Molecular Cloning of Infectious DNA from Human Papovavirus BK in *Escherichia coli*

*(Accepted 22 April 1981)*

**SUMMARY**

Recombinant DNA constructed from unit length BK virus DNA and from several defective viral forms was cloned in *Escherichia coli* HB101. The cloned unit length BK virus DNA retained its infectivity for human embryonic kidney cells, whereas the cloned defective DNA showed no infectivity. Restriction endonuclease digestion of cloned defective DNA and purified virus DNA indicated that many of the defective forms contain re-iterated sequences.

Human papovavirus BK, first isolated by Gardner *et al.* (1971), has been shown, by serological studies, to be ubiquitous in the human population. Seroconversion usually occurs in childhood, and 80% of adults have antibodies to BK (Gardner, 1973; Shah *et al.*, 1973). Primary infection with BK virus can produce a persistent or latent infection. Reactivation of the virus can occur during immunodeficiency or immunosuppressive drug therapy (Gardner *et al.*, 1971; Coleman *et al.*, 1973; Howley *et al.*, 1975), or during pregnancy (Coleman *et al.*, 1980), and the virus is then excreted in the urine.

It is not known if BK virus is associated with any clinical diseases in man. However, because of its similarities with SV40 and polyoma virus (known oncogenic viruses) its potential role in human malignancy has been examined. BK can produce tumours when injected into newborn hamsters (Shah *et al.*, 1975; Chenciner *et al.*, 1980) and can transform hamster kidney cells in tissue culture (Major & di Mayorca, 1973; Wright *et al.*, 1976). The presence of BK virus DNA has been reported in DNA isolated from several human tumours (Fiori & di Mayorca, 1976). However, further studies (Wold *et al.*, 1978) have failed to confirm these observations.

DNA extracted from purified BK virus is heterogeneous in size and includes unit length infectious molecules as well as defective molecules. The defective molecules are incapable of infecting cells without the presence of a helper function, supplied by unit length DNA (Howley *et al.*, 1975). Similar defective forms of SV40 DNA have been extensively studied (Fareed & Davoli, 1977). However, BK virus grows well only in human foetal cells and this has limited studies on the formation and molecular structure of the defective forms of BK. In this paper, we report the molecular cloning of both infectious and defective BK virus DNA. Large amounts of these molecules are now available for study.

BK virus, GS strain (obtained from Coleman *et al.*, 1973, case no. 108) was grown in cultures of human embryo lung fibroblasts. The cultures were maintained at 37 °C with twice weekly medium changes. When the cytopathic effect was well advanced the culture medium was removed and the cells frozen at −70 °C. Virus was purified from culture supernatants by centrifugation over saturated KBr solution (Wright & di Mayorca, 1975). DNA was isolated from purified virus by lysis with 1% SDS (50 °C) and by isopycnic centrifugation in CsCl–ethidium bromide (Trilling & Axelrod, 1970). The plasmid vector, pDY160 (C. Duggleby, unpublished results), was constructed by inserting the chloramphenicol transacetylase gene from pBR325 (Bolivar, 1978) into pAT153 (Twigg & Sherratt, 1980). pDY160 specifies resistance to chloramphenicol, ampicillin and tetracycline; the single *Eco*RI site lies within the chloramphenicol resistance gene.
BK virus DNA (0.2 μg) was digested with EcoRI restriction endonuclease to give linear molecules (Pater et al., 1979). pDY160 was also cleaved with EcoRI and then treated with alkaline phosphatase to prevent self ligation (Ullrich et al., 1977). EcoRI-cleaved BK virus DNA and pDY160 were mixed and ligated with T4 DNA ligase at 10 °C for 20 h in 0.06 M-tris–HCl, 0.001 M-EDTA, 0.01 M-MgCl₂, 0.01 M-dithiothreitol, 0.1 M-ATP, pH 7.2. The recombinant DNA was transformed into Escherichia coli HB101 by the method of Kushner (1978) and transformants resistant to tetracycline (20 μg/ml) were selected. The presence of recombinant DNA in bacterial colonies was strongly suggested by their acquired sensitivity to chloramphenicol (150 μg/ml). All cloning experiments were carried out under category II containment conditions with the approval of the Genetic Manipulation Advisory Group.

Plasmid DNA was isolated from clones by the cleared lysate technique of Clewell & Helinski (1969), followed by isopycnic centrifugation in CsCl–ethidium bromide. Virus and recombinant DNA was examined by digestion with different restriction endonucleases, followed by electrophoresis in agarose gels.

Sixteen putative BK clones were investigated. The size of the inserted DNA was estimated by digestion of the recombinant plasmids with EcoRI, and determination of the electrophoretic mobility of the resulting fragments (Fig. 1 a). All the plasmids yielded a fragment of molecular size 5.4 kilobases (kb), corresponding to the vector (pDY160). Three of the recombinant plasmids (pAR11, pAR16 and pAR17) contained inserts of 5.2 kb corresponding to unit length BK virus DNA, and 13 contained smaller defective forms, e.g. pAR7 (4.6 kb) and pAR9 (4.4 kb). The DNA fragments were transferred to nitrocellulose sheets by the technique of Southern (1975) and hybridized with ‘nick-translated’ BK virus DNA as described by Heinaru et al. (1978). Only the smaller EcoRI fragments (the ‘inserts’) generated from each recombinant hybridized with BK virus DNA (Fig. 1 b).

Fried et al. (1979) have shown that cloned polyoma virus DNA, when released from recombinant plasmids, retains its biological activity and produces a lytic infection in permissive tissue culture cells. Similar experiments using the cloned BK virus DNA fragments were therefore carried out after EcoRI digestion of the relevant recombinant plasmids. The infectivity of this DNA was assayed in cultured human embryonic kidney cells by transfection in the presence of DEAE-dextran (Warden & Thorne, 1968). DNA from all three unit length clones (pAR11, pAR16 and pAR17) was infectious, with a cytopathic effect being observed 13 days after infection; electron microscopy indicated that normal virus particles were produced. For technical reasons no attempt was made to count plaques. DNA from the cloned defective forms did not produce virus in human embryonic kidney cells.

Selected recombinant molecules (pAR16, pAR7 and pAR9) were characterized by HhaI restriction endonuclease digestion and by EcoRI/HindIII double-digestion (Fig. 1 c). HhaI digestion of the vector (pDY160) gave a fragment of 1.3 kb (Fig. 1 c, track 1) plus many very small fragments (not shown). The observed 1.3 kb fragment contains the EcoRI site used for insertion of BK virus DNA in each recombinant plasmid. HhaI digestion of pAR16 (Fig. 1 c, track 2) gave two observed fragments and this, by comparison with the HhaI digest of pDY160, indicated that full length cloned BK virus DNA had retained its single HhaI site (see below). HhaI digestion of the smaller plasmids pAR7 and pAR9 (Fig. 1 c, tracks 3 and 4) gave different fragmentation patterns. However, a fragment of approx. molecular size 0.6 kb (arrowed in Fig. 1 c) was present in both these plasmids (a similar 0.6 kb fragment was also seen in HhaI digests of about 50% of the other cloned defective forms). The sum of the sizes of the major fragments obtained from the HhaI digest of pAR7 (5.4 kb) was less than the expected value of 5.9 kb [BK insert (4.6 kb) + vector fragment (1.3 kb)], whereas the sum of the HhaI fragments of pAR9 corresponded to the expected value of 5.7 kb [BK insert (4.4 kb) + vector fragment (1.3 kb)].
Fig. 1. Restriction enzyme analysis of BK–pDY160 recombinant plasmids. The DNA (0.5 to 2 μg) was digested with the enzymes indicated below and the products were analysed by electrophoresis in agarose gels (1% agarose in 0.04 M Tris–HCl, 0.02 M sodium acetate, 0.001 M EDTA pH 8.2) at 3 V/cm for 14 to 16 h. The DNA was stained with ethidium bromide (1 μg/ml) and the gels photographed under u.v. illumination. (a) Electrophoresis of EcoRI digests of the vector (pDY160), selected recombinant plasmids and BK virus DNA. Molecular size markers (bacteriophage λC1857 DNA digested with EcoRI/HindIII) are shown in the margin. (b) Autoradiogram of DNA transferred from gel (a) to a sheet of nitrocellulose and hybridized with 32P-labelled BK virus DNA. (c) Electrophoresis of restriction enzyme digests of the vector and selected recombinant plasmids: track 1, HhaI digestion of pDY160; tracks 2, 3 and 4, HhaI digests of pAR16, pAR7 and pAR9 respectively; track 5, EcoRI/HindIII double-digestion of pDY160; tracks 6, 7 and 8, EcoRI/HindIII double-digests of pAR16, pAR7 and pAR9 respectively; track 9, molecular size standards from a HindIII digest of λC1857. (d) Electrophoresis of restriction enzyme digests of purified BK virus DNA: track 1, molecular size standards as in (a); tracks 2 and 3, EcoRI and HhaI digestion of BK virus DNA.
A similar discrepancy was noted following EcoRI/HindIII double-digestion of these plasmids. Double-digestion of the vector alone gave two fragments (Fig. 1c, track 5) which were also present in similar digests of the recombinant plasmids. pAR16 yielded four additional fragments (Fig. 1c, track 6), showing that the cloned unit length BK virus DNA had retained its three HindIII sites (Pater et al., 1979). Double-digestion of pAR7 and pAR9 (Fig. 1c, tracks 7 and 8) gave similar fragmentation patterns except that pAR9 yielded an additional small fragment (0.5 kb). Both double-digests yielded a 0.6 kb fragment which corresponded to the 0.6 kb fragments in the HhaI digests (Fig. 1c, tracks 3 and 4). The additional fragment observed in the double-digest of pAR9 matched the 0.5 kb fragment in the corresponding HhaI digest. Again, the sum of the sizes of the pAR7 double-digest fragments was approx. 0.6 kb less than the expected value, while the sum of the pAR9 fragments corresponded with the expected value.

In both the HhaI and EcoRI/HindIII digests of pAR7, the difference between the estimated and expected total of fragment sizes could be accounted for if the 0.6 kb fragment was present in 2 molar proportions. Therefore, photographic negatives of the gels were examined with a Gilford scanning spectrophotometer to estimate the relative proportions of each fragment. The areas under the peaks corresponding to each fragment were compared. The 0.6 kb fragment observed in both HhaI and EcoRI/HindIII digests of pAR7 was present in 2 molar proportions, but this fragment was present in 1 molar proportions in corresponding digests of pAR9. All other fragments were present in 1 molar proportions. It is therefore proposed that pAR7 contains a repeated region within the BK virus DNA which includes pairs of HindIII and HhaI sites that are each separated by 0.6 kb. The presence of 0.6 and 0.5 kb fragments in both HhaI and EcoRI/HindIII digests of pAR9 suggests that part of this repeated region may also be present in pAR9. Experiments to confirm this proposal by DNA sequence analysis are in progress.

The presence of duplicated fragments in restriction digests of cloned defective DNA prompted further investigation of the original BK virus DNA, which was digested with EcoRI or HhaI (Fig. 1d). Digestion with EcoRI cleaved unit length DNA and most of the defective forms only once (Fig. 1d, track 2); similar results (not shown) were obtained after BamHI and KpnI digestion. The defective forms of BK virus DNA had molecular sizes of 4.9 kb, 4.6 kb and 4.4 kb compared with 5.2 kb for the full length genome. HhaI cleaved full length BK virus DNA only once (Fig. 1d, track 3) but digested the defective forms to give several DNA fragments of about 3.5 kb (the predominant fragment had a molecular size of 3.2 kb) and low amounts of the expected small fragment (0.6 kb; rather faint in this gel and indicated by an arrow in Fig. 1d). The molecular sizes of the HhaI fragments of defective DNA, when added together, do not account for all of the DNA present in these molecules (4.9, 4.6 and 4.4 kb). Re-iteration of the 0.6 kb fragment or the presence of other, less predominant, small fragments would be necessary to account for the additional DNA in these defective molecules. This shows that the duplicated regions of pAR7 and pAR9 were not generated during replication in E. coli.

Re-iterated sequences have also been detected in defective SV40 DNA molecules (Fareed & Davoli, 1977). Some of these repeated sequences include highly repetitious monkey DNA. The results obtained from the molecular cloning of the defective forms of BK virus DNA (strain GS) indicate that these molecules have a similar structure to defective SV40 DNA. The possible presence of human chromosomal DNA in defective BK virus DNA is currently being investigated.

We thank Dr C. J. Duggleby for the construction of plasmid pDY 160.
REFERENCES


Short communications


(Received 29 December 1980)