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Transformation of Human Embryonic Kidney Cells by Human Papovavirus BK

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Infection of secondary human embryonic kidney (HEK) cells with human papovavirus BK (BKV) resulted in cellular lysis and degeneration within 7 days. After 30 days, multilayered colonies of transformed cells were found and subcultured for analyses. These BK-HEK cells uniformly expressed the BKV T-antigen but were only 1% V-antigen positive. They produced infectious virus and were resistant to superinfection by BKV. They reached a saturation density of 1.3×10^5 cells per cm^2 in medium with 5% fetal calf serum, were able to grow in medium containing 2% serum, and did not form colonies in soft agar or tumors in nude mice. Nonintegrated, superhelical BKV DNA was detected in the noncloned cells as expected because they were persistently infected and contained RNA transcripts complementary to both early and late regions of the BKV genome. Analysis of T-antigen-positive clonal isolates of these BK-HEK cells by the Southern technique revealed an absence of free viral DNA and the presence of integrated BKV DNA sequences corresponding to the early region of the BKV genome. These studies demonstrate the stable transformation of human cells by BKV. However, the transformed human cells which retain and express part of the BKV genome do not fully manifest the growth properties of other papovavirus-transformed cells.

Human papovavirus BK (BKV) was isolated by Gardner et al. from the urine of a renal allograft recipient (9). BKV contains a circular duplex DNA genome of approximately 5,000 base pairs and exhibits about 20 to 25% base sequence homology with the late region of simian virus 40 (SV40) (14, 21) and weaker homology in the early gene region (20). Perhaps a reflection of the close evolutionary relationship between BKV and SV40 is the remarkable similarity of their genomic sequences containing the origins of DNA replication (5). BKV and SV40 code for T-antigens which cross-react immunologically (9, 27) but different transplantation antigens (27). Recently, several somatic cell hybrids have been formed which produce antibodies specific for SV40 T-antigen without cross-reacting with BKV T-antigen (18). More recently it was shown that 7 out of 21 methionine-containing tryptic peptides from their large T-antigens were similar (23). In vitro transformation of hamster cells has been achieved with both BKV and BKV DNA (16, 22, 26), and these cells were capable of tumor production when inoculated into hamsters (22, 26). The transformation of monkey cells with BKV has also been reported (4).

Although antibodies to BKV have been found in high frequency among the populations of the U.S. and Great Britain (8, 20), conflicting reports exist with respect to its role in human neoplasia (7, 30). We have been investigating the interaction of BKV with various human cell types to define how the virus exists within its natural host. Here we report our studies on the infection of human embryonic kidney (HEK) cells with BKV.

MATERIALS AND METHODS

Cells and viruses. Primary HEK cells were purchased from Microbiological Associates, Inc., and subcultured in medium 199 supplemented with 10% fetal calf serum unless otherwise stated. BK virus from plaque isolate 218744 was kindly provided by M. Fiori and G. diMayorca. The virus was purified through KBr and CsCl density gradients and had a titer of 4.5×10^9 PFU/cm^3. Confluent monolayer cultures of secondary HEK cells were infected with BKV at a multiplicity of infection of 10 PFU per cell and maintained in medium 199 with 2% fetal calf serum at 37°C. The medium was changed every 4 to 6 days, and cells surviving the acute lytic infection formed colonies after 3 to 4 weeks. Superinfection of BK-HEK cells with BKV was carried out at a multiplicity of infection of 10, and cells were maintained in M199 with 2% fetal calf serum for 10 days. Cells on cover slips were tested for BKV capsid antigen by immunofluorescence at 4, 7, and 10 days. Infectious BKV produced by BK-HEK

cells was demonstrated by inoculating confluent secondary HEK cells in 25-cm^2 flasks or on cover slips with 2 ml of medium obtained after a 4-day incubation with BK-HEK cells. The cells were monitored for cytopathic changes and T- and V-antigens by indirect immunofluorescence assays. Antisera to BKV T- and V-antigens were obtained from K. K. Takemoto (19, 26).

DNA extraction. Total cellular DNA was prepared by washing the cells twice with NaCl-Tris (0.15 M NaCl-0.01 Tris, pH 7) and were removed from the culture dish by scraping in STE (0.01 M Tris-0.15 M NaCl-0.001 M EDTA, pH 7.2). The cells were pelleted and lysed by addition of sodium dodecyl sulfate (SDS) to a final concentration of 0.5%. Pronase was added to 100 μg/ml, and the lysate was incubated for 12 h at 37°C, extracted three times with STE-saturated phenol, and precipitated by addition of 2.5 volumes of ethanol at −20°C. The precipitate was dissolved in STD (0.01 M Tris-0.001 M EDTA-0.015 M NaCl, pH 7.2) and treated with pancreatic RNase (20 μg/ml) for 2 h at 37°C. After two additional phenol extractions, the DNA was dialyzed against STD for 24 h.

Supercoiled DNA was isolated by the method of Hirt (12). Superhelical BKV DNA preparations purified by dye-density gradient centrifugation after extraction from purified virus or infected HEK cells were generous gifts of G. diMayorca and G. Khoury (Dunlop strain).

RNA extraction. Cells were labeled in Earle phosphate-free medium supplemented with 5% dialyzed fetal calf serum containing 100 μCi of [^{32}P]orthophosphate (ICN) for 12 h at 37°C. The cells were washed twice with NaCl-Tris and removed from the culture dishes by scraping in STE. After three washes with NaCl-Tris, the cells were lysed in 0.01 M Tris (pH 8.0), 2 mM $MgCl_2$, 0.01 M NaCl, 0.5% Nonidet P-40 (Particle Data Laboratories Ltd.), and 0.5% deoxycholate for 5 min on ice. The nuclei were pelleted at 4,000 × *g* for 5 min, and the supernatant was extracted three times with phenol-chloroform-isoamyl alcohol (24:24:1) saturated with STE. After three ethanol precipitations, the RNA was chromatographed on oligodeoxythymidylic acid-cellulose (Collaborative Research, T-3 grade) as described elsewhere (15). The polyadenylic acid [poly(A)]-containing RNA was precipitated twice with ethanol, dissolved in 0.2% SDS, heated for 2 min at 65°C, and re-chromatographed on oligodeoxythymidylic acid-cellulose. The RNA which bound to the column under these conditions was used for subsequent analysis.

Analysis of cellular DNA with restriction endonucleases. Restriction endonucleases *Bam* HI and *Eco*RI were prepared as described elsewhere (11, 29). *Hin*dIII was purchased from Bethesda Research Laboratories. Digestion of DNA samples, electrophoresis through 1% agarose gels, and transfer to nitrocellulose strips (25) were as described elsewhere (28).

BKV DNA was labeled with ^{32}P to high specific activities (5 × 10^7 cpm/μg) by the "nick-translation" procedure of Maniatis et al. (17). Hybridization of labeled BKV DNA to nitrocellulose strips containing the denatured DNA samples was as described previously (28).

RNA hybridization. [^{32}P]poly(A)-containing RNA from normal and BKV-infected HEK cells was hybridized to nitrocellulose disks containing 3 μg of denatured BKV DNA (10) in 50% formamide-0.75 M NaCl-0.1 M Tris (pH 7.4)-2 mM EDTA-0.5% SDS for 16 h at 37°C. To show that the DNA on the filters was in excess, parallel hybridization experiments were performed with filters containing different amounts of BKV DNA. The fraction of RNA binding to the filters remained essentially the same with filters containing in excess of 3 μg of BKV DNA. The filters were washed five times with buffer A (0.1 M Tris-2 mM EDTA-0.5% SDS, pH 7.5) for 2 h at 23°C and incubated for another hour in hybridization buffer at 37°C. After five more washes with buffer A, the bound RNA was eluted by heating at 85°C for 3 min in 2 mM EDTA-0.01 M Tris (pH 7.4)-0.5% SDS. Yeast tRNA was added to 10 μg/ml, and the RNA was precipitated with 2.5 volumes of ethanol. Hybridization of ^{32}P-labeled poly(A)-containing RNA to nitrocellulose filters containing separated BKV DNA fragments was as described elsewhere (1).

For sedimentation analysis, the RNA was dissolved in 0.01 M Tris (pH 7.4)-0.001 M EDTA-0.2% SDS, heated at 65°C for 2 min, and sedimented through 5 to 30% sucrose gradients containing 0.15 M NaCl-0.01 M Tris, pH 7.4, for 3 h at 45,000 rpm in a Beckman SW 50.1 rotor at 5°C. The gradients were collected by bottom puncture and counted. Cellular 28S and 18S rRNA's were run in parallel gradients as sedimentation markers.

RESULTS

Establishment and growth properties of BK-HEK cells. Secondary HEK cells were infected with BKV at a multiplicity of 10. After 4 to 5 days, extensive cytopathic effects were observed, and the cell monolayer rapidly degenerated. Within 30 days, small foci of cells appeared; these cells were trypsinized, pooled, and expanded. We have been carrying these cells for 13 months at split ratios of 1:5 every 4 days in M-199 medium supplemented with 10% fetal calf serum. The establishment of persistently-infected BK-HEK cells has been repeated successfully twice with both virus and viral DNA infections. Maintenance of the acutely infected cells in medium containing 0.5% anti-BKV rabbit serum has also been effective for selection of T-antigen-positive, transformed BK-HEK cells; however, little difference was observed in the growth properties or time of appearance of these cells compared with cultures lacking the antiserum.

BK-HEK cells grew well in both 10 and 2% serum, reaching a saturation density of 1.3 × 10^5 cells per cm^2. Secondary HEK cells grew poorly in medium with serum concentrations less than 5% or at cell split ratios greater than 1 to 5. The saturation density of BK-HEK cells was generally at least fourfold greater than that of secondary HEK cells. The cells had an epithelioid

morphology and formed several dense foci of multilayered cells per 75-cm^2 flask, but they did not form colonies in soft agar (4). The cell doubling time was approximately 24 h in 10% fetal calf serum.

Expression of virus-specific antigens. The BK-HEK cells were all uniformly positive for BKV T-antigen as assayed by the indirect immunofluorescent procedure. They were about 1% positive for BKV V-antigen. The cells produced infectious virus as assayed by infecting HEK monolayers with cell extracts and testing for T- and V-antigen expression. However, they were resistant to superinfection with BKV as judged by a lack of cytopathic effects or an increase in expression of BKV V-antigen after inoculation with BKV at high multiplicities.

Arrangement of BKV DNA sequences. As a first step in analyzing the state of the BKV genome in these cells, we prepared superhelical DNA by the Hirt procedure (12) and analyzed these samples by cleavage with various restriction enzymes, electrophoresis through agarose gels, and UV illumination of ethidium bromide-stained bands. Figure 1A shows the presence of two superhelical DNA species which are cleaved to the linear form by *Eco*RI and *Bam* HI (two single-hit enzymes for BKV DNA [13, 31]). Cleavage with *Hin*dIII and *Hin*dIII + *Eco*RI gives the expected banding pattern. The *Eco*RI and *Hin*dIII cleavage sites for prototype BKV DNA are shown in Fig. 1D.

To examine the total cellular DNA, we cleaved it with restriction enzymes, fractionated the digests on agarose gels, transferred the DNA to nitrocellulose filters by the method of Southern (25), and hybridized it to ^{32}P-labeled BKV DNA. Fig. 1B shows the results of such an analysis with the enzymes *Eco*RI and *Bam* HI which cleave BKV DNA at single sites (13, 31). Track 1 shows BKV DNA cleaved with *Eco*RI. Cleavage of BK-HEK DNA with either *Eco*RI or *Bam* HI results in two major bands (tracks 2 and 3), one of which migrates with the BKV DNA marker; the other migrates just ahead of wild-type BKV DNA. Because we obtained the same pattern with two single-hit enzymes which cleave BKV DNA at different locations (13, 31), we conclude that at least a portion of these cells (presumably those producing virions) contain BKV DNA primarily in a free (nonintegrated) state. Cleavage of BK-HEK cellular DNA with *Hin*dIII and subsequent analysis of the digests as described above indicates that no gross rearrangement of the BKV DNA has taken place with respect to these cleavage sites because the pattern observed corresponds closely to that for wild-type BKV DNA (Fig. 1A to C). The *Hin*dIII-C and -D fragments do not transfer well at the low DNA concentrations used in Fig. 1C, but are observed in Fig. 1A. The fastest migrating band seen in track 1 of Fig. 1C is due to a partial digestion product and consists of fragment C + D (Fig. 1D).

In the *Hin*dIII digests, we observed that the A fragment is less intense than the B fragment (Fig. 1A, track 4, Fig. 1C, track 2). Cleavage with *Hin*dIII and *Eco*RI (Fig. 1A, track 5) shows that the A_1 fragment is less intense than the A_2 fragment. The simplest interpretation of these data is that the shortened genomes migrating more rapidly than wild-type BKV have incurred a deletion in the late gene region. These two DNA species have been maintained without additional rearrangements for over 1 year of continuous culturing of these cells.

Analyses of BKV specific RNA. When papovavirus particles are being produced in a normal lytic infection, there is a preponderance of late mRNA synthesized relative to the early mRNA species. One mechanism for a persistent virus infection such as this one might involve a translational barrier to expression of late mRNA species in a large fraction of the cells. Evidence in support of this possibility would involve demonstrating relatively normal amounts of cytoplasmic late mRNA species. To analyze the cytoplasmic viral transcripts, we purified ^{32}P-labeled poly(A)-containing RNA and hybridized it to BKV DNA immobilized on a nitrocellulose filter (10). To minimize thermal degradation of the RNA, we performed the hybridization in 50% formamide at 37°C. The RNA was then eluted from the filter and sedimented on a neutral sucrose gradient as described above. As the legend to Fig. 2 indicates, about 0.085% of the total poly(A)-containing RNA was virus specific. Figure 2 shows that this RNA sedimented at about 18S with a shoulder at 15 to 16S.

We analyzed this RNA further by hybridizing it to nitrocellulose filters containing separated fragments of BKV DNA cleaved with *Eco*RI and *Hin*dIII (Fig. 1D). Fragments A_1 + A_2 code for late mRNA, and fragments C, D, and B code for early mRNA (G. Khoury, personal communication). Hybridization of ^{32}P-labeled BK-HEK poly(A)-containing RNA to these is shown in Fig. 3. The RNA hybridized to both early and late regions, suggesting that these cells contain both species of viral mRNA. Densitometry analysis of this autoradiogram indicated that equal amounts of early and late transcripts are present (data not shown). The two bands seen above band B in Fig. 3 are due to partial digestion products of BKV DNA in this reaction. The apparent failure of hybridization to fragments C

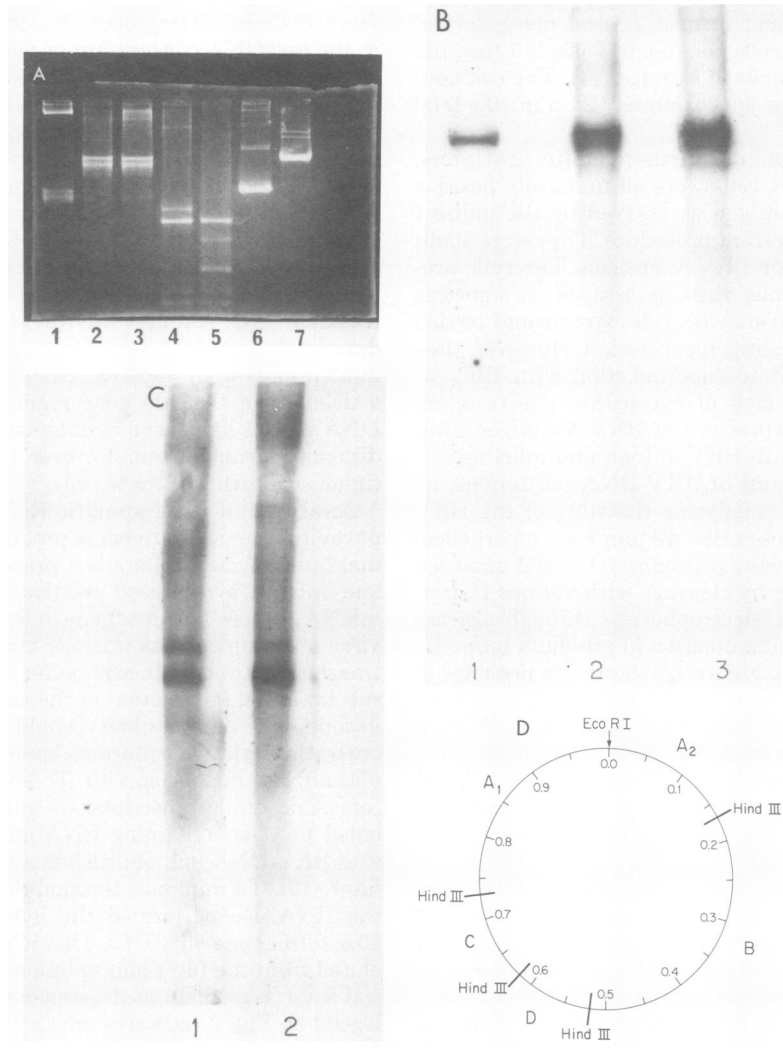

FIG. 1. *Restriction endonuclease analysis of superhelical and total cellular DNA from BK-HEK cells. (A) Superhelical BKV DNA was isolated from BK-HEK cells by selective extraction and dye-density gradient centrifugation (12, 14). Samples were cleaved with various restriction enzymes, subjected to electrophoresis through a 1% agarose gel, stained with ethidium bromide, illuminated with UV light, and photographed. Track 1, uncleaved DNA; track 2, DNA cleaved with EcoRI; track 3, DNA cleaved with Bam HI; track 4, DNA cleaved with HindIII; track 5, DNA cleaved with HindIII + EcoRI; track 6, SV40 form I DNA; track 7, SV40 DNA cleaved with EcoRI. All samples contained 1 μg of DNA except tracks 6 and 7, which contained 1.5 μg of DNA. Cleavage of wild-type BKV DNA by HindIII and EcoRI yielded fragments having the same electrophoretic mobilities as those in track 5. (B) Samples of 5 μg of total cellular DNA were digested with EcoRI, Bam HI, or HindIII, fractionated on a 1% agarose gel, transferred to nitrocellulose filters, and hybridized to ^{32}P-labeled BKV DNA. Track 1, 300 pg of BKV DNA digested with EcoRI; track 2, 5 μg of BK-HEK DNA digested with EcoRI; track 3, 5 μg of BK-HEK DNA digested with Bam HI. (C) Track 1, 300 pg of BKV DNA digested with HindIII; track 2, 5 μg of BK-HEK DNA digested with HindIII. (D) Cleavage sites for EcoRI and HindIII on the BKV (MM strain) genome (13, 31).*

and D can be accounted for by the small size of these fragments which span the proximal, 5′ parts of the early and late gene regions. Since the RNA used for hybridization was of high molecular weight, it would preferentially hybridize with the large DNA fragments containing the longest regions of homology with it (fragments B, A_1, and A_2).

Analysis of BK-HEK clones. The BK-HEK cells were cloned by serial dilution and plating in multiwell dishes. Individual colonies were subcultured as clonal isolates. All clones exam-

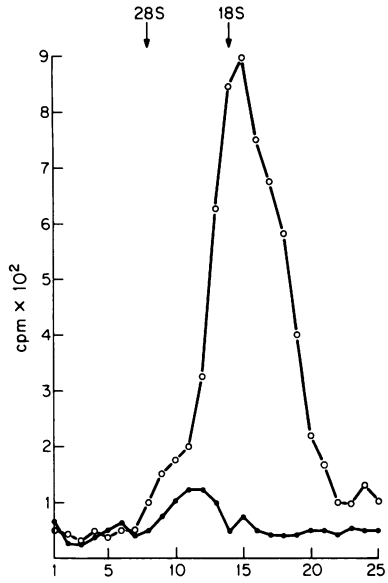

FIG. 2. *Sedimentation of BK-specific poly(A)-containing RNA from BK-HEK cells. Total poly(A)-containing ^{32}P-labeled RNA (10^7 cpm, specific activity, 5 × 10^5 cpm/μg) from normal and BKV-infected HEK cells was hybridized to filters containing denatured BKV DNA in 50% formamide at 37°C. The RNA was eluted, and a sample of each was counted: 1,300 cpm of normal HEK RNA and 9,800 cpm of BK-HEK bound to the filters. The RNA was precipitated with ethanol, dissolved in 0.01 M Tris-0.001 M EDTA-0.2% SDS (pH 7.2), heated for 2 min at 65°C, and sedimented through a 5 to 30% neutral sucrose gradient as described in the text. ^{32}P-labeled 28 and 18S were run in parallel gradients as sedimentation markers: ○, BK-HEK RNA; ●, uninfected HEK RNA.*

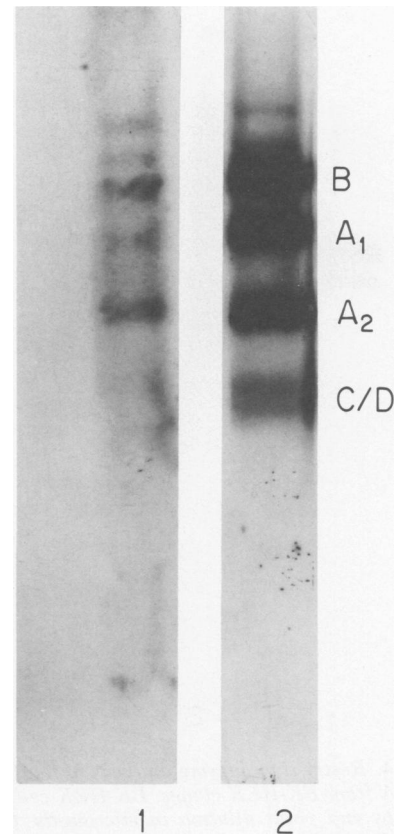

FIG. 3. *Hybridization of [^{32}P]poly(A)-containing RNA from BK-HEK cells to nitrocellulose filters containing separated fragments of BKV DNA. BKV DNA (2 μg) was digested with EcoRI and HindIII, fractionated on a 1% agarose gel, transferred to nitrocellulose filters, and hybridized to ^{32}P-BKV poly(A)-containing RNA (track 1) or ^{32}P-labeled BKV DNA (track 2).*

ined were T-antigen positive. Analysis of the BKV DNA in these cells, however, showed a striking difference from the parental BK-HEK cell line. We were unable to detect any BKV DNA in the *Bam* HI and *Eco*RI digests of total cellular DNA (data not shown) due, presumably, to the BKV sequences residing in very high-molecular-weight DNA fragments. Figure 4 shows a *Hin*dIII digest of the cellular DNA from two of our clones. Cleavage with this enzyme reveals one prominent band from both clones corresponding to part of the early region of the BKV DNA genome. Thus, these two clones appear to have lost all free BKV genomes and have retained early region sequences in an integrated form. This is supported by the detection in Fig. 4 of two *Hin*dIII fragments of high molecular weight which presumably correspond to BKV-cellular DNA junction fragments and may include sequences from the late gene region.

DISCUSSION

We have been studying the interaction between BK virus and HEK cells with the purpose of understanding how the virus functions in its natural host. Infection of HEK cells with BKV resulted in lysis of the cell monolayer and the appearance of cells capable of continuous growth within 30 days. These cells were uniformly positive for BKV T-antigen, produced infectious virus, and were able to form multiple layers in tissue culture. They grew well in 2% serum but were unable to form colonies in soft agar or form tumors in athymic nude mice (K. Takemoto, personal communication), consistent with the semitransformed nature of these cells. The inability of the BK-HEK cells to grow in soft agar is particularly interesting in view of the observation that they synthesize both BKV large T-

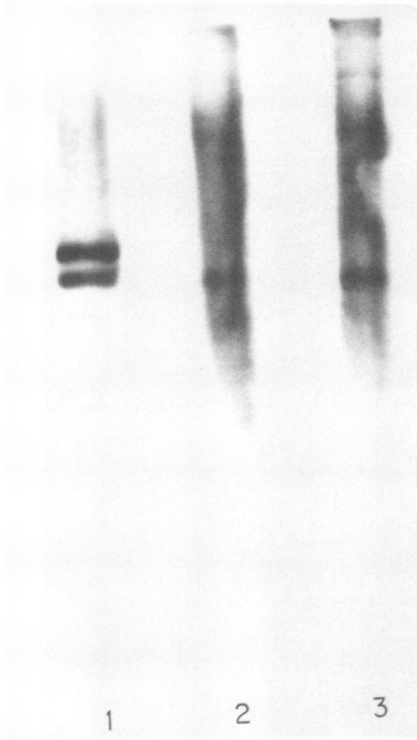

FIG. 4. *Restriction enzyme analysis of total cellular DNA from BK-HEK clones. BK-HEK cells were cloned by end point dilution in microwells. Clones from individual cells were expanded for DNA extraction and analysis as described in the text. These DNAs (20 μg) were digested with restriction enzymes, fractionated on a 1% agarose gel, transferred to a nitrocellulose filter, and hybridized to ^{32}P-labeled BKV DNA. Track 1, 300 pg of BKV DNA mixed with 20 μg of rhesus monkey cell DNA was cleaved with HindIII; track 2, clone 1 DNA cleaved with HindIII; track 3, clone 2 DNA cleaved with HindIII. In reconstruction experiments, 10 pg of BKV DNA mixed with 20 μg of rhesus monkey DNA and cleaved with EcoRI yielded an easily detectable band corresponding to BKV DNA III with this technique.*

and small t-antigens (A.F. Purchio, H. Linke and G. C. Fareed, unpublished observations). Studies of SV40-transformed rat cells suggested that a functional small t-antigen may be responsible for the ability of cells to grow efficiently in a semisolid medium (3, 24).

The uncloned BK-HEK cells carry BK viral genomes in a predominantly free or episomal state (Fig. 1). We were unable to detect integrated viral DNA sequences. Comparison of the density of the autoradiograms of cellular BKV DNA with that of known amounts of BK viral DNA allows us to estimate at least 20 copies of BKV DNA per cell. Cleavage of BK-HEK DNA with *Hin*dIII and other restriction nucleases and subsequent analyses of the digests indicate that the viral DNA is essentially wild type with respect to the cleavage sites for these enzymes (Fig. 1). The observation that these "semitransformed" cells carry free viral genomes and produce low amounts of infectious virus and are able to be maintained in tissue culture without any noticeable cytopathic effects or cell death may reflect a specific interaction of BKV with human kidney cells. The observation of a small fraction of virus-producing cells (V-antigen positive) would imply that these cells are actively replicating BKV DNA. Such cells may account for the majority of free BKV DNA detected, and the remaining nonproducing cells may contain only integrated sequences as observed in the cloned cell lines.

Our analysis of the viral RNA transcripts indicates that both early and late RNA species are present in the cytoplasm of these cells. Because our analysis was not carried out with separated strands of BK DNA, the possibility exists that we may be observing "anti-early" transcripts as have been described in the nucleus of SV40-infected monkey kidney cells (6). However, the fact that the RNA is both cytoplasmic and polyadenylated suggests that this is not the case because anti-early transcripts would not be expected to be processed and transported to the cytoplasm. Because the cells are only 1% positive for V-antigen, the late mRNA species may not be translated efficiently in these cells.

Our present analysis of the DNA from clonal isolates of the BK-HEK cells indicates a striking difference between the cloned and uncloned populations. We were unable to detect any free DNA sequences in the BK-HEK clones (Fig. 4). Because our reconstruction experiments indicate that we would easily be able to detect 0.5 copies of BKV DNA per cell, our failure to detect any bands with *Eco*RI cleavage of cellular DNA may be due to the fact that the BKV-specific fragment in the *Eco*RI digest is too large to be transferred under our conditions. Alternatively, the BKV DNA in these cells may be a tandem repeat of the "early region" which would have lost the *Eco*RI and *Bam* HI cleavage sites: these molecules could be carried in an unintegrated state but would be undetectable after incubation with *Eco*RI and *Bam* HI because supercoiled DNA does not transfer efficiently. The most plausible interpretation, however, is that these sequences are integrated within the host cell genome.

Our observation that these clones contain "early" BKV DNA sequences parallels the conclusion reached by Botchan et al. in one of their SV40-transformed rat cell clones (2). The data presented here for the state of the viral DNA in

the BK-HEK clones are in accord with independent studies of cloned HEK cell lines transformed by UV-irradiated BKV (G. Khoury and C.-J. Lai, personal communication). They differ from recent findings with clonal isolates of BKV-transformed human fetal brain cells which contain mainly free or episomal BKV genomes and are deficient in T-antigen expression (K. K. Takemoto, H. Linke, T. Miyamura, and G. C. Fareed, J. Virol., in press).

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