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Homology and relationship between the genomes of papovaviruses, BK virus and simian virus 40

(restriction enzyme analysis/heteroduplex mapping/hybridization/viral evolution)

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ABSTRACT **A number of hybridization techniques have been used to assess the homology between the genomes of BK virus (BKV) and simian virus 40 (SV40). A noncontiguous set of homologous sequences has been localized primarily within the late region of the SV40 genome, and these sequences presumably account for the cross-reaction between V-antigens of the two viruses. The reason for the relatively strong crossreaction between SV40 and BKV T-antigens is still unclear. The sequence homology and similarity in genomic organization suggest a close relationship between these papovaviruses.**

Papovavirus BK was first isolated in 1971 from the urine of a renal allograft recipient on immunosuppressive therapy (1). Isolates similar to the original BK virus (BKV) have been obtained from the urines of four of 74 renal allograft recipients (2) and from the urines of three patients with the Wiskott–Aldrich syndrome (ref. 3; unpublished results), a genetic disease characterized by deficiencies of both cellular and humoral immunity. Whereas, BKV can be distinguished from simian virus 40 (SV40) immunologically and biologically, the two viruses appear to be related (Table 1). SV40 plaque formation on African green monkey kidney (AGMK) cells can be partially neutralized by hyperimmune antiserum to BKV (4). Antisera directed against SV40 V, T, and U antigens crossreact with BKV-infected human cells when the reactions are monitored by the indirect fluorescent antibody technique (ref. 4; Takemoto, unpublished results). A cross-reaction between SV40 and BKV capsid (V) antigens has also been detected with immune electron microscopy (1, 5).

BKV DNA, like SV40 DNA, is a supercoiled genome cleaved at one unique site by the R_I restriction endonuclease, *Eco*RI (6, 7, 21, 22). Digestion of BKV DNA with the restriction endonuclease prepared from *Hemophilus influenzae* (*Hin*d) yields four DNA fragments (7), whereas digestion of SV40 DNA yields 11 fragments (13). DNA·DNA hybridization studies, using radiolabeled BKV DNA and unlabeled SV40 DNA, analyzed by hydroxyapatite chromatography, indicate that approximately 20% of the BKV nucleotide sequences are homologous to SV40 DNA (7). In the present report we have further evaluated the polynucleotide sequence homology between BKV and SV40 DNAs and have localized the common regions on the physical map of SV40 DNA. In addition to the polynucleotide sequence homology, the results of this study indicate a similarity in the organization of the genomes, suggesting an evolutionary relationship between the two viruses.

MATERIALS AND METHODS

Viruses and Viral DNA. Small plaque SV40 virus (strain 776) was grown in BSC-1 cells and the DNA was extracted from purified virions as described (8, 9).

BKV was grown in primary human embryonic kidney cells (4); the DNA was purified by differential salt precipitation (10). Polyoma viral DNA was kindly provided by T. Benjamin.

Digestion of DNA with Restriction Enzymes. ^{32}P-Labeled SV40 DNA was cleaved with *Hin*d restriction endonuclease (generously supplied by D. Nathans and M. Chen) and the 11 *Hin*d DNA fragments were isolated. Both SV40 and BKV DNA preparations (^{32}P- or ^{3}H-labeled) were cleaved by the *Eco*RI restriction endonuclease (a gift from G. Fareed) under conditions described elsewhere (11).

Separation of the strands of the 11 *Hin*d fragments of ^{32}P-labeled SV40 DNA has been outlined in detail (12).

Hybridization Experiments. DNA·DNA hybridization in solution between the minus DNA strand (0.5–1.5 ng) of the 11 ^{32}P-labeled *Hin*d SV40 fragments (13) and a 10- to 20-fold molar excess of ^{3}H-labeled sheared BKV or polyoma DNA was performed as described (14). The BKV DNA was denatured and added to a reaction mixture containing the ^{32}P-labeled SV40 fragment, 20 mM phosphate buffer, 1.0 M NaCl, and 0.06% sodium dodecyl sulfate in 0.2–0.4 ml and incubated at 68° for 24 hr. The percent DNA in duplex molecules was analyzed by hydroxyapatite (HA) chromatography (8, 9) or by digestion (12) with the single strand-specific nuclease, S_1 (18, 19).

In DNA·DNA filter hybridization experiments, approximately 1 μg of lambda, polyoma, SV40, and adenovirus 2, 7, or 12 DNA was bound to nitrocellulose filters (15). Denatured ^{32}P-labeled BKV DNA (0.01 μg = 3000 cpm) in a 0.25-ml solution containing 1 M NaCl, 10 mM Tris·HCl, 0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% bovine albumin, was allowed to react with the filters (16) for 16 hr at 68°. The filters were washed and dried, and radioactivity was determined in a toluene-based scintillation fluid.

DNA·cRNA annealing reactions were performed in solution with denatured *Eco*RI-cleaved linear molecules of ^{32}P-labeled BKV DNA and increasing amounts of SV40 cRNA. The reaction mixtures, containing 0.5 ng of BKV DNA and varying amounts of SV40 cRNA, 0.2 M NaCl, 10 mM Tris·HCl (pH 7.5) in 0.4 ml, were incubated for 1 hr at 60° and analyzed by HA chromatography.

Electron Microscopic Heteroduplex Studies. In these studies, equal amounts (10 ng) of *Eco*RI-cleaved linear BKV and SV40 DNAs were denatured in 0.05 ml of 0.1 M NaOH for 10 min at room temperature. The samples were neutralized with equimolar amounts of 1 M HCl, and an equal volume of formamide was added.

In a separate experiment, the above components were added to 0.1 μg of adenovirus 2^+ ND_2 DNA (kindly provid-

Abbreviations: SV40, simian virus 40; BKV, BK virus; HA, hydroxyapatite.

Table 1. Comparison of papovavirus antigens

Antigens	Antibody (Ab) crossreactions	
	SV40	BKV
T	ND	+(IF)(4)
U	ND	+(IF)[a]
V	+(IF)(4)	+(IF)(4)
	+(IEM)(5)	
	−(CF)(1,31)	−(CF)(1,31)
TSTA	−(BKV immunization)	ND
Neutralization	+/± (4)	ND

TSTA, tumor specific transplantation antigen; ND, not done; IF, immunofluorescence; CF, complement fixation; IEM, immune electron microscopy.
Numbers in parentheses are reference numbers.
[a] K. Takemoto, unpublished.

ed by A. S. Levine) prior to denaturation. After 30 min of incubation at 37°, the samples were diluted to the appropriate formamide concentration. The conditions for spreading these molecules have been described (17, 14).

RESULTS

Relationship of BKV DNA to other animal virus DNAs

Polynucleotide sequence homology between BKV DNA and other animal virus DNAs was first screened using DNA·DNA filter hybridization. ^{32}P-Labeled, fragmented BKV DNA was allowed to react with nitrocellulose filters on which polyoma, SV40, or adenovirus 2, 7, or 12 DNAs were immobilized. The only detectable reaction occurred between BKV DNA and the SV40 DNA filter (Table 2). In a separate experiment, the extent of this homology was evaluated by annealing a 22-fold excess of ^{3}H-labeled BKV DNA with fragmented, ^{32}P-labeled plus or minus strands of SV40 DNA. Approximately 9–10% (or 11–12% after normalization) of the ^{32}P-labeled SV40 DNA formed stable hybrid structures, resistant to the single-strand specific nuclease, S1 (Table 3). It has recently been shown that unlabeled SV40 DNA accelerates the reassociation of BKV DNA by an amount indicative of 20% polynucleotide sequence homology (7). Since hybrid formation in that study was assayed by hydroxyapatite chromatography (which does not distinguish partial from fully duplex molecules), the value of 20% is compatible with this estimate of 11–12%.

Localization of BKV DNA sequences in the SV40 genome

Two approaches were used to localize the region of homology with respect to the physical map of SV40 DNA (ref. 13; Fig. 2C). In the first, ^{32}P-labeled minus strands of SV40 *Hin*d DNA fragments were incubated with a 10- or 20-fold excess of ^{3}H-labeled, fragmented, BKV DNA. Hybrid formation was evaluated by hydroxyapatite chromatography. This procedure has been used previously to map the transcriptional templates of SV40 DNA (12, 20) and defective rearrangements of the SV40 genome (14). Under conditions where more than 80% of the BKV [^{3}H]DNA reassociated, stable hybrid structures formed with SV40 *Hin*d fragments D, G, J, and K and, to a lesser extent, with fragments C, E, F, and B (Table 4). Essentially no reassociation occurred when the BKV DNA was incubated in the presence of the minus strands of "early" *Hin*d fragments, A, H, and I, and only a low level of reannealing of the early fragment *Hin*d B was detected. Under similar reaction conditions in the absence of any added exogenous DNA, less than 5% of the minus strand DNA of any *Hin*d fragment was found in duplex molecules (Table 4). In a parallel set of experiments, the separated strands of nine of the 11 SV40 *Hin*d fragments were incubated with a 10- to 20-fold excess of unlabeled polyoma viral DNA. No annealing above background levels could be detected with any of these SV40 fragments (Table 4).

Table 2. Sequence homology between BKV DNA and the DNA of other viruses[a]

Viral DNA on filter	cpm bound per filter
Lambda	1
Polyoma	2
Adenovirus 2	0
Adenovirus 7	2
Adenovirus 12	5
SV40	115

[a] Denatured, ^{32}P-labeled BKV DNA was reacted with filters containing 1 μg of various viral DNAs under conditions described in *Materials and Methods*. Each reaction mixture contained approximately 3000 cpm (0.01 μg) of BKV [^{32}P]DNA. The values for cpm bound per filter represent the average for two filters after subtraction of background (17 cpm per filter). Under similar conditions, 40–50% of input ^{32}P-labeled SV40 DNA binds to an SV40 filter.

Electron microscopic heteroduplex mapping was used to topographically locate the regions of homology between SV40 and BKV DNA molecules. Full-length linear viral DNA molecules were prepared by treating supercoiled SV40 and BKV DNAs with the *Eco*RI restriction endonuclease (21, 22). The viral DNAs were mixed, denatured, reannealed, and spread in 40% (w/v) formamide as described (17, 14). In addition to the expected majority of homoduplex molecules, frequent heteroduplex structures were seen with a predominant central heterology bubble located approxi-

Table 3. Extent of homology between SV40 and BKV DNA[a]

SV40 DNA separated strand	Fraction of ^{3}H-labeled BKV DNA resistant to S1 nuclease	Fraction of ^{32}P-labeled SV40 separated strand resistant to S1 nuclease	Fraction of SV40 strand homologous to BKV DNA[b]
Plus	0.714	0.087	0.122
Minus	0.869	0.100	0.115

[a] Each hybridization mixture contained 0.37 μg/ml of sheared, ^{3}H-labeled BKV DNA (specific activity 3.0×10^{4} cpm/μg), 0.017 μg/ml of the indicated sheared, ^{32}P-labeled separated strand of SV40 DNA, 1.0 M NaCl, and 10 mM Tris·HCl (pH 7.5). (These quantities represent a 22-fold excess of BKV DNA, or, since only one SV40 strand was used, an effective 11-fold molar excess of BKV DNA.) The reaction mixtures were incubated at 68° for 22 hr to approximately 40 times the $C_0t_{1/2}$ of BKV DNA. The fraction of ^{32}P-labeled SV40 DNA resistant to S1 nuclease (18,19) digestion was calculated from the trichloroacetic acid-precipitable, radiolabeled nucleotides present in the reaction mixtures after digestion with S1 nuclease at 37° for 90 min. In the absence of BKV DNA, less than 0.010 of either ^{32}P-labeled SV40 DNA strand was resistant to S1 nuclease.
[b] The fraction of the SV40 strand homologous to BKV DNA was determined by normalizing the fraction of SV40 separated strand resistant to S1 nuclease by the fraction of BKV DNA resistant to S1 nuclease (71 and 87%).

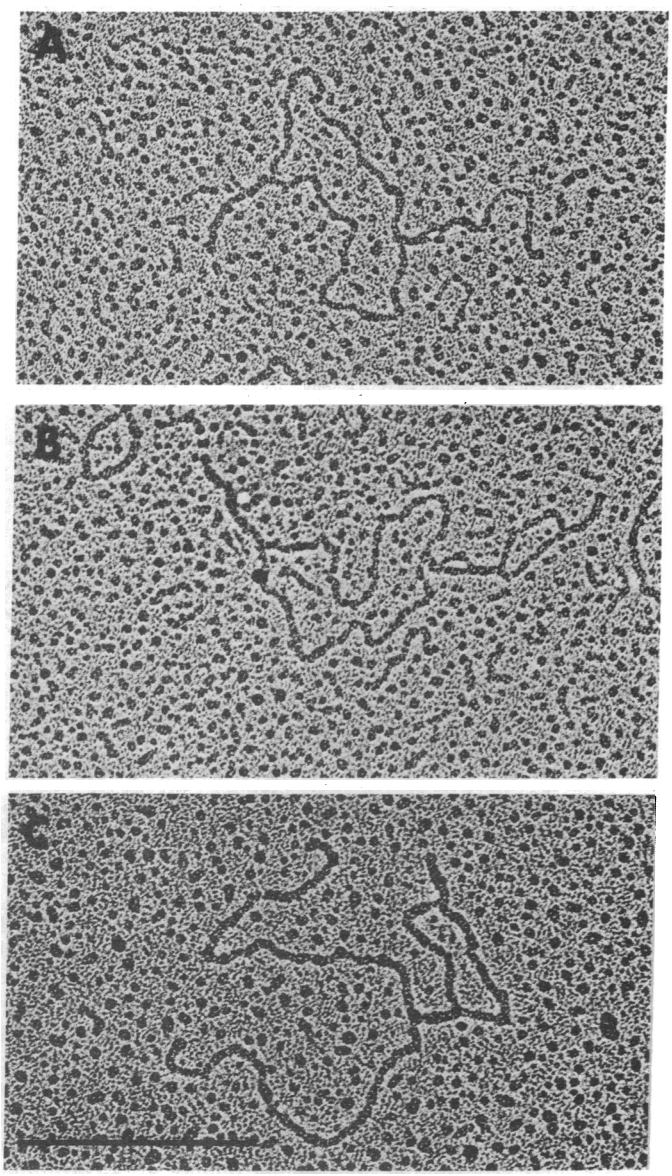

FIG. 1. Electron micrographs of SV40-BKV heteroduplexes mounted by the formamide isodenaturing technique (17) and rotary-shadowed with platinum-palladium. Panels show heteroduplex molecules spread in formamide concentrations of (A) 40%, (B) 50%, and (C) 60%. Bar represents 1 μm.

mately 0.2 to 0.7 fractional units from one end (Fig. 1A and B). Branched heteroduplex structures were observed at 60% formamide, with the only remaining homology confined to one end of the molecule (Fig. 1C). Under these conditions, homoduplex DNA molecules remain intact. We have analyzed 40 heteroduplex molecules and have determined the regions of homology and heterology (Fig. 2). The pattern observed (namely, a central region of heterology with homology localized to both ends) was consistent with the DNA·DNA hybridization experiments shown in Table 4, indicating the greatest homology with those SV40 *Hin*d fragments that were adjacent to the *Eco*RI site in the SV40 genome (*Hin*d F, G, J, K, E, D, and C) and the greatest heterology in the early region of the SV40 genome (fragments *Hin*d A, H, I, and B). In this analysis, however, the left-right orientation of heteroduplex molecules with respect to the physical map of SV40 could not be ascertained.

In order to localize the central heterology bubble in the

Table 4. Extent and location of SV40-BKV DNA sequence homology[a]

^{32}P-Labeled minus strand SV40 *Hin*d fragment	% ^{32}P-Labeled SV40 DNA in duplex molecules in the presence of unlabeled DNA[c]				
	None	SV40 (20)	Polyoma (15)	BKV (10)	BKV (20)
A	2	94	1	5	7
B	4	90	4	7	11
C	3	95	4	13	18
D	1	95	2	30	32
E	1	92	3	10	14
F	3	94	2	6	16
G	4	90	4	15	25
H	1	98	4	4	4
I	2	ND	ND[b]	5	6
J	2	ND	ND	22	25
K	2	ND	5	24	20

[a] DNA·DNA hybridization experiments were performed between the ^{32}P-labeled minus strand of the 11 SV40 *Hin*d fragments and a 10- to 20-fold molar excess of denatured unlabeled BKV or polyoma DNA. The reactions were performed as described in *Materials and Methods*, and the percent SV40 DNA in duplex molecules was assayed by HA chromatography (14). No background subtraction.

[b] ND, not done.

[c] Numbers in parentheses represent the molar ratios of unlabeled to ^{32}P-labeled SV40 DNA.

SV40-BKV heteroduplex with respect to the SV40 genome, a "heterotriplex" structure was formed by allowing the SV40-BKV DNA heteroduplexes to anneal with denatured $Ad_2^+ND_2$ viral DNA. $Ad_2^+ND_2$ is a nondefective adenovirus-SV40 hybrid virus which contains 32% of the SV40 genome (corresponding to 93% of SV40 *Hin*d fragment G and all of *Hin*d fragments B, I, and H) inserted at a site approximately 14% from one end of the adenovirus 2 DNA (23, 24). The order of the *Hin*d SV40 DNA sequences in this hybrid virus genome (beginning at 0.14 Ad_2 units) is G-B-I-H. An electron micrograph of a heterotriplex molecule is shown in Fig. 3, with a diagrammatic representation of its structure. The central region of heterology characteristic of BKV-SV40 DNA heteroduplex molecules appears to have annealed with the SV40 DNA segment present in the $Ad_2^+ND_2$ DNA at a point approximately 14% the distance from one end of the adenovirus genome. The region of greatest polynucleotide sequence homology between SV40-BKV DNA (presumably corresponding to the SV40 DNA fragment *Hin*d D) is oriented *away* from the terminal 14% of adenovirus DNA, in agreement with the assigned order of SV40 sequences in $Ad_2^+ND_2$ DNA (Figs. 2 and 3). Although only two such molecules were clearly recognized, length measurements of the various regions of these molecules were in agreement with the predicted structure.

Strand orientation of homologous sequences in BKV DNA

SV40 complementary RNA (cRNA) is complementary to one strand of SV40 DNA (25). When radiolabeled, full-length linear SV40 DNA molecules are denatured and allowed to anneal with an excess of cRNA, approximately 50% of the DNA, representing one of the two strands (the minus strand; refs. 9 and 26), forms hybrid molecules. To determine whether the BKV DNA sequences complementary to

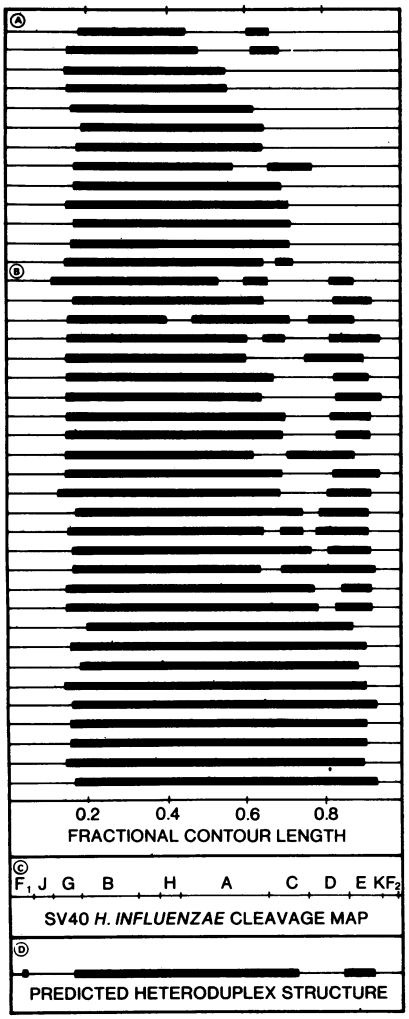

FIG. 2. Schematic diagram of 40 individual SV40-BKV heteroduplex molecules showing the map positions of regions containing visible sequence heterology (▬) and homology (——) when mounted under increasing denaturing conditions (panel A, 40% formamide; panel B, 50% formamide). Shown for comparison is the *Hin*d map of SV40 DNA (panel C) and the predicted heterology map (panel D) based on hybridization experiments of BKV DNA with the *Hin*d fragments of SV40 DNA (see Table 4).

SV40 cRNA were present on one or both BKV strands, ^{32}P-labeled, full-length linear BKV DNA molecules were hybridized with increasing amounts of unlabeled SV40 cRNA (Fig. 4). One-half of the radiolabeled BKV DNA reacted with the SV40 cRNA. Since HA chromatography selects for partial as well as complete duplex molecules, this result indicates that only one of the two BKV DNA strands contains sequences homologous to the minus SV40 DNA strand. Thus, the strand orientation of the SV40-homologous DNA sequences in the BKV genome appear to be the same as the strand orientation of these sequences in the SV40 genome. This result confirms similar data provided by the heteroduplex molecules.

DISCUSSION

The results of DNA·DNA hybridization studies and the analysis of heteroduplex molecules between *Eco*RI linear molecules of SV40 and BKV DNAs have established the portions of the SV40 genome that are homologous to BKV. The shared sequences are located principally in the late gene region of SV40 DNA, corresponding to portions of *Hin*d fragments C, D, G, J, and K, and to a lesser extent, *Hin*d fragments E and F. Little or no significant homology was detected between BKV DNA and the early region of the SV40 genome (*Hin*d fragments A, H, I, and B) by either of the techniques used, with the exception of a small segment of SV40 *Hin*d B (Table 4). Despite the small difference in the size of the genomes (7), the RI-linear BKV-SV40 heteroduplex molecules are closely aligned end-to-end. Although we now have data to suggest that this alignment is slightly staggered, the general location of homologous regions at the termini of the RI-linear molecules and the constant central region of heterology (corresponding to the location of the early SV40 genes) suggests that the BKV and SV40 genomes have the same basic organization. The studies with SV40 cRNA (Fig.

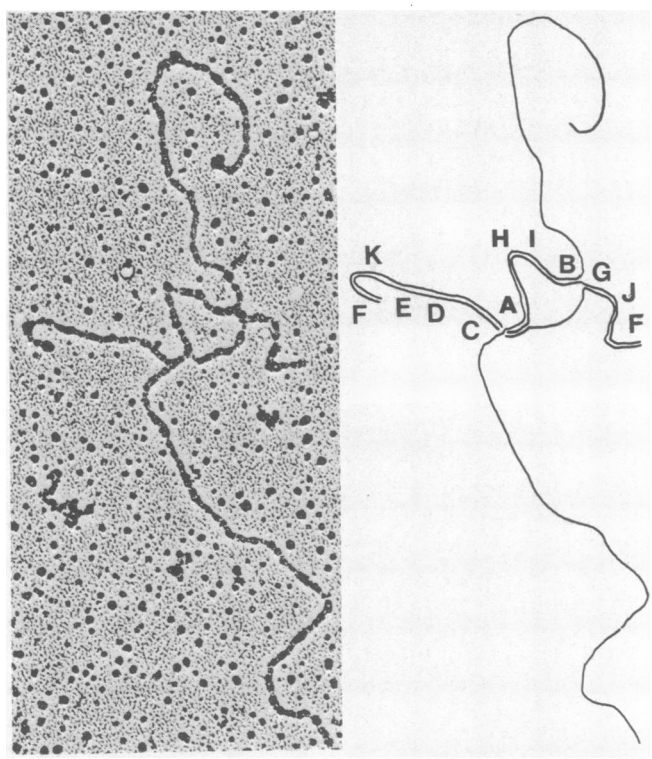

FIG. 3. Electron micrograph and schematic diagram of SV40-BKV-Ad_2 + ND_2 "heterotriplex" molecule prepared as described in *Materials and Methods*. The conditions for annealing, as well as the interpretation of this structure, are described in the *text*.

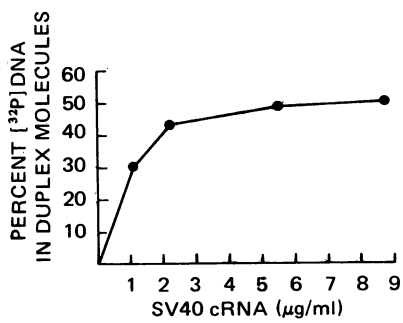

FIG. 4. Annealing of SV40 complementary RNA (cRNA) to BKV DNA. Increasing amounts of unlabeled SV40 cRNA (made *in vitro* with *E. coli* DNA-dependent RNA polymerase) were added to reaction mixtures containing 0.5 ng of ^{32}P-labeled denatured RI-linear BKV DNA (specific activity 6×10^5 cpm/μg), in 0.2 M NaCl, 10 mM Tris·HCl (0.4 ml) for 1 hr at 60°. The product was analyzed by HA chromatography to determine the percent of ^{32}P-labeled DNA in duplex molecules.

4) indicate, furthermore, that the strand orientation (plus strand versus minus strand) of the shared nucleotide sequences are the same for both SV40 and BKV. It is clear from these studies that SV40 and BKV are related to one another, sharing between 10 and 20% of their nucleotide sequences (ref. 7; Table 2). The precise evolutionary relationship of these two agents, however, is unknown.

BKV and SV40 may have evolved from a common papovavirus ancestor or diverged from one another. The factors responsible for conservation and diversification of segments of viral genomes during evolution are not well understood. It was originally postulated for the lambdoid phages, that evolution resulted from the ability to form hybrid recombinants between functional segments of various genomes (27), and this has recently been extended to the relationship between the coliphage λ, and a salmonella phage P22 (28). The similarity in the organization of the genomes of SV40 and BKV as well as the limited DNA sequence homology suggest that a similar mechanism may be responsible for the diversification of papovaviruses. One might further speculate that the specific heterologous segments are in some way responsible for the different host ranges of these viruses.

Whereas, the hybridization techniques we used have been able to localize 10–20% BKV-SV40 homology within approximately 50% of the SV40 genome, we could detect no homology between SV40 and polyoma. Using nonstringent conditions, however, Ferguson and Davis (*J. Mol. Biol.*, in press) have recently presented data suggesting that 15% of the SV40 and polyoma genomes (0.83 to 0.98 SV40 map units) have 75% sequence homology. In this regard, it appears clear that the observed DNA homology between two genomes depends on the method of analysis. In our studies, the most sensitive, and conversely, the least stringent method is electron microscopic heteroduplex analysis in 40% formamide (approximately 50% BKV-SV40 sequence homology) while the most stringent technique is liquid phase hybridization in 1.0 M NaCl at 68°, followed by S_1 nuclease analysis (11–12% homology).

It is puzzling that the nucleotide sequences shared by BKV and SV40 are located almost exclusively in the late region of the genome. Whereas, this might explain the minor cross reaction between BKV and SV40 V-antigens (1, 4, 5, 29), which presumably represent the virus-coded capsid proteins appearing late in the lytic cycle (30, 31), it does not explain the strong crossreaction of the SV40 and BKV T-antigens which appear early after infection (4). It has been suggested that if T-antigen is a virus-coded protein, the region of the SV40 genome specifying this antigen may be located between 0.43 and 0.54 map units encompassing portions of *Hin*d fragments A and H (23, 24, 32). Our experiments indicate that there is little or no homology between SV40 and BKV DNAs in these regions of the SV40 genome. Regarding this unexpected finding, one could hypothesize that SV40 T-antigen is not virus-coded but is synthesized by the cell as a consequence of some aspect of virus-cell interaction. This explanation is not entirely compatible with the observation that a wide variety of cell types infected or transformed by SV40 contain crossreacting T-antigens, whereas, the same cells infected by other viruses, including polyoma, do not. Thus, T-antigen would seem to be at least virus-specific in part. It is conceivable that T-antigen is a "hybrid" molecule, one portion coded for by the virus and the other, by cellular DNA sequences. If this is the case, our hydridization results would suggest that the crossreacting fraction of the antiserum used to monitor T-antigen could be reacting with the cell-specific portion of the molecule. It is also possible that the hybridization techniques that we have used were too stringent to detect a limited extent of homology spread throughout the early regions of the SV40 and BKV genomes. Given the degeneracy of the genetic code, it is conceivable that such gene sequences could code for similar proteins.

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