Studies on a Defective Variant of Simian Virus 40 That Is Substituted with DNA Sequences Derived from Monkey DNA

I. ORIGIN, PROPERTIES, AND PURIFICATION

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Serial passage of plaque-purified simian virus 40 at high multiplicity in monkey kidney cells produces defective virus. A particular series of such passages, in which the defective genomes contain substitutions by host (monkey) DNA sequences, is described here. The suspensions of virus obtained during the passage are shown to be defective by the usual criteria, namely, a decreased yield of plaque-forming units compared to the yield from infection with wild type virus. It is shown that the rate of viral DNA synthesis in cells infected with passaged virus, which includes the synthesis of the host-substituted variants as well as that of remaining wild type virus, can also be used as a measure of the defectiveness of the yields of the passages. For this purpose, conditions are described under which the rate of viral DNA synthesis in infected cells is proportional to the multiplicity of infection as calculated from plaque-forming units of virus. This proportionality holds both for wild type, plaque-purified virus, and for lysates containing defective variants. Further, the rate of DNA synthesis was used to study the course of infection during serial passaging. Interference with the synthesis of wild type viral DNA by the defective lysates was also demonstrated in this manner.

The nature of the viral genomes synthesized in one particular passage of the series under study was investigated. Genomes of at least three types were present: (a) wild type SV40 genomes; (b) defective genomes that are somewhat shorter than wild type DNA and give patterns of DNA fragments upon digestion with restriction endonucleases HindII and III that differ markedly from the pattern typical of wild type DNA; and (c) defective genomes that give still another pattern of DNA fragments upon digestion with HindII and III, that have lost the single cleavage site for restriction endonuclease EcoRI that is typical of wild type SV40 DNA, and that contain sequences derived from monkey DNA.

The defective simian virus 40 variants that arise upon serial high multiplicity passage of virus in permissive monkey kidney cells have been the subject of extensive study in recent years. Preparations of defective viral DNA from diverse passages have been shown to contain deletions of original viral sequences, substitutions by both repetitive and unique host DNA sequences, and reiterations of both viral and host sequences (1-23). The formation and accumulation of defective variants upon serial undiluted passage is reflected in continuously decreasing yields of plaque-forming particles with successive passages (1, 2, 6, 11, 17, 18) as well as in the formation of altered genomes. Further, stocks of some passaged, defective SV40 markedly reduce the yield of plaque-forming particles when the defectives and wild type viruses co-infect cells (1). The interfering material present in the lysates was shown to be sedimentable, and to be neutralized by SV40 antibodies (1). Closed, defective variants of SV40 have been reported to interfere with wild type SV40 DNA synthesis (25). DNA from similar defective polyoma variants (26-31) inhibits plaque formation by wild type DNA (28).

We have been interested in studying defective SV40 variants containing both unique and repetitive host (monkey) DNA sequences. The starting material for these investigations was defective virus arising from a set of high multiplicity serial passages of plaque-purified strain 777 SV40 (6, 9, 10, 14). Earlier studies (9, 10, 14) indicated that this material contained viral DNA substituted with both unique and repetitive monkey DNA sequences. The passaged virus stock is called CVB1/P3 in the earlier work.

We report here the further passaging of CVB1/P3 and the properties and changing character of the mixture of defectives upon subsequent serial passage. We also describe experiments designed to purify clones of particular defectives from the passages, and finally, the purification from a mixture of defectives of a particular variant genome that is resistant to endo-

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nuclease R-EcoRI (32). Further, conditions under which the rate of viral DNA synthesis in infected cells is proportional to the multiplicity of infected (plaque-forming units (pfu) per cell) are described. The rate of DNA synthesis is used to study the defectiveness of the passaged virus and the course of infection during the passages.

In the accompanying paper (33) we report an analysis of the structure of this endonuclease R-EcoRI resistant genome by restriction endonuclease mapping.

EXPERIMENTAL PROCEDURES

Cells and Virus Stocks—All experiments were carried out with the BSC-1 line of monkey kidney cells: cells were cultured as described previously (34). A stock of plaque-purified SV40, strain 777, was kindly provided by Dr. Ernest Winocour of the Weizmann Institute of Science. The plaque from which the 777 stock was derived was named CVB (6). New stocks were prepared in this laboratory by infecting confluent monolayers of BSC-1 cells at multiplicities of less than 1 × 10^3 plaque-forming units per cell. When the cytopathic effect was complete, the lysate was collected and treated as previously described (6). Virus was titrated by plaque assay on BSC-1 monolayers (6).

Defective virus stocks were all derivatives of the passage series designated CVB Series 1 (called CVB/1 in previous papers; the series was initiated with a stock of CVB (see above) (6, 9). The various stocks were obtained as follows. CVB Series 1, passage 3 (CVB/1/P3) was obtained from Dr. Ernest Winocour. This material was subjected to further serial undiluted passages in our laboratory, yielding the procedures previously described (6). These stocks will be referred to as CVB/1/P4 through CVB/1/P8 (see Table I). All viral stocks were sonicated for 45 s at full power in a 10 kc Raytheon sonifier prior to use.

Additional defective viral stocks were obtained by isolation of plaques from the stocks just described according to the procedure of Boussinault and Nasmyth (36). In brief, wells were prepared in solidified plaque, and plaques arising after infection with dilutions of the passaged stocks, were picked, suspended, and used to infect cell monolayers. Adsorption was carried out with the plates at a slight angle to insure co-infection by a defective variant and accompanying wild type virions. After complete cytopathic effect (11 days) the lysate was collected, sonicated, and used as stock. In the experiments described in this paper, only a single such isolate was used. It was obtained from a plaque recovered after infection with CVB/1/P8. The resulting stock (CVPS) had a titer of 2 × 10^9 pfu/ml, and was used to initiate a series of undiluted passages as before (6); this series will be referred to as CVPS1/P8, where n designates the passage number (see Table I).

Labeling of Cultures and Preparation of Labeled DNA for Rate Studies—At various times after infection (times indicated with each experiment), the medium was removed from plates and replaced with modified Eagle's medium (37) containing 2% fetal bovine serum in addition to the 0.05 M Tris/HCl, pH 7.5, and 0.15 M NaCl. The [3H]-labeled virus was then analyzed by centrifugation to equilibrium in CsCl. Solid CsCl was added to the suspension of virus to give a density of 1.33 g/cm^3 prior to centrifugation. After centrifugation (SW50 rotor, Beckman L5-65B centrifuge, 35,000 rpm, 62 h, 20°C) drop fractions were collected from the bottom of the tube and radioactivity was determined.

Preparation of Purified Wild Type and Defective Viral DNA—Cells were infected as described above. A multiplicity of infection of about 90 pfu/cell was used with wild type strain 777 SV40, and infections with passaged virus were made with 1 ml of undiluted lysate per plate (100-mm diameter) (approximately 2 × 10^6 cells). At 24 h after infection [3H]-labeled DNA (about 20 Ci/mmol, 10 μCi/ml of medium, New England Nuclear Corp.) or [14C]-labeled DNA (5 μCi/ml, about 50 Ci/mmol, Schwarz/Mann) was added: in other instances the cells were washed with phosphate-free medium and phosphate-free medium containing 60 μCi of [3H]-labeled inorganic phosphate per ml were added. At 48 h after infection viral DNA was isolated by the Hirt procedure (35). The Hirt supernatant fractions were extracted with phenol and the DNA precipitated with two volumes of ethanol at −20°C. The precipitated DNA was dissolved in 0.1 M Tris/HCl, pH 7.8, 0.01 M EDTA and dialyzed against the same buffer. It was then purified by centrifugation to equilibrium in CsCl containing ethidium bromide, as described below. Fractions containing closed circular duplex DNA (DNA I) were pooled, ethidium was removed (see below), and the DNA was dialyzed against 0.1 M NaCl, 1.5 mM Tris/HCl, pH 7.5, 0.01 M EDTA. DNA was precipitated with two volumes of ethanol at −20°C, the precipitates were collected by centrifugation and dissolved in 0.1 M NaCl, 1.5 mM Tris/HCl, pH 7.8, 1 mM EDTA, and treated for 30 min at 57°C with 50 μg/ml of heated (100°C, 10 min) pancreatic DNase I. The material was then centrifuged through a neutral sucrose gradient, as described below, and DNA A (approximately 21 S) was collected. In general, [3H]-labeled DNA had a specific radioactivity of about 200,000 cpm/μg, and [14C]-labeled DNA had a specific radioactivity of about 30,000 cpm/μg. For the preparation of DNA resistant or sensitive to endonuclease R-EcoRI, DNA I was purified by sucrose gradient centrifugation and treated with endonuclease R-EcoRI as described below.

The mixture was extracted with phenol, and dialyzed and precipitated as above. DNA A (EcoRI resistant) was separated from the EcoRI-sensitive linear duplex DNA (DNA III) by equilibrium centrifugation in CsCl in the presence of ethidium bromide, followed by centrifugation in neutral sucrose gradients. The resulting DNA III (EcoRI sensitive) was generally a mixture of wild type and defective molecules. These were subsequently separated from each other by electrophoresis in 1.4% agarose gels as described below.

The DNA was fragmented by sonication, unless otherwise indicated, and hybridization of labeled DNA to unlabeled DNA on nitrocellulose filters was carried out as previously described (6). Data are expressed as hybridization index, which is the percentage of a given sample of DNA that hybridized to 35 μg of DNA per ml of monkey DNA A of control No. 2 with 10 μg of DNA. A sample of DNA that hybridized to 10 μg of SV40 DNA from plaque-purified virus on a filter, multiplied by 100. This procedure detects the presence of monkey reiterated sequences in the labeled DNA preparation, but not monkey unique sequences (6, 9).

Centrifugation—DNA was centrifuged to equilibrium in CsCl gradients in the presence of ethidium bromide (38) as previously described (6). Ethidium bromide was removed from pooled fractions with Dowex 50 (38). Centrifugation in 10 to 30% neutral sucrose gradients was carried out with 10 μl Tris/HCl, pH 7.8, 10 μl EDTA, 0.1 ml from cell cultures and washed once in a SW50 rotor (Beckman L5-65B centrifuge). Alkaline sucrose gradients (5 to 20%) contained 0.3 M NaOH, 0.7 M NaCl, 2.5 M EDTA, and 0.05% sodium lauryl sarcosinate (Chemical Additives Co.) and centrifugation was at 49,000 rpm for 90 min at 5°C in the SW50.1 rotor. For analytical purposes gradients were collected dropwise directly into vials and counted in a triton/toluene scintillation fluid. For preparative purposes, fractions were collected in tubes and samples counted as above prior to pooling appropriate fractions.

Gel Electrophoresis—Electrophoresis was carried out in 20-cm-long, 6-mm-wide, cylindrical 1.4% agarose gels (Sigma Chemical Co.) room temperature for 20 h. The medium contained 0.04 M Tris/HCl, pH 7.8, 0.02 M sodium acetate and 2 mM EDTA. Samples were applied after addition of sucrose to 20% and about 0.1 volume of 1% bromophenol blue. The running buffer was the same as that used to prepare the gels. For analytical purposes slices (2 mm) were cut
and minced with a Gilson gel slicer and suspended in a triton/toluene scintillation fluid and counted. For preparative purposes bands were visualized with ethidium bromide (39), appropriate sections were then cut out and homogenized with 0.015 M NaCl, 1.5 mM sodium citrate or a 10-fold dilution thereof in a Dounce homogenizer. The suspension was passed through a column containing a layer of Sephadex G-25 to remove bits of agarose. Pulverized material was pooled and passed over Sephadex G-25 to remove bits of agarose.

Polyacrylamide gel electrophoresis (6% acrylamide, acrylamide:bisacrylamide, 40:1), was carried out in 6-mm-wide cylinders, 19 cm long, for 15 h at 50 V and room temperature in the presence of 0.2% sodium dodecyl sulfate. The buffer mixture also contained 0.04 M Tris/HCl, pH 7.8, 0.02 M sodium acetate, 2 mM EDTA. Samples were made 6% in glycerol and 0.2% in sodium dodecyl sulfate prior to layering on the gel. Bromphenol blue was used as the tracking dye. At the end of the run, 1-mm slices were cut (as above) and the dried, minced slices were incubated overnight at 50-60°C in 5 ml of 30% H2O2, before counting in triton/toluene scintillation fluid.

Digestion With Restriction Endonucleases - Purified DNA I (5 pg/ml) was treated with endonuclease R. EcoRI (Miles Chemical Co.) in 0.1 M Tris/HCl, pH 7.5, 0.05 M NaCl, 0.01 M MgCl2, for 2 h at 37°C. Preliminary experiments were carried out to determine, for each experiment, the amount of enzyme required to obtain complete digestion of all susceptible molecules. The reaction was stopped by the addition of concentrated EDTA to 20 mM.

Digestions with the mixture of endonucleases R-(HindII/HindIII) (40) were carried out with about 20 µg of DNA per ml in 0.05 M NaCl, 1.5 mM Tris/HCl, pH 7.5, 8.7 mM MgCl2, for 2 h at 37°C. The reaction was stopped with EDTA as above. The mixture of endonucleases R-(HindII/HindIII) was prepared according to Smith (40). With this preparation, 5 µl of enzyme gave complete digestion of 1 µg of DNA under the conditions specified above.

Electron Microscopy - Supercoiled viral DNA was treated briefly with D1ase to form relaxed circular duplexes and prepared for electron microscopy by a modification of the aqueous Kleinschmidt technique (41). Grids were examined in a Siemens Elmskop 101 at an accelerating voltage of 40 kV and photographed on Kodak electron image plates at a magnification of ×6000. Length measurements were made on a PDP-10 digital computer equipped with a Rand tablet and stylus. Magnification was determined by using a grating replica (Ernest Fullam, Inc., Schenectady, N.Y.). We are grateful to Dr. Claude Garon for carrying out the electron microscopy.

RESULTS

Characterization of the DNA Produced Upon Infection with CVPS/1/P2 - As described above, in order to obtain a single species of defective substituted SV40 for further study, we attempted to select a single species by the plaque isolation technique of Brockman and Nathans (12). A single plaque, called CVPS, was isolated after infection of BSC-1 cells with passage CVB/1/P8. As shown in Table I, high multiplicity passage of the stock CVPS (CVPS/1/P) afforded a lysate which yielded viral DNA that hybridized efficiently to monkey DNA (hybridization index 17). (Several other plaque isolates obtained from CVPS did not yield substituted molecules upon one or two passages, indicating that they contained only wild type SV40.) This finding suggested that substituted defective molecules were present in CVPS. However, as the following series of experiments indicated, several different defective species were probably present in the plaque rather than a defective of a single type.

SV40 DNA I present in the Hirt supernatant fraction obtained after infection with CVPS/1/P2 was purified by sedimentation to equilibrium in CsCl density gradients containing ethidium bromide. SV40 DNA I isolated from cells infected with wild type strain 777 SV40 and cells infected with CVPS/1/P2 both gave narrow symmetrical peaks at the density expected for superhelical SV40 DNA. On neutral sucrose gradients (Fig. 4a) the DNA I from cells infected with CVPS/1/P2 sedimented as a broader band than did the wild type marker DNA and contained some material smaller than full length molecules. The size heterogeneity and presence of small molecules was also apparent when the DNA I was sedimented on alkaline sucrose gradients (Fig. 4b), when the DNA I was subjected to electrophoresis on agarose gels (Fig. 4c) and when the length of nicked circular molecules was measured on electron micrographs. SV40 strain 777 DNA had an average length of 1.68 ± 0.05 µm (103 molecules measured) while CVPS/1/P2 DNA had an average length of 1.58 ± 0.08 µm (107 molecules measured).

Upon treatment of DNA I isolated from cells infected with CVPS/1/P2 with endonuclease R. EcoRI, the DNA was resolved into several species, some of them being sensitive to and others resistant to the endonuclease R. EcoRI. This conversion was demonstrated by centrifugation in neutral (Fig. 4d) or alkaline (Fig. 4e) sucrose gradients or by electrophoresis on agarose gels (Fig. 4f). Independent experiments at various concentrations of endonuclease R. EcoRI showed that the conditions used in Fig. 4 afforded essentially complete cleavage of all susceptible circular duplexes (with the exception of Fig. 4 where insufficient enzyme was present). Thus, exhaustive digestion with endonuclease R. EcoRI yielded (a) a small amount of DNA III of the size expected for for wild type SV40, (b) some DNA III that is significantly shorter than wild type DNA III and represents defective molecules that are sensitive to endonuclease R. EcoRI (CVPS/1/P2 (EcoRI sens)) and (c) closed circular DNA resistant to EcoRI cleavage (CVPS/1/P2 (EcoRI res)). The CVPS/1/P2 (EcoRI res) DNA I appears, from the data in Fig. 4 to be slightly smaller than full length SV40, a conclusion confirmed by measurement of the contour lengths of relaxed circles prepared from CVPS/1/P2 (EcoRI res) (data not shown).

In general, approximately 50% of the total circular duplex DNA isolated from cells infected with CVPS/1/P2 was resistant to cleavage by endonuclease R. EcoRI, although the numbers varied from 40 to 65% from preparation to preparation (see Table I). The amount of full length, EcoRI-sensitive DNA presumed to be wild type (DNA III) also varied and was generally between 5 and 10% of the total, or less (see, for example, Fig. 4f).

Presence of Host Repetitive Sequences in CVPS/1/P2 (EcoRI res) DNA I - The presence of DNA sequences derived from monkey repetitive sequences in total CVPS/1/P2 DNA is indicated by the data in Table I. The data in Table I were obtained using DNA fragmented by sonication. When full length linear strands of total CVPS/1/P2 DNA were tested for hybridization to monkey DNA on filters rather than sonicated fragments, 48% as much of the DNA hybridized to monkey DNA as to SV40 wild type DNA (Table V, line 3). At the separated EcoRI-resistant and EcoRI-sensitive species present in CVPS/1/P2 were tested for hybridization, only the EcoRI-resistant
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Fig. 1. Effect of multiplicity of infection on the rate of viral DNA synthesis. Confluent monolayers of BSC-1 cells were infected with SV40 strain 777 or CVB/1/P4 at the indicated number of plaque-forming units per cell (MOI). Infected cells were labeled with \(^{3}H\)dTd for 15 min at 24, 37, and 48 h after infection (A) or 35 hours after infection (B). A, SV40 strain 777; O, 24 h; ©, 37 h; A, 48 h; inset, 24 h; ©, 37 h. (35 h) O, SV40 strain 777; •, CVB/1/P4. The values have been corrected for the incorporation of \(^{3}H\)dTd in mock infected cells. See p. 5123 for a description of this experiment.

Fig. 2. Growth cycle of CVB/1/P8. Confluent monolayers of BSC-1 cells were infected with 1 ml of undiluted stock of CVB/1/P8. At indicated times after infection the medium (10 ml) was removed and 5 ml of phosphate-buffered saline was added to the monolayer. The cell monolayer was scraped off with a sterile rubber policeman. The resulting cell suspensions were sonicated at full power in a Raytheon 10 kc sonifier for 1 min. The plaque-forming units in the supernatant medium and sonicated cell extracts were measured. The total plaque-forming units in the medium \((1.7 \times 10^5)\) and sonicated cell extract \((3.8 \times 10^5)\) at 2 days after infection were taken as equal to 1 and the units in other samples were calculated relative to those in the 2 day samples. O, plaque-forming units released into medium, •, plaque-forming units in cell monolayer. On the ordinate, pfu is plaque-forming units. See p. 5123 for a description of this experiment.

Fig. 3. Production of \(^{3}H\)dTd-labeled viral particles with passaged virus. Confluent monolayers of BSC-1 cells were infected with 1 ml of undiluted passaged virus stock or a mixture of SV40 strain 777 (10 plaque-forming units per cell) and 1 ml of undiluted CVP8/1/P4 stock in a volume of 2 ml and \(^{3}H\)dTd was added at 24 h as described under “Experimental Procedures.” For each stock, two plates of cells were used and equivalent aliquots of the \(^{3}H\)dTd-labeled virus concentrated by the polyethylene glycol procedure were mixed with \(^{3}C\)labeled SV40 strain 777 virus and banded to equilibrium on CsCl gradients. ---, \(^{3}H\)dTd labeled passaged virus; ——, \(^{3}C\)labeled SV40 strain 777 virus as marker. Infecting virus was: A, CVP8/1/P1; B, CVP8/1/P2; C, CVP8/1/P3; D, CVP8/1/P4; E, CVP8/1/P6; F, mixed infection of SV40 strain 777 and CVP8/1/P4. The individual points are not shown; every fraction was counted. See p. 5123 for a description of this experiment.

Fig. 5a shows the results obtained when total \(^{3}P\)-labeled CVP8/1/P2 DNA I, and \(^{3}H\)-labeled CVP8/1/P2 (EcoRI res) DNA I were mixed, digested to completion with endonucleases R-HindIII/HindIII, and then analyzed by polyacrylamide gel electrophoresis. The pattern of fragments obtained from the total defective DNA closely resembled previously published patterns obtained with CVB/1/P3 (10); only low levels of wild type fragments were produced (the typical wild type pattern

species hybridized to monkey DNA (Table VI, lines 4 and 5). The CVP8/1/P2 (EcoRI res) DNA hybridized almost equally well to monkey and SV40 DNA. The data presented in Table VI and Fig. 4 suggest that about one-half of the total defective DNA is resistant to endonuclease R-EcoRI and that all the host repetitive sequences are present in that species of DNA.
Defective SV40 DNA Substituted with Host (Monkey) Sequences

Table VI

Hybridization of CVP8/1/P2 DNA to SV40 and monkey DNA on filters

<table>
<thead>
<tr>
<th>DNA on filter</th>
<th>DNA on</th>
<th>Hybridization index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SV40</td>
<td>monkey</td>
</tr>
<tr>
<td></td>
<td>cpn</td>
<td>% hybridized</td>
</tr>
<tr>
<td>Wild type 777</td>
<td>13,720</td>
<td>71</td>
</tr>
<tr>
<td>CVP8/1/P2 (sonicated)</td>
<td>13,400</td>
<td>57</td>
</tr>
<tr>
<td>CVP8/1/P2</td>
<td>5,820</td>
<td>27</td>
</tr>
<tr>
<td>CVP8/1/P2 (EcoRI res)</td>
<td>4,674</td>
<td>41</td>
</tr>
<tr>
<td>CVP8/1/P2 (EcoRI sens)</td>
<td>29,180</td>
<td>21</td>
</tr>
</tbody>
</table>

Except for the sample indicated as sonicated, all labeled DNA samples were full length molecules: 777, CVP8/1/P2 and CVP8/1/P2 (EcoRI res) strands were obtained from closed circular molecules by irradiation damage during storage and separation of the resulting nicked circles (DNA II) by centrifugation. Full length linear molecules (DNA III) of CVP8/1/P2 (EcoRI sens) were obtained by endonuclease R.EcoRI digestion and purification by centrifugation. Hybridization index is defined under "Experimental Procedures."

FIG. 4. Physical characterization of viral DNA isolated from BSC-1 cells infected with passage CVP8/1/P2, before and after treatment with restriction endonuclease R.EcoRI. Viral DNA was isolated from infected cells and purified by centrifugation to equilibrium in CsCl-ethidium bromide and neutral sucrose gradient centrifugation, as described under "Experimental Procedures." The abscissas are all the same, except for c', which is as indicated. O — O, 1C-labeled wild type marker DNA; •—•, 3H-labeled CVP8/1/P2 DNA. a, b, and c are before endonuclease R.EcoRI treatment, a', b', and c' are after. a, Neutral sucrose: 3H-labeled CVP8/1/P2 DNA in the presence of W-labeled SV40 strain 777 component I DNA I to generate the DNA III marker. The wild type DNA, was evident. Fig. 5a shows that CVP8/1/P2 (EcoRI sens) DNA was purified by agarose gel electrophoresis as shown in Fig. 4c; only the faster moving component III was eluted. The pattern obtained from CVP8/1/P2 (EcoRI sens) was again markedly different from the original mixture of defectives and was different from CVP8/1/P2 (EcoRI res) as well. In particular, no fragment the size of fragment E was produced, a finding which is consistent with the observation (Table VI) that none of the host repetitive sequences present in the mixture of defectives was in the species sensitive to endonuclease R.EcoRI. The data in Fig. 5d demonstrate that the fragments produced by the digestion of the EcoRI-sensitive species also differed from those obtained upon digestion of wild type DNA I.

The experiments presented in Fig. 5 thus confirm the conclusion that the endonuclease R.EcoRI effects a separation of two different types of defective variants from the initial mixture.

Further Evolution of CVP8/1/P2 upon Subsequent Passaging — On the basis of hybridization to monkey DNA on filters as well as the characteristic pattern of digestion by endonucleases R Hinellll/HindIII (especially the presence of fragment E), the DNA produced upon infection with CVP8/1/P2 was markedly similar to that produced upon infection with CVP8/1/P3 (9, 10). It therefore appeared that this particular mixture of defectives was relatively stable upon passaging. Indeed this was one reason for choosing CVP8/1/P2 for detailed study. Nevertheless, as shown in Fig. 6, the mixture of defectives changes in character upon subsequent passaging of CVP8/1/P2. The pattern of DNA fragments produced by endonucleases R Hinellll/HindIII upon digestion of DNA isolated from cells infected with CVP8/1/P3, CVP8/1/P6, and CVP8/1/P7 is shown in Fig. 6. They are compared with the digests obtained with the wild type plaque-purified strain 777 SV40 (plaque CBV) as well as CVP8, CVP8/1/P1 and CVP8/1/P2. The fragments produced by the progeny of low multiplicity infection by CVP8 were essentially identical with those produced by the progeny of wild type virus. The progeny of CVP8/1/P1 and CVP8/1/P2 gave similar arrays of fragments although in different relative amounts, consistent with the increased hybrid-
sequences were also lost during the subsequent passaging of the virus. As shown by the hybridization indices reported in Table I, host repetitive sequences of CVP8/1/P2 DNA used in this experiment (Fig. 6) appeared to have a relatively low proportion of those fragments expected from the defective, endonuclease R-EcoRI-sensitive species. Using the smallest fragment (E) as an indicator, the fragment began to disappear at passage CVP8/1/P3 and was totally absent by CVP8/1/P6. The rest of the digest pattern also changed markedly during these passages. As shown by the hybridization indices reported in Table I, host repetitive sequences were also lost during the subsequent passaging of CVP8/1/P2, although the bulk of the DNA I remained resistant to endonuclease R-EcoRI (Table I).

**DISCUSSION**

We report here studies designed to establish conditions under which the rate of viral DNA synthesis in infected cells can be used to follow events during serial passaging and as a quantitative measure of the defectiveness of SV40. Conditions under which the rate of wild type viral DNA synthesis was essentially proportional to the multiplicity of infection are described. Proportionality was observed at relatively low multiplicities of infection, and we assume that it is related to the number of cells which are productively infected. At relatively high multiplicities of infection the rate became constant, presumably because the cells are maximally infected. Kato *et al.* (45) demonstrated with primary African green monkey kidney cells that even at an input multiplicity of 10 plaque-forming units per cell of standard virus, only about 20% of the cells were actively synthesizing DNA. We cannot tell from our results whether or not a single virus particle infecting a cell results in the maximal rate of infection, or whether there is an increase in rate when more than one virion infects the cells. It is of some interest that, although it differs in detail, the time course of viral DNA synthesis reported here in confluent cultures of BSC-1 cells is similar in outline to the results reported by Manteuil *et al.* (36) with growing CV-1 cells. As pointed out by Manteuil *et al.* (36), the onset of viral DNA synthesis in confluent monolayers is delayed compared to the onset in growing cells, and similarly, other characteristics, such as the time for achieving maximal rates of viral DNA synthesis, are also delayed. Comparing our results with the earlier work, the several aspects are all delayed by about 10 h in the confluent BSC-1 system compared to the growing CV-1 system.

We then studied the rate of viral DNA synthesis during the standard procedures used for serial passage of SV40, procedures known to result in the accumulation of defective virions of various types. Each lysate, that is crude stock of virus, was used undiluted to infect new cells. Those lysates with significant numbers of measurable plaque-forming units induced the synthesis of viral DNA at rates similar to those predicted on the basis of the number of plaque-forming units. Those lysates which could be characterized as highly defective on the basis of the plaque assay gave very low rates of viral DNA synthesis and the rates did not increase even over many days. Full cytopathic effect was never observed in cells infected with, for example, CVPB/1/P4. As predicted by the low or undetectable rates of viral DNA synthesis, the defective stocks were also defective in yielding detectable viral particles although some infective virus was being produced. The data in Fig. 2 show that plaque-forming virus was formed continually over a period of many days during such an infection and the data suggest that multiple cycles of viral replication occur.

The correlation between plaque-forming units in a given lysate and the rate of viral DNA synthesis induced by the lysate is of some interest. Since plaque assays were carried out with highly diluted samples, each plaque should represent infection with a wild type virus or, perhaps infrequently, complementation by two defectives. Therefore it appears that the overall rate of viral DNA synthesis is determined by the wild type virus present in the lysate. This interpretation is supported by the data in Fig. 1B. Over a 200-fold range of multiplicity of infection, the rate of DNA synthesis observed after infection with wild type SV40 was essentially the same as the rate observed after infection with CVPB/1/P4, at a given multiplicity. Since it is known that even with wild type viruses only a small proportion of virion particles result in plaque formation (46) it is difficult to interpret the results with the
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Defective SV40 DNA appears to reflect the concentration of plaque-forming virus in the infecting stock, the DNA that is being made may still be largely defective as shown by hybridization to monkey DNA and analysis with restriction endonucleases (Refs. 9 and 10, and this paper). The DNA sequence corresponding to the origin of SV40 DNA replication is conserved in various SV40 defec-
tives. This defective species, termed CVP8/1/P2 (EcoRI res) DNA, has been studied in detail (33). Information on the localization in monkey chromosomes of the repetitive monkey DNA sequences present in fragment E of CVP8/1/P2 (EcoRI res) DNA I has been studied in detail (33). Information on the localization in monkey chromosomes of the repetitive monkey DNA sequences present in fragment E of CVP8/1/P2 (EcoRI res) DNA I has been studied in detail (33).

Earlier work suggested that the stocks of passaged, defective SV40 from which those used in the present study were derived contained at least some molecules carrying DNA sequences derived from the host cells. An effort was made to select such molecules and prepare a pure clone of defective by applying the method of Brockman and Nathans (12) to the passage called CVPB/1/P8. However, the plaque CVP8 did not yield a single type of defective upon one or two passages. The presence of a high per cent of substituted molecules even after one high multiplicity passage of CVP8 (CVPB/1/P1, Table I) suggested that the defective genomes originated from the plaque rather than resulting from the evolution of new defec-
tives in the passaging. Further, the digestion of the DNA I obtained after infection with CVPB/1/P1 or CVPB/1/P2 with restriction endonucleases R-HindII/HindIII gave a pattern of fragments very similar to that obtained with the DNA from the CVB series (10).

Defective SV40 genomes containing host sequences were then purified from the total DNA I obtained upon infection with CVPB/1/P2 by taking advantage of the resistance of the host substituted genomes to cleavage by the endonuclease R-EcoRI. Wild type SV40 DNA I contains one cleavage site for this enzyme at map position 0 and full length DNA III is produced (39, 52, 53). The resistance of certain defective species to cleavage by restriction endonucleases known to cleave wild type SV40 has been used previously for the purification of defective species (16). In the present instance, treatment with endonuclease R-EcoRI revealed that at least three species were present in the mixture of DNA I produced by infection with CVPB/1/P2. A small per cent of wild type SV40 DNA I was present as determined by the full length DNA III produced and the presence of small amounts of characteristic fragments upon cleavage with endonuclease R-HindII/HindIII digestion. Second, another DNA I species, sensitive to endonuclease R-EcoRI, but yielding relatively short DNA III molecules was detected: this species, CVPB/1/P2 (EcoRI sens) DNA, yielded a characteristic pattern of fragments upon digestion with endonuclease R-HindII/HindIII as shown in Fig. 5. Third, a DNA I species that is resistant to endonuclease R-EcoRI cleavage is also present in the DNA produced by CVB/1/P2. This DNA I species gives its own characteristic pattern of fragments upon cleavage with endonucleases R-HindII/HindIII and further, contains all of the detectable repetitive monkey sequences that were present in the original mixture of defec-
tives. This defective species, termed CVP8/1/P2 (EcoRI res) DNA I, has been studied in detail (33). Information on the localization in monkey chromosomes of the repetitive monkey DNA sequences present in fragment E of CVPB/1/P2 (EcoRI res) DNA I has also been presented (44).

It has been observed that the particular forms of defective SV40 genomes present in serial passages frequently change upon subsequent passaging (occ, for example, Refs. 11, 12, 16). Therefore it is of interest that the mixture of defec-
tives originally noted in CVB/1/P3 (10) was remarkably stable through passage CVB/1/P6, upon the isolation of the plaque CVP8, and through passage number CVPB/1/P2. Nevertheless, as demonstrated by the data in Fig. 6 and Table I, the defective changed markedly upon further passaging. Thus, in confirmation of the observations of several other workers, it seems that constant alterations and rearrangements of the defective SV40 variants occur during lytic infection with subsequent selection of certain species for efficient replication. Neither the mecha-
nisms involved in these changes nor the factors influencing...
them are understood and the nature of the defective variants obtained in any given passage remains unpredictable.

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