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Electron Microscope Study of the Base Sequence Homology Between Simian Virus 40 and Human Papovavirus BK

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The base sequence homology between the genomes of simian virus 40 (SV40) and human papovavirus BK (BKV) was studied by the heteroduplex method of Ferguson and Davis (J. Mol. Biol. 94:135-149, 1975). When mounted for microscopy in 30% formamide (T_m -35°C), BKV/SV40 heteroduplexes were an average of 92% double-stranded and contained only two small nonhomologous regions that mapped near the junctions between the early and late regions of the SV40 genome. At higher formamide concentrations, the fraction of duplex DNA in the BKV/SV40 heteroduplexes decreased, indicating significant base mismatching in the homologous regions. The strongest regions of homology were located in the late region.

The human papovavirus BK (BKV) was originally isolated from the urine of a renal allograft recipient on immunosuppressive therapy (11). Viruses similar to the prototype have subsequently been isolated from the urines of 30 to 40% of immunosuppressed renal allograft recipients and from the urines of three patients with Wiskott-Aldrich syndrome, a disease characterized by a deficiency of humeral and cell-mediated immunity (1, 7, 13, 16, 20, 21, 39). Although BKV has not yet been isolated from immunocompetent individuals, serological surveys suggest that infection with BKV is very common in humans. About 60 to 80% of persons living in Europe and the United States have detectable antibodies to BKV (10, 23, 35).

Several recent studies indicate an evolutionary relationship between BKV and the more extensively studied simian papovavirus SV40. Antisera directed against intact SV40 virions are weakly cross-reactive with BKV virion antigens by immunofluorescence tests (36, 38) or immune electron microscopy (11, 30). Antisera directed against disrupted SV40 virions or against the intranuclear T antigen induced early in SV40 infection show strong cross-reactions in immunofluorescence tests with the corresponding BKV antigens (27, 31, 34, 36, 38). BKV can complement an early SV40 mutant, *tsA*, in monkey cells under nonpermissive conditions (24). The genome of BKV is a closed circular duplex DNA molecule with a molecular weight slightly less than that of the SV40 genome (13, 14, 29). DNA-DNA hybridization studies employing a variety of techniques (13, 14, 18, 28) have revealed significant sequence homology between the genomes of BKV and SV40. Depending on the stringency of the hybridization conditions, estimates for the extent of homology between the two genomes have ranged from 10 to 50%. The homologous sequences detected in these studies were localized within the late region of the SV40 genome.

We have studied the sequence homology between the genomes of BKV and SV40 by the electron microscope heteroduplex method (5, 6, 8). DNA molecules of the two viruses were cleaved once with endonuclease R · *Eco*RI, and heteroduplexes were constructed by the method of Ferguson and Davis (8). The heteroduplexes were then mounted for microscopy in various concentrations of formamide to achieve various effective temperatures. We have detected a greater extent of homology between the genomes of BKV and SV40 than that previously reported. At the lowest effective temperature tested (T_m -35°C), the BKV/SV40 heteroduplexes were about 90% duplex and contained only two single-stranded regions, whose locations mapped near the junctions between the early and late regions of the SV40 genome. At higher effective temperatures, the fraction of duplex DNA in the BKV/SV40 heteroduplexes decreased, indicating that the base sequences in the homologous regions were not perfectly matched. The strongest regions of homology between the genomes of the two viruses were located in the late region.

MATERIALS AND METHODS

Cells and viruses. SV40 virus (small plaque, strain 776) was grown in monolayers of BSC-1 cells as pre-

viously described (17). BKV (plaque-purified prototype strain) was grown in human embryonic kidney cells as previously described (14).

Viral DNA. SV40 and BKV DNAs were prepared by the method of Hirt (12) as modified by Danna and Nathans (3).

Restriction endonuclease cleavage of viral DNA. (i) Endonuclease R•*Eco*RI. SV40 or BKV DNA at a concentration of 8 μg/ml was incubated with endonuclease R · *Eco*RI for 2 h at 37°C in a buffer containing 0.1 M Tris-hydrochloride (pH 7.2), 0.05 M NaCl, 0.005 M $MgCl_2$, and 0.002 M 2-mercaptoethanol. The amount of enzyme required for complete digestion was determined in trial reaction mixtures. 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 1.5 mM NaCl–0.15 mM sodium citrate. The endonuclease R · *Eco*RI was a gift from Paul Geshelin.

(ii) Endonuclease R•*Bam*HI. SV40 DNA (20 μg/ml) was incubated with 50 U of endonuclease R · *Bam*HI (Bethesda Research Laboratories) per ml for 4 h at 37°C in a buffer containing 0.02 M Tris-hydrochloride (pH 7.5), 0.007 M $MgCl_2$, and 0.002 M 2-mercaptoethanol.

Preparation of "snapback" heteroduplexes. BKV/SV40 heteroduplexes were prepared by the method of Ferguson and Davis (8) with minor modifications. A mixture of linear SV40 and BKV DNA molecules produced by restriction endonuclease digestion was incubated with *Escherichia coli* DNA ligase to form mixed oligomers. The reaction mixture contained 20 to 30 μg of DNA per ml, 0.03 M Tris-hydrochloride (pH 8.0), 0.001 M EDTA, 0.01 M $MgCl_2$, 0.01 M $(NH_4)_2SO_4$, 80 μM nicotinamide adenine dinucleotide, 50 μg of bovine serum albumin per ml, and 200 U of *E. coli* DNA ligase (a gift from 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 sodium citrate.

The products of the ligase reaction were denatured with alkali and then neutralized to produce "snapback" molecules. Denaturation was accomplished by addition of NaOH to a final concentration of 0.1 M. After 5 min at room temperature, the solution was neutralized by addition of one-fifth volume of 1 M Tris-hydrochloride (pH 7.1). Formamide was then added to a final concentration of 30, 40, 50, or 60%, depending on the effective temperature desired. After 5 min at room temperature, the DNA was mounted for electron microscopy.

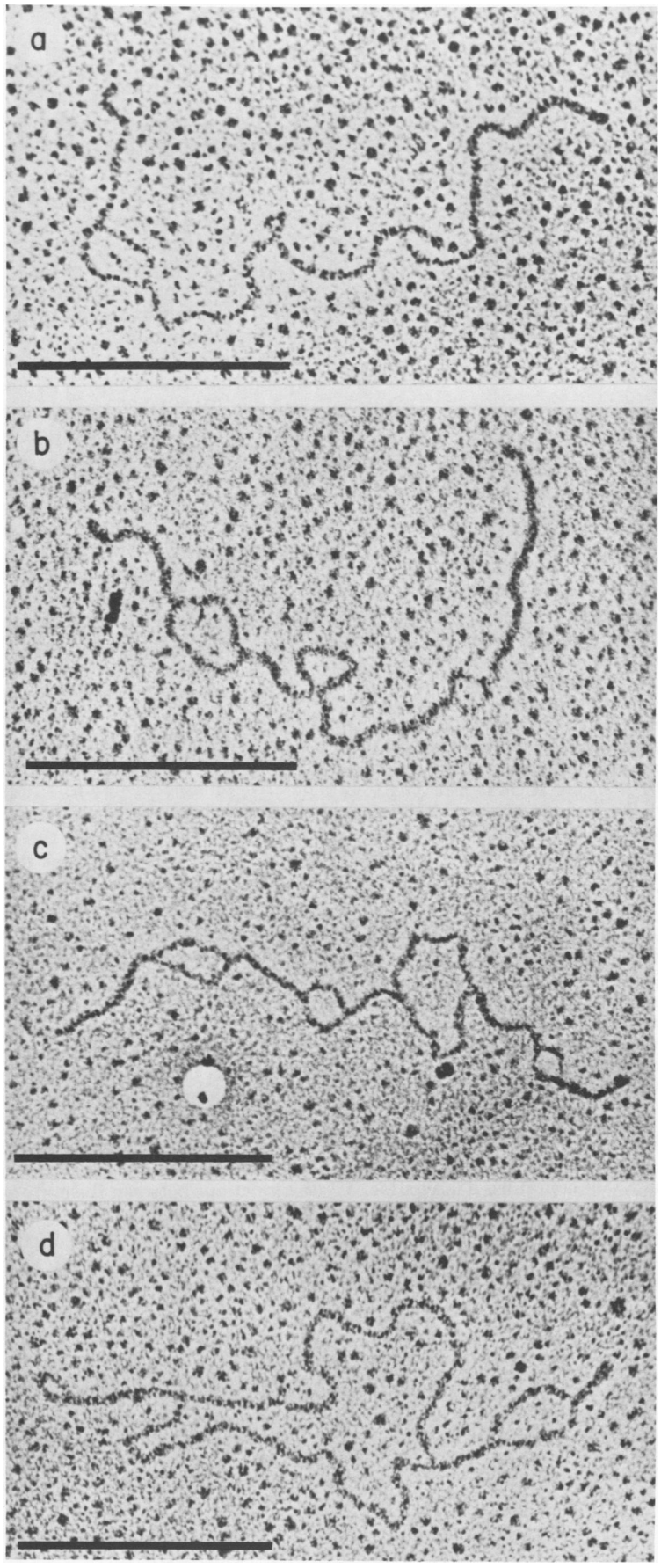
Electron microscopy. Samples were mounted for electron microscopy as described by Davis et al. (6). The salt and formamide concentrations in the spreading solution and the hypophase were adjusted to achieve various effective temperatures as described by Davis and Hyman (5). In each case, the T_m of SV40 DNA was calculated from the equation (22, 33): $T_m = 81.5 + 16.6 \cdot (\log M) + 0.41 \cdot (\% G+C) - 0.72 \cdot (\%$ 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 with 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 BKV and SV40 DNA molecules were compared. Again no significant difference could be detected.

RESULTS

Structure of BKV/SV40 heteroduplexes at T_m −35°C. BKV/SV40 heteroduplexes were prepared by the Ferguson and Davis method (8). BKV and SV40 DNA molecules were digested with endonuclease R · *Eco*RI, which is known to cleave each genome at a single site within a homologous portion of the late region (18). The resulting unit-length linear molecules were covalently joined at their cohesive termini with *E. coli* ligase. Among the products of this reaction were BKV/SV40 dimers in which complementary strands of the two viral DNA molecules were covalently joined. Alkaline denaturation and neutralization of mixed dimers of this type resulted in the formation of BKV/SV40 heteroduplexes by intramolecular renaturation. These molecules were mounted for electron microscopy in 30% formamide, corresponding to an effective temperature of T_m −35°C. Under these conditions, the SV40/BKV heteroduplexes were unit-length linear molecules containing one or two small substitution loops (Fig. 1a). Figure 2a summarizes length measurements on the SV40/BKV heteroduplexes. The molecules were 92% double-stranded on the average, and contained two small regions of nonhomology separated by about 0.5 SV40 map unit. In control experiments, homodimers of SV40 or BKV DNA were constructed and examined by electron microscopy after alkaline denaturation and neutralization. Molecules with substitution loops were not observed in either control preparation. In the SV40 preparation, all of the renatured

FIG. 1. *Electron micrographs of BKV/SV40 heteroduplexes. BKV and SV40 DNAs were cleaved to unit-length linear molecules with endonuclease R · EcoRI. Heteroduplexes were prepared by the method of Ferguson and Davis (8) and mounted for microscopy in (a) 30% formamide (T_m −35°C), (b) 40% formamide (T_m −28°C), (c) 50% formamide (T_m −20°C), and (d) 60% formamide (T_m −13°C). Bar, 0.5 μm.*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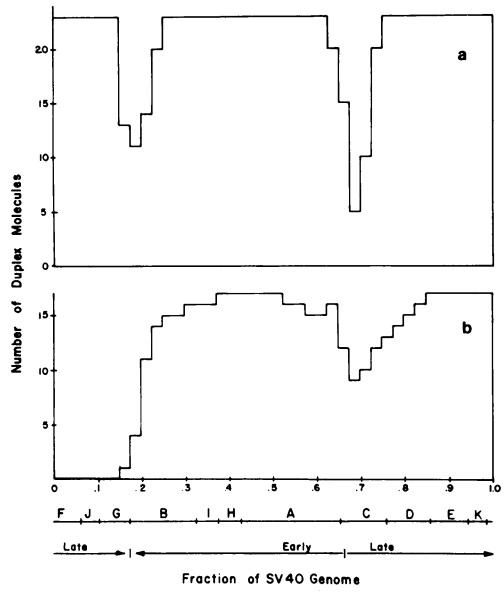


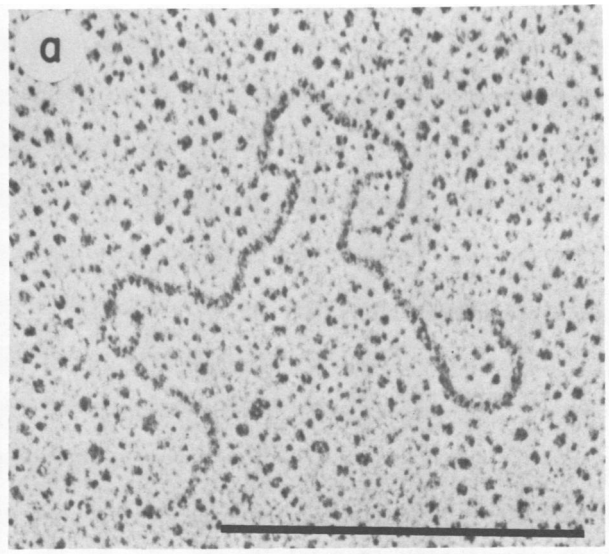
FIG. 2. *BKV/SV40 homology maps at T_m −35°C. (a) Length measurements were performed on BKV/SV40 heteroduplexes of the type shown in Fig. 1a. The molecules were aligned relative to one another by inspection to produce the homology map shown. (b) Length measurements were performed on BKV/SV40 heteroduplexes of the type shown in Fig. 3a. The SV40 DNA strand in these heteroduplexes did not contain the segment of the SV40 genome between SV40 map positions 0 and 0.16 (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 homology map shown in (a) was aligned with that shown in (b) by inspection. 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 HindII + III cleavage map of SV40 and the locations of the early and late regions are shown below the abscissa.*

molecules were completely duplex. In the BKV preparation, a small fraction of the renatured molecules (about 20%) contained deletion loops whose average lengths were less than 5% of the genome length. Attempts to eliminate the deletions from the BKV population by cloning the virus stock were unsuccessful. The accumulation of deletions during productive infection has been observed with other papovaviruses, albeit at lower frequencies.

Orientation of BKV/SV40 heteroduplexes with respect to the SV40 physical map. The ends of the BKV/SV40 heteroduplexes must lie at SV40 map position zero, the cleavage site of the *Eco*RI restriction enzyme (25, 26). However, there are two possible ways to orient the heteroduplexes with respect to the SV40 physical map. To determine the correct orientation, the following experiment was carried out. SV40 DNA was digested with endonuclease R · *Bam*HI in addition to endonuclease R · *Eco*RI. The *Bam*HI enzyme is known to cleave SV40 DNA at map position 0.16, so the products of the double digestion were a large fragment, 0.84 SV40 map unit in length, and a small fragment, 0.16 SV40 map unit in length (M. Mathews and J. Sambrook, unpublished data). These fragments were covalently joined to unit-length linear BKV DNA molecules produced by *Eco*RI digestion, and heteroduplexes were formed as described above. When this preparation was mounted for electron microscopy, heteroduplexes containing one substitution loop and two single-stranded tails of unequal length at one terminus were observed (Fig. 3a). These molecules were interpreted as heteroduplexes between a unit-length BKV strand and a strand derived from the large SV40 fragment produced by digestion with the *Eco*RI and *Bam*HI enzymes. The longer single-stranded tail represented the BKV strand and the shorter single-stranded tail represented the SV40 strand. The end of the shorter tail marked the *Bam*HI cleavage site (SV40 map position 0.16), so these heteroduplexes could be aligned absolutely with respect to the SV40 physical map, yielding the homology map shown in Fig. 2b. The correct orientation of the complete homology map shown in Fig. 2a was then determined by comparison with Fig. 2b. The data show that the two regions of nonhomology in BKV/SV40 heteroduplexes mounted at T_m −35°C map near the junctions between the early and late regions of the SV40 genome.

Structure of BKV/SV40 heteroduplexes at higher effective temperatures. When the BKV/SV40 heteroduplexes were mounted for

FIG. 3. *Electron micrographs of BKV/SV40 heteroduplexes used to orient homology maps with the physical map of SV40. BKV DNA molecules were cleaved to unit-length linear molecules with endonuclease R · EcoRI. SV40 DNA molecules were cleaved twice by sequential digestion with endonuclease R · EcoRI (cleavage site, 0) and endonuclease R · BamHI (cleavage site, 0.16). The figure shows representative heteroduplexes composed of a unit-length BKV 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 0.16 and 0 (clockwise on the SV40 map). The heteroduplexes were mounted for microscopy in (a) 30% formamide (T_m −35°C), (b) 50% formamide (T_m −20°C), and (c) 60% formamide (T_m −13°C). Bar, 0.5 μm.*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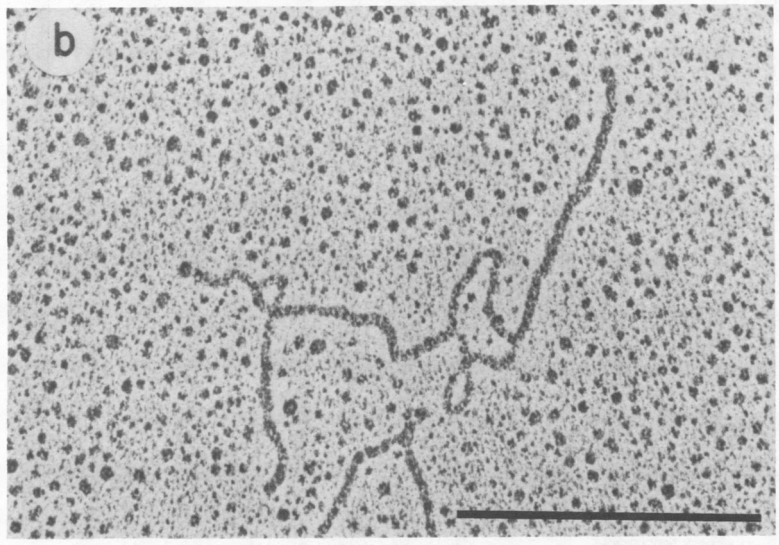
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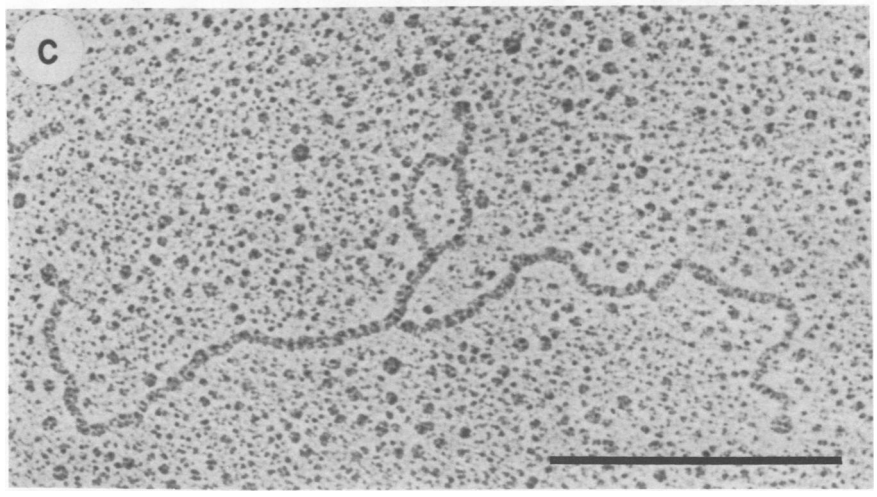
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electron microscopy in 40% formamide (T_m −28°C), the molecules observed were similar to those seen at T_m −35°C except that the two substitution loops were slightly larger (Fig. 1b and 4b). The average amount of duplex DNA per molecule was 83%.

At an effective temperature of T_m −20°C (50% formamide), the BKV/SV40 heteroduplexes contained between two and five substitution loops (Fig. 1c), and the average duplex content per molecule decreased to 42%, indicating that the nucleotide sequences in the duplex regions observed at lower effective temperatures were not perfectly matched. The heteroduplexes were oriented with respect to the SV40 physical map as described above (Fig. 3b and 5a, b). Much of the decrease in duplex content of the heteroduplexes mounted at T_m −20°C occurred within the early region of the SV40 genome; however, a small melted segment was also observed in the late region.

The most stringent condition employed in these experiments corresponded to an effective temperature of T_m −13°C (60% formamide). At this effective temperature, the BKV/SV40 heteroduplexes contained only 20% duplex DNA on the average (Fig. 1d). When the heteroduplexes were oriented with respect to the SV40 physical map as before (Fig. 3c and 6a, b), it was found that the residual duplex DNA was confined entirely to the late region of the SV40 genome. In control experiments, BKV and SV40 homoduplexes were mounted for electron microscopy at T_m −13°C. No substitution loops were observed, indicating that these conditions are not sufficiently stringent to produce detectable melting in either genome when both strands are perfectly matched.

DISCUSSION

SV40 and BKV are closely related viruses with a similar genetic organization. Under the least stringent conditions employed in the present work (T_m −35°C), the two genomes showed homology over about 90% of their lengths. This extent of homology is significantly greater than that detected in previous studies (13, 14, 18, 28), which employed more stringent hybridization conditions. At higher effective temperatures, the fraction of duplex DNA in BKV/SV40 heteroduplexes decreased, indicating considerable base mismatching in the homologous regions. Figure 4 summarizes the data for all four effective tem-

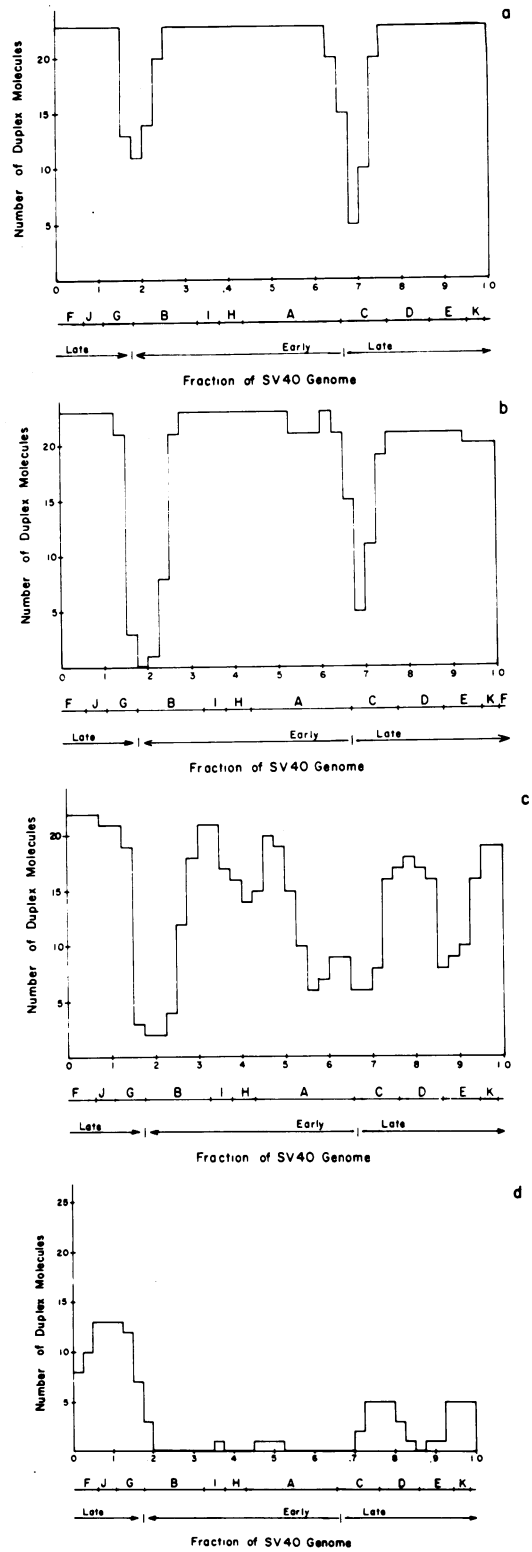

FIG. 4. *Summary of BKV/SV40 homology maps at the four effective temperatures studied. (a) T_m −35°C, (b) T_m −28°C, (c) T_m −20°C, and (d) T_m −13°C.*

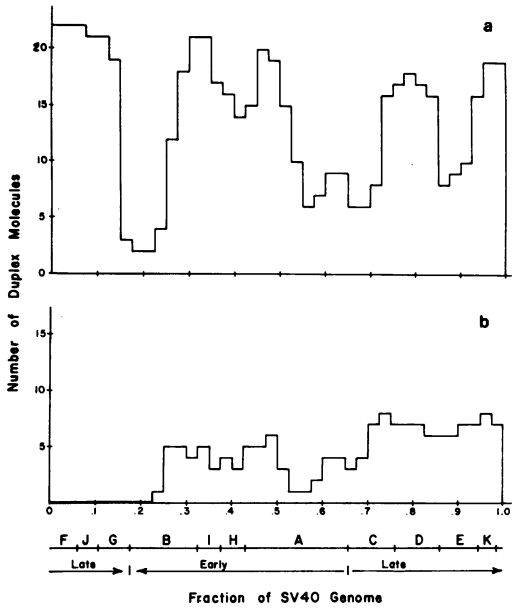

FIG. 5. *BKV/SV40 homology maps at T_m −20°C. (a) Homology map obtained from length measurements of BKV/SV40 heteroduplexes of the type shown in Fig. 1c. (b) Homology map obtained from length measurements of BKV/SV40 heteroduplexes of the type shown in Fig. 3b. The maps were aligned and oriented with respect to the SV40 physical map as described in the legend to Fig. 2.*

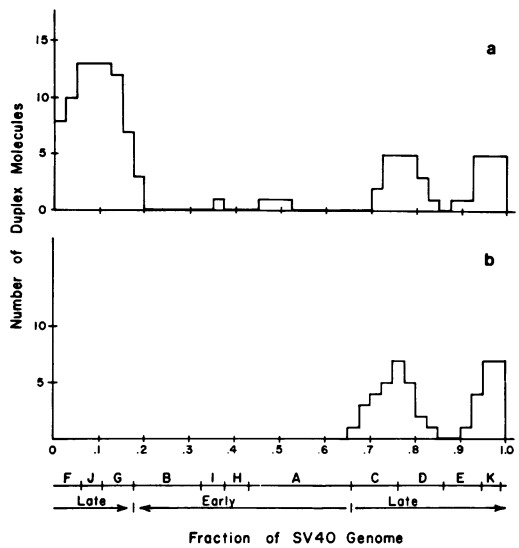

FIG. 6. *BKV/SV40 homology maps at T_m −13°C. (a) Homology map obtained from length measurements of BKV/SV40 heteroduplexes of the type shown in Fig. 1d. (b) Homology map obtained from length measurements of BKV/SV40 heteroduplexes of the type shown in Fig. 3c. The maps were aligned and oriented with respect to the SV40 physical map as described in the legend to Fig. 2.*

peratures studied. The heteroduplexes were an average of 50% duplex at an effective temperature of about T_m −21°C. If every 1% of mismatch in the DNA sequence lowers the T_m by 1.4°C (15), then the average amount of mismatch between the sequences of the two genomes is about 15%.

The degree of homology between the BKV and SV40 genomes differed markedly in different regions of the two genomes. Two small regions, centered at SV40 map positions 0.18 and 0.69, respectively, were single-stranded in BKV/SV40 heteroduplexes even under the least stringent conditions. These regions of weak or absent homology are located near the junctions between the early and late regions of the SV40 genome. In the case of SV40, at least a portion of each nonhomologous region appears to be dispensable for virus multiplication, since viable deletions mapping within them have been isolated (37). This may account for the more rapid divergence of the nucleotide sequences in these two regions.

Under more stringent conditions (T_m −20°C), the BKV/SV40 heteroduplexes were about 40% duplex on the average. A substantial portion of the early region, mapping roughly between SV40 map positions 0.27 and 0.53, remained largely duplex under these conditions. Most of the temperature-sensitive mutations (*tsA*) that affect the function of the early region of SV40 have been mapped in the segment between SV40 map positions 0.32 and 0.43, which lies within this duplex region (19). At T_m −20°C, the portion of the early region distal to SV40 map position 0.53 is largely melted. Viable deletions of SV40 that map within this region (0.54 to 0.59) have been isolated (37). With the exception of one segment lying between SV40 map positions 0.85 and 0.93, the late region remained double-stranded at T_m −20°C. The melted segment corresponds to the region where *tsD* mutants of SV40 have been mapped (19).

Under the most stringent conditions employed in these studies (T_m −13°C), the early region was completely melted. Two parts of the late region retained detectable duplex DNA. One of these, lying between SV40 map positions 0.73 and 0.83, represents the region coding for the N-terminal portion of the SV40 virion polypeptide VP2 (32). The other part (SV40 map position 0.95 to 0.17) represents the region coding for the major capsid polypeptide VP1 (9, 32). In this context, 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).

The finding of extensive homology between the early regions of SV40 and BKV is consistent with the findings that the T antigens of the two viruses are strongly cross-reactive (27, 31, 34, 38), that the T antigens of the two viruses have similar peptide maps (32a, 37a), and that BKV can complement an SV40 *tsA* mutant in monkey cells at the nonpermissive temperature (24). On the other hand, the finding in the present and in previous studies (18, 28) that the strongest homology between the two viruses maps in the late region appears to be inconsistent with the fact that the virion antigens of BKV and SV40 are only weakly cross-reactive (11, 30, 36, 38). This apparent discrepancy may be due to the fact that the antivirion sera used in the immunological studies were prepared against intact virions. Such sera may detect only that subset of antigenic determinants that reside at the surface of the virion. These surface determinants may evolve faster than the portions of the capsid proteins that are internal to the virion, due to selective pressures related to the needs of the two viruses to interact with different cell surfaces in their respective hosts. The observation that the late antigens of BKV and SV40 are strongly cross-reactive when sera directed against detergent- or alkali-disrupted capsids are used is consistent with this general hypothesis (36).

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