Evolutionary Relationships of the Primate Papovaviruses: Base Sequence Homology Among the Genomes of Simian Virus 40, Stump-Tailed Macaque Virus, and SA12 Virus

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Physical maps of the genomes of the two newly discovered primate papovaviruses, SA12 and stump-tailed macaque virus (STMV), were generated by restriction endonuclease analysis. The base sequence homologies among the genomes of SA12, stump-tailed macaque virus, and simian virus 40 (SV40) were studied by heteroduplex analysis. Heteroduplexes between SA12 and SV40 DNAs and stump-tailed macaque virus and SV40 DNAs were constructed and mounted for electron microscopy in various amounts of formamide to achieve a range of effective temperatures. At each effective temperature, the regions of duplex DNA in the heteroduplexes were measured and localized on the SV40 physical and functional maps. By analyzing the data from this study and from our previous study (N. Newell, C. J. Lai, G. Khoury, and T. J. Kelly, Jr., *J. Virol.* **25**:193-201, 1978) on the base sequence homology between the genomes of BK virus and SV40, some general conclusions have been drawn concerning the evolutionary relationships among the genomes of the primate papovaviruses. The extent of homology among the viral genomes does not reflect the phylogenetic relationships of their hosts. At comparable effective temperatures ($T_m - 33^\circ\text{C}$), the heteroduplexes between the DNAs of BK virus and SV40 contained the largest amount of duplex (about 90%). The heteroduplexes made between SA12 and SV40 DNAs were slightly less homologous, containing about 80% duplex. The heteroduplexes made between SV40 and stump-tailed macaque virus DNAs were only 20% duplex under the same conditions. When the various heteroduplexes were mounted for microscopy at effective temperatures greater than $T_m - 33^\circ\text{C}$, the fraction of the duplex DNA decreased in each case, indicating the existence of considerable base mismatching in the homologous regions. When specific coding or noncoding regions of the viral genomes were compared, the data indicated that the extent of sequence divergence differed markedly from one region to another. In all the heteroduplexes studied, there were two regions, located near the junctions between the early and late regions on the SV40 map, which were essentially nonhomologous. All of the heteroduplexes studied showed significantly greater homology in the late region than in the early region. Within the late region, the sequences coding for the major capsid polypeptide, VP1, were the most highly conserved.

There are five known primate papovaviruses of the SV40-polyoma subgroup: simian virus 40 (SV40) from the rhesus macaque, stump-tailed macaque virus (STMV) from the stump-tailed macaque, SA12 virus from the chacma baboon, and BK virus (BKV) and JC virus (JCV) from man. There exist much data which suggest that these papovaviruses evolved from a common ancestor. All of the viruses have naked,

icosahedral capsids which measure 40 to 45 nm in diameter (1, 10, 27, 32, 47). Each of the viral genomes is a double-stranded, covalently closed circular DNA molecule whose molecular weight is 3.0×10^6 to 3.5×10^6 (13, 14, 26, 31, 32, 47). These viruses share one or more common structural antigens which have been shown to reside on the major structural capsid protein, VP1 (38, 40). The early antigen (T antigen) of all of the primate papovaviruses cross-react to some extent with the early antigen of SV40 (23, 29, 32, 39, 40, 46-48). The nucleic acid homology rela-

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tionships among the genomes of SV40, BKV, and JCV have been characterized in previous studies. By using DNA filter hybridization techniques, estimates for the extent of homology between the DNAs of JCV and SV40 and BKV and SV40 ranged from 10 to 50%, depending on the stringency of the hybridization conditions (13, 14, 16, 25). The homologous sequences detected in these studies were localized within the late region of the SV40 genome. By using the electron microscope heteroduplex method of Ferguson and Davis (8) for the mapping of homologous regions of DNA, we have reported 90% homology between the genomes of BKV and SV40 under nonstringent conditions ($T_m - 35^\circ\text{C}$) (24). The genomes of the more recently discovered papovaviruses, SA12 and STMV, are less well characterized.

SA12 was originally isolated from an uninoculated primary culture of vervet monkey kidney cells (19), but its probable natural host is the African chacma baboon (*Papio ursinus*) as indicated by the presence of neutralizing antibodies in sera from these animals (47). The T antigens of SA12 and SV40 show strong and reciprocal cross-reactivities in immunofluorescence tests, but the virion antigens of SA12 show no cross-reaction in immunofluorescence or immune electron microscopy tests with sera directed against any of the other papovavirus virions (47). SA12 has been shown to transform hamster kidney cells (J. D. Valis and K. V. Shah, unpublished observations).

STMV was isolated from uninoculated cultures of kidney cells of the stump-tailed macaque (*Macaca speciosa*) (30). It does not appear to be closely related to the other primate papovaviruses by immunological criteria. It shows no virion cross-reactivity in immune electron microscopy tests with sera directed against JCV, BKV, polyoma, or SV40 virions, and its T antigen is only slightly cross-reactive with those of SV40 and BKV (32). STMV has an unusual biology in that it seems to be transmitted as a congenital infection and to persist throughout the life of the animal. The virus has been recovered from cultures of every adult and fetal kidney that has been grown satisfactorily (41). All other papovaviruses, primate and nonprimate, are transmitted as postnatal infections, and they are rarely, if ever, recovered from fetal tissue (7). In addition, antibodies to STMV are not apparent in the sera of animals whose kidneys, when cultured, yield STMV (Shah et al., Fed. Proc., 34:4278, 1975). All other papovaviruses evoke a long-lasting antibody response in an infected host. Transformation by STMV has not yet been demonstrated, although a variety

of primate and nonprimate cells have been tested (K. V. Shah, unpublished data).

In our studies, we have attempted to define the relationships among the genomes of the primate papovaviruses by heteroduplex mapping of the homologous regions of the viral DNAs in the electron microscope. In a previous communication we reported on the regions of homology between BKV and SV40 DNAs (24). In the present study we have analyzed the regions of homology between SA12 and SV40 DNAs and STMV and SV40 DNAs. It might be expected that the extent of homology among the viral genomes would reflect the phylogenetic relationships of their hosts but, in fact, this was shown not to be the case. The genomes of BKV and SV40 appear to be the most closely related of the viruses studied. The genomes of SA12 and SV40 are slightly less related, whereas the genomes of STMV and SV40 are quite dissimilar. When specific coding or noncoding regions of the viral genomes are compared, the extent of sequence divergence differs markedly from one region to another. In all the heteroduplexes studied, there were two regions, located near the junctions between the early and late regions of the SV40 map, which were essentially nonhomologous. All of the heteroduplexes studied showed significantly greater homology in the late region than in the early region. Within the late region, the sequences coding for the major capsid polypeptide, VP1, were the most highly conserved.

MATERIALS AND METHODS

Cells and viruses. SV40 virus (small plaque, strain 776) was grown in monolayers of BSC-1 cells as previously described (15). Prototype SA12 (47) was grown in a rhesus embryonic kidney cell line MA104 (Microbiological Associates, Bethesda, Md.) in Eagle minimal essential medium supplemented with 2% fetal calf serum (Microbiological Associates). Virus stock was prepared from the progeny of a single plaque. STMV was grown in primary rhesus kidney cells as described previously (32).

Viral DNA. Unlabeled SV40, STMV, and SA12 DNAs and ^{32}P -labeled SV40 and SA12 DNAs were prepared by the method of Hirt (12) as modified by Danna and Nathans (3).

Restriction endonuclease cleavage of viral DNA. Viral DNA was digested at concentrations between 1 and 10 $\mu\text{g}/\text{ml}$ in 25- or 50- μl reaction mixtures. The amount of enzyme required for complete digestion was determined in trial reaction mixtures. All incubations were performed at 37°C , with the exception of the *TaqI* digestions, which were incubated at 50°C . Viral DNA was incubated with *HpaII*, *BamHI*, *BglII*, or *TaqI* in a buffer containing 20 mM Tris-hydrochloride (pH 7.4), 7 mM MgCl_2 , and 7 mM 2-mercaptoethanol. *HaeII* digestions were done in a buffer containing

6 mM Tris-hydrochloride (pH 7.4), 6 mM MgCl₂, and 6 mM 2-mercaptoethanol. Incubations with *Pst*I and *Hha*I were done in a buffer containing 6 mM Tris-hydrochloride, (pH 7.4), 6 mM MgCl₂, 6 mM 2-mercaptoethanol, and 50 mM NaCl. DNA was incubated with *Eco*RI in 100 mM Tris-hydrochloride (pH 7.2), 5 mM MgCl₂, 2 mM 2-mercaptoethanol, and 50 mM NaCl. Incubations with *Hpa*I were done in a buffer containing 10 mM Tris (pH 7.5), 10 mM MgCl₂, 5 mM 2-mercaptoethanol, and 50 mM NaCl. Incubations with *Bgl*II were carried out in a buffer containing 50 mM Tris-hydrochloride (pH 8), 10 mM MgCl₂, 10 mM 2-mercaptoethanol and 50 mM KCl.

The reactions were stopped by the addition of EDTA to a final concentration of 10 mM. The resulting solutions were extracted twice with buffer-saturated phenol and dialyzed against TE (10 mM Tris-hydrochloride, pH 8.6, 1 mM EDTA) unless otherwise stated. *Bam*HI, *Hpa*II, *Hpa*I, *Hha*I, and *Bgl*II were purchased from Bethesda Research Laboratories, Inc. *Bam*HI, *Hae*II, *Eco*RI, *Hha*I, and *Pst*I were purchased from New England Biolabs. *Eco*RI, *Bgl*II, and *Taq*I were gifts from Paul Geshelin, David Shortle, and Gary Ketner, respectively. When double digestions were done, the enzymes were added sequentially, and appropriate buffer and salt adjustments were made.

5'-Terminal labeling of STMV and SV40 restriction fragments. Solutions containing restriction fragments were adjusted to 20 mM Tris-hydrochloride (pH 7.8)–50 mM NaCl and incubated at 37°C for 30 min with 1/20 volume of bacterial alkaline phosphatase (1 mg/ml in 0.5 M NH₄HCO₃, 25 mM MgSO₄, 0.1 mM ZnSO₄, heated to 65°C for 30 min; Worthington Biochemicals Corp.) (33, 49). The reaction mixtures were then adjusted to 50 mM EDTA. After incubation at room temperature for 10 min, the reaction mixtures were extracted twice with buffer-saturated phenol and dialyzed against TE. The 5'-termini of the DNA were labeled by using T4 polynucleotide kinase (PL Biochemicals, Inc.) in the presence of [γ -³²P]ATP (18, 33, 49). The reaction mixture (100 μ l) contained 50 mM Tris-hydrochloride (pH 9.5), 10 mM MgCl₂, 5 mM dithiothreitol, 1 μ M spermidine, 1 μ M [γ -³²P]ATP, 1 to 2 U of T4 polynucleotide kinase, and 30 ng of DNA. After incubation at 37°C for 30 min, the reaction mixture was dialyzed extensively against 10 mM Tris-hydrochloride (pH 7.5), 5 mM EDTA, and 1 M NaCl to remove the free ATP. The [γ -³²P]ATP was prepared by the method of Glynn and Chappell (11) as modified by Maxam and Gilbert (21).

Gel electrophoresis. Restriction endonuclease digests of viral DNA were subjected to electrophoresis on both 1.4% agarose (SeaKem) and 4% acrylamide–0.2% bisacrylamide (BioRad Laboratories) slab gels in a buffer containing 40 mM Tris-hydrochloride (pH 7.8), 20 mM sodium acetate, and 2 mM EDTA. Agarose gel electrophoresis was carried out at 2.5 V/cm for 24 h; acrylamide gel electrophoresis was carried out at 3.75 V/cm for 16 to 20 h. After electrophoresis, gels containing radioactive DNA were dried and placed in close contact with a sheet of Kodak X-ray film (RP/S-54) for 12 h to 4 days. Gels containing unlabeled DNA were stained in electrophoresis buffer containing 0.5 μ g of ethidium bromide per ml for 1 h

and photographed under shortwave UV illumination.

Preparation of standard heteroduplexes. SA12/SV40 heteroduplexes were prepared by the method of Davis et al. (6). A mixture of linear SV40 and SA12 DNA molecules produced by restriction endonuclease digestion (each at a concentration of 3 μ g/ml) was denatured by incubation in 0.1 N NaOH for 5 min at room temperature. The solution was neutralized by the addition of 1/5 volume of 1 M Tris-hydrochloride (pH 7.1), and formamide (Matheson, Coleman, and Bell) was added to a final concentration of 50%. The DNA was allowed to renature at room temperature for 2.5 h. After extensive dialysis against TE at 4°C, the DNA was mounted for electron microscopy in 30, 40, or 50% formamide, depending on the effective temperature desired (see below).

Preparation of snapback heteroduplexes. STMV/SV40 heteroduplexes were prepared by a modification of the method of Ferguson and Davis (8). A mixture of linear SV40 and STMV DNA molecules (in 1.5 mM NaCl, 0.15 mM sodium citrate) produced by restriction endonuclease digestion was incubated with *Escherichia coli* DNA ligase to form mixed oligomers. The reaction mixture contained 20 to 30 μ g of DNA per ml, 0.03 M Tris-hydrochloride (pH 8.0), 0.001 M EDTA, 0.01 M MgCl₂, 0.01 M (NH₄)₂SO₄, 80 μ M nicotinamide adenine dinucleotide, 80 μ g of bovine serum albumin per ml, and 200 U of *E. coli* DNA ligase (a gift of Nicholas Muzyczka) per ml. After incubation at 15°C for 4 h, the reaction was stopped by addition of EDTA to a final concentration of 0.01 M. The reaction mixture was extracted twice with phenol and dialyzed against 0.015 M NaCl, 0.0015 M Na citrate.

The products of the ligase reaction were denatured with alkali and then neutralized to produce "snapback" molecules. Denaturation was accomplished by the addition of NaOH to a final concentration of 0.1 M. After 5 min at room temperature, the solution was neutralized by the addition of 1/5 volume of 1 M Tris-hydrochloride (pH 7.1). Formamide was then added to a final concentration of 30 or 35%, depending on the effective temperature desired (see below). After 5 min at room temperature, the DNA was mounted for electron microscopy.

Electron microscopy. Samples were mounted for electron microscopy as described by Davis et al. (6). The salt and formamide concentrations in the spreading solution and the hypophase were adjusted to achieve various effective temperatures as described by Davis and Hyman (5). In each case, the T_m of SV40 DNA was calculated from the following equation (22, 37). $T_m = 81.5 + 16.6 (\log M) + 0.41 (\%G + C) - 0.72 (\% \text{ formamide})$, where M is the molarity of monovalent salt, and $\%G + C$ is the percentage of guanine plus cytosine residues in the DNA (41% for SV40 [2, 4]). The effective temperature was then expressed as $T_m - \Delta T$, where ΔT is the difference between the T_m and the temperature at which the DNA was mounted for microscopy. The grids were observed and photographed with a JEM 100B electron microscope. Contour length measurements were made by using a digital length calculator (Numonics, North Wales, Pa.).

In control experiments the lengths of single-stranded and double-stranded circular SV40 DNA

molecules mounted on the same grid were compared. At all effective temperatures studied, the two lengths were the same within experimental error. In another control, the lengths of double-stranded SA12 and SV40 DNA molecules were compared. Again, no significant difference could be detected. In agreement with previous observations (32), the length of STMV DNA was 91% that of SV40 DNA.

RESULTS

Construction of a physical map of SA12 DNA. Five restriction endonucleases, *Bgl*II, *Pst*I, *Hha*I, *Eco*RI and *Hpa*II, were used to digest SA12 DNA to construct a physical map. The resultant fragments were sized on 1.4% agarose and 4% acrylamide gels (data not shown). In all cases, SV40 DNA was digested and subjected to electrophoresis under the same conditions, and the SV40 DNA fragment mobilities were used as molecular weight standards. To obtain information on the relative positions of the restriction fragments produced by each of the five enzymes, the ten possible double digestions were done. Table 1 gives the relative sizes of the fragments produced by all the single and double enzyme digestions. Only one physical map was consistent with all the data (Fig. 1). Restriction enzymes *Bam*HI, *Hae*II, and *Bgl*II do not cleave SA12 DNA.

Structure of SA12/SV40 heteroduplexes at $T_m - 33^\circ\text{C}$. SV40 DNA and SA12 DNA were digested to unit length linear with *Taq*I and *Bgl*II, respectively. The DNAs were denatured and hybridized to form heteroduplexes and were mounted for microscopy in 30% formamide, corresponding to an effective temperature of $T_m - 33^\circ\text{C}$. The SV40/SA12 heteroduplexes were unit length linear molecules that were about 80% duplex and contained two or three small substi-

TABLE 1. Fragment sizes produced by restriction endonuclease digestion of SA12 DNA

Endonuclease	No. of cuts	% of SA12 DNA length ^a
<i>Bgl</i> II	1	100
<i>Pst</i> I	1	100
<i>Hha</i> I	1	100
<i>Eco</i> RI	2	76, 24
<i>Hpa</i> II	3	58, 32, 10
<i>Bgl</i> II + <i>Pst</i> I	2	66, 34
<i>Bgl</i> II + <i>Hha</i> I	2	80, 20
<i>Pst</i> I + <i>Hha</i> I	2	54, 46
<i>Bgl</i> II + <i>Eco</i> RI	3	48, 28, 24
<i>Pst</i> I + <i>Eco</i> RI	3	76, 17, 7
<i>Hha</i> I + <i>Eco</i> RI	3	49, 27, 24
<i>Bgl</i> II + <i>Hpa</i> II	4	43, 32, 15, 10
<i>Pst</i> I + <i>Hpa</i> II	4	53, 32, 10, 5
<i>Hha</i> I + <i>Hpa</i> II	4	58, 32, 8.5, 1.5
<i>Eco</i> RI + <i>Hpa</i> II	5	46, 18, 15, 11, 10

^a The molecular weight of SA12 DNA is 101% that of SV40 DNA.

FIG. 1. Physical map of SA12 DNA. The map was constructed as described in the text and was then aligned with respect to the physical and functional maps of SV40 DNA (see text). The SV40 map is shown conventionally with the endonuclease *Eco*RI cleavage site at 0. The early and late regions of the genomes are denoted.

tution loops. An example is shown in Fig. 2a, and a summary of the length measurements of the heteroduplexes is shown in Fig. 3a. The two larger substitution loops were separated by about 0.5 SV40 map units. It is clear from the observed structure of the SA12/SV40 heteroduplexes that the two viral genomes have a similar genetic organization. The *Bgl*II and *Taq*I restriction enzymes were chosen for these experiments because they cleave the two genomes at similar places on their respective genetic maps. Preliminary experiments in which other combinations of enzymes were employed yielded heteroduplexes that were circular or contained single-stranded regions at both ends.

In control experiments, homoduplexes of SA12 and SV40 were constructed and examined in the electron microscope. In neither case were substitution loops observed. The SV40 homoduplexes appeared completely duplex, whereas 5% of SA12 homoduplexes contained small (less than 5% of the genome length) deletion loops. These deletions were not further characterized.

Orientation of SA12/SV40 heteroduplexes with respect to the SV40 physical map. The ends of the SA12/SV40 heteroduplexes must lie at SV40 map position 0.57, the cleavage site of the *Taq*I restriction enzyme (31). However, there are two ways to orient the heteroduplexes with respect to the SV40 physical map. To determine the correct orientation, the

FIG. 2. Electron micrographs of SA12/SV40 heteroduplexes. SA12 and SV40 DNAs were cleaved to unit-length linear molecules with endonucleases *Bgl*II and *Taq*I, respectively. Heteroduplexes were prepared by the method of Davis et al. (6) and mounted for microscopy in (a) 30% formamide ($T_m - 33^\circ\text{C}$), (b) 40% formamide ($T_m - 25^\circ\text{C}$), and (c) 50% formamide ($T_m - 18^\circ\text{C}$). The length of each bar corresponds to 0.5 μm .

following experiment was carried out. SV40 DNA was digested with both the *Taq*I and the *Hpa*II restriction enzymes. *Hpa*II is known to cleave SV40 DNA at map position 0.73 (42), so that the double digest yielded DNA fragments of 84% and 16% of the SV40 genome length.

These fragments were hybridized to the linear SA12 DNA produced by *Bgl*II enzyme digestion. After mounting for microscopy, heteroduplexes with two substitution loops and a single-stranded tail at one terminus were observed (Fig. 4c). These molecules were interpreted as heteroduplexes between the SA12 full-length linear strand and a strand derived from the large SV40 fragment produced by enzyme digestion with *Taq*I and *Hpa*II. The long single-stranded tail represented the SA12 strand, and the end of the duplex proximal to the single stranded tail marked the *Hpa*II cleavage site on the SV40 strand. These heteroduplexes could be aligned absolutely with respect to the SV40 physical map, giving the homology map shown in Fig. 4b. The correct orientation of the complete homology map (Fig. 3a and 4a) was determined by comparison with Fig. 4b. The data show that the two larger regions of nonhomology lie near the junctions between the early and late regions of SV40. The third region of nonhomology in the SA12/SV40 heteroduplexes lies in the late region around map position 0.85.

Structure of SA12/SV40 heteroduplexes at higher effective temperatures. When the heteroduplexes were spread at $T_m - 25^\circ\text{C}$ (40% formamide), they contained between three and six substitution loops (Fig. 2b). A summary of the length measurements of these molecules is shown in Fig. 3b. The average amount of duplex at this temperature was 53%, indicating that there was a significant amount of base mismatching in the duplex regions seen at the lower effective temperature. The heteroduplexes were oriented with respect to the SV40 physical map by an experiment identical to that described in the preceding section (data not shown). Most of the decrease in duplex content at this temperature occurred in the early region.

At an effective temperature of $T_m - 18^\circ\text{C}$ (50% formamide), the heteroduplexes remained 23% duplex on the average (Fig. 2c and 3c). Only a small portion of the early region remained duplex, and the majority of the duplex was in the late region. In control experiments, SA12 and SV40 homoduplexes were mounted for microscopy at $T_m - 18^\circ\text{C}$. No substitution loops were observed, indicating that these conditions were not sufficiently stringent to produce any melting of duplex DNA when the two strands were perfectly matched.

Alignment of the physical and functional maps of SA12 DNA. Because of the high degree of homology between the DNAs of SA12 and SV40, it was assumed that the functional organization of SA12 was similar to SV40, i.e., that about 50% of its genome was devoted to early functions and the other 50% devoted to

FIG. 3. Summary of SA12/SV40 homology maps at the three effective temperatures studied. (a) Homology map at $T_m - 33^\circ\text{C}$ obtained from length measurements of 38 SA12/SV40 heteroduplexes of the type shown in Fig. 2a. (b) Homology map at $T_m - 25^\circ\text{C}$ obtained from length measurements of 28 SA12/SV40 heteroduplexes of the type shown in Fig. 2b. (c) Homology map at $T_m - 18^\circ\text{C}$ obtained from length measurements of 29 SA12/SV40 heteroduplexes of the type shown in Fig. 2c. The molecules were aligned relative to one another by inspection to produce the homology maps. To construct the histograms, each heteroduplex was divided into 40 equal segments. The number of molecules that were duplex within a given segment (ordinate) was then plotted as a function of the position of the segment within the SV40 physical map (abscissa). The heteroduplexes were aligned with respect to the SV40 physical map as described in the text. The Hind II + III cleavage map of SV40 DNA and the locations of the early and late regions are shown below the abscissa.

late functions. To correlate the presumed early and late regions of SA12 DNA with its physical map, the following experiment was done. SA12 DNA was digested with *Pst*I and *Bgl*II, both of which cleave SA12 DNA once. The products of the double digestion were a large fragment, 66% of the genome length, and a small fragment, 34% of the genome length. These fragments were hybridized to unit length linear DNA produced by digestion with *Taq*I and mounted for electron microscopy at $T_m - 25^\circ\text{C}$. Molecules were observed which had two or three substitution loops and two single-stranded tails of unequal length at one terminus (Fig. 5a). These molecules were interpreted as heteroduplexes between a unit length linear strand of SV40 and a strand from

the larger fragment of SA12. The longer single-stranded tail represented the SV40 strand, and the shorter single-stranded tail represented the SA12 strand. The end of the shorter single-stranded tail marked the site where *Pst*I cleaved the SA12 DNA. The homology map for these heteroduplexes is shown in Fig. 5b. This histogram was aligned with the histogram shown in Fig. 3b to locate the presumed early and late functional regions of SA12 DNA with respect to the *Bgl*II and *Pst*I cleavage sites. Once the absolute position of these sites was known, the other restriction enzyme cleavage sites were positioned accordingly. The relationship between the physical and functional map of SA12 DNA as determined in this manner is shown in Fig. 1.

FIG. 4. SA12/SV40 homology maps at $T_m - 33^\circ\text{C}$. (a) Homology map obtained from length measurements of SA12/SV40 heteroduplexes of the type shown in Fig. 2a. (b) Homology map obtained from length measurements of 17 SA12/SV40 heteroduplexes of the type shown in c. The SV40 DNA strand did not contain the segment of the SV40 genome between SV40 map positions 0.57 and 0.73 (clockwise on the SV40 map). This fact made it possible to deduce the absolute orientation of the heteroduplexes with respect to the SV40 physical map. The homology map shown in (a) was aligned with that shown in (b) by inspection. The histograms were constructed as described in the legend to Fig. 3. The *HindII* + *III*

Construction of a physical map of STMV DNA. STMV DNA was digested with each of four restriction endonucleases, *HaeII*, *EcoRI*, *HpaI*, and *HpaII*, and the resulting fragments were sized on 1.4% agarose and 4% acrylamide gels (data not shown). In all cases, SV40 DNA was digested and subjected to electrophoresis under the same conditions, and the SV40 fragment mobilities were used as molecular weight standards. To obtain information on the relative positions of the restriction fragments produced by each of the four enzymes, the six possible double digestions were carried out. Table 2 gives the relative sizes of the fragments produced by all the single- and double-enzyme digestions. Only one physical map was consistent with all the data (Fig. 6). Restriction enzyme *BamHI* does not digest STMV DNA.

Structure and orientation of STMV/SV40 heteroduplexes. STMV/SV40 heteroduplexes were prepared by the method of Ferguson and Davis (8). STMV and SV40 DNA molecules were digested with endonuclease *HaeII* which has been shown to cut each genome once. The resulting unit length linear molecules were joined at their cohesive terminal with *E. coli* DNA ligase. Among the reaction products were STMV/SV40 dimers in which complementary strands of the two DNAs were covalently joined. Alkaline denaturation and renaturation of this type of molecule resulted in the formation of snapback heteroduplexes by intramolecular renaturation. These molecules were mounted for microscopy in 30% formamide, corresponding to an effective temperature of $T_m - 32^\circ\text{C}$. Under these conditions, one type of heteroduplex molecule was observed which had a loop at one end, a central duplex region containing 1 to 4 small substitution loops, and two single-stranded tails of unequal length at the other end (Fig. 7a). The molecules were about 20% duplex on the average. It was somewhat surprising that only one

cleavage map of SV40 DNA and the locations of the early and late regions are shown below the abscissa. (c) Electron micrograph of SA12/SV40 heteroduplex used to orient the homology maps with the physical map of SV40. SA12 DNA molecules were cleaved to unit-length linears with endonuclease *BglII*. SV40 DNA molecules were cleaved twice by sequential digestion with endonuclease *TaqI* (cleavage site, 0.57) and endonuclease *HpaII* (cleavage site, 0.73). The figure shows a representative heteroduplex composed of a unit length SA12 strand and an SV40 strand derived from the larger of the products of the double restriction endonuclease digestion. The latter includes the segment of the SV40 genome between map positions 0.73 and 0.57 (clockwise on the SV40 map). The length of the bar corresponds to 0.5 μm.

FIG. 5. (a) Electron micrograph of SA12/SV40 heteroduplex used to align the physical and functional maps of SA12 DNA. SV40 DNA was cleaved to unit-length linears with endonuclease *TaqI*. SA12 DNA was cleaved twice by sequential digestion with endonucleases *BglII* and *PstI*, giving fragments of 66 and 34% of SA12 DNA. The figure shows a representative heteroduplex composed of a unit-length SV40 strand and an SA12 strand derived from the larger of the products of the double restriction endonuclease digestion. The heteroduplexes were prepared by the method of Davis et al. (6) and mounted for microscopy in 40% formamide ($T_m = 25^\circ\text{C}$). The length of the bar corresponds to 0.5 μm . (b) SA12/SV40 homology map used to align the physical and functional maps of SA12. Length measurements were performed on 13 SA12/SV40 heteroduplexes of the type shown in Fig. 5a. The molecules were aligned relative to one another to produce the homology map shown. These heteroduplexes were oriented with respect to the SV40 physical map by comparison with the homology map in Fig. 3b.

type of heteroduplex was observed. In the most general case, the Ferguson and Davis procedure (8) should yield two distinctly different snapback heteroduplexes as a consequence of the fact that complementary strands of SV40 and STMV DNAs can be joined in two possible orientations via the *HaeII* cohesive termini (Fig. 8). In the present case, it appears that the locations of the *HaeII* sites in the SV40 and STMV genomes are such that the two heteroduplexes generated were indistinguishable within the error of the electron microscopic method.

To locate the regions of homology between

SV40 and STMV within the SV40 physical map, an experiment was performed to identify a specific end of the SV40 strands in the STMV/SV40 heteroduplexes. A schematic outline of this experiment is shown in Fig. 9. The SV40 DNA, in addition to being cut with *HaeII* (map position 0.83) (34) was also cut with *TaqI*, which cleaves at 0.57 map units (31). The products of the double digestion, a large fragment 74% of unit length, and a small fragment 26% of unit length, were separated on a 5 to 20% neutral sucrose gradient. The purified 74% fragment of SV40 DNA and the STMV linear DNA (generated by digestion with *HaeII*) were joined at their cohesive termini. Instead of the two different heterodimers made previously, only one type of heterodimer was produced because there is only one possible way to join complementary strands of the two genomes to generate a snapback heteroduplex. The heterodimers were denatured, neutralized, and mounted for microscopy at $T_m = 32^\circ\text{C}$. By comparison of these heteroduplexes with those described above, the SV40 strand could be identified. The heteroduplexes appeared similar to the ones seen previ-

TABLE 2. Fragment sizes produced by restriction endonuclease digestion of STMV DNA

Endonuclease	No. of cuts	% of STMV DNA length ^a
<i>HaeII</i>	1	100
<i>EcoRI</i>	2	54, 46
<i>HpaI</i>	2	82, 18
<i>HpaII</i>	2	89, 11
<i>HaeII</i> + <i>EcoRI</i>	3	57, 26, 17
<i>HaeII</i> + <i>HpaI</i>	3	66, 18, 16
<i>HaeII</i> + <i>HpaII</i>	3	73, 16, 11
<i>EcoRI</i> + <i>HpaI</i>	4	49, 33, 13, 5
<i>EcoRI</i> + <i>HpaII</i>	4	45, 45, 9, 1
<i>HpaI</i> + <i>HpaII</i>	4	39, 32, 18, 11

^a The molecular weight of STMV DNA is 91% that of SV40 DNA.

FIG. 6. Physical map of STMV DNA. See text for details.

FIG. 7. Electron micrographs of STMV/SV40 heteroduplexes. (a) STMV and SV40 DNAs were cleaved to unit length linear molecules with endonuclease *HaeII*. Heteroduplexes were prepared by the method of Ferguson and Davis (8) and mounted for microscopy in 30% formamide ($T_m - 32^\circ\text{C}$). (b) STMV DNA molecules were cleaved to unit length linears with endonuclease *HaeII*. SV40 DNA molecules were cleaved twice by sequential digestion with endonuclease *HaeII* (cleavage site, 0.83) and endonuclease *TaqI* (cleavage site, 0.57). The figure shows a representative heteroduplex composed of a unit length STMV strand and an SV40 strand derived from the larger of the products of the double restriction endonuclease digestion. The latter includes the segment of the SV40 genome between map positions 0.83 and 0.57 (clockwise on the SV40 map). The heteroduplexes were mounted for microscopy in 30% formamide ($T_m - 32^\circ\text{C}$). (c) STMV/SV40 heteroduplex of the type described in b, but mounted for microscopy in 35% formamide ($T_m - 26^\circ\text{C}$). The length of each bar corresponds to 0.5 μm .

ously, except that the long single-stranded tail was now shortened (Fig. 7b). The shortened tail was presumed to be the SV40 strand whose end

was at 0.57 map units (the *TaqI* cleavage site), so these heteroduplexes were aligned absolutely with respect to the SV40 physical map, yielding the homology map shown in Fig. 10a. The average amount of duplex DNA at this effective temperature was 19%, significantly less than the amount of duplex DNA seen between SA12 and SV40 DNAs at an equivalent temperature. The data show that the majority of the homology is in the late region, and there is also a small region of homology in the early region distal to the origin of replication. Again, there appears to be very little homology near the joints between the early and the late regions (SV40 map positions 0.17 and 0.67).

FIG. 8. Schematic outline of the experimental procedure used to generate STMV/SV40 heteroduplexes. The letters a, b, c, x, y, and z represent specific regions of the two viral genomes. The four possible types of heterodimers generated by the procedure are shown in the center of the figure. The symbols + and - represent the two complementary strands of the double-stranded DNA molecules. Of the four different heterodimers, only two have complementary strands of the two viral genomes covalently joined, and can form "snapback" molecules. If the intramolecular renaturation occurred between the x sequences and the c sequences as shown, the two resulting heteroduplexes would have similar structures (see text).

FIG. 9. Schematic outline of the experimental procedure used to locate the regions of homology between STMV and SV40 DNAs within the SV40 physical map. Because SV40 DNA was digested with two different endonucleases, only the end of the large fragment near the c sequences could be joined to linear STMV DNA. Two types of heterodimers were generated, but only one type had complementary strands of the two viral genomes joined, and therefore, could form a "snapback" molecule. The end of the long single-stranded tail of the heteroduplex was at the TaqI cleavage site (SV40 map position 0.57). (See text and legend to Fig. 8.)

When the heterodimers were mounted for microscopy in 35% formamide ($T_m = 26^\circ\text{C}$), the average amount of duplex decreased slightly to 17% (Fig. 7c and 10b). The remaining duplex was localized mainly in the late region.

DISCUSSION

Physical maps of the genomes of the two newly characterized primate papovaviruses, SA12 and STMV, were constructed by restriction endonuclease analysis. The maps were generated by comparison of the fragments resulting from single and double digestions with endonucleases which made few cuts in the DNAs. During the restriction endonuclease analyses, at least one single-cut enzyme was found for both SA12 and STMV DNAs. These single-cut enzymes were used to linearize the viral DNAs so that heteroduplexes could be constructed.

To study the homology relationships, heteroduplexes between SV40 and SA12 DNAs and between SV40 and STMV DNAs were constructed and mounted for electron microscopy at various concentrations of formamide to achieve a range of effective temperatures. All of the heteroduplexes exhibited some base sequence homology and, at each effective temperature, the regions of duplex DNA in the heteroduplexes were measured and localized on the SV40 physical and functional maps. When the various heteroduplexes were mounted for microscopy at the higher effective temperatures, the fraction of duplex DNA decreased in each case, indicating the existence of considerable base mismatching in the homologous regions.

The SA12/SV40 heteroduplexes showed about 80% homology at $T_m = 33^\circ\text{C}$. They contained two nonhomologous regions mapping

FIG. 10. STMV/SV40 homology maps at $T_m = 32^\circ\text{C}$ and $T_m = 26^\circ\text{C}$. (a) Homology map obtained from the length measurements of 28 STMV/SV40 heteroduplexes of the type shown in Fig. 7b. (b) Homology map obtained from the length measurements of 17 STMV/SV40 heteroduplexes of the type shown in Fig. 7c. The SV40 strand did not contain the segment of the SV40 genome between SV40 map positions 0.57 and 0.83 (clockwise on the SV40 map). This fact made it possible to deduce the absolute orientation of the heteroduplexes with respect to the SV40 physical map (see text). The histograms were constructed as described in the legend to Fig. 3. The HindII + III cleavage map of SV40 DNA and the locations of the early and late regions are shown below the abscissa.

near the junctions between the early and late regions on the SV40 genome and also a nonhomologous region lying between SV40 map positions 0.80 and 0.90. This melted region corresponds to the place where *tsD* mutants of SV40 have been mapped (17). At a higher effective temperature ($T_m - 25^\circ\text{C}$), the average amount of duplex decreased to 53% in the heteroduplexes. The melted regions occurred in both the early and late portions of the genome.

Under the most stringent conditions used ($T_m - 18^\circ\text{C}$), most of the remaining duplex was in two portions of the late region. One portion, lying between SV40 map positions 0.65 and 0.85, represents the region coding for the N terminal end of the SV40 polypeptide VP2 (35). The other portion (SV40 map positions 0.90 to 0.17) represents the region coding for the SV40 protein, VP1. A small part of the early region remained duplex in the SA12/SV40 heteroduplexes at $T_m - 18^\circ\text{C}$ (SV40 map positions 0.40 to 0.56). Some of the SV40 *tsA* mutants map within this region (17).

Only a small amount of homology was detected between the genomes of SV40 and STMV. At $T_m - 32^\circ\text{C}$, the heteroduplexes were 20% duplex. There was a small amount of homology in the early region (SV40 map position 0.17 to 0.38) distal to the SV40 origin of replication. The majority of the duplex was in the late region and was entirely localized to the coding region of the VP1 gene. When the heteroduplexes were mounted for microscopy at $T_m - 26^\circ\text{C}$, the average amount of duplex decreased slightly to 17%. This remaining duplex was localized mainly in the late region. Figures 3 and 10 summarize the data for all effective temperatures studied for the SA12/SV40 and STMV/SV40 heteroduplexes, respectively.

By analyzing the data presented in this study and in our previous study on BKV (24), some general conclusions can be drawn concerning the homology relationships among the genomes of the primate papovaviruses. Since the regions of duplex DNA in the heteroduplexes were localized on the SV40 map at all effective temperatures studied, it was possible to compare the degree of homology in specific coding or noncoding regions of the viral genomes. In all the heteroduplexes studied, there were two regions (SV40 map positions 0.18 and 0.69), located near the junctions between the early and late regions on the SV40 map, which were essentially nonhomologous. In the case of SV40, at least a portion of each nonhomologous region appears to be dispensable for virus multiplication, since deletions mapping within them have been isolated (43). This may account for the more rapid

divergence of the nucleotide sequences in these two regions. All of the heteroduplexes studied showed significantly greater homology in the late region than in the early region. Within the late region, the sequences coding for the major capsid protein, VP1 (SV40 map positions 0.95 to 0.16) (9, 31, 35, 50) were the most highly conserved. It is interesting to note that the region of strongest homology between the distantly related papovaviruses SV40 and polyoma has been shown to lie between SV40 map positions 0.93 and 0.98, which includes the coding region for the N terminus of VP1 (8).

A summary of the melting behavior of heteroduplexes made with SV40 DNA is shown in Fig. 11. At comparable effective temperatures ($T_m - 33^\circ\text{C}$), the heteroduplexes made between BKV and SV40 DNAs contained the largest amount of duplex (about 90%). The heteroduplexes made between SA12 and SV40 DNAs were slightly less homologous, containing about 80% duplex. The heteroduplexes made between SV40 and STMV DNAs were only 20% duplex under the same conditions.

It might be expected that the extent of homology among the viral genomes would reflect the phylogenetic relationships of their hosts but, in fact, this was shown not to be the case. The virus whose genome is most closely related to that of SV40 is the human papovavirus BKV. Slightly less homologous are the DNAs of SA12 and SV40 yet, according to the fossil record, the

FIG. 11. Summary of the melting behavior of heteroduplexes made with SV40 DNA and DNA from BKV, SA12, STMV, or polyoma. The percent duplex is graphed versus the effective temperature. The data for the SV40/SA12 and SV40/STMV heteroduplexes are from the text. The data for the polyoma/SV40 heteroduplexes are taken from Ferguson and Davis (8). The data for the SV40/SV40 and SV40/BKV heteroduplexes are taken from Newell et al. (24).

two viral hosts, the chacma baboon, and the rhesus macaque, diverged much more recently than man and the rhesus macaque. The least amount of homology was found between STMV and SV40 DNAs, yet the two viral hosts are from the same genus, *Macaca*. It is difficult to interpret these findings in the absence of additional information on the rates of evolution of the viral genomes. On the one hand, if the various primate papovaviruses diverged from one another at the same time as their respective hosts diverged, the data would suggest that there has been a wide variation in the rates at which base changes have been fixed in the different viral genomes. On the other hand, if the rates of fixation of base changes have been similar for all of the viral genomes studied, then at least in some cases the divergence of the viruses cannot have coincided with the divergence of their modern hosts. For example, it is possible that the two distantly related viruses STMV and SV40 diverged long before the divergence of their modern hosts by occupying separate niches in some progenitor of the macaques. Another possibility is that the two closely related viruses BKV and SV40 diverged after the divergence of their modern hosts as a consequence of a chance cross-species transmission from monkey to human or vice versa. Studies of additional papovaviruses isolated from diverse species may help to determine which, if any, of these possibilities is correct.

The findings of homology between the early regions of the viral genomes studied is consistent with immunological and biochemical data. The extensive homology between the early regions of SV40 and BKV correlates with the findings that the T antigens of the two viruses are strongly cross-reactive (23, 29, 39, 46), that the T antigens of the two viruses have similar peptide maps (36, 44, 45), and that BKV can complement an SV40 *tsA* mutant in monkey cells at the nonpermissive temperature (20). The early regions of SA12 and SV40 also showed extensive homology which is consistent with the fact that the T antigens of the two viruses are strongly cross-reactive (47). The weak cross-reaction found between the T antigens of STMV and SV40 (32) correlates with the finding of very little homology between the early regions of the two viral genomes.

The findings in the present study that the strongest homologies between the viruses map in the late regions would appear to be inconsistent with the immunological data showing that the virion antigens of the papovaviruses are not cross-reactive (with the exception of the virion antigens of BKV and SV40 which are slightly cross-reactive) (10, 28, 32, 40, 46, 47). This ap-

parent discrepancy may be due to the fact that the anti-V sera used in these studies were prepared against intact virions. Such sera may detect only that subset of antigenic determinants which reside at the surface of the virion. These surface determinants may evolve faster than the portions of the capsid proteins which are internal to the virion due to selective pressures related to the needs of the various viruses to interact with different cell surfaces in their respective hosts and to evade the host's immune response. The observation that the late antigens of all the papovaviruses are strongly cross-reactive when sera directed against detergent- or alkali-disrupted capsids are used is consistent with this general hypothesis (38, 40).

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