JOURNAL OF VIROLOGY, Oct. 1975, p. 959-973

Vol. 16, No. 4
Printed in U.S.A.

Physical Map of the BK Virus Genome

PETER M. HOWLEY,* GEORGE KHOURY, JANET C. BYRNE, KENNETH K. TAKEMOTO, AND MALCOLM A. MARTIN

Laboratory of Biology of Viruses and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014*

Received for publication 20 May 1975

Two new human papovavirus isolates (JMV and MMV) from the urines of patients with Wiskott-Aldrich syndrome were morphologically and serologically identical to BK virus (BKV). The genomes of these two new isolates were found to be indistinguishable from prototype BKV DNA in a variety of nucleic acid hybridization experiments. Like BKV DNA, JMV and MMV DNAs share approximately 20% of their polynucleotide sequences with simian virus 40 DNA. The genome of JMV was indistinguishable from that of BKV by restriction endonuclease analysis; MMV DNA contained three instead of four R·*Hin*d cleavage sites and one rather than no R·*Hpa*II cleavage sites. Physical maps of the BKV and MMV genomes were constructed using restriction endonucleases, and these maps were oriented to the map of simian virus 40 DNA.

Human papovavirus BK (BKV) was initially isolated from the urine of a renal allograft recipient (5). The relationship of BKV to another well-studied papovavirus, simian virus 40 (SV40), and its oncogenic potential have generated considerable interest. A cross-reaction between the structural proteins of BKV and SV40 by immune electron microscopy and partial neutralization of SV40 plaque formation by BKV hyperimmune serum have been described (5, 17, 24), although a recent report indicates that the tryptic digest patterns are quite different (26). The tumor antigens induced by SV40 and BKV have been shown to share determinants when monitored by indirect immunofluorescence (24). We have recently reported a 20% polynucleotide sequence homology between the genomes of SV40 and the prototype BKV (10). The genomes of the two viruses appear to have a similar organizational arrangement, with the major regions of homology localized to portions of the SV40 genome which are transcribed late in the lytic cycle (G. Khoury, P. M. Howley, C. Garon, M. F. Mullarkey, K. K. Takemoto, and M. A. Martin, Proc. Natl. Acad. Sci. U.S.A., in press). BKV, like SV40, is capable of transforming mammalian cells in vitro (12, 19). Tumor formation has also been observed after the inoculation of transformed hamster kidney cells into suckling hamsters (19). Primary hamster kidney cells, transformed with purified BKV DNA, are also tumorogenic in hamsters (K. K. Takemoto and M. A. Martin, manuscript in preparation).

In this study we have characterized the genomes of two new human papovavirus isolates which are morphologically and immunologically indistinguishable from BKV. Unlike BKV, which was initially passaged in African green monkey kidney cells, the new isolates were propagated solely in human cells. The source of both new isolates was the urine of two patients with the Wiskott-Aldrich syndrome (WAS), an X-linked recessive disorder characterized by defects of both cellular and humoral immunity. This disease has also been associated with a high incidence of malignancies of the reticuloendothelial system (1, 6). Both patients from whom the BKV-like isolates were made developed reticulum cell sarcomas and have died.

MATERIALS AND METHODS

Cells. BSC-1 cells were grown in Eagle medium supplemented with 10% fetal calf serum. Human embryonic kidney cells, purchased from Flow Laboratories (Rockville, Md.), were propagated in Eagle medium containing 2× vitamins and amino acids supplemented with 10% fetal bovine serum.

Viruses. BSC-1 cells were infected with the small plaque variant of SV40 at an input multiplicity of approximately 0.1 PFU/cell, and virus was purified 7 to 9 days after infection as previously described (7). ^{32}P-labeled virus was prepared by carrying out the infection in phosphate-free Eagle medium containing carrier free [^{32}P]orthophosphate (100 μCi/ml). ^{3}H-labeled SV40 was purified from cells continuously exposed to [^{3}H]thymidine (10 μCi/ml) beginning 30 h after infection.

Plaque-purified BKV was used to infect HEK cells at a multiplicity of approximately 0.1 PFU/cell. Virus

was purified from cells 8 to 10 days after infection, at which time the hemagglutination titer of virus is the supernatant fluid was 1:80 or greater as previously described (14).

The viruses from the urines of the two patients with WAS (JMV and MMV) were propagated in primary human embryonic kidney cells. Stocks of JMV and MMV were obtained from these initial cultures and used for subsequent infections of HEK cells. Virus was purified as described above for BKV 8 to 12 days after infection, at which time the hemagglutination titer of the medium was 1:80 or greater.

^{32}P-labeled BKV, JMV, and MMV were prepared as described for SV40.

Viral DNA. Unlabeled and radiolabeled SV40 DNAs were prepared from purified virions following an incubation with 1% sodium dodecyl sulfate at 50 C and isopycnic centrifugation in cesium chloride-ethidium bromide as previously described (7). Unlabeled and radiolabeled DNAs from BKV, JMV, and MMV were either prepared in an identical manner from purified virions or directly from infected cells by differential salt precipitation (9). Viral DNAs were mechanically sheared at 50,000 lbs/in^2 in a Ribi cell fractionator (Ivan Sorvall) to a molecular size of 3.1×10^5 daltons prior to DNA-DNA reassociation experiments (7). ^{32}P-labeled adenovirus 2 DNA was generously supplied by S. Straus.

DNA-DNA reassociation. Denatured DNAs were allowed to reassociate in either 0.14 M sodium phosphate buffer at 60 C or 0.60 M sodium phosphate buffer at 68 C, and aliquots removed at various times were analyzed for single- and double-stranded DNA by hydroxyapatite chromatography as previously described (7).

Restriction endonuclease cleavage of viral DNA. **(i) R·EcoRI.** Reaction mixtures (0.2 ml) containing 0.1 M Tris-hydrochloride, pH 7.5, 0.005 M $MgCl_2$, 10 to 30 ng of supercoiled viral DNA, and 0.1 to 0.5 U of R·*Eco*RI (a generous gift of G. Fareed) were incubated at 37 C for 30 min (3). Enzymatic digestion was stopped after the addition of EDTA to a final concentration of 0.02 M, and the cleavage products were subsequently monitored by sedimentation through 5 to 30% (wt/vol) neutral sucrose or by electrophoresis in 1.4% agarose gels.

(ii) R·Hind. Unfractionated R·*Hin*d was the generous gift of M. Chen. Mixtures (0.05 ml) containing 6.6 mM Tris-hydrochloride, pH 7.5, 6.6 mM $MgCl_2$, 50 mM NaCl, 5 to 50 ng of ^{32}P-labeled viral DNA, and sufficient enzyme (approximately 0.03 U) to effect limit digestion of substrate DNA were incubated at 37 C for 16 h. To obtain partial digestion products of ^{32}P-labeled R·*Eco*RI linear BKV DNA, both the time of incubation and enzyme to DNA ratio were varied. Analysis of limit digestions and separation of the incomplete digestion products were carried out in composite polyacrylamide-agarose slab gels.

(iii). R·HpaI and R·HpaII. These enzymes were generous gifts of D. Nathans and M. Mann. For limit digestions with R·*Hpa*I, 0.02-ml reaction mixtures containing 20 mM Tris-hydrochloride, pH 7.5, 10 mM $MgCl_2$, 1 mM mercaptoethanol, 1 μg of gelatin, 1 μg of supercoiled viral DNA, and sufficient enzyme were incubated for 3 h at 37 C. For limit digestions with R·*Hpa*II, 0.20-ml reaction mixtures containing 20 mM Tris-hydrochloride, pH 7.4, 7 mM $MgCl_2$, 7 mM mercaptoethanol, 1 μg of supercoiled viral DNA and sufficient enzyme were incubated for 2 h at 37 C.

Agarose gel electrophoresis. Electrophoresis through 1.4% (wt/vol) agarose (Seakem) slab gels (17 by 12 by 0.3 cm) at 60 V for 16 h at 20 C was carried out in a buffer containing 40 mM Tris-hydrochloride, pH 7.8, 5 mM sodium acetate, and 1 mM EDTA (8). Sliced millimeter fractions were dissolved in 0.2 ml of 30% H_2O_2 and assayed for radioactivity in Triton X-100 toluene scintillation fluid.

Polyacrylamide-agarose electrophoresis. Analytical electrophoretic analysis of restriction endonuclease cleavage fragments of viral DNA in slab gels consisting of 3% polyacrylamide-0.5% agarose was performed as previously described (10). Preparative electrophoresis of partial restriction endonuclease cleavage products of BKV DNA was carried out in 2.2% polyacrylamide-0.5% agarose slab gels (17 by 12 by 0.3 cm) for 11 h at 60 V in a buffer consisting of 40 mM Tris-hydrochloride, pH 7.2, 20 mM sodium acetate, and 1 mM EDTA. The DNA bands of the partial digestion products were visualized after staining for 30 min with ethidium bromide (0.5 μg/ml) under UV light (22).

RESULTS

Characterization of the DNAs of the WAS isolates. Takemoto et al. reported the isolation of a papovavirus (MMV) from a patient with WAS which shared numerous properties with papovavirus BK (25). Recently, a second isolate (JMV), from another patient with the same disease, was also found to be similar to BKV (Takemoto et al., unpublished observations). Inoculation of HEK, human fetal brain, WI-38, or Vero cells with the two new isolates caused cytopathic changes characteristic of a BKV lytic infection (25; Takemoto et al., unpublished observations). Both MMV and JMV agglutinated human type O erythrocytes, and the reaction was specifically inhibited by anti-BKV rabbit antiserum, indicating close antigenic similarity (26; Takemoto et al., unpublished observations). Furthermore, cells infected with JMV or MMV synthesized a T antigen immunologically similar to that of BKV and SV40 (Takemoto et al., unpublished observations).

The relationship of the two new isolates to BKV was further evaluated by examining the genomes of these viruses. The DNA, isolated from purified MMV or JMV, co-banded with the supercoiled forms of SV40 or BKV DNAs after cesium chloride-ethidium bromide isopycnic centrifugation. In a previous publication we noted that prototype BKV DNA was heterogeneous, consisting of at least four discrete DNA species which could be resolved by electrophore-

sis through 1.4% agarose (Fig. 1A) (10). The homogeneity and size of JMV DNA (Fig. 1B) and MMV DNA (Fig. 1C) relative to SV40 DNA were evaluated similarly. Under the conditions employed, the electrophoretic mobilities of viral DNA, in descending order, were: supercoiled molecules, full-length linear molecules, and nicked circular molecules. The DNAs of both JMV and MMV were significantly more homogeneous than the DNA of BKV and, in fact, appeared to be as pure as the ^{3}H-labeled SV40 DNA marker. In each case only one size species of DNA could be identified. Both the supercoiled and nicked circular forms of JMV and

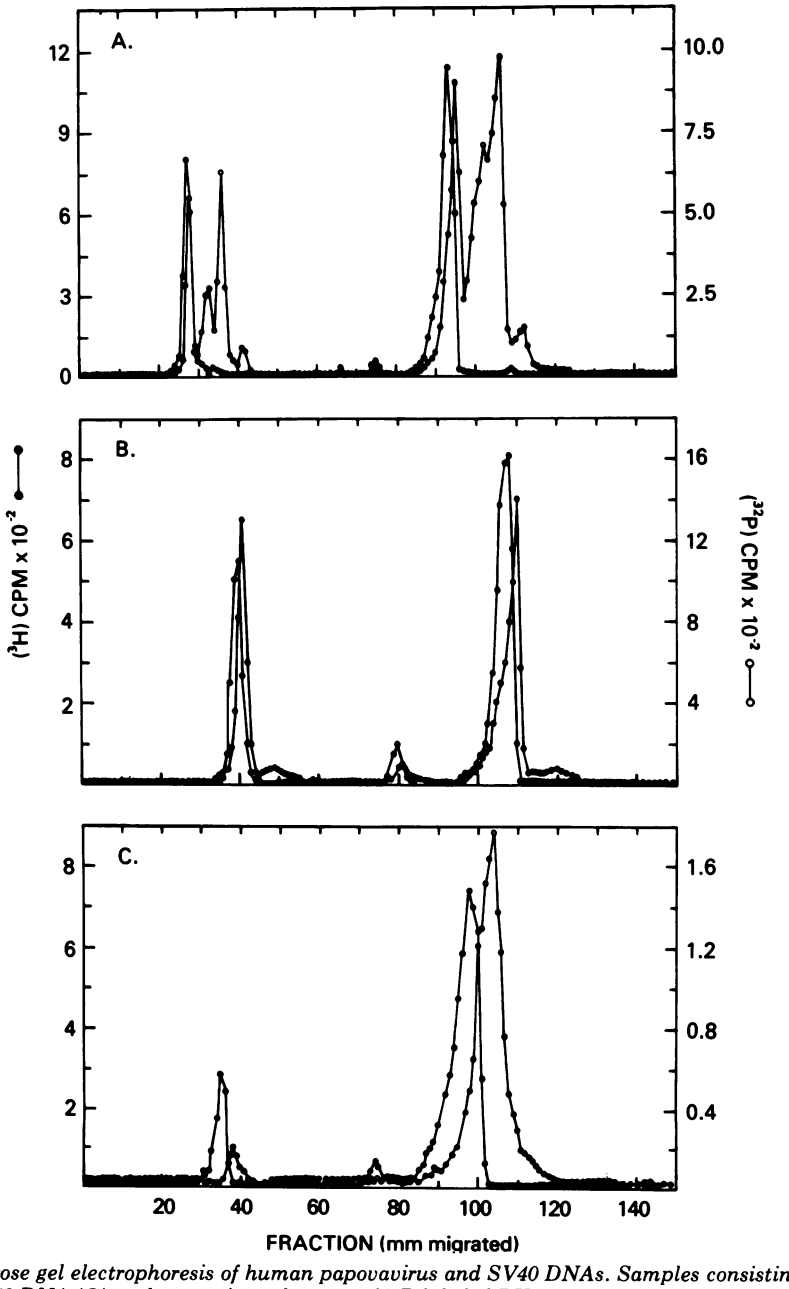

FIG. 1. *Agarose gel electrophoresis of human papovavirus and SV40 DNAs. Samples consisting of 200 ng of ^{3}H-labeled SV40 DNA (●) and approximately 50 ng of ^{32}P-labeled BKV (A), JMV (B), or MMV (C) DNAs (○) were subjected to electrophoresis in 1.4% agarose slab gels for 16 h at 60 V as described. Each gel was cut into 1.0-mm slices which were individually dissolved in 0.2 ml of 30% H_2O_2 and assayed for radioactivity.*

MMV DNAs migrated further than their SV40 DNA counterparts, indicating that the genomes of each were somewhat smaller than that of SV40 (3.6×10^6 daltons). JMV DNA (Fig. 1B) appeared to have the same electrophoretic mobility as the largest (slowest migrating) supercoiled species of BKV DNA (Fig. 1A), which we previously have shown to be the only infectious BKV DNA species (10). Its molecular weight is approximately 3.45×10^6 (see below). MMV DNA (Fig. 1C), however, migrates even faster than either JMV DNA or the largest BKV DNA species, relative to the SV40 markers, indicating that MMV DNA is slightly smaller (3.26×10^6 daltons, see below) than either the JMV or the infectious BKV genomes.

To determine whether the two WAS isolates were essentially identical to the prototype BKV or were members of a related but distinct group of human papovaviruses, a series of DNA-DNA reassociation experiments were carried out to evaluate the extent of polynucleotide sequence homology among the three viral genomes. ^{32}P-labeled JMV DNA (Fig. 2A) and MMV DNA (Fig. 2B) were reassociated alone or in the presence of a 3- or 10-fold excess of ^{3}H-labeled BKV DNA. In both cases the reassocation of radiolabeled DNA follows the theoretical curves for 100% polynucleotide sequence homology (slopes 4X and 11X), suggesting that the genomes of BKV and each of the WAS isolates are indistinguishable. These isolates will therefore be referred to as BKV(JM) and BKV(MM).

Polynucleotide sequence homology between the genomes of BKV(JM) or BKV(MM) and SV40 DNA. We have previously reported that the genomes of SV40 and BKV share approximately 20% of their polynucleotide sequences when DNA reassociation is analyzed by hydroxyapatite chromatography (10). Since the prototype BKV was initially grown in Vero cells, a continuous line of African green monkey cells in which SV40 undergoes a lytic infection, it was necessary to examine other isolates of BKV which had been passaged solely in human cell cultures to confirm the observed polynucleotide sequence homology between SV40 and BKV DNAs.

Such an analysis was carried out by examining the effect of unlabeled SV40 DNA on the kinetics of reassociation of ^{32}P-labeled BKV(JM) and BKV(MM) DNAs. We examined the resulting biphasic reactions using the equations developed by Sharp et al. for two simultaneous second-order reactions occurring at different rates (23). ^{32}P-labeled BKV(JM) DNA was reassociated in the presence of a 171-fold molar

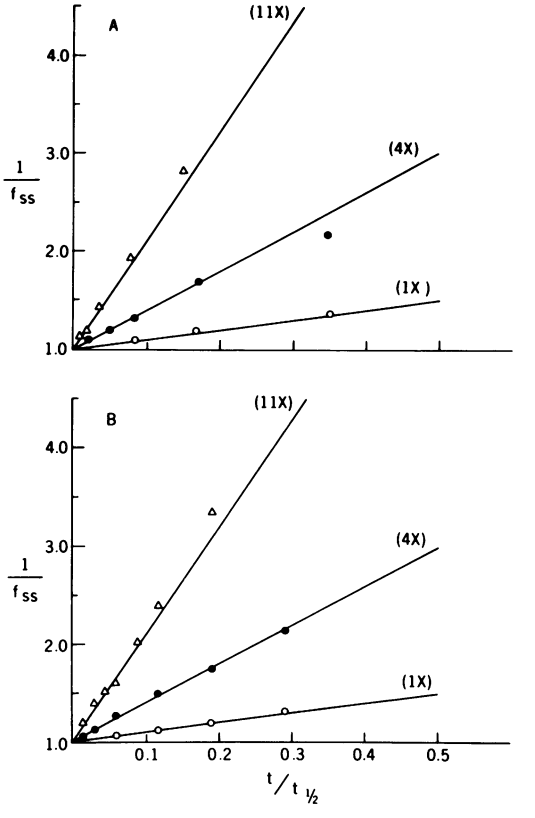

FIG. 2. *Kinetics of reassociation of JMV and MMV DNAs in the presence or absence of excess BKV DNA. (A) Mechanically fragmented ^{32}P-labeled JMV DNA (specific activity, 1.0×10^5 counts/min per μg) was allowed to reassociate at a concentration of 1.25×10^{-2} μg/ml alone (○) or in the presence of a threefold (●) or a tenfold (△) excess of ^{3}H-labeled BKV DNA fragments in 0.14 M sodium phosphate buffer for varying periods of time at 60 C. The fraction of radiolabeled DNA remaining single stranded was determined by hydroxyapatite column chromatography as described. The $t_{½}$ for ^{32}P-labeled JMV DNA reassociating alone (17.1 h) was the average of six determinations. (B) Mechanically fragmented ^{32}P-labeled MMV DNA (specific activity, 1.0×10^6 counts/min per μg) was allowed to reassociate at a concentration of 8.60×10^{-4} μg/ml alone (○) or in the presence of a threefold (●) or a tenfold (△) excess of ^{3}H-labeled fragmented BKV DNA in 0.60 M sodium phosphate buffer for varying periods of time at 68 C. The $t_{½}$ for ^{32}P-labeled MMV DNA reassociating alone (58.7 h) was the average of six determinations. The straight lines in each panel with the slope of one (1X) represent the theoretical second-order reaction curves for each of the radiolabeled DNAs reassociating alone. The straight lines with slopes four (4X) and eleven (11X) are the theoretical curves for each of the radiolabeled DNAs reassociating in the presence of a threefold and tenfold excess of unlabeled homologous DNA, respectively.*

excess of unlabeled SV40 DNA (Fig. 3A); the experimental data (open circles) approximate the theoretical curves calculated for 20 and 25% polynucleotide sequence homology. In Figure 3B, the experimental data for ^{32}P-labeled BKV(MM) DNA reassociating in the presence of a 288-fold molar excess of SV40 DNA fall along the theoretical curve for 20% polynucleotide sequence homology.

To ascertain whether the genomes of the three BKV isolates contained human DNA sequences, ^{32}P-labeled viral DNA was allowed to reassociate in the presence of a 1.2×10^6-fold excess of DNA from a human fibroblast strain, Flow 5000 (a molar ratio of diploid host cell DNA to viral DNA of approximately 0.9). No detectable acceleration in the rate of reassociation of BKV, BKV(JM), or BKV(MM) DNAs was observed.

Restriction endonuclease cleavage of BKV(JM) and BKV(MM) DNAs. The prototype BKV genome has been shown to contain one R·*Eco*RI cleavage site (10, 16) and four R·*Hin*d cleavage sites (10).

The DNAs of the new isolates BKV(JM) and BKV(MM) were each digested with bacterial restriction endonucleases to further assess their similarity to the prototype BKV genome. After exposure to R·*Eco*RI, supercoiled BKV(JM) and BKV(MM) DNAs were converted to full-length linear molecules when the cleavage products were analyzed by agarose gel electrophoresis. Like prototype BKV DNA, the genomes of the two WAS isolates each contain a single R·*Eco*RI cleavage site.

Digestion of BKV(JM) DNA with R·*Hin*d resulted in four cleavage products which had the same electrophoretic mobilities as the R·*Hin*d prototype BKV restriction fragments (Fig. 4, panels 3 and 5). The four cleavage products were 1.5×10^6, 1.2×10^6, 0.41×10^6, and 0.34×10^6 daltons in size as previously

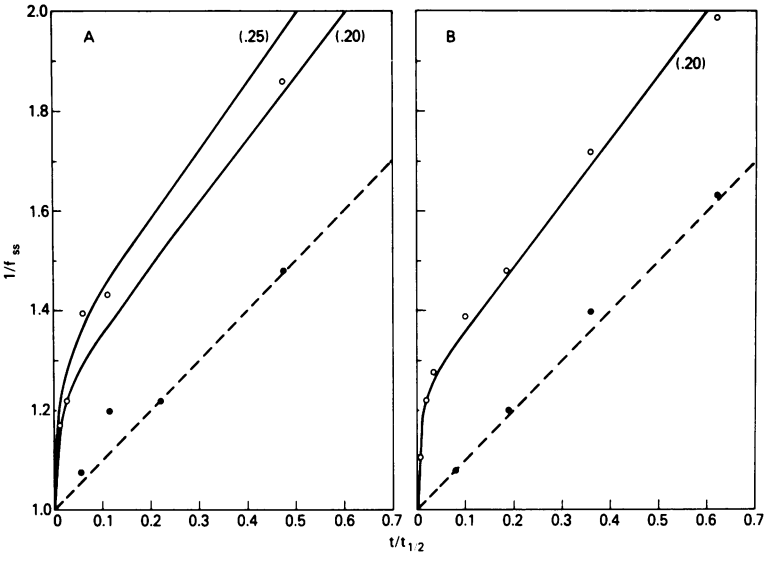

FIG. 3. *Kinetics of reassociation of BKV(JM) and BKV(MM) DNAs alone and in the presence of an excess of unlabeled SV40 DNA. (A) Mechanically fragmented, ^{32}P-labeled BKV(JM) DNA (specific activity, 3.0×10^5 counts/min per μg) was allowed to reassociate at a concentration of 1.25×10^{-2} μg/ml alone (●) or in the presence of a 171-fold excess of unlabeled SV40 DNA fragments (○) for varying periods of time in 0.14 M sodium phosphate buffer at 60 C. The fraction of radiolabeled DNA remaining single stranded (f_{ss}) was assayed by hydroxyapatite column chromatography. The $t_{1/2}$ for BKV(JM) DNA reassociating alone (17.7 h) was the average of five separate determinations. (B) Mechanically fragmented, ^{32}P-labeled BKV(MM) DNA (specific activity, 1.0×10^6 counts/min per μg) was allowed to reassociate at a concentration of 8.60×10^{-4} μg/ml alone (●) or in the presence of a 288-fold excess of unlabeled SV40 DNA fragments (○) for varying periods of time in 0.60 M sodium phosphate buffer at 68 C. The $t_{1/2}$ for ^{32}P-labeled BKV(MM) DNA reassociating alone (58.7 h) was the average of six determinations. The dashed line in each panel is the theoretical curve for the reassociation of the radiolabeled DNAs alone. The solid lines in panel A represent the theoretical curves for the renaturation of labeled BKV(JM) DNA in the presence of a 171-fold excess of SV40 DNA, assuming a 0.20 or 0.25 fraction polynucleotide sequence homology. The solid line in panel B represents the theoretical curve for the renaturation of BKV(MM) DNA in the presence of a 288-fold excess of SV40 DNA, assuming a 0.20 fraction polynucleotide sequence homology.*

reported (10). These molecular weights were calculated from the electrophoretic mobilities of known SV40 and adenovirus 2 DNA cleavage products in composite 3% polyacrylamide-0.5% agarose gels (Fig. 5). Successive digestion of either the prototype BKV or BKV(JM) DNA with R·*Eco*RI and R·*Hin*d resulted in cleavage of the *Hin*d fragment A into two additional fragments, A_1 and A_2 (Fig. 4, panels 4 and 6), which had electrophoretic mobilities corresponding to DNA molecules 0.92×10^6 and 0.60×10^6 daltons in size. The genomes of the prototype BKV and BKV(JM) were therefore indistinguishable with respect to the number and location of restriction endonuclease cleavage sites.

Digestion of BKV(MM) DNA with R·*Hin*d, on the other hand, resulted in three, instead of four, cleavage products (Fig. 4, and panel 7) with molecular weights of 1.5×10^6, 1.3×10^6, and 0.46×10^6 (Table 2). The sum of the molecular weights of the three *Hin*d cleavage fragments, 3.26×10^6, indicates that the genome of BKV(MM) is 94.5% the size of prototype BKV or BKV(JM) DNAs and 90.5% the size of SV40 DNA. Whereas the largest *Hin*d cleavage product of BKV(MM) appeared to co-migrate with the *Hin* A fragment of prototype BKV DNA, the two smaller fragments had electrophoretic mobilities which differed from prototype BKV *Hin* B, C, or D (Fig. 4, panel 7). As was observed with prototype BKV and BKV(JM) DNAs, successive digestion of BKV(MM) DNA resulted in the cleavage of DNA fragment *Hin* A into two new fragments previously described (Fig. 4, panels 4 and 6). This result indicates that the *Hin* A DNA segment (1.5×10^6 daltons), which contains the R·*Eco*RI cleavage site, is common to all three BKV genomes examined thus far.

Incubation of prototype BKV or BKV(JM) DNAs with R·*Hpa*II did not result in detectable cleavage of the respective supercoiled DNA forms. The BKV(MM) genome, however, was

TABLE 1. *Enzyme cleavage sites*

Isolate	R·*Eco*RI	R·*Hin*d	R·*Hpa*II	R·*Hpa*I
BKV	1	4	0	0
BKV(JM)	1	4	0	0
BKV(MM)	1	3	1	0

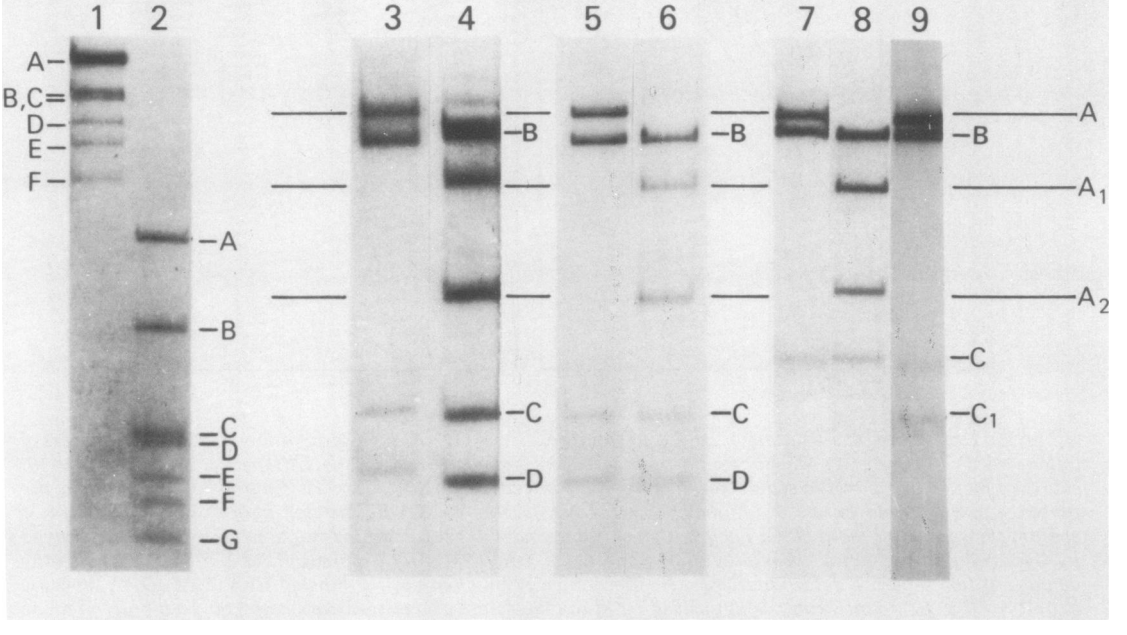

FIG. 4. *Restriction endonuclease cleavage patterns of BKV DNAs. Samples (50 μl) containing* ^{32}*P-labeled prototype BKV (panel 3), BKV(JM) (panel 5), or BKV(MM) (panel 7) DNAs were digested with R·Hind and analyzed by electrophoresis in composite 3% polyacrylamide-0.5% agarose slab gels as described. Successive digestion of prototype BKV, BKV(JM) or BKV(MM) DNAs with R·EcoRI and R·Hind is depicted in panels 4, 6, and 8, respectively. Successive cleavage of BKV(MM) DNA with R·HpaII and R·Hind is presented in panel 9. The six R·EcoRI fragments of adenovirus 2 DNA(panel 1) and the seven largest R·Hind fragments of SV40 (panel 2) are included as markers. Samples were subjected to electrophoresis under similar conditions for 16 h at 60 V, and migration is from top to bottom.*

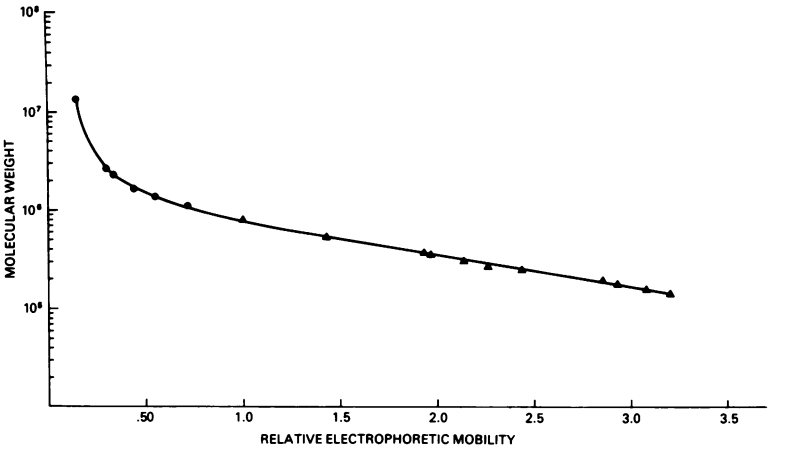

FIG. 5. *A comparison of the relative electrophoretic mobilities and the molecular weights of various restriction endonuclease cleavage DNA fragments subjected to electrophoresis in composite 3% polyacrylamide-0.5% agarose gels. Relative electrophoretic mobilities are expressed as the ratio of the distance migrated by a specific DNA fragment to the distance migrated by R·Hind SV40 DNA fragment A. Symbols: ●, R·EcoRI fragments of adenovirus 2 DNA; ▲, the eleven R·Hind fragments of SV40 DNA.*

cleaved into full-length linear molecules which co-migrated with the R·*Eco*RI-generated linear molecules in 1.4% agarose gels (see Fig. 9). To localize the R·*Hpa*II cleavage site in BKV-(MM) DNA, ^{32}P-labeled DNA was successively digested with R·*Hpa*II and R·*Hin*d, and the products were analyzed by composite 3% polyacrylamide-0.5% agarose slab gel electrophoresis (Fig. 4, panel 9). A new cleavage product, C_1, was observed with a molecular weight of 0.37×10^6 coincident with a decrease in the amount of BKV(MM) fragment *Hin* C. When the double-digestion reaction was analyzed in a composite 5% polyacrylamide-0.5% agarose gel, an additional cleavage product, C_2, with a molecular weight of approximately 8.5×10^4, was detected. The sum of the molecular weights of fragments C_1 and C_2 (0.455×10^6) corresponds closely to that calculated for BKV(MM) fragment *Hin* C (0.46×10^6).

Physical map of the BKV genome. A restriction endonuclease map of BKV DNA was obtained by analysis of partial R·*Hin*d cleavage products. Full-length linear, ^{32}P-labeled BKV DNA molecules, prepared by cleavage with R·*Eco*RI, were digested with a 1/10 dilution of R·*Hin*d for 30 min; the resultant partial cleavage products were separated by electrophoresis in a composite 2.2% polyacrylamide-0.5% agarose gel (Fig. 6). Each of the partial cleavage products was eluted from the gel as previously described (10) and then digested with R·*Hin*d under conditions which would have completely cleaved untreated BKV DNA. Such products were then analyzed by electrophoresis in a composite 3% polyacrylamide-0.5% agarose gel (Fig. 7, panels 2–5). Although complete digestion was not achieved, limit digestion products of the reactions could be identified, making it possible to construct a physical map of the prototype BKV genome. The partial digest fragment which migrated 74 mm in the preparative 2.2% polyacrylamide-0.5% agarose gel (Fig. 6) and had an estimated molecular weight of 1.35×10^6 was cleaved into fragments A_1 and *Hin* C after further digestion with R·*Hin*d (Fig. 7, panel 5). Therefore, fragments A_1 and *Hin* C are located adjacent to one another in the BKV genome. The sum of their molecular weights, 1.33×10^6, corresponds well with the estimated molecular weight (1.35×10^6) of the partial R·*Hin*d digest product A_1 C. A second partial digest fragment, which migrated 49 mm in the preparative gel (Fig. 6), was digested into prototype BKV *Hin*d fragments B, A_2, C, and D as well as several partial fragments (Fig. 7, panel 2). Similarly, the partial digest fragment migrating 55 mm in the preparative gel (Fig. 6) yielded fragments B, A_2, and D upon further digestion with R·*Hin*d (Fig. 7, panels 3 and 4). These data are summarized in Table 3 and allow the construction of the physical map of the prototype BKV genome which is shown in Fig. 8.

We have previously demonstrated that the heteroduplex structures formed by reassociating R·*Eco*RI linear SV40 and prototype BKV DNA molecules appear to line up end to end, with the regions of major homology located in those sequences adjacent to the R·*Eco*RI cleavage sites in each of the viral genomes (Khoury et al.,

TABLE 2. *Molecular weights of BKV restriction endonuclease DNA fragments*

Fragment	Relative electrophoretic mobility[b]	Mol wt[c]
BKV DNA fragment[a]		
R·*Hin*d A	0.54	1.50×10^6
R·*Eco*RI, R·*Hin*d A_1	0.85	0.92×10^6
R·*Eco*RI, R·*Hin*d A_2	1.30	0.60×10^6
R·*Hin*d B	0.65	1.20×10^6
R·*Hin*d C	1.78	0.41×10^6
R·*Hin*d D	2.05	0.34×10^6
Sum of R·*Hin*d fragments	—	3.45×10^6
BKV(MM) DNA fragment		
R·*Hin*d A	0.54	1.50×10^6
R·*Eco*RI, R·*Hin*d A_1	0.85	0.92×10^6
R·*Eco*RI, R·*Hin*d A_2	1.30	0.60×10^6
R·*Hin*d B	0.61	1.30×10^6
R·*Hin*d C	1.68	0.46×10^6
R·*Hpa*II, R·*Hin*d C_1	1.92	0.37×10^6
R·*Hpa*II, R·*Hin*d C_2		0.85×10^5[d]
Sum of R·*Hin*d fragments		3.26×10^6

[a] Restriction endonuclease cleavage fragments of BKV(JM) DNA are identical to those of BKV DNA.

[b] The ratio of the electrophoretic mobility of the specified DNA fragment to that of SV40 *Hin* A in a composite 3% polyacrylamide-0.5% agarose gel.

[c] Molecular weights calculated from electrophoretic mobilities of known standards (Fig. 5).

[d] Molecular weight estimated from its relative electrophoretic mobility in a composite 5% polyacrylamide-0.5% agarose gel.

in press). In the SV40 genome, this region of homology comprises those sequences which are transcribed late in the lytic infection and includes sequences corresponding to SV40 *Hin* fragments C, D, E, and K, on one side of the R·*Eco*RI site, and SV40 *Hin* fragments G, J, and possibly a small portion of *Hin* B to the other side of the R·*Eco*RI cleavage site (Khoury et al., in press).

The physical map of the prototype BKV genome, shown in Fig. 8, was oriented with respect to the R·*Eco*RI site of SV40 DNA by reassociating BKV *Hin*d-*Eco*RI fragments A_1 and A_2 alone, or in the presence of mixtures of specific SV40 *Hin* fragments (Table 4). Under the hybridization conditions employed, unlabeled SV40 *Hin* fragments D and K enhanced the reassociation of ^{32}P-labeled BKV fragment A_1; the reannealing of ^{32}P-labeled BKV fragment A_2 was unaffected in the presence of these SV40 *Hin* fragments. Unlabeled SV40 Hin fragments J and G, on the other hand, enhanced the reassociation of ^{32}P-labeled fragment A_2 with little if any effect on the reannealing of ^{32}P-labeled BKV fragment A_1.

The results of this experiment indicate that SV40 *Hin* fragments. Unlabeled SV40 *Hin* fragportions of BKV fragment A_1 and that SV40 *Hin* fragments J and G share nucleotide sequences with BKV fragment A_2. After the convention used for SV40 DNA, which locates the R·*Eco*RI cleavage site at 12 o'clock and the adjacent *Hin* fragments J and G positioned in a clockwise direction from this site, BKV fragment A_2 has been assigned a position next to and clockwise from the R·*Eco*RI cleavage site of BKV DNA (Fig. 8).

Physical map of BKV(MM) genome. Whereas the genome BKV(JM) cannot be distinguished from that of BKV by restriction endonuclease cleavage patterns, BKV(MM) DNA is slightly smaller (Fig. 1) and contains one less R·*Hin*d cleavage site (Table 1). Since the BKV(MM) genome is circular, each of the three R·*Hin*d fragments must border each other. It was necessary, however, to localize the R·*Eco*RI site within *Hin*d fragment A with respect to BKV(MM) *Hin*d fragments B and C. As described above, full-length R·*Eco*RI-generated linear molecules of BKV(MM) DNA were partially digested with R·*Hin*d and analyzed by electrophoresis in composite 3% polyacrylamide-0.5% agarose gels. Molecular weights for the partial cleavage fragments were determined from their electrophoretic mobilities (Fig. 5) and are presented in Table 5, as are the calculated molecular weights of restriction endonuclease-generated fragments for the two possible orders. The experimental data are compatible with the sequence A_2 BCA_1.

Heteroduplex mapping studies involving R·*Eco*RI linear BKV(MM) and SV40 DNAs indicated terminal regions of homology and a central area of heterology (Howley and Garon, unpublished observations). These structures were indistinguishable from the heteroduplex structures formed between SV40 and BKV DNAs (Khoury et al., in press). The BKV(MM) genome was oriented to SV40 DNA by reassociating radiolabeled, BKV(MM) fragments A_1 and A_2 with mixtures of unlabeled SV40 *Hin* fragments as described above (Table 4). BKV(MM)

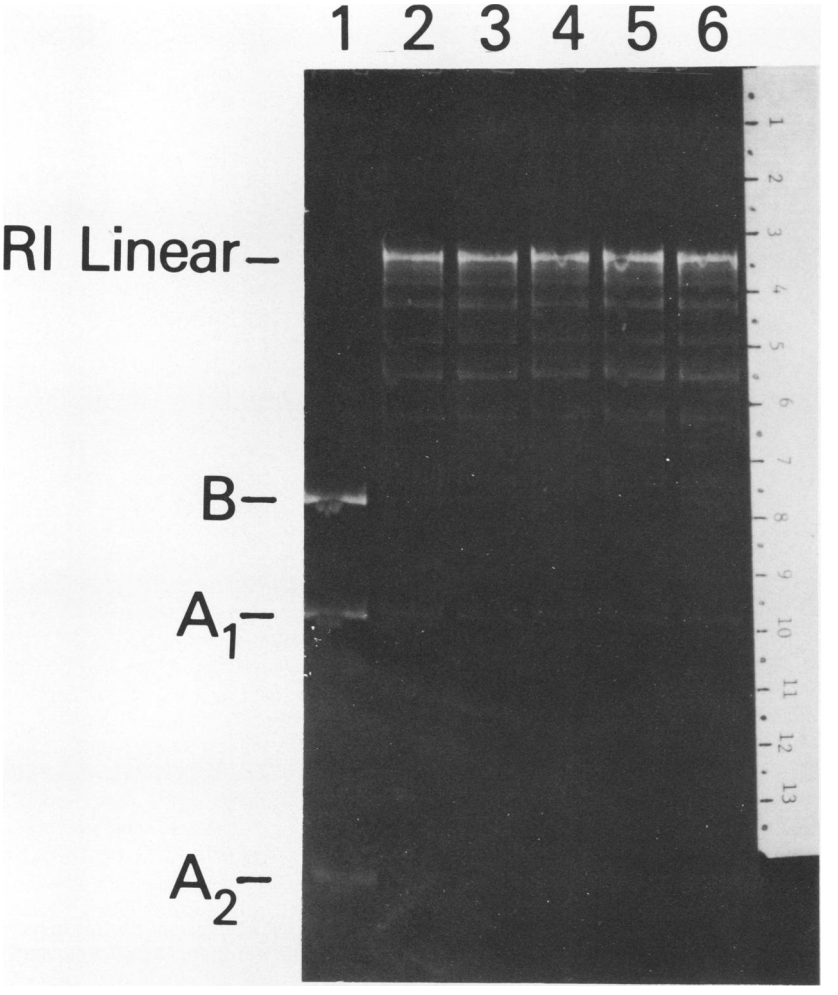

FIG. 6. *Partial R·Hind cleavage products of R·EcoRI linear BKV DNA. Partial cleavage products were generated as described. Samples (50 μl) containing approximately 0.5 μg of ^{32}P-labeled BKV partial cleavage products (specific activity, 10^4 counts/min per μg) were incubated for 30 min at 37 C in 1% sodium dodecyl sulfate before application to a composite 2.2% polyacrylamide–0.5% agarose slab gel in slots 2 to 6. Samples were subjected to electrophoresis for 11 h at 60 V as described. Slot 1 contains 1.0 μg of unlabeled BKV DNA completely cleaved by R·EcoRI plus R·Hind. DNA bands were visualized with UV light after 30 min of staining with ethidium bromide (0.5 μg/ml) (22). Migration is from top to bottom; the ruler at the right is marked in centimeters.*

fragment A_1, like prototype BKV fragment A_1, shared sequences with SV40 *Hin* D and K, and BKV(MM) fragment A_2 had homology with sequences in SV40 *Hin* J and G. The arrangement of polynucleotide sequences in fragment *Hin* A of the three BKV genomes examined to date is indistinguishable.

R·*Hpa*II cleaves the BKV(MM) genome at one unique site in fragment *Hin* C (Fig. 4), whereas it recognizes no sites in the DNAs of either prototype BKV or BKV(JM) (Table 1). BKV(MM) DNA was successively cleaved with R·*Hpa*II and R·*Eco*RI, and the resulting fragments were analyzed by electrophoresis in a composite 3% polyacrylamide-0.5% agarose gel (Fig. 9, panel 4). The molecular weights of the resulting two fragments, calculated from their respective electrophoretic mobilities, indicated that the R·*Hpa*II cleavage site was 0.31 map units from the R·*Eco*RI site, corroborating our previous assignment to BKV(MM) *Hin* fragment C (Fig. 4, panel 9). The physical map of the BKV(MM) genome is presented in Fig. 10 with the R·*Eco*RI site at 12 o'clock. This orien-

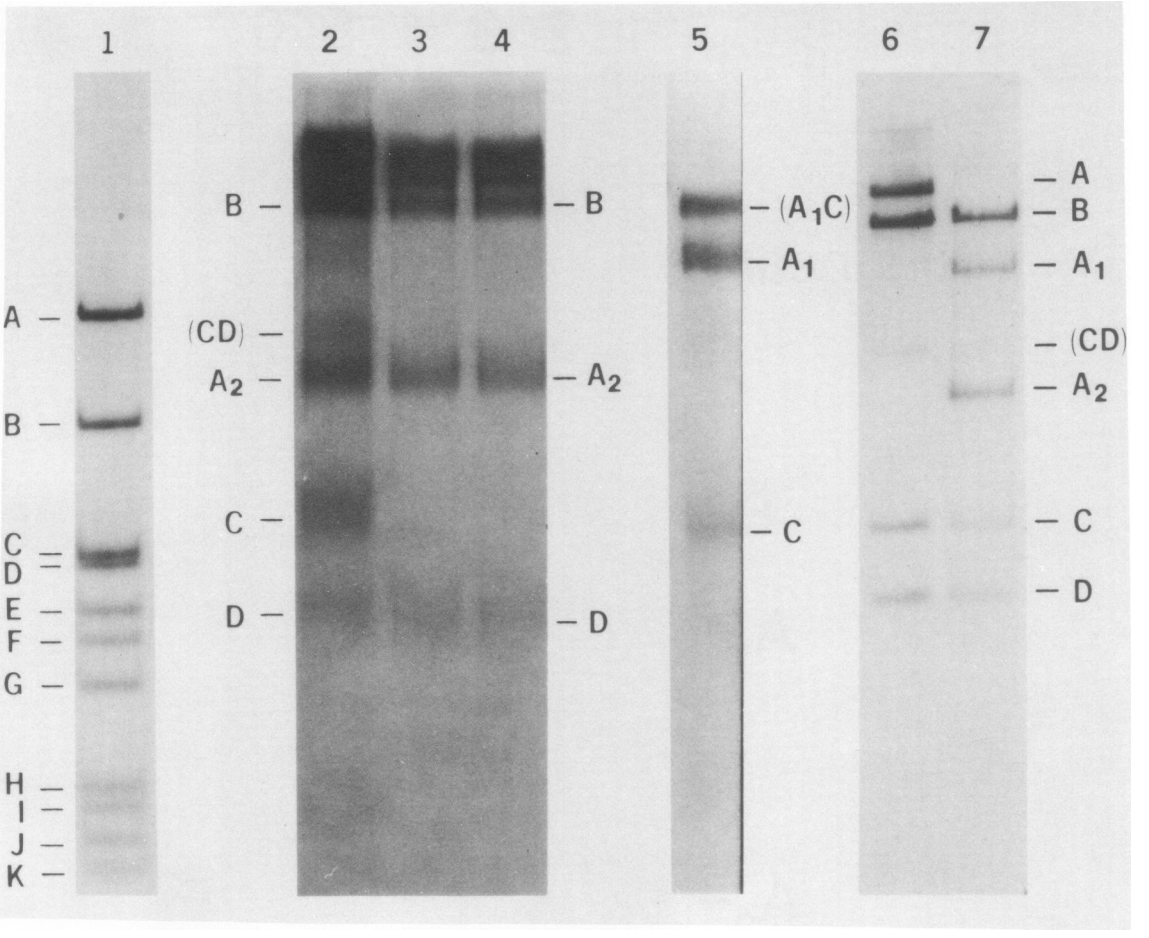

FIG. 7. *Electropherograms of restriction endonuclease redigestion products of partial digest fragments of BKV DNA.* ^{32}*P-labeled partial digestion products which migrated 49 mm (panel 2), 55 mm (panels 3 and 4), or 74 mm (panel 5) in the preparative gel shown in Fig. 6 were redigested with R·Hind under the standard conditions and subjected to electrophoresis in composite 3% polyacrylamide-0.5 agarose gels as described. Cleavage of* ^{32}*P-labeled SV40 (panel 1) and BKV (panel 6) DNAs with R·Hind as well as BKV DNA, successively cleaved with R·EcoRI and R·Hind (panel 7), are included as reference standards. Migration is from top to bottom.*

tation was confirmed by reassociating the two fragments, generated by the successive digestion of BKV(MM) DNA with R·*Hpa*II and R·*Eco*RI, in the presence or absence of specific SV40 *Hin* fragments (Table 4). Under the conditions employed, reassociation of the smaller fragment (1.0×10^6 daltons) was accelerated by the mixture of SV40 *Hin* fragments D and K and not by the mixture of SV40 *Hin* fragments J and G. Conversely only the mixture of SV40 *Hin* fragments J and G influenced the reassociation of the larger BKV(MM) fragment. Thus the smaller *Eco*RI-*Hpa*II BKV(MM) fragment must lie to the left of the R·*Eco*RI site extending 0.31 map units from the R·*Eco*RI site into fragment *Hin* C (Fig. 10).

DISCUSSION

In this study we have characterized the genomes of two new human papovavirus isolates which are serologically and morphologically indistinguishable from prototype BKV. Both isolates were obtained from the urines of patients with WAS, a genetic disease characterized by cell-mediated and humoral immunological deficiencies. Despite the ubiquity of BKV antibodies in the sera of adult humans tested (4, 13, 21), extensive efforts to isolate this agent from immunologically competent individuals have not been successful. Instead, this virus has been isolated from the urines of humans who are either genetically immunologically deficient

(patients with WAS) or exogenously immunosuppressed (renal allograft recipients). Renal transplant patients appear to excrete BKV intermittently, whereas individuals with WAS whose urines have been tested continuously shed virus (Takemoto and Mullarkey, unpublished observations). Of interest is the fact that renal allograft recipients and young males with WAS have a high incidence of malignancy, particulary reticulum cell sarcomas. However, a pathogenic relationship between BKV and any human disease has yet to be determined.

TABLE 3. *Order of BKV DNA restriction endonuclease cleavage fragments*

Mol wt of partial fragments[a]	Limit products	Sum of mol wts of limit products	Order
1.35×10^6	A_1,C	1.33×10^6	A_1C
1.7×10^6	A_1,C,D	1.67×10^6	A_1CD
2.2×10^6	A_2,B,D	2.14×10^6	DBA_2
2.5×10^6	A_2,B,C,D	2.53×10^6	$CDBA_2$
0.71×10^6	C,D	0.75×10^6	CD

[a] Estimated from the electrophoretic mobility in a composite 2.2% polyacrylamide-0.5% agarose gel.

The results presented here indicate that the genomes of these two new isolates are significantly more homogeneous than that of the prototype BKV previously examined (Fig. 1) (10). Both were indistinguishable from the prototype BKV in nucleic acid hybridization experiments, and hence have been referred to as BKV(JM) and BKV(MM). Each of the new isolates was passaged exclusively in human cells, and the genomes of each share approximately 20% of their sequences with the SV40 genome, confirming our previous report of a 20% polynucleotide sequence homology between prototype BKV and SV40 DNAs (10). This result indicates that prototype BKV is distinct from SV40 and did not evolve from the latter by

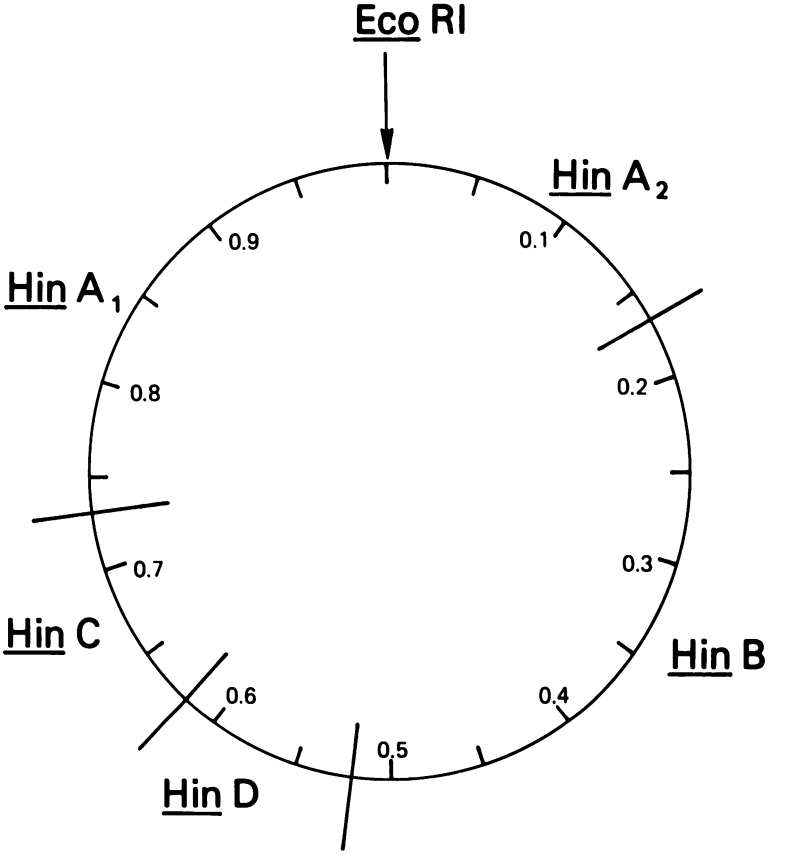

FIG. 8. *Restriction endonuclease cleavage map of BKV DNA. Map units, expressed as fractions of the circular BKV genome commencing at the R·EcoRI cleavage site, were determined from the molecular weights of the individual cleavage fragments.*

TABLE 4. *Orientation of the BKV and BKV(MM) genomes*[a]

Fragment	Percent duplex molecules		
	Reassociated alone	Reassociated in the presence of SV40 *Hin* D and K	Reassociated in the presence of SV40 *Hin* G and J
BKV DNA			
A_1	23.9	47.2	19.5
A_2	20.9	24.3	47.3
BKV(MM) DNA			
A_1	15.2	52.1	14.3
A_2	10.0	12.1	30.0
R·*Eco*RI, R·*Hpa*II (2.3×10^6-dalton fragment)	19.6	21.2	31.9
R·*Eco*RI, R·*Hpa*II (1.0×10^6-dalton fragment)	15.2	49.7	18.8

[a] Reassociation of radiolabeled restriction endonuclease cleavage fragments of BKV DNA or BKV(MM) DNA in the presence or absence of mixtures of unlabeled SV40 *Hin*d fragments. ^{32}P-labeled fragments of BKV DNA or BKV(MM) DNA (2.5×10^5 counts/min per μg) were allowed to reassociate alone (1.2×10^{-3} μg/ml) or in the presence of a 20-fold molar excess of a mixture of either unlabeled SV40 *Hin* fragments D and K or unlabeled SV40 *Hin* fragments G and J. Each reaction mixture, containing 0.60 M sodium phosphate buffer and 0.0025 M EDTA, was incubated for approximately ⅓ the Cot_{50} of the radiolabeled DNA reassociating alone. The percent ^{32}P-labeled DNA in duplex molecules was assayed by hydroxyapatite column chromatogrphy.

recombination during its early propagation in monkey cells.

The DNA of BKV(JM) was identical in size and in number and location of restriction endonuclease cleavage sites to the largest of the prototype BKV DNA species, which we have previously shown to be the only infectious DNA form (10). Whereas BKV(MM) DNA could not be distinguished from the prototype BKV DNA, in appropriate nucleic acid hybridization experiments its genome was found to be 6% smaller (Fig. 1) and contained several different restriction endonuclease cleavage sites (Fig. 4).

We have determined the order of the restriction endonuclease cleavage products of BKV and BKV(MM) DNAs and constructed physical maps for both viral genomes (Fig. 8 and 10). Since the areas of homology in BKV and SV40 DNAs span the respective R·*Eco*RI sites (Khoury et al., in press), it has been possible to orient the two genomes around this locus. BKV *Hin* A, which contains the R·*Eco*RI cleavage site, has homology with those SV40 *Hin* fragments which are transcribed late in the lytic infection. Therefore it should contain sequences that are expressed late in cells productively infected by BKV. Since the early region in SV40 extends from 0.18 to 0.68 map units (11), the corresponding region in the BKV genome, including BKV DNA fragments *Hin* B, D, and part of C, is probably transcribed early during the lytic cycle. Experiments to confirm these predictions are currently in progress in this laboratory.

A comparison of the cleavage maps shown in Fig. 8 and 10 suggests that the region extending from 0.52 to 0.73 map units in the BKV genome

TABLE 5. *Order of BKV(MM) R·Hind DNA fragments*

Model one (A_2BCA_1)		Model two (A_2CBA_1)		Mol wt of partial cleavage products of BKV(MM)
Predicted partial cleavage products	Mol wt	Predicted partial cleavage products	Mol wt	
A_2BCA_1	3.26×10^6	A_2CBA_1	3.26×10^6	3.26×10^6
BCA_1	2.68×10^6	CBA_1	2.68×10^6	2.73×10^6
A_2BC	2.36×10^6	A_2CB	2.36×10^6	2.30×10^6
A_2B[a]	1.90×10^6	BA_1[a]	2.22×10^6	1.91×10^6
BC	1.76×10^6	CB	1.76×10^6	1.74×10^6
CA_1[a]	1.38×10^6	B	1.30×10^6	1.30×10^6
B	1.30×10^6	A_2C[a]	1.06×10^6	
A_1	0.92×10^6	A_1	0.92×10^6	0.92×10^6
A_2	0.60×10^6	A_2	0.60×10^6	0.60×10^6
C	0.46×10^6	C	0.46×10^6	0.46×10^6

[a] Predicted partial cleavage products which allow differentiation between the two possible models. The fragment migrating with the mobility of a 1.91×10^6-dalton duplex DNA molecule found experimentally must represent the fragment A_2B in the first model. Partial cleavage product CA_1 most probably co-migrates experimentally with fragment B. No fragments migrating with the molecular weights of BA_1 and A_2C, predicted partials for model two, were observed.

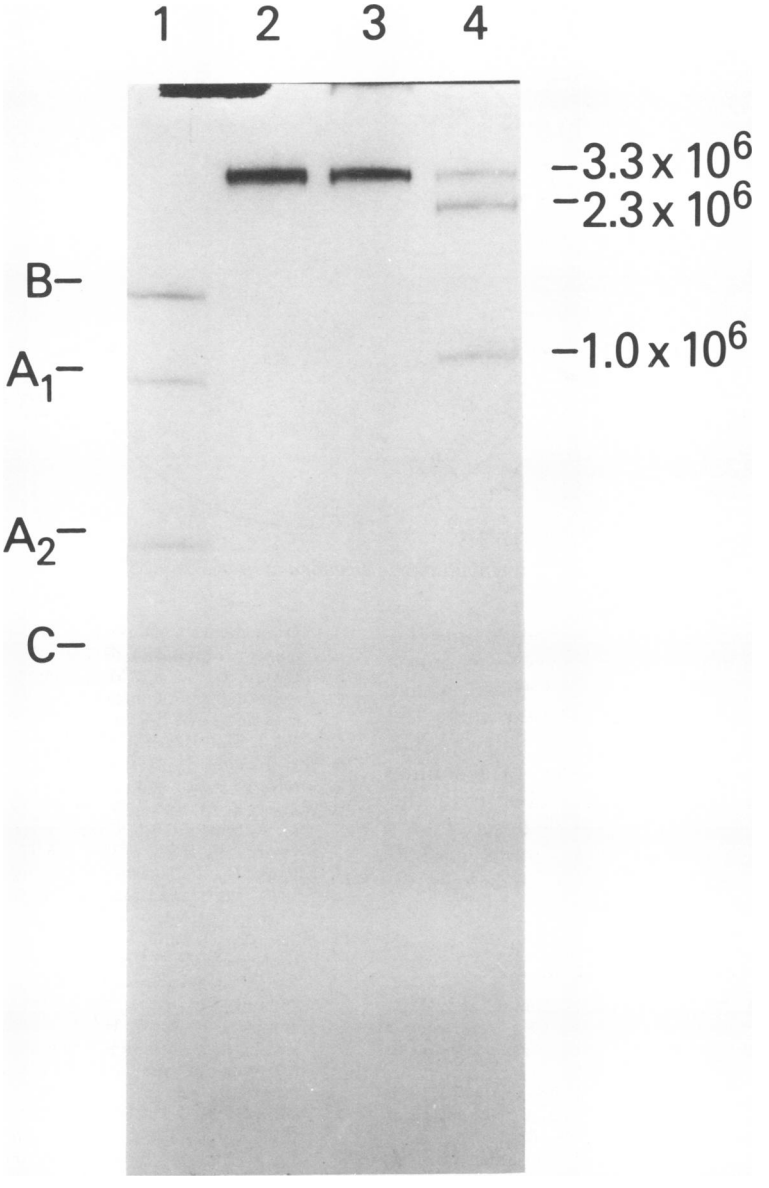

FIG. 9. *Electropherogram of restriction endonuclease cleavage products of BKV(MM) DNA. Samples (50 μl) containing ^{32}P-labeled supercoiled BKV(MM) DNA fragments were subjected to electrophoresis in a composite 3% polyacrylamide-0.5% agarose slab gel for 16 h at 60 V after cleavage with the following restriction enzymes: (1) R·EcoRI plus R·HindIII; (2) R·EcoRI; (3) R·HpaII; and (4) R·HpaII plus R·EcoRI. Migration is from top to bottom; uncleaved supercoiled molecules remain at the origin.*

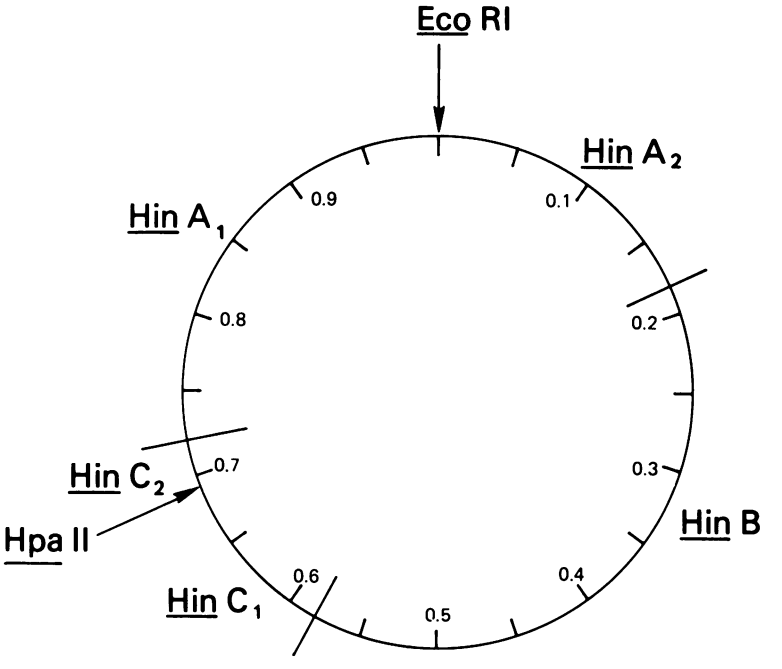

FIG. 10. *Restriction endonuclease cleavage map of BKV(MM) DNA.*

can vary from isolate to isolate. The genome of a third WAS isolate of BKV differs from prototype BKV DNA only in fragment *Hin* C, which is located between 0.64 to 0.76 map units (S. Bond, unpublished observations). This variable region is topographically located in a similar portion of the BKV genome, relative to the R·*Eco*RI cleavage site, as the one described for different isolates of SV40, which maps in SV40 fragment *Hin* C encompassing 0.655 to 0.760 map units (2, 15, 20).

LITERATURE CITED

1. Blaese, R. M., W. Strober, R. S. Brown, and T. A. Waldman. 1968. The Wiskott-Aldrich syndrome. A disorder with a possible defect in antigen processing or recognition. Lancet **1**:1056-1061.
2. Danna, K. J., G. H. Sack, Jr., and D. Nathans. 1973. Studies in simian virus 40 DNA. VII. A cleavage map of the SV40 genome. J. Mol. Biol. **78**:363-376.
3. Fareed, G. C., C. F. Garon, and N. P. Salzman. 1972. Origin and direction of simian virus 40 deoxyribonucleic acid replication. J. Virol. **10**:484-491.
4. Gardner, S. D. 1973. Prevalence in England of antibody to human polyomavirus (B. K.). Br. Med. J. **1**:77-78.
5. Gardner, S. D., A. M. Field, D. V. Coleman, and B. Hulme. 1971. New human papovavirus (B. K.) isolated from urine after renal transplantation. Lancet **1**:1253-1257.
6. Gatti, R. A., and R. A. Good. 1971. Occurrence of malignancy in immunodeficiency disease. A literature review. Cancer **28**:89-98.
7. Gelb, L. D., D. E. Kohne, and M. A. Martin. 1971. Quantitation of simian virus 40 sequences in African green monkey, mouse and virus-transformed cell genomes. J. Mol. Biol. **57**:129-145.
8. Hayward, G. S., and M. G. Smith. 1972. The chromosome of bacteriophage T5. I. Analysis of the single stranded DNA fragments by agarose gel electrophoresis. J. Mol. Biol. **63**:383-395.
9. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365-369.
10. Howley, P. M., M. F. Mullarkey, K. K. Takemoto, and M. A. Martin. 1975. Characterization of human papovavirus BK DNA. J. Virol. **15**:173-181.
11. Khoury, G., P. Howley, D. Nathans, and M. Martin. 1975. Posttranscriptional selection of simian virus 40-specific RNA. J. Virol. **15**:433-437.
12. Major, E. O., and G. di Mayorca. 1973. Malignant transformation of BHK_{21} clone 13 cells by BK virus—a human papovavirus. Proc. Natl. Acad. Sci. U.S.A. **70**:3210-3212.
13. Mäntyjärvi, R. A., O. H. Meurman, L. Vihma, and B. Berglund. 1973. A human papovavirus (B. K.), biological properties and seroepidemiology. Ann. Clin. Res. **5**:283-287.
14. Mullarkey, M. F., J. F. Hruska, and K. K. Takemoto. 1974. Comparison of two human papovaviruses with simian virus 40 by structural protein and antigenic analysis. J. Virol. **13**:1014-1019.
15. Nathans, D., and K. J. Danna. 1972. Studies of SV40 DNA. III. Differences in DNA from various strains of SV40. J. Mol. Biol. **64**:515-518.
16. Osborn, J. E., S. M. Robertson, B. L. Padgett, G. M. ZuRhein, D. L. Walker, and B. Weisblum. 1974. Comparison of JC and BK human papovaviruses with simian virus 40: restriction endonuclease digestion and gel electrophoresis of resultant fragments. J. Virol. **13**:614-622.
17. Penney, J. B., and O. Narayan. 1973. Studies of the antigenic relationships of the new human papovaviruses by electron microscopy agglutination. Infect.

Immun. 8:299-300.
18. Pettersson, U., C. Mulder, H. Delius, and P. A. Sharp. 1973. Cleavage of adenovirus type 2 DNA into six unique fragments by endonuclease R·RI. Proc. Natl. Acad. Sci. U.S.A. 70:200-204.
19. Portolani, M., G. Barbanti-Brodano, and M. LaPlaca. 1975. Malignant transformation of hamster kidney cells by BK virus. J. Virol. 15:420-422.
20. Sack, G. H., Jr., O. Narayan, K. J. Danna, L. P. Weiner, and D. Nathans. 1973. The nucleic acid of an SV40-like virus isolated from a patient with progressive multifocal leukoencephelopathy. Virology 51:345-350.
21. Shah, K. V., R. W. Daniel, and R. M. Warszawaski. 1973. High prevalence of antibodies to BK virus, an SV40-related papovavirus, in residents of Maryland. J. Infect. Dis. 128:784-787.
22. Sharp, P. A., B. Sugden, and J. Sambrook. 1973. Detection of two restriction endonucleases in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry 12:3055-3063.
23. Sharp, P. A., U. Pettersson, and J. Sambrook. 1974. Viral DNA in transformed cells. I. A study of the sequences of adenovirus 2 DNA in a line of transformed rat cells using specific fragments of the viral genome. J. Mol. Biol. 86:709-726.
24. Takemoto, K. K., and M. F. Mullarkey. 1973. Human papovavirus, B. K. strain: biological studies including antigenic relationship to simian virus 40. J. Virol. 12:625-631.
25. Takemoto, K. K., A. S. Rabson, M. F. Mullarkey, R. M. Blaese, C. F. Garon, and D. Nelson. 1974. Isolation of papovavirus from brain tumor and urine of a patient with Wiskott-Aldrich syndrome. J. Natl. Cancer Inst. 53:1205-1207.
26. Wright, P. J., and G. DiMayorca. 1975. Virion polypeptide composition of the human papovavirus BK: comparison with simian virus 40 and polyoma virus. J. Virol. 15:828-835.