

Key words: BK virus/transformation/blotting/DNA integration/episomal state

Transformation of Human Embryonic Fibroblasts by BK Virus, BK Virus DNA and a Subgenomic BK Virus DNA Fragment

By M. P. GROSSI,¹ A. CAPUTO,¹ G. MENEGUZZI,² A. CORALLINI,¹ L. CARRA,¹ M. PORTOLANI,³ M. BORGATTI,⁴ G. MILANESI² AND G. BARBANTI-BRODANO^{1*}

¹ *Institute of Microbiology, University of Ferrara, via Luigi Borsari 46, I-44100 Ferrara,*

² *Institute of Biochemical Genetics, National Research Council, via S. Epifanio 14, I-27100 Pavia,*

³ *Institute of Microbiology, University of Ancona, via Grotte di Posatora, I-60100 Ancona and*

⁴ *Institute of Microbiology and Virology, University of Bologna, via San Giacomo 12, I-40126 Bologna, Italy*

(Accepted 3 August 1982)

SUMMARY

Human embryonic fibroblasts (HEF) have been transformed by BK virus (BKV) DNA and by u.v.-inactivated or live BKV alone or in association with methylcholanthrene (MTC). The transformed cells produced BKV large T and small t antigens as well as the cellular 53 kdal protein, detected by immunofluorescence and immunoprecipitation. After an initial phase of lysis and virus shedding, virus or its coat protein antigen could not be detected in transformed cells. All human transformed cell lines could be superinfected by BKV or BKV DNA, but their susceptibility to superinfection was 20- to 500-fold lower than normal HEF. BKV could be rescued by fusion of transformed cells with normal HEF or Vero cells and by transfection of normal HEF with total DNA and DNA extracted from the Hirt supernatant of transformed cells. Blot hybridization analysis of DNA from transformed cells showed a considerable amount of free BKV DNA in monomeric and polymeric forms. Integrated BKV DNA was absent in most cell lines but present in only small amounts in BKV-transformed cells treated with MTC. Analysis of free BKV DNA with various restriction endonucleases and by blot hybridization showed that monomeric forms were complete BKV genomes, whereas polymers contained both complete and defective or rearranged BKV DNA. Transformation of HEF was also obtained with a 3.7 kilobase (kb) fragment of the BKV genome, produced by sequential digestion of BKV with the restriction endonucleases *Hha*I and *Eco*RI. This fragment extends clockwise on the virus genome from 0 to 72.2 map units and contains the entire early region. Blot hybridization analysis of cells transformed by the *Hha*I/*Eco*RI 3.7 kb fragment showed two separate integrations of BKV sequences without free virus DNA.

INTRODUCTION

Cells permissive to papovaviruses support virus replication and lyse upon infection with virus or virus DNA. Nevertheless, mouse cells have been transformed by polyoma virus (Dulbecco & Vogt, 1960) and monkey and human cells by simian virus 40 (SV40) (Fernandes & Moorhead, 1965; Carp & Gilden, 1966; Koprowski *et al.*, 1962; Shein & Enders, 1962). BK virus (BKV), a human papovavirus isolated by Gardner *et al.* (1971), is oncogenic for hamsters and mice and transforms hamster, mouse, rat, rabbit and monkey cells in culture (for reviews see Howley, 1980; Padgett, 1980). Although human cells are permissive for BKV replication (Takemoto & Mullarkey, 1973), they have been transformed by BKV. In addition to abortive transformation of human foreskin and embryonic fibroblasts (Shah *et al.*, 1976; Portolani *et al.*, 1978), stable transformation of human embryonic kidney and foetal brain cells has been described (Purchio & Fareed, 1979; Takemoto *et al.*, 1979). In these reports, however, transformation was always

associated with persistent BKV infection and virus production. Moreover, BKV-transformed foetal brain cells were unable to produce BKV tumour (T) antigen (Takemoto *et al.*, 1979). Blot hybridization analysis of BKV-transformed human cells detected mainly, if not exclusively, full-length free BKV DNA in circular monomeric form, whereas the evidence for integrated virus DNA was uncertain (Purchio & Fareed, 1979; Takemoto *et al.*, 1979). In this report we describe stable transformation of human embryonic fibroblasts (HEF) by BKV and BKV DNA and characterize the state of virus DNA in transformed cells.

METHODS

Cells and virus. HEF were grown and propagated in Eagle's minimal essential medium (MEM) containing 2 × vitamins and amino acids and supplemented with 10% foetal bovine serum. Vero cells were grown and maintained in the same medium supplemented with 5% calf serum. Prototype BKV (Gardner strain) was grown in Vero cells and purified on to a cushion of KBr saturated solution, followed by two cycles of equilibrium density gradient centrifugation in CsCl (Maraldi *et al.*, 1975). Virus was titrated by haemagglutination of type O human erythrocytes and by the fluorescent antibody (FA) focus assay (Aaronson & Todaro, 1970) in HEF.

Virus inactivation by ultraviolet irradiation. To inactivate virus infectivity, purified BKV stocks in Petri dishes were exposed to u.v. light (wavelength 253.7 nm, surface dose 72 J/m²/s) for 16 min at 10 cm from the lamp.

Immunofluorescence. The indirect immunofluorescent (IF) test to detect BKV T and virus coat protein (VP) antigens was performed as described (Portolani *et al.*, 1975). Specific antiserum to BKV T was obtained from hamsters bearing tumours induced by BKV-transformed hamster cells. Specific antiserum to BKV VP was produced in rabbits hyperimmunized with purified BKV.

Immunoprecipitation and SDS-PAGE. Immunoprecipitation to detect BKV large T and small t antigens in transformed cells was carried out by the method of Kress *et al.* (1979) with minor modifications. Immune complexes were eluted from Sepharose beads and analysed by SDS-PAGE in composite gels of 7.5 and 15% acrylamide (Laemmli, 1970). Electrophoresis was carried out at room temperature at 20 mA for 6 to 8 h. The gels were stained with Coomassie Brilliant Blue, destained, dried and exposed to Kodak X-Omat R films for 2 to 5 days. The following molecular weight standards were used to calibrate the gels: phosphorylase B, mol. wt. 95 kilodaltons (K), bovine serum albumin (67K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21K) and lysozyme (14.5K).

Transformation of cells with BKV or BKV DNA. Sub-confluent HEF were infected with live or u.v.-irradiated BKV at 100 FA focus-forming units per cell. One day after infection of cells with live BKV, methylcholanthrene (MTC) was added to the medium of some cultures (5 µg/ml) and kept for 20 days. Control cell cultures were treated with MTC alone. The calcium phosphate precipitation technique (Graham & Van der Eb, 1973) as modified by Wigler *et al.* (1979) was used to infect cells with supercoiled BKV DNA molecules (FI) or with the *HhaI/EcoRI* BKV DNA fragments. Infection was carried out at 1 µg of virus DNA (made up to 10 µg with salmon sperm DNA) per 10⁶ cells. One day after infection with BKV or BKV DNA FI, the medium of some cell cultures was supplemented with hyperimmune rabbit serum to BKV (100 haemagglutination-inhibiting units per ml).

DNA extraction and purification. Virus DNA and total cell DNA were extracted as described (Chenciner *et al.*, 1980). Separation of low mol. wt. from high mol. wt. cell DNA was obtained by selective precipitation according to the method of Hirt (1967). To further purify the high mol. wt. DNA from contaminating molecules of monomeric virus DNA, the DNA extracted from the Hirt pellet was centrifuged in 5 to 20% continuous sucrose gradients at 26000 rev/min for 6 h in the SW27 rotor of a Spinco ultracentrifuge, according to the method of Rigby & Berg (1978).

Blot hybridization analysis. Blot hybridization analysis of transformed cells DNA was as described (Chenciner *et al.*, 1980).

Molecular weight and band intensity measurements. In blot hybridization experiments, the mol. wt. of cellular or virus DNA restriction fragments were measured using *EcoRI*-generated fragments of SPP1 phage DNA (McIntosh *et al.*, 1978) or BKV DNA fragments produced by digestion with various endonucleases as internal standards. To determine the number of genome equivalents of BKV DNA per diploid cell genome, various amounts of *EcoRI*-digested DNA from transformed cells were electrophoresed and blot-hybridized in the same gel together with increasing amounts of *EcoRI*-digested BKV DNA. Band intensities were evaluated by scanning the autoradiographs with a Kipp and Zonen densitometer. The number of genome equivalents was then determined by calculation of the ratio of weights of the peaks obtained.

Rescue of BKV from transformed cells. Two different techniques were employed to rescue BKV from transformed cells. Normal HEF or Vero cells were always used as indicators. (i) Fusion by polyethylene glycol (PEG) was performed in monolayers by seeding equal amounts of transformed and indicator cells. Twenty-four h later, 50% (w/w) PEG-1000 (Fluka) in Eagle's MEM was added for 1 min. Cells were then processed by the method of O'Malley & Davidson (1977). (ii) Transfection was performed by the calcium precipitation technique with 20 µg of

total transformed cell DNA per 10^6 indicator cells. Cell cultures set up for virus rescue were monitored for the presence of BKV in the supernatant medium by haemagglutination and in the cell monolayer by IF for BKV VP antigen.

RESULTS

Establishment of transformation

A few days after infection of HEF with BKV or BKV DNA FI, cells started to show cytopathic effects. Cell lysis was delayed in cultures infected with u.v.-irradiated virus or treated with anti-BKV serum. About 2 months after infection, when the cell monolayer was extensively affected by the virus cytopathic effect, small colonies began to grow up. They were isolated, propagated and finally gave rise to massive cell cultures. Several cell lines were established and were given the following designations: HEF-BKV, transformed by live BKV; HEF-BKV-UV, transformed by u.v.-irradiated BKV; HEF-BKV-MTC, transformed by live BKV in the presence of MTC; and L552, transformed by BKV DNA FI. Cells treated with MTC alone showed a transient toxic effect, but were not transformed. HEF were also infected with BKV DNA fragments obtained by a double digestion with *HhaI* and *EcoRI*. These enzymes cut BKV DNA once each, at 72.2 and 0 map units (m.u.) respectively. One of the two fragments was 3.7 kilobases (kb) long and contained the entire early region, spanning 0 to 72 m.u. in the clockwise direction. The other *HhaI/EcoRI* fragment was 1.5 kb long and extended clockwise from 72.2 to 100 m.u. HEF infected with either BKV DNA *HhaI/EcoRI* fragment did not show cytopathic effects. About 1 month after inoculation of cells with the *HhaI/EcoRI* 3.7 kb fragment, dense colonies began to appear over the cell monolayer. Single colonies were isolated which gave rise to several cell lines, one of which (L603) was studied in detail. No cell transformation was induced by the *HhaI/EcoRI* 1.5 kb fragment.

General properties of transformed cells

Transformed cells had an epithelioid morphology and grew well in both 10% and 2% serum, reaching a saturation density of 1 to 1.5×10^5 cells per cm^2 . Approximately 5 to 10% of giant polynucleated cells were distributed in the cell monolayer. The cell doubling time was 20 to 24 h in 10% foetal bovine serum. When plated on plastic flasks, transformed cells formed dense foci of multi-layered cells with a plating efficiency of 5 to 30%, but they did not form colonies in medium containing agar or methylcellulose. They did not induce tumours in nude mice when inoculated at the ratio of 5×10^6 to 10×10^6 cells per animal. Cytogenetic analysis showed the presence of the large metacentric chromosomes which are typical markers of human cells. Transformed cells were quasi-diploid with a modal chromosome number of 53. Ten to 15% of cells in the different cell lines had a sub-tetraploid chromosome content. The confluent monolayers were divided in the ratio of 1:2 and, between the 40th and 60th culture passages, transformed cells underwent a period of crisis comparable to that observed in SV40-transformed human cells (Girardi *et al.*, 1965), from which they recovered. Each cell line is now beyond the 100th culture passage and has been carried in culture for over 2 years.

Expression of virus-specific antigens

BKV T antigen was detected by IF in 98 to 100% of cell nuclei in all transformed cell lines. The 97K large T and the 17K small t antigens as well as the cellular 53K protein (Lane & Crawford, 1979) were detected in transformed cells by immunoprecipitation of cell homogenates with anti-BKV T serum followed by SDS-PAGE of the immune complexes (Fig. 1*a, b*). The electrophoretic pattern was very similar in all transformed cell lines. Several bands migrating ahead of large T antigen or just before small t antigen were observed in the immunoprecipitates of cell homogenates treated with anti-T serum (Fig. 1*a, b*). These bands probably represent truncated large T antigens and/or modified small t antigens. The latter were more prominent in the HEF-BKV-MTC cell line (Fig. 1*b*).

Before entering crisis, HEF transformed by BKV or BKV DNA FI produced VP antigen, detected by IF in 0.1 to 1% of cell nuclei. Haemagglutinating activity for human type O

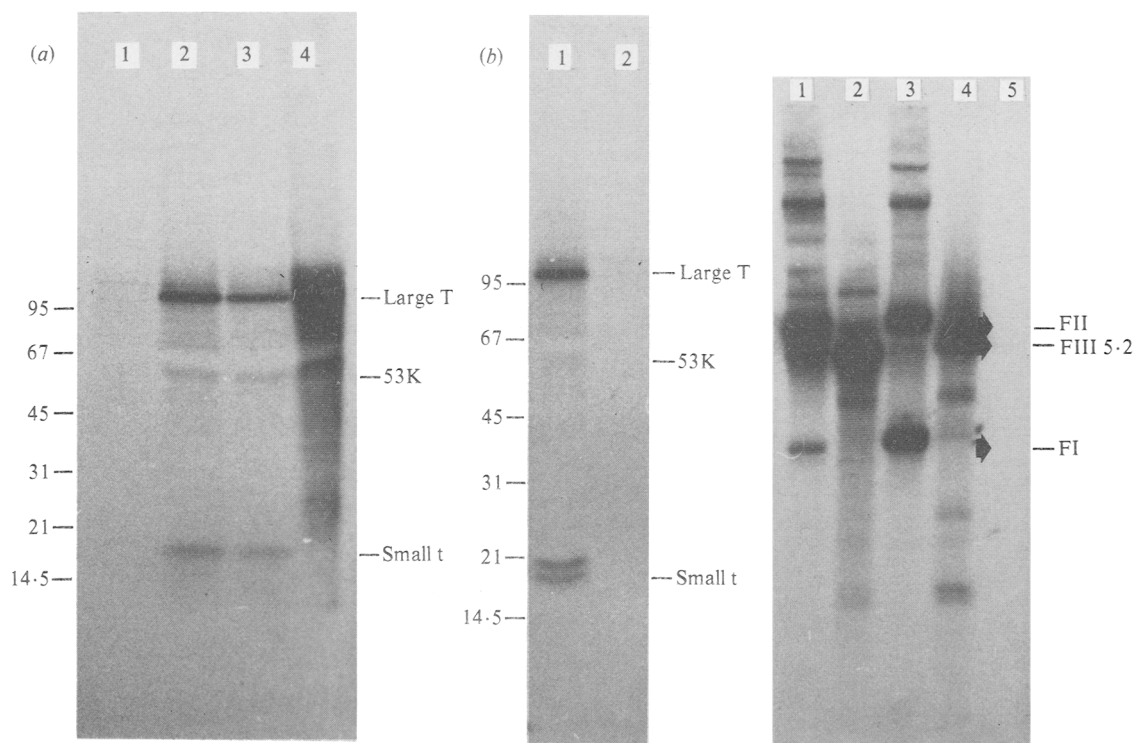


Fig. 1

Fig. 2

Fig. 1. (a) Immunoprecipitation of HEF-BKV cell homogenate with 30 μ l (lane 2) or 15 μ l (lane 3) of hamster serum to BKV tumour antigens and with 30 μ l of normal hamster serum (lane 1). The homogenate of BKV-transformed hamster cells was immunoprecipitated with hamster serum to BKV tumour antigens and electrophoresed in lane 4 as a positive control. Three bands corresponding to 97K, 53K and 17K appear in lanes 2 to 4, whereas they are absent in lane 1. Mol. wt. markers are, from top to bottom: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme. (b) Immunoprecipitation of HEF-BKV-MTC cell homogenate with hamster serum to BKV tumour antigens (lane 1) and with normal hamster serum (lane 2). Experimental conditions and labelling are as in (a). (a) and (b) show independent experiments.

Fig. 2. Blot hybridization of 32 P-labelled BKV DNA FI to total cellular DNA from HEF-BKV-UV (lanes 1, 2) and L552 (lanes 3, 4). Cellular DNA (20 μ g per lane) was analysed uncut (lanes 1, 3) or after cleavage with *Eco*RI (lanes 2, 4). Lane 5 contains 2.5 genome equivalents of BKV DNA FI, FII and FIII. These are barely visible because the large amount of virus DNA in transformed cell lines made necessary a short exposure of the autoradiogram (5 h). Their positions are indicated by arrows. The mol. wt. standard is expressed in kb.

erythrocytes was never found either in the supernatant medium or cell homogenates of BKV- or BKV DNA FI-transformed cells. After recovering from crisis, transformed cells remained uniformly negative for VP antigen synthesis. HEF transformed by the *Hha*I/*Eco*RI 3.7 kb fragment of BKV DNA produced neither VP antigen nor haemagglutinating activity.

Superinfection of transformed cells with BKV or BKV DNA

All human transformed cell lines could be superinfected by BKV or BKV DNA, but their susceptibility to superinfection was much lower than that of normal HEF. The results of superinfection experiments are presented in Table 1. Transformed cells were seeded on coverslips and superinfected with BKV or BKV DNA FI. At various time intervals after superinfection, monolayer cultures were tested by IF for the production of VP antigen; a small percentage of cells gave positive results. The proportion of VP-positive cells was highest 8 or 10 days after infection and decreased 15 days after infection. After superinfection with BKV or BKV DNA, HEF transformed by BKV DNA *Hha*I/*Eco*RI 3.7 kb fragment and normal HEF

Table 1. Superinfection of transformed HEF with BKV or BKV DNA

Cells	BKV*				BKV DNA*			
	Day 4	Day 8	Day 12	Day 15	Day 4	Day 8	Day 12	Day 15
HEF-BKV	0	4.1	2.3	0.2	0	0	0.8	0.2
HEF-BKV-UV	0	1.4	3.4	1.2	0	0	1.4	1.7
HEF-BKV-MTC	0.6	2.5	1.1	0	ND†	ND	0.6	0.6
L552	ND	3.2	3.6	0.8	ND	0	1.1	0
L603	2.4	16.3	35.8	46.1	1.4	11.8	25.6	38.2
Normal HEF	18.3	56.7	82.4	97.8	1.5	12.6	31.7	52.8

* Cells were superinfected with 100 focus-forming units of BKV per cell or with 1 µg of BKV DNA FI per 10⁶ cells. Results are expressed as the percentage of VP-positive nuclei after infection at the day indicated.

† ND, Not done.

showed 5 to 100 times and 20 to 500 times respectively more VP antigen-producing cells than HEF transformed by BKV or BKV DNA FI.

Rescue of BKV from transformed cells

BKV was rescued from all BKV- or BKV DNA FI-transformed cell lines upon fusion with normal HEF. In addition, virus rescue was obtained by transfection of normal HEF and Vero cells with total cellular DNA or Hirt supernatant DNA extracted from transformed cells. No virus was rescued from the L603 cell line transformed by BKV DNA *HhaI/EcoRI* 3.7 kb fragment.

State and arrangement of BKV DNA in transformed cells

Analysis by blot hybridization of the state and arrangement of BKV DNA in HEF transformed by BKV or BKV DNA FI showed a similar pattern in all transformed cell lines. When uncut total cellular DNA was analysed (Fig. 2, lanes 1 and 3), free, full-length supercoiled BKV DNA molecules (FI) and relaxed molecules (FII) were detected. The band migrating ahead of BKV DNA FII probably represented free defective virus DNA. In addition, hybridization bands were observed in the area of high mol. wt. DNA, suggesting the presence either of integrated virus sequences or polymeric forms of BKV DNA. Digestion of total cellular DNA with *EcoRI* produced a prominent hybridization band of full-length linear BKV DNA (FIII) (Fig. 2, lanes 2 and 4). The significance of the bands slower or faster than BKV DNA FIII in this digest will be discussed below.

To characterize the monomeric and high mol. wt. virus sequences better, blot hybridization analysis was performed on DNA extracted from the Hirt supernatant (HS-DNA) and pellet (HP-DNA) of the various transformed cell lines. Blot hybridization analysis of HS-DNA from HEF-BKV-UV is presented in Fig. 3. Uncut DNA showed two hybridization bands comigrating with BKV DNA FI and FII, while digestion with *EcoRI* or *HhaI* produced BKV DNA FIII. Digestion of supernatant DNA with *HindIII* (an enzyme which produces four cuts on BKV DNA) yielded the four fragments A, B, C and D, indicating that all the *HindIII* cleavage sites had been conserved in free monomeric BKV DNA. Analysis by blot hybridization of HS-DNA from HEF-BKV, HEF-BKV-MTC and L552 gave similar results, suggesting that in cells transformed by BKV or BKV DNA FI, free monomeric forms of the BKV genome are mostly complete, non-defective BKV DNA molecules.

We then analysed by blot hybridization the HP-DNA from transformed cells. When HP-DNA of HEF-BKV-UV was hybridized uncut or after digestion with *HincII*, *HpaII* or *HpaI* (three enzymes which do not cut BKV DNA) three slow-migrating bands were detected (Fig. 4, lanes 2 to 5). These had the same relative position in the uncut DNA and in the DNA preparations digested by the three non-cutting enzymes, indicating that they do not represent virus sequences integrated into cellular DNA, but, rather, polymeric forms of free BKV DNA. Digestion of HP-DNA with *BamHI*, *PstI* (which cut BKV DNA once) and *CfoI* (an isoschizomer of *HhaI*) produced a prominent band of full-length BKV DNA FIII (Fig. 4, lanes 7

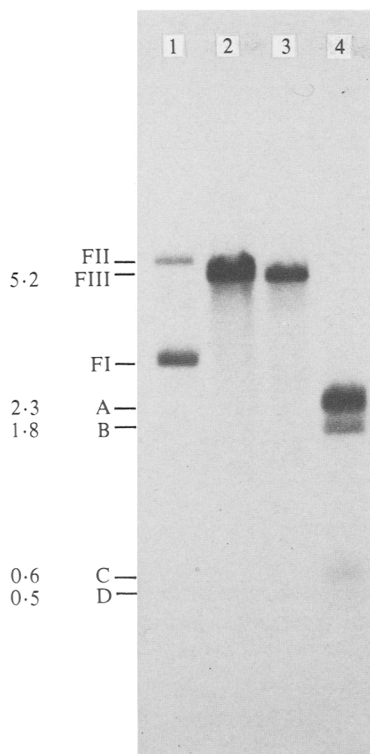


Fig. 3

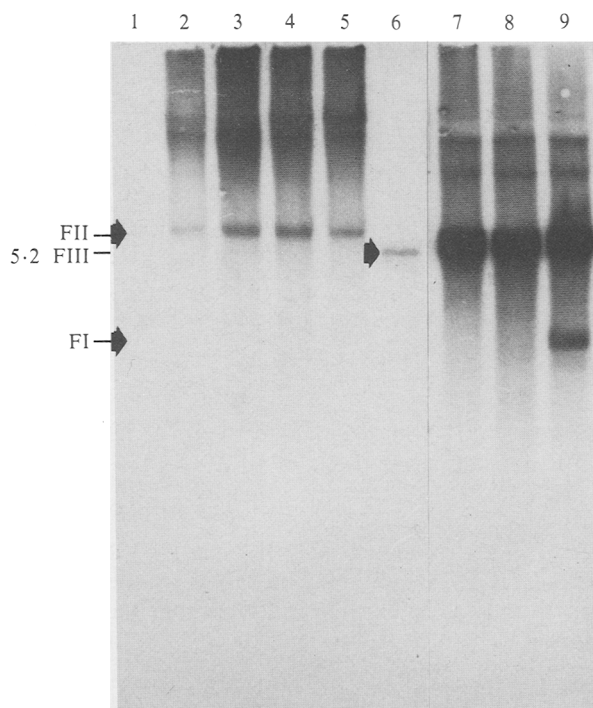


Fig. 4

Fig. 3. Blot hybridization of ^{32}P -labelled BKV DNA FI to the DNA extracted from the Hirt supernatant of HEF-BKV-UV. DNA was analysed uncut (lane 1), or after cleavage with *EcoRI* (lane 2), *HhaI* (lane 3) and *HindIII* (lane 4). Mol. wt. standards are expressed as kb. A, B, C and D are the four *HindIII* fragments, with mol. wt. of 2.3, 1.8, 0.6 and 0.5. The autoradiogram was exposed 5 h.

Fig. 4. Blot hybridization of ^{32}P -labelled BKV DNA FI to the DNA extracted from the Hirt pellet of HEF-BKV-UV. DNA (10 μg per lane) was analysed uncut (lane 2) or after cleavage with *HincII* (lane 3), *HpaII* (lane 4), *HpaI* (lane 5), *BamHI* (lane 7), *PstI* (lane 8) and *CfoI* (lane 9). Lane 1 contains 10 genome equivalents of BKV DNA FI and FII; lane 6 contains 10 genome equivalents of BKV DNA FIII. Mol. wt. standards are expressed in kb. The band comigrating with BKV DNA FII in lanes 2 to 5 is probably a residual free monomeric form of BKV DNA contaminating the DNA extracted from the Hirt pellet. The autoradiogram was exposed 13 h. Arrows point to weak bands in lanes 1 and 6.

to 9), indicating that virus genomes are organized in the polymers mostly as tandem head-to-tail arrays of full-length BKV DNA molecules. Only a few weak bands still migrated in the area of high mol. wt. DNA, suggesting that some of the polymeric BKV DNA forms had lost the cleavage sites for *BamHI*, *PstI* and *CfoI*.

HP-DNA from HEF-BKV-MTC showed a different pattern of hybridization. In addition to the slow-migrating polymeric forms, one band was present in the *HincII* digest and two bands in the *HpaII* digest (Fig. 5, lanes 3, 4) which were absent in the uncut DNA and in *HpaI*-digested DNA (Fig. 5, lanes 2, 5). These hybridization bands most likely contain BKV DNA sequences integrated into cellular DNA. Digestion of HEF-BKV-MTC HP-DNA with *EcoRI*, *BamHI*, *PstI* and *CfoI* produced full-length BKV DNA FIII as well as several bands migrating more slowly or ahead of linear BKV DNA (Fig. 5, lanes 7 to 10). These bands may originate from the virus integrations and represent junctions of virus and cellular DNA. Densitometric analysis, however, showed that some of these bands contain between five and ten times the amount of DNA present in the bands corresponding to integrated virus sequences. Most of the DNA of these hybridization bands should therefore derive from polymeric forms of BKV DNA. In this case, the bands migrating more slowly than BKV DNA FIII probably represent defective virus polymers that have lost the cleavage sites for *EcoRI*, *BamHI*, *PstI* and *CfoI*. The bands

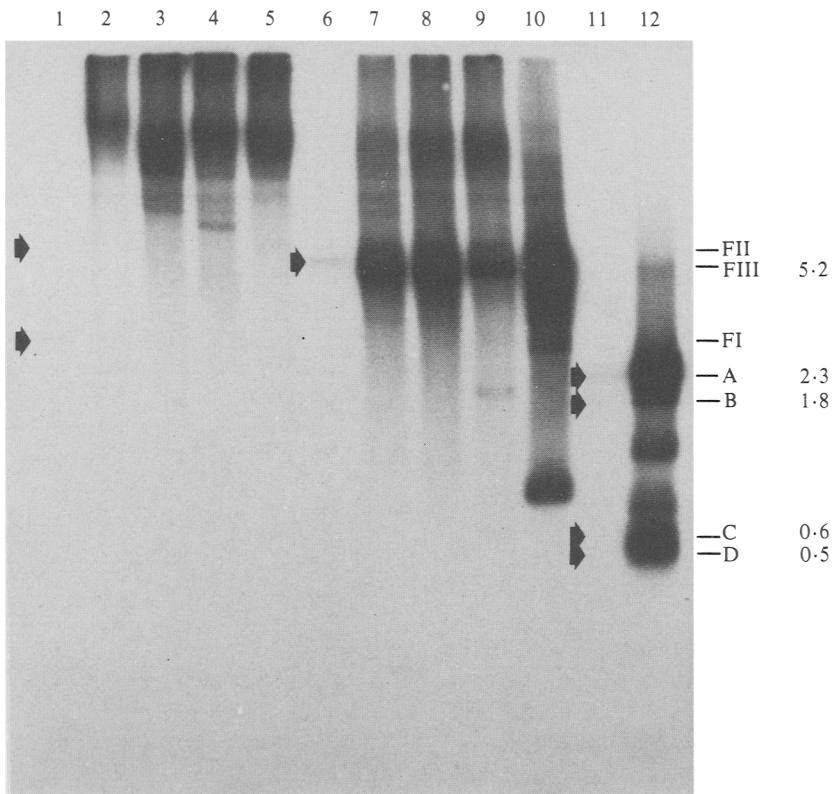


Fig. 5. Blot hybridization of ^{32}P -labelled BKV DNA FI to the DNA extracted from the Hirt pellet of HEF-BKV-MTC. DNA (10 μg per lane) was analysed uncut (lane 2) or after cleavage with *HincII* (lane 3), *HpaII* (lane 4), *HpaI* (lane 5), *EcoRI* (lane 7), *BamHI* (lane 8), *PstI* (lane 9), *CfoI* (lane 10) and *HindIII* (lane 12). Lane 1 contains 10 genome equivalents of BKV DNA FI and FII, lane 6 contains 10 genome equivalents of BKV DNA FIII and lane 11 contains 20 genome equivalents of BKV DNA cleaved with *HindIII*. Mol. wt. standards and the four *HindIII* fragments are as in Fig. 3. The autoradiogram was exposed 6 h. Arrows point to weak bands in lanes 1, 6 and 11.

migrating ahead of BKV DNA FIII may originate from virus polymers containing partially duplicated virus genomes with more than one cleavage site for the four enzymes which cut single-unit virus DNA only once. The bands migrating faster than BKV DNA FIII were more numerous and prominent in the *CfoI* digest of HP-DNA (Fig. 5, lane 10). The *CfoI* cleavage site at 72.2 m.u. is close to the origin of BKV DNA replication at 67.0 m.u. This suggests a duplication of this region of virus DNA, since during replication of papovavirus DNA in permissive cells rearrangement of virus sequences frequently leads to reiteration of the replication origin (Kelly & Nathans, 1977; Winocour *et al.*, 1980; Carroll *et al.*, 1981). The hybridization pattern of the *HindIII* digest of HEF-BKV-MTC HP-DNA was also rather complex (Fig. 5, lane 12): in addition to the four *HindIII* fragments, two bands were observed which may derive from virus integrations and/or from defective polymeric BKV DNA molecules lacking one or more *HindIII* restriction sites.

The single-strand specific nuclease S1 converts circular monomeric and catenated polymeric forms of papovavirus DNA to monomeric linear molecules (Germond *et al.*, 1974; Beard *et al.*, 1973; Chowdhury *et al.*, 1975; Law *et al.*, 1981). We found that BKV DNA FI and FII were converted to full-length linear molecules by digestion with nuclease S1 (data not shown). After S1 treatment of the HP-DNA from HEF-BKV-UV, HEF-BKV-MTC and L552, some of the high mol. wt. virus DNA sequences were converted to FIII (data not shown), indicating that some of the polymeric BKV DNA was probably arranged in supercoiled and/or relaxed catenated circular forms.

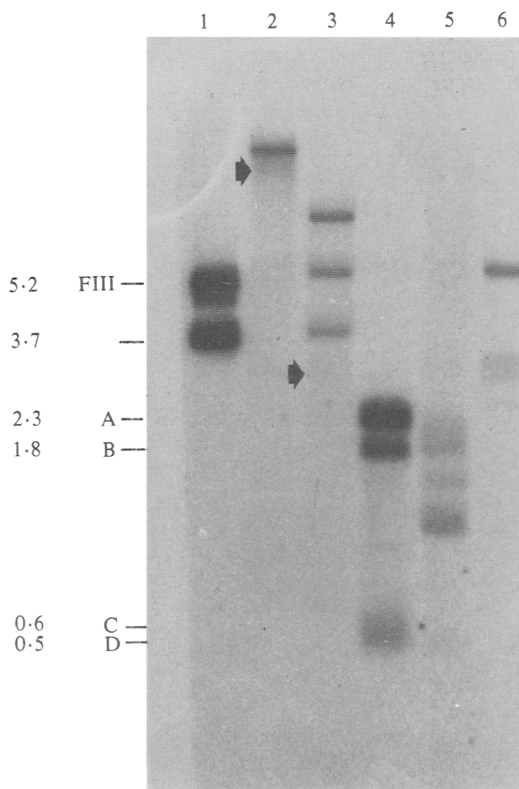


Fig. 6. Blot hybridization of ^{32}P -labelled BKV DNA FI to total cellular DNA from L603, transformed by the *HhaI/EcoRI* 3.7 kb fragment of BKV DNA. Cellular DNA (10 μg per lane) was analysed after cleavage with *HincII* (lane 2), *PstI* (lane 3), *HindIII* (lane 5) and *PvuII* (lane 6). Lane 1 contains 5 genome equivalents of BKV DNA FIII and of the *HhaI/EcoRI* fragment. The band migrating ahead of FIII represents defective virus DNA which is more apparent in this autoradiogram due to over-exposure. Lane 4 contains 10 genome equivalents of BKV DNA cleaved with *HindIII*. Mol. wt. standards and the four *HindIII* fragments are as in Fig. 2 and 3. Arrows point to weak bands in lanes 2 and 3. The autoradiogram was exposed 20 days.

To determine the quantity of the total (monomeric and polymeric) free BKV DNA in transformed cells, cellular DNA was digested with *EcoRI* and hybridized in the same gel together with different amounts of BKV DNA FIII. Densitometric analysis showed that HEF-BKV and HEF-BKV-MTC contained approximately 550 genome equivalents per diploid cell genome, while HEF-BKV-UV and L552 contained between 60 and 380 genome equivalents per diploid cell genome, depending on the different DNA preparation analysed.

Blot hybridization of L603 DNA digested with *HincII* showed the presence of two slow-migrating bands of integrated virus sequences (Fig. 6, lane 2). Digestion with *PstI* produced four bands (Fig. 6, lane 3), indicating that the *PstI* cleavage site at 31.9 m.u. was conserved in both virus integrations. Digestion with *HindIII* showed the presence of *HindIII* fragments B and D, whereas fragments A and C were lacking (Fig. 6, lane 5). The other bands present in the *HindIII* digest most probably represent junctions of virus and cellular DNA. Digestion of cellular DNA with *PvuII* produced four hybridization bands (Fig. 6, lane 6). *PvuII* has two cleavage sites on BKV DNA, at 9.9 and 72.3 m.u. Since the BKV DNA fragment used for transformation of L603 extends from 0 to 72.2 m.u., only the *PvuII* cleavage site at 9.9 m.u. was present in the transforming DNA and was maintained in both virus integrations. Therefore, in L603 one site of the virus insertions maps in *HindIII* fragment A between the *EcoRI* site at 0 m.u. and the first *PvuII* site at 9.9 m.u. Since fragment C is absent in the *HindIII* digestion pattern and the

integrated virus DNA contains all the information necessary to code for a complete large T antigen, the other sites of insertion of the integrated molecules should map in *Hind*III fragment C between 72.2 and 64.0 m.u., the latter site representing the region of the virus genome transcribing the 5' end of the early mRNA.

DISCUSSION

HEF have been transformed by BKV, BKV DNA FI and a BKV DNA fragment containing the early region of the BKV genome. The cells have many characteristics of the transformed state, although they are unable to grow in semi-solid media or induce tumours in nude mice. It is reasonable to assume that transformation was specifically induced and maintained by BKV, since (i) transformed cells produced both BKV large T and small t antigens, (ii) BKV was rescued from transformed cells and (iii) BKV DNA was detected in transformed cells by blot hybridization.

The state and organization of BKV DNA in HEF transformed by BKV and BKV DNA FI were unusual, in that the great majority of the virus DNA was detected in free circular monomeric and polymeric forms. No conclusive evidence was obtained of integrated BKV DNA except in HEF-BKV-MTC. Since polymers of SV40 DNA are formed during virus replication in permissive cells (Martin *et al.*, 1976; Rigby & Berg, 1978), it is likely that BKV DNA polymers were synthesized in human cells by DNA replication. Chia & Rigby (1981) have shown that in mouse cells infected by SV40 polymers of virus DNA are formed by replication and then recombine with one another. Recombination of BKV DNA polymers could contribute to the formation of the defective and rearranged virus DNA molecules observed in all BKV-transformed human cell lines. These molecules were more evident in HEF-BKV-MTC, as shown by reiterations of the replication origin, which could be correlated to the presence of truncated large T antigens and modified small t antigen in this cell line (Fig. 1*b*). In HEF-BKV-MTC, integration and rearrangement was probably favoured by treatment with methylcholanthrene, a carcinogen which interacts with DNA. Indeed, carcinogen-mediated amplification and rearrangement of SV40 integrated virus sequences have been recently described (Lavi, 1981). Transformation of HEF by the *Hha*I/*Eco*RI 3.7 kb virus DNA fragment resulted in a simple pattern of integration, since two independent insertions of virus sequences into cellular DNA were observed, both probably containing the entire early region of the BKV genome.

Our results, in agreement with previous reports (Purchio & Fareed, 1979; Takemoto *et al.*, 1979), indicate that stable transformation can be maintained in human cells by BKV in a free state, without any requirement for integration. The question arises why complete, infectious BKV DNA actively replicates in transformed human cells without VP antigen and virus production. Owing to extensive cell lysis after infection, cells are probably subjected to a strong selection allowing a few non-permissive or low-permissive survivors to develop the transformed phenotype. These transformed cells replicate BKV DNA and produce BKV T antigen without production of structural antigens and virus, possibly due to a block in the expression of late virus genes. The limited permissiveness of BKV-transformed human cells to BKV was confirmed by their low sensitivity to superinfection with BKV and BKV DNA at high multiplicity.

BKV-transformed human cells are not the only example of maintenance of stable transformation by free papovavirus DNA. In fact, transformation of human cells by BKV is reminiscent of the bovine papillomavirus system. This virus induces tumours in natural or experimental hosts and transforms rodent and bovine cells. Tumours and transformed cells do not contain integrated virus sequences, but only multiple copies of free monomeric and polymeric virus DNA (Lancaster & Olson, 1980; Moar *et al.*, 1981; Law *et al.*, 1981; Pfister *et al.*, 1981).

The episomal state of BKV DNA in BKV-transformed human cells may be relevant to the possible role of papovaviruses in human neoplasia. In fact, the presence of episomal BKV or SV40 DNA in human tumours and tumour cell lines has been reported (Pater *et al.*, 1980; Krieg *et al.*, 1981). Recently, we too have detected free monomeric BKV DNA in a human tumour of pancreatic islets (Corallini *et al.*, 1983).

This work was supported by Consiglio Nazionale delle Ricerche-Progetto Finalizzato 'Controllo della Crescita Neoplastica' and by N.A.T.O. Research Grant No. 284.81. The skilful technical assistance of Mrs Wilma Rossi-Pusinanti and Mr Cesare Mussi is gratefully acknowledged.

REFERENCES

- AARONSON, S. A. & TODARO, G. J. (1970). Infectious SV40 and SV40 DNA: rapid fluorescent focus assay. *Proceedings of the Society for Experimental Biology and Medicine* **134**, 103–106.
- BEARD, P., MORROW, J. F. & BERG, P. (1973). Cleavage of circular, superhelical simian virus 40 DNA to a linear duplex by S1 nuclease. *Journal of Virology* **12**, 1303–1313.
- CARP, R. I. & GILDEN, R. V. (1966). A comparison of the replication cycles of simian virus 40 in human diploid and African green monkey kidney cells. *Virology* **28**, 150–162.
- CARROLL, D., HANSEN, J. L., MARYON, E. B. & O'NEILL, F. J. (1981). SV40 defectives selected during low multiplicity passage on A172 human glioblastoma cells. *Virology* **112**, 461–471.
- CHENCINER, N., GROSSI, M. P., MENEGUZZI, G., CORALLINI, A., MANSERVIGI, R., BARBANTI-BRODANO, G. & MILANESI, G. (1980). State of viral DNA in BK virus-transformed rabbit cells. *Virology* **103**, 138–148.
- CHIA, W. & RIGBY, P. W. J. (1981). Fate of viral DNA in nonpermissive cells infected with simian virus 40. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 6638–6642.
- CHOWDHURY, K., GRUSS, P., WALDECK, W. & SAUER, G. (1975). Action of S1 nuclease on nicked circular simian virus 40 DNA. *Biochemical and Biophysical Research Communications* **64**, 709–716.
- CORALLINI, A., GROSSI, M. P., CARRA', L., CAPUTO, A., BALBONI, P. G., NEGRINI, M., MILANESI, G., FEDERSPIL, G. & BARBANTI-BRODANO, G. (1983). Episomal DNA of a BK virus variant in a human insulinoma. *Journal of Medical Virology* (in press).
- DULBECCO, R. & VOGT, M. (1960). Significance of continued virus production in tissue culture rendered neoplastic by polyoma virus. *Proceedings of the National Academy of Sciences of the United States of America* **46**, 1617–1632.
- FERNANDES, M. & MOORHEAD, P. (1965). Transformation of African green monkey kidney cultures infected with simian vacuolating virus 40 (SV40). *Texas Reports on Biology and Medicine* **23**, 242–258.
- GARDNER, S. D., FIELD, A. M., COLEMAN, D. V. & HULME, B. (1971). New human papovavirus (B.K.) isolated from urine after renal transplantation. *Lancet* *i*, 1253–1257.
- GERMOND, J. E., VOGT, V. M. & HIRT, B. (1974). Characterization of the single-strand-specific nuclease S1 activity on double-stranded supercoiled polyoma DNA. *European Journal of Biochemistry* **43**, 591–600.
- GIRARDI, A. J., JENSEN, F. C. & KOPROWSKI, H. (1965). SV40-induced transformation of human diploid cells: crisis and recovery. *Journal of Cellular and Comparative Physiology* **65**, 69–84.
- GRAHAM, F. L. & VAN DER EB, A. J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456–467.
- HIRT, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *Journal of Molecular Biology* **26**, 365–369.
- HOWLEY, P. M. (1980). Molecular biology of SV40 and the human polyomaviruses BK and JC. In *Viral Oncology*, pp. 489–550. Edited by G. Klein. New York: Raven Press.
- KELLY, T. J. JR. & NATHANS, D. (1977). The genome of simian virus 40. *Advances in Virus Research* **21**, 85–173.
- KOPROWSKI, H., PONTEN, J. A., JENSEN, F., RAVDIN, R. G., MOORHEAD, P. & SAKSELA, E. (1962). Transformation of cultures of human tissue infected with simian virus 40. *Journal of Cellular and Comparative Physiology* **59**, 281–292.
- KRESS, M., MAY, E., CASSINGENA, R. & MAY, P. (1979). Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. *Journal of Virology* **31**, 472–483.
- KRIEG, P., AMTMANN, E., JONAS, D., FISCHER, H., ZANG, K. & SAUER, G. (1981). Episomal simian virus 40 genomes in human brain tumors. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 6446–6450.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LANCASTER, W. D. & OLSON, C. (1980). State of bovine papilloma virus DNA in connective-tissue tumors. In *Viruses in Naturally Occurring Cancers*, pp. 223–232. Edited by M. Essex, G. Todaro & H. zur Hausen. New York: Cold Spring Harbor Laboratory.
- LANE, D. P. & CRAWFORD, L. V. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature, London* **278**, 261–263.
- LAVI, S. (1981). Carcinogen-mediated amplification of viral DNA sequences in simian virus 40-transformed Chinese hamster embryo cells. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 6144–6148.
- LAW, M. F., LOWRY, D. R., DVORETZKY, I. & HOWLEY, P. (1981). Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 2727–2731.
- MCINTOSH, P. K., DUNKER, R., MULDER, C. & BROWN, N. C. (1978). DNA of *Bacillus subtilis* bacteriophage SPP1: physical mapping and localization of the origin of replication. *Journal of Virology* **28**, 865–876.
- MARALDI, N. M., BARBANTI-BRODANO, G., PORTOLANI, M. & LA PLACA, M. (1975). Ultrastructural aspects of BK virus uptake and replication in human fibroblasts. *Journal of General Virology* **27**, 71–80.

- MARTIN, M. A., HOWLEY, P. M., BYRNE, J. C. & GARON, C. F. (1976). Characterization of supercoiled oligomeric SV40 DNA molecules in productively infected cells. *Virology* **71**, 28–40.
- MOAR, M. H., CAMPO, M. S., LAIRD, H. & JARRET, W. F. H. (1981). Persistence of non-integrated viral DNA in bovine cells transformed *in vitro* by bovine papillomavirus type 2. *Nature, London* **293**, 749–751.
- O'MALLEY, K. A. & DAVIDSON, R. L. (1977). A new dimension in suspension fusion techniques with polyethylene glycol. *Somatic Cell Genetics* **3**, 441–448.
- PADGETT, B. (1980). Human papovaviruses. In *Molecular Biology of Tumor Viruses II. DNA Tumor Viruses*, pp. 339–370. Edited by J. Tooze. New York: Cold Spring Harbor Laboratory.
- PATER, M. M., PATER, A., FIORI, M., SLOTA, J. & DI MAYORCA, G. (1980). BK virus DNA sequences in human tumors and normal tissues and cell lines. In *Viruses in Naturally Occurring Cancers*, pp. 329–341. Edited by M. Essex, G. Todaro and H. zur Hansen. New York: Cold Spring Harbor Laboratory.
- PFISTER, H., FINK, B. & THOMAS, C. (1981). Extrachromosomal bovine papillomavirus type 1 DNA in hamster fibromas and fibrosarcomas. *Virology* **115**, 414–418.
- PORTOLANI, M., BARBANTI-BRODANO, G. & LA PLACA, M. (1975). Malignant transformation of hamster kidney cells by BK virus. *Journal of Virology* **15**, 420–422.
- PORTOLANI, M., BORGATTI, M., CORALLINI, A., CASSAI, E., GROSSI, M. P., BARBANTI-BRODANO, G. & POSSATI, L. (1978). Stable transformation of mouse, rabbit and monkey cells and abortive transformation of human cells by BK virus, a human papovavirus. *Journal of General Virology* **38**, 369–374.
- PURCHIO, A. F. & FAREED, G. C. (1979). Transformation of human embryonic kidney cells by human papovavirus BK. *Journal of Virology* **29**, 763–769.
- RIGBY, P. W. J. & BERG, P. (1978). Does simian virus 40 DNA integrate into cellular DNA during productive infection? *Journal of Virology* **28**, 475–489.
- SHAH, K. V., HUDSON, C., VALIS, J. & STRANDBERG, J. D. (1976). Experimental infection of human foreskin cultures with BK virus, a human papovavirus. *Proceedings of the Society for Experimental Biology and Medicine* **153**, 180–186.
- SHEIN, H. M. & ENDERS, J. F. (1962). Multiplication and cytopathogenicity of simian vacuolating virus 40 in cultures of human tissues. *Proceedings of the Society for Experimental Biology and Medicine* **109**, 495–500.
- TAKEMOTO, K. K. & MULLARKEY, M. F. (1973). Human papovavirus BK strain: biological studies including antigenic relationship to simian virus 40. *Journal of Virology* **12**, 625–631.
- TAKEMOTO, K. K., LINKE, H., MIYAMURA, T. & FAREED, G. C. (1979). Persistent BK papovavirus infection of transformed human fetal brain cells. *Journal of Virology* **29**, 1177–1185.
- WIGLER, M., PELLICER, A., SILVERSTEIN, S., AXEL, R., URLAUB, G. & CHASIN, L. (1979). DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 1373–1376.
- WINOCCOUR, E., KESHET, I., NEDJAR, G. & VOGEL, T. (1980). Origins of SV40 genetic variation. *Annals of the New York Academy of Sciences* **354**, 43–52.

(Received 11 May 1982)