (iii) capable of replication under the conditions of undiluted serial passage. The influence of these factors on the development of serially passaged SV40 DNA will be discussed. In the accompanying paper (11) the substituted SV40 DNA is characterized with respect to the type of host sequences found.

MATERIALS AND METHODS

Cells. The BS-C-1 line of African green monkey kidney cells was cultured as previously described (6). The confluent monolayers contained approximtely 4 $\times 10^6$ cells.

Virus. Plaque-purified stocks of SV40, strains 777 (2) and 776 (16), were prepared from single plaque isolates subjected to three sequential plaque-purification steps (6). The high-titer stocks were grown from these isolates by infecting cells at low multiplicity as described in Table 1 (footnote *a*). Twelve to 14 days later, when cytopathic effects were complete, the virus was harvested (see below). SV40 stock X-8 is a non-plaque-purified stock of SV40 (strain 777) maintained in this laboratory by serial passage of the virus at input multiplicities in the range of 0.1 to 1 PFU per cell. Virus was titered by plaque assay on BS-C-1 monolayers and purified by procedures reported elsewhere (6).

DNA infectivity. The plaque-forming ability of SV40 DNA was measured on BS-C-1 monolayers by the DEAE-dextran procedure of McCutchan and Pagano (8, 9, 12).

Serial, undiluted passage of SV40. Passage 1 was initiated by infecting a confluent monolayer of BS-C-1 cells (approximately 4 $\times 10^6$) with the plaque-purified virus stocks at high multiplicity as specified in Table 1 (footnote *a*). After a 2-h period for virus absorption, 8 ml of medium containing 2% calf serum was added. When strong cytopathic effects were observed, the medium and cells were collected and sonically treated for 3 min at full power in a 10 kcycle/s Raytheon sonic vibrator. One sample of this crude virus suspension was used undiluted (1 ml per 4 $\times 10^6$ cells) to infect a new culture of cells for the next serial passage; a second 1-ml sample was used, undiluted, to infect cells to isolate labeled viral DNA progeny; a third sample was used to determine, by plaque assay, the number of plaque-forming particles in the yield. The same procedure was repeated at each passage level. In general, all crude virus suspensions were stored at −60 C until completion of a given set of undiluted serial passages; thereafter, the number of

TABLE 1. *Serial undiluted passage of different plaque-purified SV40 stocks on monkey BS-C-1 cells*

Origin of plaque-purified stocks[a]	Passage no.	Days to harvest	PFU/ml in yield	Hybridization of progeny ^{3}H-SV40 DNA[b]				
				Input counts/min	% of input bound to filters containing:			(BS-C-1/SV40) $\times$ 100
					SV40 DNA	BS-C-1 DNA	No DNA	
777, CVB, series 1	0	12	4×10^9	19,744	56	0.2	0.1	0.4
	5	13	4×10^6	6,315	45	35	0.1	77.8
777, CVB, series 2	1	4	3×10^9	87,500	66	0.3	0.1	0.5
	5	4	3×10^7	93,350	76	9	0.3	11.8
777, CV1	0	11	1×10^9	18,065	51	0.2	0.2	0.4
	7	5	3×10^7	11,128	93	0.7	0.1	0.8
	10	14	1×10^6	4,285	81	3	0.2	3.7
777, CVG	1	4	2×10^9	28,259	52	0.5	0.2	1.0
	7	4	1×10^7	28,359	59	19	0.3	32.2
776, CV8	1	4	2×10^8	—[c]	—	—	—	—
	4	4	5×10^6	23,173	45	0.7	0.1	1.6
	8	9	3×10^5	5,432	53	8	0.5	15.1

[a] The virus strains and individual plaque isolates from which the stocks were derived (see Materials and Methods). The CV1 and CV8 stocks were prepared by infecting 4 $\times 10^6$ BS-C-1 cells with 1 ml of medium containing 1,000 PFU of the respective plaque isolates (after the 2-h absorption period, an additional 8 ml of medium was added); the CVG and CVB (series 2) stocks were prepared by infecting 4 $\times 10^6$ cells with 8 ml of medium containing 8 PFU of the respective plaque isolates. To prepare the CVB series 1 stock, cells were infected with 1 ml of plaque-isolate CVB (100 PFU) and the yield was purified by centrifugation through a cushion of CsCl solution (6); the purified virus was then diluted and used as inoculum (10,000 PFU per 1 ml of medium per 4 $\times 10^6$ cells) for the preparation of the stock. In all cases, passage 1 was initiated by infecting cells with the different plaque-purified stocks at high multiplicity (4 $\times 10^8$ to 9 $\times 10^8$ PFU per 1 ml of medium per 4 $\times 10^6$ cells). The yield of passage 1 was then used, *undiluted*, to infect cells for the subsequent passages as described in Materials and Methods.

[b] Hybridization tests with the radioactive SV40 DNA I synthesized (24 to 48 h postinfection) in cells infected either with plaque-purified virus (passage 0) at an input multiplicity of 4 $\times 10^8$ to 9 $\times 10^8$ PFU per 1 ml of medium per 4 $\times 10^6$ cells, or with a 1-ml, undiluted, sample of the virus yield at the respective passage level. The isolation and purification of ^{3}H-SV40 DNA I, and the subsequent hybridization tests, are described in Materials and Methods.

[c] —, Not tested.

plaque-forming particles produced at each passage level was determined in a single set of assays.

Extraction and purification of radioactive closed circular SV40 DNA. Infected cells were labeled, 24 to 48 h postinfection, with approximately 10 μCi of ^{3}H-TdR (thymidine-*methyl-^{3}H*, 18-26 Ci/mmol, obtained from the Radiochemical Centre, Amersham, England) or 50 μCi of ^{32}P-inorganic phosphate per ml of medium. SV40 DNA was extracted from labeled, infected cells by the procedure of Hirt (4). Closed circular viral DNA (component I) in the Hirt supernatant fraction was then purified either by band sedimentation in alkaline CsCl (19) as previously described (6) or by centrifugation to equilibrium in a CsCl gradient supplemented with ethidium bromide (10). In the latter case, the material in the Hirt supernatant fraction was extracted with phenol, precipitated with alcohol, and resuspended in 3 ml of a solution containing CsCl (final density 1.55 g/ml), 0.01 M Tris (pH 7.4), and 300 μg of ethidium bromide per ml. The solution was then centrifuged in the Spinco rotor SW50 or SW50.1 for 40 to 48 h at 35,000 rpm. The fractions containing the circular duplex SV40 DNA molecules (component I) were pooled, and the ethidium bromide was removed by multiple extractions with chloroform-isoamyl alcohol (24:1, vol/vol). The DNA was then dialyzed extensively first against 0.1 $\times$ SSC and then 0.01 $\times$ SSC (1 $\times$ SSC, standard saline citrate, is 0.15 M NaCl-0.015 M sodium citrate). When the infected cells had been labeled with ^{32}P-inorganic phosphate, the material in the Hirt supernatant fraction was treated with pancreatic RNase (50 μg/ml, 30 min at 37 C) prior to phenol extraction, alcohol precipitation, and equilibrium centrifugation. The specific activities of the ^{3}H-SV40 DNA were in the range 10^{5} to 2×10^{5} counts per min per μg of DNA, and the activity of the ^{32}P-SV40 DNA was 4×10^{5} counts per min per μg.

Isolation of SV40 DNA replicative forms. BS-C-1 cells were infected with 1 ml of serially passaged SV40 (passage 3 of CVB virus described in Table 2) diluted to contain 2×10^{8} PFU/ml. Forty-eight hours postinfection, the medium was replaced with fresh prewarmed medium, and after a further 2 h at 37 C the infected cultures were labeled with ^{3}H-TdR (10 μCi/ml of medium) for short periods of time (5-15 min) as indicated in the text. At the end of the pulse the medium was removed and the cells were quickly lysed by the Hirt procedure (4). The material in the Hirt supernatant fraction was extracted with phenol, precipitated with ethanol, and resuspended in 1 $\times$ SSC. After the addition of purified ^{32}P-SV40 DNA component I (from infected cells labeled 24 to 50 h postinfection) as a marker, the material was sedimented through a 5 to 20% (wt/wt) sucrose gradient, containing 1 $\times$ SSC, at 4 C for 3.5 h at 40,000 rpm in a Spinco SW65 rotor (7, 15). The ^{3}H-labeled fractions in the 25 to 26*S* region of the gradient (see Fig. 2) were pooled (the level of ^{32}P counts per minute in the pooled fractions was 5%, or less, that of the ^{3}H radioactivity); the material was then precipitated with ethanol and resuspended in 0.01 $\times$ SSC. To confirm that the material from the 25 to 26*S* region of the neutral sucrose gradient represents virus RF-DNA, a sample was recentrifuged through alkaline CsCl solutions (6); all of the ^{3}H-labeled DNA sedimented at 16*S* or less, depending upon the pulse time (7, 15).

Alkaline sucrose gradients. SV40 DNA I molecules were fractionated in 5 to 20% (wt/wt) alkaline sucrose gradients containing 1 M NaCl, 0.3 N KOH, and 1 mM EDTA (pH 12.8). Centrifugation was in the Spinco SW41 rotor at 38,000 rpm for 3.5 at 20 C. Eighty fractions of 5 drops each were collected from the bottom of the tube.

DNA-DNA hybridization. Hybridization was carried out in 50% formamide at 37 C as described previously (6). The SV40 DNA immobilized on filters was component I derived from plaque-purified virus grown at very low multiplicity and displayed no detectable homology to host cell DNA. The radioactive SV40 DNA used in the hybridization reaction was denatured by heating at 100 C for 15 min in 0.01 SSC and then rapidly cooled in an ice-salt bath. This produces fragmented, denatured DNA approximately 30% the molecular weight of intact single strands of SV40 DNA (6). In some experiments, the radioactive SV40 DNA, after heat denaturation as above, or radioactive SV40 component II DNA (see Table 5), was denatured by exposure to a solution containing 90% (vol/vol) formamide, 10 mM Tris-hydrochloride (final pH 9.1), for 1 to 2 h at 37 C (11). Thereafter, the salt and formamide concentrations were adjusted to those used for hybridization by appropriate dilution and addition (11). To recover the hybridized ^{3}H-DNA, the nitrocellulose membrane filter was incubated at 37 C for 1 to 2 h in 3 ml of the formamide-denaturing solution described above. Over 90% of the hybridized ^{3}H-DNA was recovered from the filter. The amount of DNA immobilized on the filter (10 μg of SV40 DNA or 50 μg of BS-C-1 cell DNA) was greatly in excess (at least 30-fold) of the amount of radioactive DNA in solution. The percentage of serially passaged ^{3}H-SV40 DNA bound to filters containing SV40 DNA or BS-C-1 DNA did not vary over the range of ^{3}H-SV40 DNA concentrations used (10^{-3} μg/ml to 2×10^{-1} μg/ml). The percent of the radioactive DNA that binds to control filters containing no DNA is reported in each instance but is not subtracted from experimental values. For the purpose of comparison, the hybridization data is also expressed as an index [(percent of radioactive SV40 DNA bound to BS-C-1 DNA/percent of radioactive SV40 DNA bound to SV40 DNA) $\times$ 100] which is a measure of the relative efficiency of hybridization to host DNA corrected for the efficiency of hybridization obtained in the SV40 DNA-SV40 DNA control reaction (6).

As discussed previously (6), the percent hybridization between the large radioactive SV40 DNA fragments and the cell DNA reflects the relative proportion of molecules which contain sequences homologous to reiterated cell DNA and not the proportion of such sequences in any one molecular fragment. Experiments to determine the average proportion of host sequences in a given molecule, and the nature of those cell DNA sequences, will be reported in the accompanying paper (11).

RESULTS

Accumulation of substituted SV40 DNA during serial passage of different virus

clones. Previously (6), a single plaque-purified isolate of SV40, strain 777, was used to demonstrate that serial undiluted passage of SV40 results in the synthesis of SV40 DNA I molecules containing sequences homologous to host DNA. This finding has now been extended to four plaque-purified virus clones, derived from two virus strains. The data are summarized in Table 1. As previously shown (6), SV40 DNA I synthesized in cells infected at high multiplicity with the plaque-purified virus stocks hybridizes to only a marginal degree with cell DNA immobilized on a filter. However, upon serial passage, each of the virus clones displays a similar behavior; with increasing passage number there is a striking decrease in the yield of plaque-forming virus and an increase in the relative proportion of SV40 DNA I molecules which hybridize to DNA derived from the host BS-C-1 cells.

The data in Table 1 also show that the percentage of virus DNA which hybridizes to host DNA at a given passage level varies widely in the different sets of serial passages. This variation is even apparent in independent sets of passages initiated by virus from the same plaque isolate (cf. CVB series 1 and 2). In the case of CV1 virus, only a relatively small proportion of SV40 DNA I molecules with measurable homology to host DNA accumulated during 10 sequential serial passages, despite a 1,000-fold decrease in the yield of plaque-forming virus. In contrast, when multiple sets of serial passages were reinitiated from earlier passages, rather than from the plaque-purified stock or original plaque isolate, the extent of host homology of the progeny SV40 DNA was more reproducible. As shown in Table 2, three different preparations of passage 3 virus, each of which was independently derived from earlier passages, produced progeny DNA with approximately the same high level of host homology. We will later discuss the possible explanations for the variations observed in the yields of substituted SV40 DNA produced in different sets of serial passages.

To determine if the serial passaging procedure selects for independently replicating virus with altered properties, three individual plaques were isolated from BS-C-1 assay plates infected with CVB passage 3 virus (Table 2) at high dilution. After two further sequential plaque purification steps, high-titer viral stocks were prepared from each of these isolates. When these plaque-purified virus stocks were used to infect BS-C-1 cells, the SV40 DNA synthesized showed little or no homology to host DNA, as is the case for all other plaque-purified viruses tested. When one of these isolates was re-subjected to the serial passaging procedure, the general pattern of accumulation of substituted SV40 DNA molecules and decreased yields of plaque-forming particles was again obtained.

Size and infectivity of substituted SV40 DNA. Yoshiiki (21) has described defective SV40 particles that arise during serial undiluted passage of the virus and which contain closed-circular DNA molecules slightly shorter than those of plaque-forming SV40. In electron microscopy studies on denatured and renatured SV40 DNA from serially passaged virions, Tai et al. (17) observed heteroduplex molecules which were shorter than the native SV40 length and which contained substitution "loops." It was therefore of interest to study the size and plaque-producing capacity (specific infectivity)

TABLE 2. *Multiple serial passages initiated from earlier passages of the same plaque-purified stock*[a]

Passage no.	Yield (PFU/ml)	Hybridization of progeny ^{3}H-SV40 DNA				
		Input counts/min	% of input bound to filters containing:			(BS-C-1/SV40) × 100
			SV40 DNA	BS-C-1 DNA	No DNA	
1	1×10^9	8,840	75	2	0.1	2.7
2	2×10^9	16,164	51	18	0.1	35.4
3	1×10^9	21,678	48	36	0.1	75.0
3′	3×10^8	34,000	38	22	0.2	58.0
3″	1×10^9	17,208	43	35	0.1	81.3
4	5×10^7	14,926	44	31	0.4	70.5

[a] Passage 1 was initiated by infecting cells with the plaque-purified stock CVB, series 1, described in Table 1 (the hybridization tests with the progeny ^{3}H-SV40 DNA I synthesized in cells infected with passages 0 and 5 of this same virus are also described in Table 1). Passage 3′ was obtained by infecting cells with another undiluted sample of passage 2. Passage 3″ was obtained by a separate series of undiluted infections starting with the yield of passage 1.

of the virus DNA which hybridizes to host cell DNA. Two types of passages SV40 were examined (Fig. 1; and Table 3); X-8 virus, a non-plaque-purified stock of SV40 maintained by multiple serial passaged at a medium multiplicity (0.1 to 1 PFU/cell), and SV40 subjected to three undiluted serial passages subsequent to plaque purification (clone CVB, passage 3 in Table 2). Plaque-purified virus, prior to serial passage, served as a control. The radioactive SV40 DNA I molecules extracted from cells infected with each of these virus preparations were sedimented through alkaline sucrose gradients, using the plaque-purified SV40 DNA I as a marker. From the sedimentation patterns shown in Fig. 1, it can be seen that, in both cases, the majority of serially passaged SV40 DNA I molecules sediment slower than the marker DNA, indicating that they are somewhat smaller in size. It should, however, be noted that the peak for the plaque-purified virus DNA marker is narrower and more symmetrical than that for the serially passaged DNA.

The fractionated SV40 DNA I molecules were next assayed for specific infectivity and homology to host DNA. In the case of the virus DNA synthesized in cells infected with X-8 virus (Fig. 1A, Table 3), the DNA at the lighter side of the peak (fractions 28 and 29) differs from that at the heavier side of the peak (fraction 17) in two respects: a 45-fold lower specific infectivity and a 2- to 3-fold increase in the level of hybridization with host DNA (the hybridization index increases from 12 to 34). Thus the sedimentation of X-8 SV40 DNA I through an alkaline sucrose gradient resulted in a partial separation of normal, plaque-producing molecules, from shorter, defective molecules which hybridize to host DNA. The control experiment with plaque-purified SV40 DNA (prior to serial passage) supports this interpretation in that the specific infectivity is uniformly high (10^5 to 5×10^5 PFU/μg of DNA) across the gradient and none of the fractionated DNA molecules hybridize detectably with host DNA (Table 3). Compared to X-8-infected cells, a larger proportion of the SV40 DNA I molecules synthesized in cells infected with the third serial undiluted passage of CVB virus contain host DNA (cf. unfractionated DNA samples in Table 3) and the hybridization index of the fractionated DNA molecules is roughly constant across the gradient. By the more sensitive test of specific infectivity, however, it is apparent that some normal-sized plaque-forming molecules are still present since the specific infectivity of the heavier fractions (nos. 19–21) is 10-fold greater than that of the lighter fractions (nos. 23, 25, 28, and 30). That these plaque-forming DNA molecules do not contain detectable host DNA sequences is confirmed by the finding that three plaque isolates, picked from assay plates infected with third-passage CVB virus at high

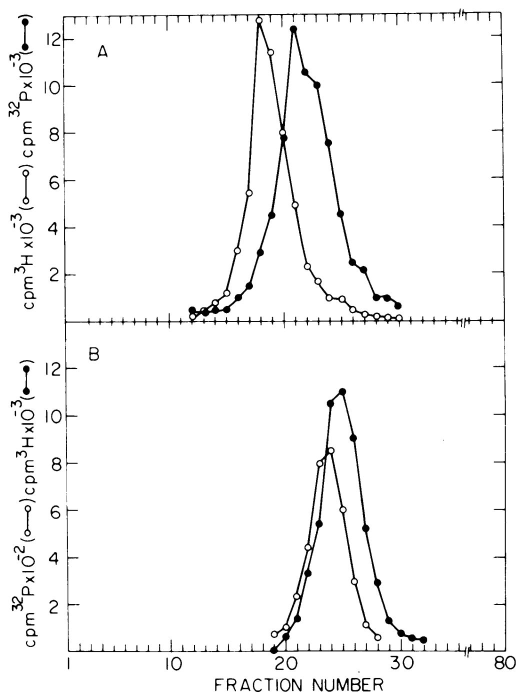

FIG. 1. *Alkaline sucrose gradient fractionation of component I SV40 DNA isolated from cells infected with plaque-purified SV40, non-plaque-purified SV40, and serially passaged SV40. A, BS-C-1 cells were infected with either a stock of plaque-purified virus (CVG, Table 1) or non-plaque-purified stock X-8 at an input multiplicity of 300 PFU/cell. The infected cells were labeled with ^{3}H-TdR or ^{32}P, and component I viral DNA was extracted and purified by CsCl-ethidium bromide centrifugation, as described in Materials and Methods. After reprecipitation in 67% alcohol, the two viral DNAs were mixed and centrifuged on an alkaline sucrose gradient (sedimentation is from right to left). Only the region showing component I (53S), the fastest sedimenting species, is shown. A small amount of material representing component II, arising from degradation of component I, was seen near the top of the gradient. Symbols: ○, ^{32}P-DNA isolated from cells infected with plaque-purified CVG; ●, ^{3}H-DNA isolated from cells infected with non-plaque-purified stock X-8. B, BS-C-1 cells were infected with either a stock of plaque-purified virus (CVB) at an input multiplicity of 50 PFU/cell or with CVB, passage 3 virus (Table 2) at 35 PFU/cell. The procedure was as described in A. Symbols: ○, ^{32}P-DNA isolated from cells infected with plaque-purified CVB; ●, ^{3}H-DNA isolated from cells infected with CVB, passage 3.*

TABLE 3. *Hybridization and specific infectivity of component I SV40 DNA after fractionation on alkaline sucrose gradients*[a]

^{3}H-SV40 DNA	Fraction from gradient	Specific infectivity [(PFU per μg of DNA) × 10^{-3}]	Hybridization				
			Input counts/min	% of input bound to filters containing:			(BS-C-1/SV40) × 100
				SV40 DNA	BS-C-1 DNA	No DNA	
Plaque-purified CVG	14	530	6,700	55	0.1	0.1	0.2
	18	NT	31,000	39	0.1	0.1	0.3
	22	110	5,900	61	0.1	0.1	0.2
	24 + 25	390	NT				
	Unfractionated	NT	29,500	71	0.1	0.1	0.1
X-8	17	136	7,600	74	9	0.1	12
	19	135	20,500	59	11	0.1	19
	21	9	31,900	54	13	0.2	24
	24	5	22,200	50	15	0.2	30
	28 + 29	3	4,400	53	18	0.1	34
	Unfractionated	5	47,200	54	14	0.1	26
Plaque-purified CVB	21	14	7,847	103	0.1	0.3	0.1
	24	26	21,184	45	0.1	0.1	0.2
	27	9	7,933	96	0.1	0.1	0.1
	Unfractionated	16	27,382	90	0.8	0.2	0.9
CVB, passage 3	19-21	30	22,246	66	35	0.3	53
	23	3	21,034	58	38	0.2	66
	25	2	20,885	74	44	0.1	59
	28	3	20,970	60	38	0.2	63
	30	3	3,407	109	76	0.3	70
	Unfractionated	1	30,980	66	34	0.1	51

[a] SV40 component I DNA was prepared and fractionated on alkaline sucrose gradients as described in Fig. 1 except that the DNA from each infection was sedimented separately. In each case ^{3}H-DNA was used. The alkaline sucrose runs were carried out simultaneously with the related one described in Fig. 1. The sedimentation patterns of X-8 and CVG virus DNAs were identical to those shown in Fig. 1A; and those of CVB and CVB passage 3 virus DNAs were identical to those shown in Fig. 1B. Samples of the indicated gradient fractions were assayed for specific infectivity of the DNA and for hybridization. The lines labeled "unfractionated" state the data for the DNA prior to alkaline sucrose sedimentation. NT, Not tested.

dilution, failed to produce progeny with measurable homology to host DNA prior to the serial passaging procedure. We have also examined the specific infectivity of virus DNA molecules extracted from virions rather than from the infected cells; the specific infectivity of the third serial passage of clone CVB virus was 10-fold lower than that of the DNA from the parent plaque-purified stock used to initiate the serial passages. These experiments, therefore, indicate that the majority of substituted SV40 DNA I molecules are defective with respect to plaque production and somewhat smaller in size than the nondefective molecules which display no detectable homology to host DNA.

Evidence for the replication of substituted SV40 DNA. Although the majority of substituted SV40 DNA molecules are defective with respect to the formation of plaques, the accumulation of such molecules during serial passages suggested that they may replicate under the high multiplicity conditions used in the serial passaging procedure. To explore this possibility, two types of experiments were performed. In the first, we studied the possible presence of host sequences in the replicative forms of SV40 DNA; in the second approach, we isolated the host sequences from randomly fragmented substituted DNA to determine if these sequences had incorporated a radioactive precursor of DNA.

To determine if host sequences are present in replicative forms (RF) of the virus DNA, we isolated SV40 DNA from cells infected with CVB, passage 3 virus (Table 2), and labeled with ^{3}H-TdR for short periods of time. The sedimentation of material from infected cells labeled for 7 min is shown in Fig. 2. The material sedimenting in the 25 to 26S region of the neutral sucrose gradient is considered to be

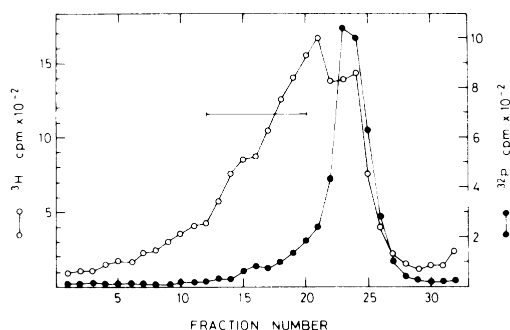

FIG. 2. *Sucrose gradient centrifugation of pulse-labeled SV40 DNA from cells infected with serially passaged virus. The ^3H-labeled SV40 DNA (O) was isolated from cells infected with CVB passage 3 virus (Table 2) after a 7-min pulse with ^3H-TdR at 50 h postinfection and sedimented in a 5 to 20% neutral sucrose gradient, as described in Materials and Methods. ^{32}P-SV40 DNA component I (●), prepared from cells infected with plaque-purified virus and labeled 24 to 50 h postinfection, serves as the marker. The fractions designated by the horizontal bar, in the 25 to 26S region of the gradient, were pooled. These fractions are considered to contain the replicative forms (RF) of the ^3H-SV40 DNA.*

RF-DNA (7, 15) since its proportion relative to that of mature SV40 DNA I (which sediments at 21*S*) decreased with increasing pulse time and it was totally converted into material sedimenting at 16*S* or less in alkaline CsCl solutions (data not shown). The hybridization results with various RF-DNA preparations are shown in Table 4. In experiment 2, the RF-DNA was further purified by a second cycle of sedimentation in a neutral sucrose gradient. In both experiments, the high levels of hybridization to host DNA indicate the presence of host sequences in the replicative forms of the virus DNA.

The isolation of the host sequences by random fragmentation of substituted SV40 DNA to small pieces is shown by the experiment described in Table 5. Intact strands of ^{3}H-labeled substituted virus DNA and 4*S* fragments derived from these intact strands were hybridized with BS-C-1 cell DNA. The radioactive material bound to the filters containing the host DNA was then eluted and tested for its ability to rehybridize back to plaque-purified SV40 DNA or cell DNA. In confirmation of previous results (6), the covalent linkage between the viral and cellular sequences in the intact chains is demonstrated by the data in lines 1 and 2 of Table 5; 67% of the material eluted from the BS-C-1 filter rehybridized back to plaque-purified SV40 DNA, and the BS-C-1/SV40 hybridization index (48%) was similar to that obtained in the first cycle of hybridization (57%). In contrast, the data in lines 3 and 4 (Table 5) show that the covalent linkage between viral and cellular sequences was disrupted in the majority of 4*S* fragments; of the 12% which hybridized to BS-C-1 DNA, only 5% rehybridized back to SV40 DNA whereas 65% rehybridized back to cellular DNA. The host sequences were thus effectively separated from the viral sequences in the population of 4*S* fragments. Since both types of fragments are radioactive (lines 3 and 4), the data demonstrate that ^{3}H-TdR was incorporated into both the viral and host sequences present in the intact substituted SV40 DNA chain.

DISCUSSION

Previously we reported that serial undiluted passage of one plaque-purified SV40 isolate resulted in the accumulation of SV40 DNA molecules containing polynucleotide sequences homologous to sequences found in the DNA of the host cell (6). The results summarized in Table 1 extend these observations to four plaque-purified SV40 isolates, derived from two different virus strains, and the data in Table 5 supply further confirmation of the covalent linkage between the viral and host sequences. The phenomenon of host substitution in the viral genome may be a general feature of DNA tumor viruses, since recent studies with polyoma show that plaque-purified isolates of this virus also generate substituted progeny, after serial undiluted passage in primary mouse kidney cells (S. Lavi and E. Winocour, manuscript in preparation).

It is known from the work of others that serial undiluted passage of SV40 results in the accumulation of defective virions (18) containing closed-circular viral DNA which is slightly smaller than the DNA of plaque-forming particles (21). No data relevant to the possible presence of host sequences in this DNA was presented. In the present communication we have shown that the majority of substituted SV40 DNA molecules are, similarly, defective as plaque producers and somewhat smaller and more heterogeneous in size compared to plaque-forming DNA. Nevertheless, it is clear from the studies reported here and in the accompanying paper (13) that substituted SV40 DNA does replicate during serial undiluted passage of the virus. This conclusion is based upon the findings that host DNA sequences are also present in replicative forms of SV40 DNA and that ^{3}H-thymidine is incorporated into both the cellular and viral sequences of the substituted genome. Shortened replicative forms of what is

presumably defective SV40 DNA have been observed by Yamamoto and Oda (20) in electron micrographs of DNA from undiluted passaged virus stocks. In cells infected with serially passaged virus at high multiplicity, the replication of defective DNA may proceed by complementation with nondefective molecules or by complementation between different types of defective SV40 DNA molecules.

It is possible that substituted SV40 DNA has a selective advantage during the serial passaging procedure. Preferential replication of defective molecules might arise either from an inhibition of nondefective DNA synthesis (5, 18) or from an absolute increase in the rate of defective DNA replication compared to nondefective. The smaller average size of the substituted molecules and the concomitant shorter time required for replication could well account for preferential synthesis. Even if these molecules are only 10 to 20% shorter than the DNA of the plaque-purified type, they could overtake the longer molecules after only a few rounds of replication. A second possible explanation is that replication of the host sequences is more rapid than replication of SV40 sequences and

TABLE 4. *Presence of host sequences in SV40 DNA replicating forms from cells infected with serially passaged virus*

Expt[a]	Pulse time (min)	Hybridization of ^{3}H-SV40 RF-DNA				
		Input (counts/min)	% of input bound to filters containing:			(BS-C-1/SV40) × 100
			SV40 DNA	BS-C-1 DNA	No DNA	
1	5	3,271	92	56	0.2	61
	10	8,038	85	44	0.4	52
	15	17,249	66	52	0.5	79
2	7	6,545	63	48	0.3	76

[a] Expt 1: The ^{3}H-labeled replicative forms (RF) of SV40 DNA were isolated from cells infected with CVB, passage 3 virus, as described in Fig. 2, except that the pulse times were 5, 10, and 15 min, and the mature component I SV40 ^{32}P-DNA used as a sedimentation marker was derived from cells infected with CVB, passage 3 virus. The ^{32}P counts per minute in the pooled ^{3}H-labeled RF-DNA fractions was no more than 5% of the ^{3}H counts per minute. The data for the hybridization experiments were, however, all obtained under double-label counting conditions in which the spillover of ^{32}P counts into ^{3}H counts was insignificant. The RF-DNA was denatured by the formamide procedure (see Materials and Methods) prior to hybridization. Expt 2: The RF-DNA was that preparation described in Fig. 2, recentrifuged a second time under the same conditions. The hybridization procedure was as for expt 1.

TABLE 5. *Separation of the host and viral sequences in 4S random fragments of substituted SV40 DNA*

^{3}H-SV40 DNA[a]	Input counts/min	% of input hybridized to filters containing:			(BS-C-1/SV40) × 100
		SV40 DNA	BS-C-1 DNA	No DNA	
1. Component II	41,965	53	30	0.2	57
2. Component II eluted from BS-C-1 filter line 1	1,260	67	32	0	48
3. Random 4*S* fragments	17,600	81	12	0.2	14
4. 4*S* fragments eluted from BS-C-1 filter line 3	495	5	65	0	1300

[a] Cells were infected with CVB passage 3 virus (Table 2), labeled, and the virus DNA was isolated as described in Materials and Methods. Component I was purified by CsCl-ethidium bromide equilibrium centrifugation. Component I ^{3}H-SV40 DNA was treated with dilute pancreatic DNase (5×10^{-5} µg/ml, 15 min, 37 C) and component II (16*S* plus 18*S*) was isolated by sedimentation through a 5 to 20% alkaline sucrose gradient (line 1). Component I ^{3}H-SV40 DNA was sonically treated at full power in a Raytheon 10 kcycle/s sonic vibrator for 120 min, and centrifuged through a 5 to 20% neutral sucrose gradient. The fragments that sedimented together with 4*S* tRNA were pooled and used for hybridization (line 3). ^{3}H-DNA hybridized to BS-C-1 DNA was recovered from the hybrid complex (lines 2 and 4) by treating the filters with the formamide denaturing solution described in Materials and Methods.

that the higher the proportion of host sequences in a given molecule the greater its kinetic advantage. Thirdly, if, in fact, initiation is the rate-limiting step in replication, the advantage of the substituted molecules could also be ascribed to preferential initiation.

We turn now to the variations in the yields of substituted SV40 DNA described in Table 1. It should first be noted that the host sequences detected in the present report and earlier ones (1, 6) are homologous to the reiterated or repetitive sequences in the host DNA since the conditions of hybridization do not permit detection of unqiue sequences. However, as indicated by the experiments in the accompanying paper (11), unique host sequences are also likely to be incorporated into substituted SV40 DNA. Hence, the apparently lower yields of substituted SV40 detected in some sets of serial passages could be partly due to the production of substituted molecules containing solely unique host sequences (in part, the variations could also be due to the synthesis of defective DNA with deletions but no host substitutions). Work currently in progress on the CV1 series of passages (Table 1) suggests that many of the defective viral DNA molecules produced do, in fact, contain a substitution with solely unique host sequences. It thus appears that the serial passaging procedure can generate different types of substituted SV40 DNA. The data in Table 2 suggest that events which occur during an early passage may dictate the nature of the substituted viral progeny produced during subsequent passages.

The present experiments do not afford any insight into the mechanism by which the substituted viral DNA molecules are initially formed. Lavi and Winocour (6) suggested recombinational events between cellular and virus DNAs as one possible mechanism, and evidence supporting the concept of viral integration during SV40 lytic infection has recently been reported (3, 13). The primary recombination events may continue throughout the serial passaging procedure. However, in addition to these primary events, selection on the basis of preferential replication may play a dominant role in determining the types of defective, including substituted, SV40 DNA molecules that accumulate during any given set of serial passages.

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