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Recurring Defective Variants of Simian Virus 40 Containing Monkey DNA Segments

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Four independently and newly isolated defective variants of simian virus 40 have been characterized. All four are very similar, if not identical, to two previously and independently isolated variants (Wakamiya et al., J. Biol. Chem. **254**:3584-3591, 1979; J. Papamatheakis, E. Kuff, E. Winocour, and M. F. Singer, J. Biol. Chem. **255**:8919-8927, 1980). The documented similarities include restriction endonuclease maps and the presence of the same monkey DNA segments covalently linked to simian virus 40 DNA sequences. Each of the newly described variants was first detected upon serial passaging of wild-type simian virus 40 at a high multiplicity of infection at 33°C as recently described (M. F. Singer and R. E. Thayer, J. Virol. **35**:141-149, 1980). A variety of experiments support the idea that the various isolates were independent and do not reflect inadvertent cross-contamination. Two of the new isolates arose during passage of wild-type strain 777 virus in BSC-1 cells, one during passage of strain 776 in BSC-1 cells, and one during passage of strain 776 in primary African green monkey kidney cells. The two variants obtained after passage of strain 776 were shown to contain a particular recognition site for restriction endonuclease *Mbo*II within their simian virus 40 DNA segments, as do the two previous isolates. This site is not present in wild-type strain 776 DNA but is shown here to be present in wild-type strain 777 DNA. The surprising recurrence of closely related variants and particularly the unexpected presence of the endo R·*Mbo*II site in variants derived from passaging strain 776 suggest that these variants may arise by mechanisms other than recombination between the initial infecting viral genome and the host DNA.

A variety of defective variants of simian virus 40 (SV40) are formed upon serial passage of plaque-purified virus in permissive cells at a high multiplicity of infection (3, 11). The genomes of one group of variants are characterized by the presence of covalently linked segments of viral DNA and monkey DNA derived from the permissive host cells. Several members of this group have been characterized to varying extents, and recently nucleotide sequence data for some of the variants have been reported (6, 7, 14, 29). Many of these variants contain several (from 3 to about 10) tandem repetitions of basic repeat units comprising viral and monkey DNA segments. The repetitions are arranged in a circle and together constitute a molecule approximately the size of an SV40 genome. Different variants commonly include the region of the SV40 genome identified with the origin of replication, but they contain different total amounts of SV40 DNA and different parts and amounts of the monkey genome. Earlier experiments (5, 16) suggested that certain monkey segments might be preferred. Several questions remained unanswered, including whether the set of monkey sequences that can recombine with SV40 comprises the entire monkey genome or is more limited and whether the structure of the variants reflects a specific interaction between the virus and host. It has been assumed that many variants may be formed, whereas only those with an advantage for replication and packaging into SV40 capsids become sufficiently abundant to be detected.

In addition, the mechanism by which variants of this type are formed is unknown. It is also not known whether the observed variants represent unique structures resulting from chance recombinations or whether they recur and thus reflect specific events during the infectious cycle. One such variant, termed CVP8/1/P2(*Eco*RI resistant) (abbreviated to CVP8/1/P2), consists of four tandem repeats of a DNA segment 1,210 base pairs (bp) long (18, 29). Figure 1A shows a schematic drawing of this repeat unit. The entire nucleotide sequence is known (29). The repeat

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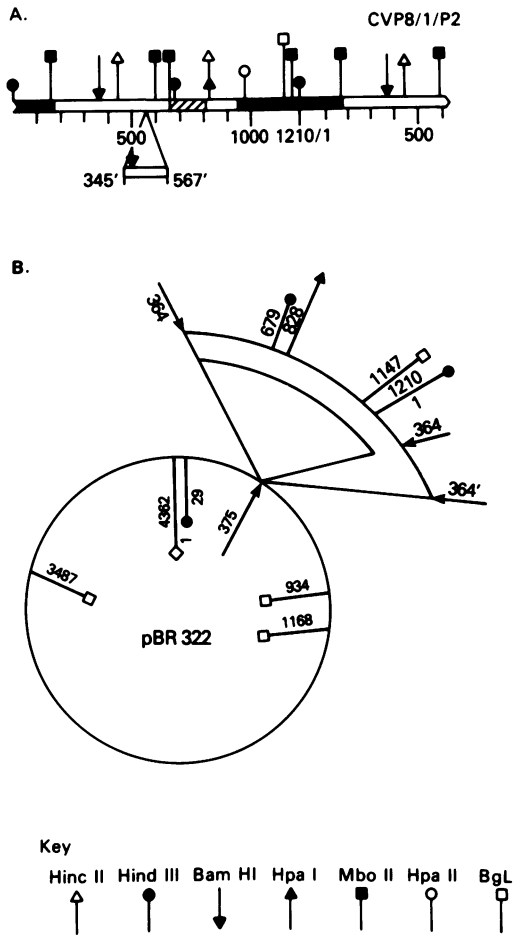

FIG. 1. *(A) Structure of the host-substituted SV40 variant designated CVP8/1/P2. One complete repeat unit (1-1,210) and a portion of an adjacent repeat unit (1-600) are shown. The entire nucleotide sequence is known (29). Four head-to-tail tandem repeats of this segment constitute the whole genome. Sites of cleavage by several restrictions endonucleases are shown. The dark bars represent SV40 sequences, the open bars represent monkey sequences that occur infrequently in the monkey genome, and the slashed bar represents sequences homologous to the highly repetitive α-component DNA of the monkey genome. One of every three to four repeating units contains a reiteration of residues 345 to 567, inserted after residue 567, as shown at the bottom of the diagram. (B) Structure of recombinant DNA molecules predicted to result from the insertion of one 1,210-bp repeat unit of CVP8/1/P2 or one 1,210-bp repeat unit of CVP8/1/P2 plus the extra 223-bp reiteration into pBR322. The variant segments are shown ligated into the single endo R·BamHI site of pBR322 by means of the endo R·BamHI sites within the variant. The diagram shows restriction endonuclease sites used in the present work. The placement of the sites in pBR322 is according to Sutcliffe (27, 28). The positions of endo R·BglI sites were deduced from the nucleotide sequence of pBR322 (27) and the recognition site of endo R·BglI (24; Lautenberger et al., Fed. Proc. 38: 293, 1979) and confirmed by appropriate double digestions (unpublished data). The placement of the sites in CVP8/1/P2 is from the published sequence (29), shown in (A).*

unit is made up of SV40 DNA sequences, sequences homologous to the highly reiterated DNA of African green monkeys called the α-component (13, 22), and sequences homologous to infrequently reiterated African green monkey DNA. One portion of the latter sequences, residues 364 to 679 (Fig. 1A), will be referred to here as fragment C. One in every three to four repeat units differs from the others by the insertion of a 223-bp-long repetition of a portion of the sequence. Variant CVP8/1/P2 DNA is replicated very efficiently compared with its wild-type SV40 helper in infected monkey kidney cells. Furthermore, the relative efficiency of synthesis of the variant DNA, compared with that of the wild type, is increased at 33°C compared to 37°C (25). Some earlier experiments suggested that variants containing monkey DNA sequences homologous to those in CVP8/1/P2 do indeed recur (16). Given the replicative advantage of CVP8/1/P2 genomes at 33°C, it might be expected that such genomes, if formed, would become more abundant and therefore more easily detectable at 33°C than at 37°C. Accordingly, independent serial passaging experiments were carried out at 33°C, using two different stocks of plaque-purified wild-type SV40 to initiate passages as well as several different cell lines. Preliminary characterization of the resulting viral stocks suggested that in each case variants containing the monkey sequences found in CVP8/1/P2 had accumulated to a significant extent after four passages (25). Evidence indicating that the starting cells and virus were not detectably contaminated with CVP8/1/P2 genomes was presented.

We report here the further characterization of variant genomes related to CVP8/1/P2 from four of the independently passaged stocks previously described (25). It should be stressed at the outset that, whereas these passaged stocks contained several different detectable variant genomes, only those related to CVP8/1/P2 have been studied in detail.

Some of the results presented here have been summarized elsewhere (M. F. Singer, E. L. Kuff, T. N. H. Lee, T. McCutchan, J. Papamatheakis, R. E. Thayer, and E. Winocour, *in Mobilization and Reassembly of Genetic Information*, in press).

MATERIALS AND METHODS

Unless indicated otherwise, all materials and methods were as previously described (17a, 25, 29, 30).

Viruses and cells. The CV-1 line of cells was obtained recently from Paul Berg, Stanford University, Stanford, Calif. The passaged viral stocks used to prepare the variant viral DNAs are designated CV371/1/P4, CV372/1/P4, CV776/3/P4, and CV776/4/P4 (see Table 1 and reference 25 for explanation of nomenclature, the history of these stocks, and the initial characterizations of the defective variants contained in them). In brief, stocks of wild-type SV40 were prepared at a low multiplicity of infection, starting with virus that had been purified by three successive plaque isolations. Both the continuous BSC-1 cell line (9) and primary African green monkey kidney cells were used. Several independent passage series were initiated with each of the stocks, and four serial passages at high multiplicities of infection were carried out at 33°C. The earlier paper (25) reported a variety of experiments designed to detect the presence of the CVP8/1/P2 variant as a contaminant either in the cells or in the wild-type SV40 stocks used to initiate the serial passages. Within the sensitivity of the methods used, no evidence of such contamination was obtained. Additional experiments concerning purity of the initiating SV40 strain 776 stocks have now been carried out. DNA I was isolated from cells infected with CV776/3/P1 and CV776/4/P1. These lysates are the yields from the very first high-multiplicity passages in the series ultimately yielding (after four serial passages) CV776/3/P4 and CV776/4/P4 (Table 1). The DNA was digested with both endo R·*Mbo*II and a mixture of endo R·*Hin*cII and endo R·*Hin*dIII. In each case the digestion pattern obtained after electrophoresis on 4% polyacrylamide gels was identical to that ofstrain 776 DNA (10), indicating that the passages had not been grossly contaminated with strain 777 virus. The marked difference in the *Mbo*II cleavage patterns of these two strains is documented below.

Preparation of viral DNA. Confluent plates of BSC-1 cells were infected with approximately 1 ml of the passaged stocks for every 3×10^6 cells. Absorption was carried out at 37°C for 2 h, and the plates were then shifted to 33°C. Infected cells were labeled with [^{3}H]thymidine, 10 μCi/ml. In different experiments labeling was either for 48 h between 48 and 96 h after infection or for 24 h between 72 and 96 h after infection. Viral DNA was then isolated (8), deproteinized by repeated treatments with phenol and chloroform-isoamyl alcohol (24:1, vol/vol), and purified by centrifugation to equilibrium in CsCl containing ethidium bromide.

^{32}P-labeled probes. Three different DNAs were used as probes. (i) Wild-type SV40 DNA was prepared from virions. (ii) A DNA fragment termed AGMr (*Hin*dIII)-1 and representing the repeating monomer unit of highly repetitive α-component DNA of the African green monkey was prepared as described previously (22). This fragment has no detectable contamination by other monkey DNA sequences. (iii) A DNA segment 315 bp long (termed fragment C) derived from the previously characterized SV40 variant CVP8/1/P2 (see Fig. 1A) and containing sequences homologous to infrequently reiterated African green monkey DNA was prepared as described previously (17a, 30). The fragment contains 283 bp (residues 364 to 646, Fig. 1A) of low-reiteration-frequency monkey DNA and 33 bp of highly reiterated monkey α-component DNA (residues 647 through 679). These 33 bp do not hybridize significantly to α-component sequences under the conditions used in the present experiments. For some experiments the fragment C probe was contained within a pBR322 vector. This plasmid, pSVDp3C3, was kindly made available by Thomas McCutchan. All probes were labeled with ^{32}P by nick translation (20) to a specific radioactivity of about 10^8 cpm/μg.

Molecular cloning in *Escherichia coli*. Segments of variant SV40 genomes were purified by cloning in *E. coli* K-12, using the plasmid pBR322 (1) as a vector. The methods have been described in detail previously (17a). All ligations were into the endo R·*Bam*HI site of pBR322. All experiments were carried out under the containment conditions specified by the National Institutes of Health Guidelines for Recombinant DNA Research.

RESULTS

Characterization of defective viral DNAs. Preliminary characterization of viral DNA I's isolated from BSC-1 cells infected with CV371/1/P4, CV372/1/P4, CV776/3/P4, and CV776/4/P4 (Table 1) indicated that a portion of each DNA was composed of molecules in which SV40

TABLE 1. *Description of SV40 stocks passaged at 33°C*[a]

Initiating plaque-purified viral stock	Independent passage series no.	Cell type in which passaged	Lysate obtained after four serial passages
CV371	1	BSC-1	CV371/1/P4
CV372	1	BSC-1	CV372/1/P4
CV776	3*	BSC-1	CV776/3/P4
CV776	4*	AGMK	CV776/4/P4

[a] The characterization of the plaque-purified wild-type stocks used to initiate the passage series, the procedure used for high-multiplicity passaging, and the initial characterization of the passaged lysates have been described (see text; 25). CV371 and CV372 are strain 777 wild-type stocks; CV776 is a strain 776 stock. Several independent passage series were initiated with each stock and were numbered as shown in column 2. Those passage series marked with an asterisk were carried out in another laboratory at the National Institutes of Health to avoid possible contamination of the cultures with material present in our laboratory. The lysates are named by a tripartite nomenclature (see column 4) in which the first term (e.g., CV371) indicates the initiating plaque-purified viral stock, the second term (e.g., 1 or 3) indicates an independent passage series initiated with that stock, and the third term (e.g., P4) indicates the number of serial passages before collection of the lysate in question (in the example, four). AGMK, African green monkey kidney.

sequences were linked to monkey DNA sequences (25). In the present experiments, BSC-1 cells were infected with each of the four passaged stocks, and intracellular viral DNA I was isolated and analyzed by gel electrophoresis before and after treatment with various restriction endonucleases.

The results for CV371/1/P4 are shown in Fig. 2. The DNA preparation contained DNA I as well as DNA II and a small amount of DNA III (Fig. 2A, lane d), and all three appeared to be mixtures of molecules the same size and somewhat smaller than the size of wild-type SV40 (Fig. 2A, lane a). Digestion with endo R·*Eco*RI (Fig. 2A, lane e), endo R·*Bam*HI (lane g), and endo R·*Bgl*I (lane h) yielded some full-length linear molecules (DNA III) as expected for wild-type SV40 DNA. However, a substantial portion of the DNA was resistant to endo R·*Eco*RI (lane e), and a predominant band about 1,200 bp in length as well as several other fragments were produced by endo R·*Bam*HI (lane g) and endo R·*Bgl*I (lane h). Digestion of the DNA with endo R·*Hin*dIII (lane f) also gave a variety of products: bands with the mobilities expected from digestion of wild-type SV40 DNA (lane b) were present only in small amounts. These results indicated that the viral DNA synthesized in cells infected with CV371/1/P4 was a mixture of a relatively small amount of wild-type SV40 and a variety of defective variants. The variant molecules included substantial amounts of material that was resistant to endo R·*Eco*RI and produced a major 1,200-bp product upon treatment with endo R·*Bam*HI or endo R·*Bgl*I.

The DNA on the agarose gel shown in Fig. 2A was transferred to a nitrocellulose sheet (26) and hybridized with ^{32}P-labeled α-component DNA (Fig. 2B). The α-component probe hybridized with the CV371/1/P4 DNA I, II, and III (Fig. 2B, lane d), and most of the material that hybridized was resistant to cleavage with endo R·*Eco*RI (Fig. 2B, lane e). The α-component probe also hybridized with (i) a *Hin*dIII fragment about 530 bp in length (Fig. 2B, lane f) corresponding to one of the major bands seen on the ethidium-stained gel (Fig. 2A, lane f), (ii) the predominant 1,200-bp fragment produced upon digestion with endo R·*Bam*HI (Fig. 2B, lane g), and (iii) the predominant 1,200-bp fragment produced upon digestion with endo R·*Bgl*I as well as several additional *Bgl*I fragments, including one about 1,400 bp in length (Fig. 2B, lane h).

Figure 2C shows the hybridization obtained when a nitrocellulose blot of a gel similar to that in Fig. 2A was hybridized with ^{32}P-labeled SV40 DNA. The CV371/1/P4 DNA I and II hybridized (Fig. 2C, lane d), and a large portion of the material hybridizing with the wild-type DNA was resistant to cleavage with endo R·*Eco*RI (Fig. 2C, lane e). In addition to fragments of the size expected from wild-type DNA, endo R·*Hin*dIII digestion yielded a smear of additional fragments that hybridized to the SV40 DNA probe (Fig. 2C, lane f). Some of the material hybridizing with the SV40 DNA probe was converted to DNA III by endo R·*Bam*HI, as expected for wild-type DNA, but some was converted to fragments between 1,200 and 1,700 bp in length.

Figure 2D shows a second nitrocellulose filter made from the same gel as the filter shown in Fig. 2C. The two filters were stacked one upon the other during the transfer. The filter shown in Fig. 2D was hybridized with the ^{32}P-labeled fragment C probe. The CV371/1/P4 DNA I hybridized with the fragment C probe (Fig. 2D, lane d), and all of the hybridizing material was resistant to digestion with endo R·*Eco*RI (Fig. 2D, lane e). Treatment with endo R·*Hin*dIII yielded two fragments containing the fragment C sequence, about 900 and 700 bp in length, respectively (Fig. 2D, lane f). Treatment with endo R·*Bam*HI (Fig. 1D, lane g) yielded a major hybridizable band of about 1,200 bp in length and a second band, very faint on the gel, of approximately 300 bp in length.

These data were consistent with the preliminary observations mentioned above (25), and the results can be summarized as follows. Infection of BSC-1 cells with CV371/1/P4 yields viral DNA containing a relatively small amount of wild-type SV40 DNA and substantial amounts of several variant genomes: one type of variant is slightly smaller than a full-length wild-type genome in size; it is resistant to endo R·*Eco*RI cleavage; the main product of digestion with endo R·*Bam*HI is a fragment about 1,200 bp in length; the main product of digestion with endo R·*Bgl*I is a fragment about 1,200 bp in length, and an additional fragment about 1,400 bp in length occurs with some frequency; SV40, α-component, and fragment C sequences are covalently linked, and all three are present in the 1,200-bp *Bam*HI fragment; α-component sequences are also present in both the 1,200- and 1,400-bp-long fragments produced by endo R·*Bgl*I; α-component sequences are present in the 530-bp fragment produced by endo R·*Hin*dIII, whereas both SV40 and fragment C sequences are present in somewhat larger fragments produced by endo R·*Hin*dIII. When these data are compared with the known structure of variant CVP8/1/P2 (Fig. 1A), they strongly suggest that a major variant produced by infection with CV371/1/P4 has a repeat unit very similar to that of CVP8/1/P2. Furthermore, the experiments presented in Fig. 2 also suggest that some

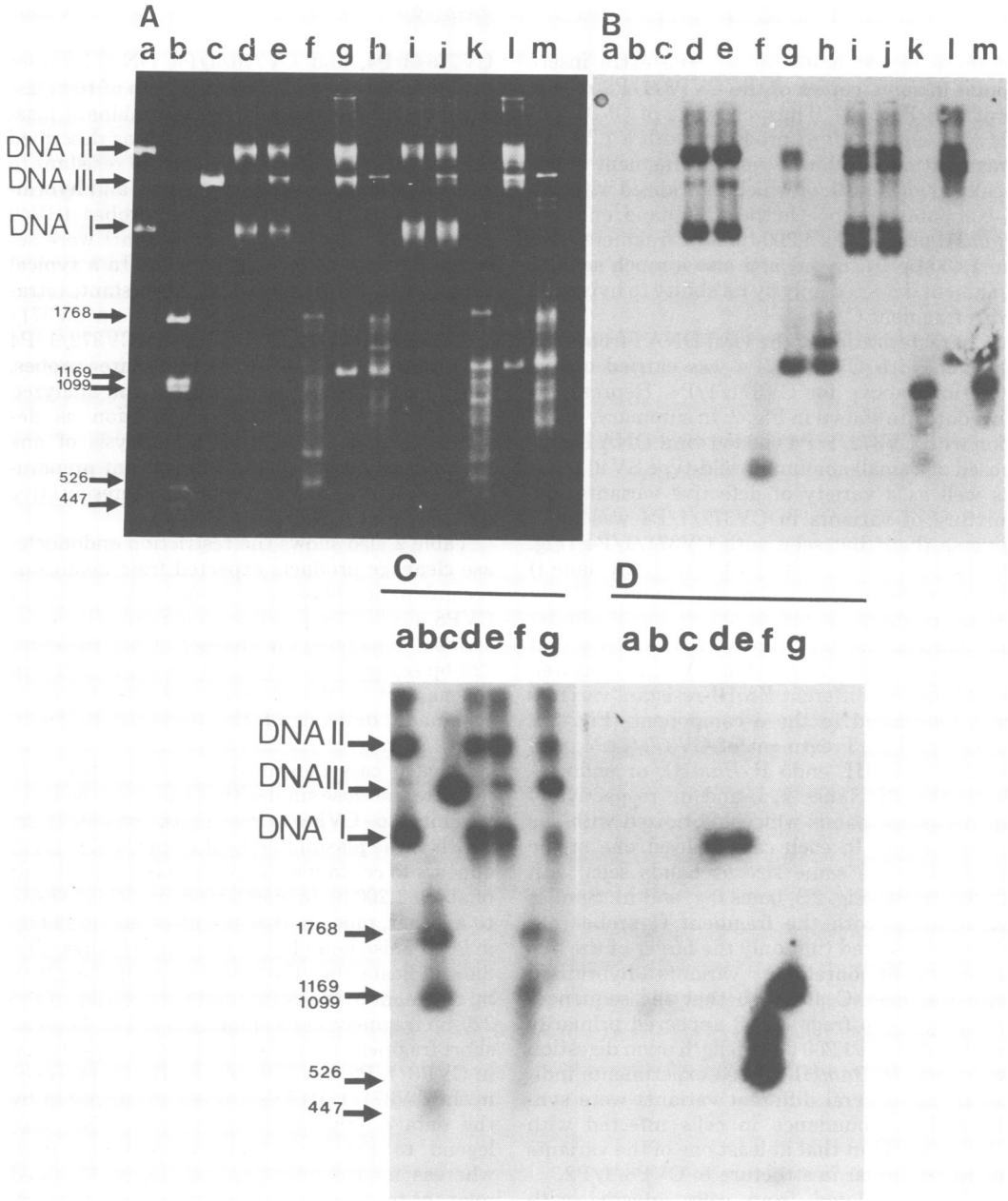

FIG. 2. *Analysis of CV371/1/P4 and CV372/1/P4 viral DNAs by gel electrophoresis, restriction endonuclease digestion, and hybridization. (A) Photograph of a 1.4% agarose gel stained with ethidium bromide: lane a, wild-type SV40 DNA I and II; lane b, as (a) treated with endo R·HindIII; lane c, as (a) treated with endo R·BglI; lane d, CV371/1/P4 DNA I, II, and III; line e, as (d) treated with endo R·EcoRI; lane f, as (d) treated with endo R·HindIII; lane g, as (d) treated with endo R·BamHI; lane h, as (d) treated with endo R·BglI; lane i, CV372/1/P4 DNA I and II; lane j, as (i) treated with endo R·EcoRI; lane k, as (i) treated with endo R·HindIII; lane l, as (i) treated with endo R·BamHI; lane m, as (i) treated with endo R·BglI. (B) Nitrocellulose blot of gel shown in (A) hybridized with the ^{32}P-labeled α-component probe. (C) Nitrocellulose blot of another 1.4% agarose gel hybridized with ^{32}P-labeled SV40 DNA: lane a, wild-type SV40 DNA I, II, and III; lane b, as (a) treated with endo R·HindIII; lane c, as (a) treated with endo R·BamHI; lane d, CV371/1/P4 DNA I and II; lane e, as (d) treated with endo R·EcoRI; lane f, as (d) treated with endo R·HindIII; lane g, as (d) treated with endo R·BamHI. (D) Second nitrocellulose blot made from the same gel as in (C) and hybridized with ^{32}P-labeled fragment C.*

of these repeat units contain the extra insert found in some copies of the CVP8/1/P2 repeat unit (see Fig. 1A). Thus, cleavage of CV371/1/P4 with endo R·*Bgl*I produced both a 1,200-bp fragment and, in lower yield, a fragment about 1,400 bp long, both of which hybridized with the α-component probe. On the other hand, endo R·*Bam*HI produced a 1,200-bp-long fragment (and no 1,400-bp fragment) and also a much smaller fragment detected only by its ability to hybridize with fragment C.

Characterization of the viral DNA I from cells infected with CV372/1/P4 was carried out as described above for CV371/1/P4. Representative data are shown in Fig. 2. In summary, infection with CV372/1/P4 yielded viral DNA I composed of a small amount of wild-type SV40 DNA as well as a variety of defective variants; the mixture of variants in CV372/1/P4 was more diverse than that seen with CV371/1/P4 (Fig. 2). Thus, CV372/1/P4 DNA I (Fig. 2A, lane i) was more heterogeneous in size than CV371/1/P4 and contained substantial amounts of at least one variant shorter than wild-type DNA that contained an *Eco*RI site (Fig. 2A, lane j), as well as at least two different *Eco*RI-resistant variants that hybridized to the α-component (Fig. 2B, lanes i and j). Treatment of CV372/1/P4 with endo R·*Hin*dIII, endo R·*Bam*HI, or endo R·*Bgl*I (Fig. 2B, lanes k, l, and m, respectively) yielded some bands which hybridized with the α-component. In each case at least one of the bands was the same size as bands seen with CV371/1/P4 (Fig. 2B, lanes f, g, and h). Similar experiments with the fragment C probe (not shown) indicated that only the larger of the two α-component-containing variants hybridized with fragment C and also that the sequences homologous to fragment C appeared primarily in a band about 1,200 bp in length upon digestion with endo R·*Bam*HI. These experiments indicated that several different variants were synthesized in abundance in cells infected with CV372/1/P4 but that at least one of the variants might be similar in structure to CVP8/1/P2.

DNA I isolated from cells infected with CV776/3/P4 and CV776/4/P4 was analyzed in the same way as described for CV371/1/P4 and CV372/2/P4. The data (not shown) again indicated that the viral DNA I was a mixture of wild-type genomes and several different variants. In each case, one relatively abundant variant appeared to be similar to the prototypic CVP8/1/P2 variant and thus to variants present in CV371/1/P4 and CV372/1/P4.

Further characterization of variant viral DNAs by molecular cloning in *E. coli*. To facilitate purification of the variant DNAs, endo R·*Bam*HI digests of CV371/1/P4, CV372/1/P4, CV776/3/P4, and CV776/4/P4 DNA I's were ligated into the endo R·*Bam*HI site of the plasmid vector pBR322, and the recombinant plasmids were transfected into *E. coli* as described previously (17a). Colonies that were resistant to ampicillin, sensitive to tetracycline, and hybridized with all three ^{32}P-labeled probes (SV40 DNA, fragment C, and α-component) were selected and grown on a large scale. In a typical experiment, 25 of 42 ampicillin-resistant, tetracycline-sensitive colonies derived from CV371/1/P4 and 21 of 42 derived from CV372/1/P4 gave a positive hybridization to all three probes. The plasmid DNAs were purified and analyzed by restriction endonuclease digestion as described previously (17a). The analysis of one such plasmid from each of the variant preparations is summarized in Table 2. Representative data are shown in Fig. 3 and 4.

Table 2 also shows the restriction endonuclease cleavage products expected from analogous recombinant plasmids made from variant CVP8/1/P2. Two possible recombinants are described, depending on whether or not the extra 223-bp segment accompanies the major 1,210-bp repeat unit in the plasmid (see Fig. 1B for a schematic drawing of the expected recombinant).

In each case described in Table 2 the data indicate the close similarity of the newly isolated variants to CVP8/1/P2. Thus, pSVD371-25, which is the plasmid derived from CV371/1/P4, appears to contain a complete major repeat unit of about 1,200 bp in length from one *Bam*HI site to another, plus another fragment about 220 bp in length also bounded by two *Bam*HI sites. The data indicate that the orientation of the 1,200-bp fragment in the vector and the position of the 220-bp fragment are as indicated in Fig. 1B. The short fragment is similar to the extra repeat seen in CVP8/1/P2 (see Fig. 1A and B). Its presence in the CV371/1/P4 DNA was also suggested by the data on the uncloned variant DNA (see legend to Fig. 2). It should be noted that, whereas no hybridization data were obtained relevant to the presence of residues 804 through 953 of CVP8/1/P2 in pSVD371-25, the restriction endonuclease analysis supports such a conclusion.

Similarly, pSVD372-6, pSVD776-175, and pSVD776-123, derived from CV372/1/P4, CV776/3/P4, and CV776/4/P4, respectively, contain fragments about 1,200 bp in length with restriction endonuclease sites consistent with a close similarity between the cloned fragment and the major repeating unit of CVP8/1/P2. All three cloned fragments appear to be single major repeat units without the extra 220-bp fragment that was observed in pSVD371-25.

TABLE 2. *Summary of restriction endonuclease fragments obtained by digestion of recombinant molecules constructed by joining pBR322 and BamHI fragments derived from the SV40 variants*[a]

Enzyme	Expected for insert from CVP8/1/P2		Fragment size (bp) observed for recombinants			
	Fragment size (bp)	Composition	pSVD371-25 (CV371/1/P4)	pSVD372-6 (CV372/1/P4)	pSVD776-175 (CV776/3/P4)	pSVD776-123 (CV776/4/P4)
*Bam*HI	4,362	−	4,480	4,360	4,360	4,360
	1,210	+	1,220	1,200	1,210	1,210
	(223)	(+)	240			
*Bgl*I	2,319	−	2,300	2,300	2,460	2,450
	2,033	+	2,030	2,050	2,130	2,110
	(1,209)	(+)	1,200			
	986*	+		900	970	990
	234	−	240	230	NS	260
*Hin*dIII	(4,603)	(+)	4,700			
	4,380*	+		4,500	4,210	4,360
	661	+	640	690	620	670
	531	+	530	580	520	550
*Bam*HI/*Bgl*I	2,319	−	2,150	2,400	2,300	2,470
	1,250	−	1,260	1,230	1,320	1,280
	783	+	760	780	760	800
	559	−	560	530	560	580
	427	+	460	430	450	460
	234	−	NS	230	NS	260
	(223)	(+)	NS			
*Bam*HI/*Hpa*I	4,362	−	4,520	4,360	4,360	4,450
	744	+	740	700	740	780
	466	+	490	450	480	490
	(223)	(+)	NS			
*Eco*RI/*Hpa*I	(4,962)	(+)	5,150			
	4,729*	+		5,000		
	843	+	820	860		
*Bam*HI/*Hin*dIII	4,016	−	4,200			
	531	+	530			
	364	+	a			
	346	−	a			
	315	+	a			
	(223)	(+)	230			

[a] Column 1 gives the enzymes used to degrade the various plasmid DNAs. Column 2 shows the size of fragments expected for digestion of recombinant plasmids containing *Bam*HI fragments derived from CVP8/1/P2 inserted into the single *Bam*HI site of pBR322 in the direction shown in Fig. 1B. Because of the small 223-bp repeat in some monomer units of CVP8/1/P2, two different types of recombinants are possible (see Fig. 1B). Fragments expected only from insertion of the major 1,210-bp repeat unit of CVP8/1/P2 are marked with an asterisk. Fragments expected only from insertion of the major repeat unit plus the extra 223-bp fragment are given in parentheses. The fragments expected from both are listed with no special designation. Column 3 shows a plus (+) if the fragment in question contains SV40 variant sequences with or without joined pBR322 sequences and a minus (−) if the fragment is expected from pBR322 alone. Columns 4 through 7 show the data obtained upon analysis of the indicated recombinant molecules: representative gels showing how such data were obtained are shown in Fig. 3 and 4. The sizes of the various DNA fragments were determined by comparison of their mobilities on the gels with the mobilities of the marker fragments on a plot of the logarithm of size in base pairs against mobility measured in centimeters (15). The sizes of the markers, which are noted on the figures, were calculated from the nucleotide sequence of SV40 DNA (4) and pBR322 DNA (27). The cleavage sites for endo R·*Bgl*I within pBR322 were identified by using the known recognition site for the enzyme (24; J. A. Lautenberger, C. T. White, M. H. Edgell, and C. A. Hutchinson III, Fed. Proc. **38**:293, 1979), the primary nucleotide sequence of pBR322 (28, 29), and a computerized search (12). NS indicates a fragment expected on the basis of the other data but not observed probably because the electrophoresis was run too long. No entry indicates experiments that were not carried out. An a in column 4 denotes the fact that a smear of several unresolved fragments was observed in this region of the gel: the largest fragment in the smear was calculated to be about 380 bp long and the shortest was about 300 bp long.

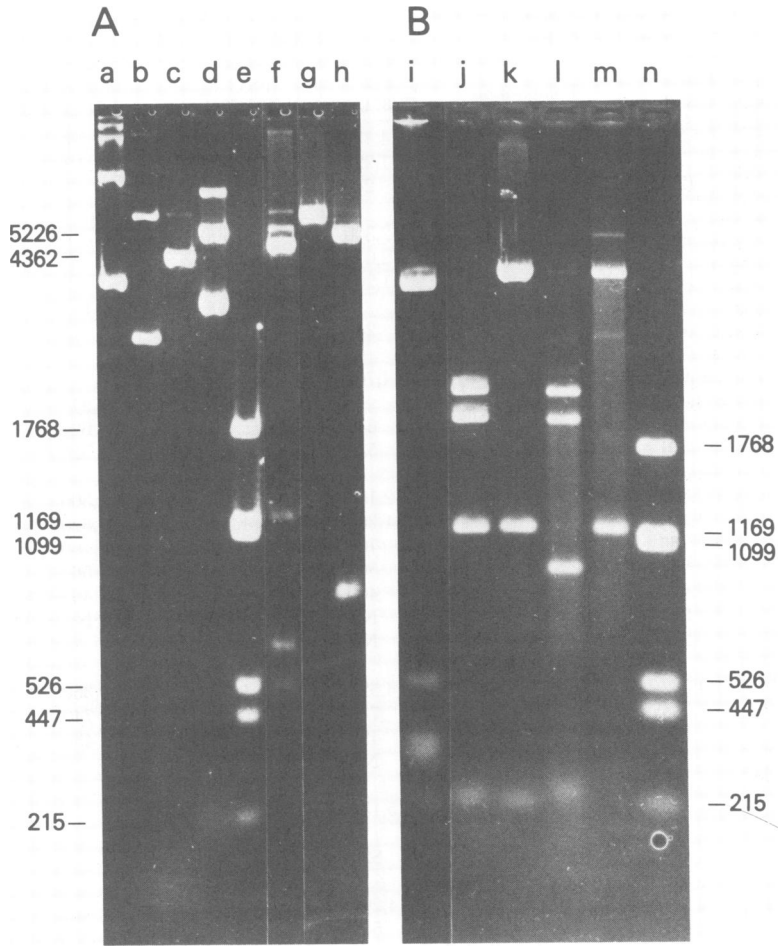

FIG. 3. *Characterization of pSVD371-25 by digestion with restriction endonucleases. Photographs of 1.4% agarose gels stained with ethidium bromide are shown. A and B were two separate experiments. The sample in each lane was as follows: a, pSVD371-25; b, pBR322 DNA I and II; c, pBR322 DNA III produced by cleavage with endo R·EcoRI; d, SV40 strain 776 DNA I, II, and III; e, SV40 strain 776 DNA treated with endo R·HindIII; f, as (a) treated with endo R·HindIII; g, as (a) treated with endo R·EcoRI; h, as (a) treated with endo R·EcoRI and endo R·HpaI; i, as (a) treated with endo R·BamHI and endo R·HindIII; j, as (a) treated with endo R·BglI; k, as (a) treated with endo R·BamHI; l, pSVD372-6 treated with endo R·BglI; m, pSVD372-6 treated with endo R·BamHI; n, as (e). The data are summarized in Table 2.*

Unusual property of the variants in CV776/3/P4 and CV776/4/P4. Wild-type virus strain 777 was used to initiate the sets of passages leading to CVP8/1/P2, CV371/1/P4, and CV372/1/P4, whereas passage of strain 776 produced CV776/3/P4 and CV776/4/P4 (25). Various differences in the restriction endonuclease digests of these two wild-type strains have been reported previously (10, 15). An additional difference occurs in the region around the origin of replication. Samples of wild-type strain 777 and 776 DNA I were digested with endo R·*Mbo*II, and the products were analyzed by electrophoresis (Fig. 5). A 691-bp-long fragment expected from the region around the origin of replication of strain 776 (4, 19) is evident in strain 776 DNA but is missing from the strain 777 digest. Instead, two fragments approximately 190 and 500 bp long are produced, exactly as expected if strain 777 contained an adenine·thymine base pair at residue 5,127 (21) instead of a guanine·cytosine base pair. The single-base-pair change results in the formation of a new recognition site for endo R·*Mbo*II.

Since the variants contain SV40 sequences around the origin of replication, including the endo R·*Bgl*I site, the presence or absence of the endo R·*Mbo*II site in the variants is relevant to

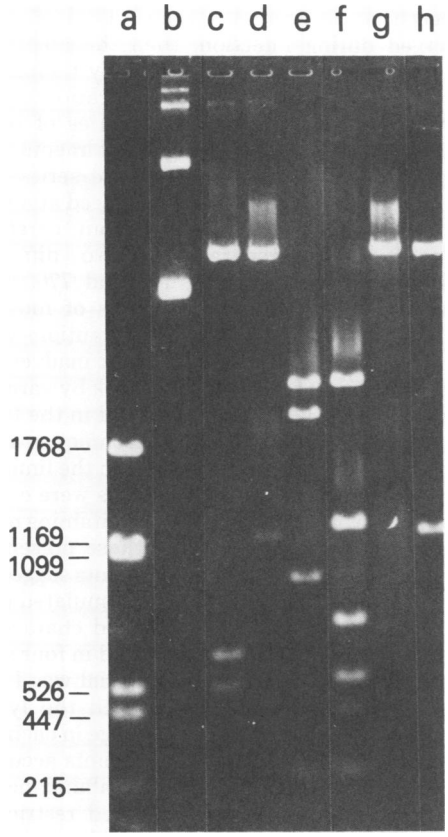

FIG. 4. *Characterization of pSVD776-123 by digestion with restriction endonucleases. The procedures were as described for Fig. 3. The lanes contain the following samples: a, SV40 wild-type strain 776 DNA treated with endo R·HindIII; b, pSVD776-123; c, as (b) treated with endo R·HindIII; d, as (b) treated with endo R·BamHI; e, as (b) treated with endo R·BglI; f, as (b) treated with endo R·BglI and endo R·BamHI; g, as (b) treated with endo R·BamHI and endo R·HpaI; h, control of pSVD372-6 treated with endo R·BamHI. The results are summarized in Table 2.*

the provenance of the variant structure. The sequence analysis of CVP8/1/P2 (29), which arose during passaging of strain 777, showed the type 777 sequence, that is, the endo R·*Mbo*II recognition site. In the experiment shown in Fig. 6, lane c, the *Bam*HI fragment isolated from pSVD776-175 was digested with endo R·*Mbo*II. A *Bam*HI repeat unit containing this *Mbo*II site is expected to produce five products 540, 212, 195, 191, and 71 bp in length, respectively (Fig. 1A). Were the *Mbo*II site in question (at approximately residue 1,177 in CVP8/1/P2) missing, the 540- and 191-bp fragments would be fused to produce a single fragment 731 bp in length. The data in Fig. 6, lane c, clearly show that the extra *Mbo*II site is indeed present in the cloned fragment. As a control, the digestion of a *Bam*HI fragment derived from another variant (termed 290) which arose during infections with strain 777 (17a, 30) is included for comparison and gave the same products as did pSVD776-175. Thus, the variant in CV776/3/P4 (pSVD776-175) contains a restriction endonuclease site associated with strain 777 but not with strain 776, even though strain 776 was used to initiate the passage series.

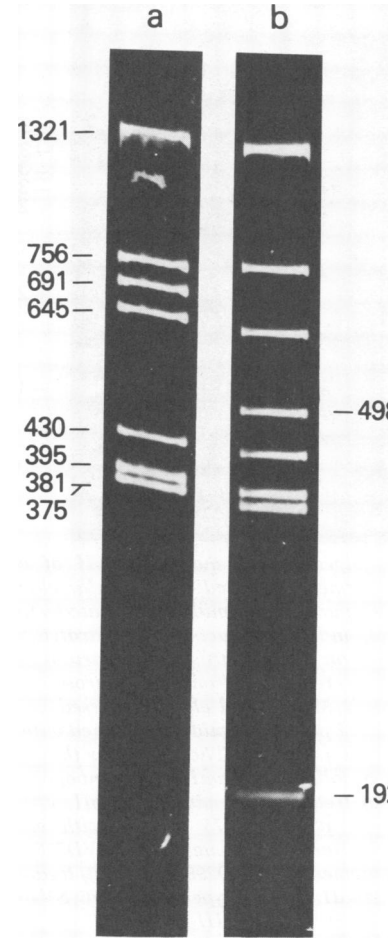

FIG. 5. *Digestion of wild-type SV40 DNA with endo R·MboII. The photograph is a 4% polyacrylamide gel stained with ethidium bromide. (a) Strain 776; (b) strain 777. The sizes of the fragments expected from strain 776 are shown on the left. The sizes were calculated from the primary nucleotide sequence of strain 776 (4) and the known recognition and cleavage sites for endo R·MboII (23). The distance between the recognition and cleavage sites was taken into account in the calculations. The sizes of the new fragments produced from strain 777 DNA are shown on the right.*

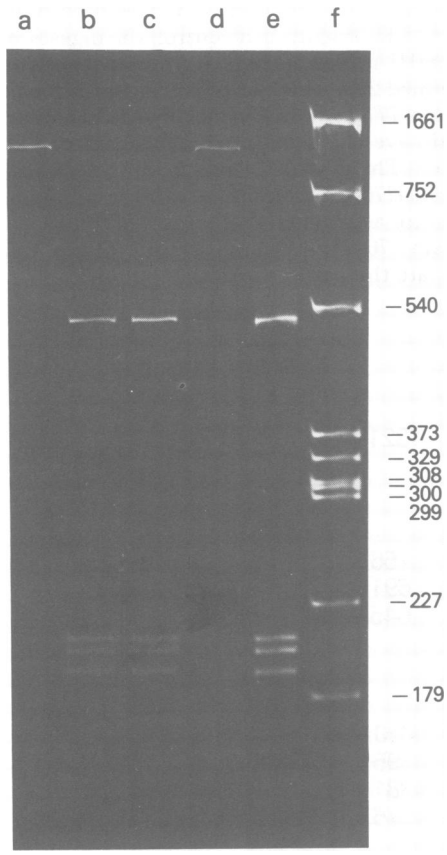

FIG. 6. *Digestion by endo R·MboII of variant BamHI fragments purified by molecular cloning in E. coli. The purified plasmids were treated with endo R·BamHI, and the products were separated by electrophoresis on 1.4% agarose. The variant BamHI fragments were eluted from the agarose, digested with endo R·MboII, and electrophoresed. A photograph of a 4% polyacrylamide gel stained with ethidium bromide is shown. The DNAs in the different lanes are as follows: a, BamHI fragment of pSVD776-123; as (a) treated with endo R·MboII; c, BamHI fragment of pSVD776-175 treated with endo R·MboII; d, BamHI fragment of pSVD776-175; e, BamHI fragment of pSVD290-2 (see text) treated with endo R·MboII; f, wild-type strain 776 SV40 DNA treated with endo R·HaeIII.*

Similarly, the variant fragment in pSVD776-123 (derived from CV776/4/P4) also contained the *Mbo*II site typical of wild-type strain 777, as described for pSVD776-175 (Fig. 6, lane b).

DISCUSSION

As noted above, CVP8/1/P2 replicates readily in infected monkey cells in the presence of wild-type helper, and the relative replicative advantage of the variant is enhanced at 33°C compared to 37°C (25). It seemed reasonable to expect that if variants of the CVP8/1/P2 type emerged during infection, then the ability to detect them would be increased by taking advantage of their preferential replication at the low temperature. Therefore, a series of high-multiplicity serial passaging experiments was carried out at 33°C. Each passage series was initiated with a stock of virus prepared at a very low multiplicity of infection from carefully plaque-purified preparations. Two different strains of wild-type SV40, 777 and 776, were used, as were two different types of monkey kidney cells (Table 1). Various precautions were taken to minimize the possibility of inadvertent contamination of the initiating stock by variants such as CVP8/1/P2 that are stored in the laboratory, and several different techniques indicated no such contamination within the limits of detection (25). The passaged stocks were examined for the presence of variants containing monkey sequences homologous to those present in CVP8/1/P2. Preliminary experiments suggested that such variants might have accumulated (25).

This paper presents the detailed characterization of variants that accumulated in four such passage series. The starting material was DNA I isolated from cells infected with the lysate produced in the fourth serial passage in each set. In each case several different variants accumulated. One type, which varied in abundance in the four different samples, yielded restriction endonuclease fragments and hybridization patterns consistent with a close similarity to CVP8/1/P2. Further purification of variant DNA by molecular cloning in *E. coli* confirmed the structural similarities. In summary, the data indicate that similar variants recur regardless of whether strain 777 or strain 776 is used to initiate the passages and regardless of whether BSC-1 cells or primary African green monkey kidney cells are used. The similarity extends to the homology of the monkey sequences and the restriction endonuclease maps. Any differences between the variants in question appear to be minor. In at least one case (CV371/1/P4) the similarity to CVP8/1/P2 extends to the extra 223-bp reiteration. In addition, several other 33°C passages which have not been studied further may also contain variants similar to CVP8/1/P2 (25).

Recently a related variant was detected after a single infection of BSC-1 cells with SV40 strain 777 (30) by using ^{32}P-labeled fragment C as a probe with an in situ plaque hybridization procedure applied to cellular monolayers in an infectious-center type of assay. Variants hybridizing with fragment C occurred rarely: in one experiment two positive plaques were detected out of 10^6 infectious centers. The variant was characterized by procedures similar to those de-

scribed in this paper (17a). Some primary nucleotide sequence information was obtained. All of the data indicated that the newly isolated variant (termed 290) was essentially identical to CVP8/1/P2, including the detailed structure of the SV40-monkey junction at residues 953 and 954, the deletion of 153 bp of wild-type SV40 DNA between residues 987 and 988 of the variant, the inversion of SV40 sequences (residues 954 through 987) in the variant compared with their orientation in the wild-type genome, and the occasional presence of the extra 223-bp reiteration (see Fig. 1A).

The data in this paper as well as the characterization of variant 290 suggest that variants of the CVP8/1/P2 type have emerged repeatedly and independently. The conclusion must be tentative because, in spite of the various precautions and contraindications concerning contamination, that possibility cannot be rigorously excluded. The recurring emergence of such similar variants suggests that this class of variants may not arise by simple nonspecific recombination between viral and host DNA. In this respect those variants that arose during passage of strain 776 are particularly striking. The CVP8/1/P2-type variants isolated after four serial passages of 776 in either BSC-1 or African green monkey kidney cells contained a particular *Mbo*II site identified as characteristic of strain 777 but not of strain 776. Restriction endonuclease digestion of wild-type DNA I isolated after infecting cells with the yields of the first passages in each series confirmed the fact that the initiating stock was indeed strain 776. It is possible that the single-base-pair change that leads to the introduction of the *Mbo*II site in the variants occurs repeatedly during infection, but this seems unlikely since strain 776 is stable in this regard. Therefore, it is necessary to consider the possibility that the CVP8/1/P2-type variants do not arise through a series of recombinational events. In addition to the odd fact concerning the *Mbo*II site, it seems unlikely that the multiple hypothetical events that need to be postulated if the variant arises solely through recombination between wild-type viral genomes and the monkey genome (even assuming that the monkey sequences in the variant are contiguous in the monkey genome, a fact which is not established) would occur repeatedly. One possible alternative explanation is that monkey kidney cells contain a DNA segment whose structure is in whole or in part that found in the CVP8/1/P2-type variants. The segment may be genomic or may be present in a nonchromosomal DNA molecule. It would have to be present in low copy number or only in rare cells since the fragment C sequence occurs only infrequently in monkey DNA (17, 21, 29; T. McCutchan, unpublished data). SV40 might then serve as a helper, allowing efficient replication and packaging of this small DNA. Experiments designed to investigate this hypothesis are in progress.

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