Cooperation in Oncogenesis between BK Virus Early Region Gene and the Activated Human c-Harvey ras Oncogene

By A. CORALLINI, M. PAGNANI, A. CAPUTO, M. NEGRINI, G. ALTAVILLA, L. CATOZZI AND G. BARBANTI-BRODANO.

Institutes of 1Microbiology, 2Pathologic Anatomy and 3Radiology, School of Medicine, University of Ferrara, Via Luigi Borsari 46, I-44100 Ferrara, Italy

(Accepted 7 May 1988)

SUMMARY

Rapidly growing, undifferentiated brain tumours were induced in newborn Syrian hamsters by intracerebral inoculation of a recombinant DNA (pBK/c-rasA) carrying the BK virus (BKV) early region gene and the activated human c-Harvey-ras (c-Ha-ras) oncogene. Neither of the two genes inoculated alone nor recombinant DNA of the BKV early region gene and the normal human c-Ha-ras proto-oncogene were tumourigenic. Tumour-derived cell lines propagated in culture were immortalized and had growth characteristics consistent with a fully transformed phenotype. Tumours and tumour cell lines contained pBK/c-rasA sequences integrated into cellular DNA and expressed BKV- and c-Ha-ras-specific transcripts as well as BKV T antigen and c-Ha-ras p21. These findings are discussed in relation to a possible cooperation or synergism between BKV and cellular oncogenes in human neoplasia.

Human viruses displaying transforming activity and experimental oncogenicity are widely investigated for their potential association with human tumours. BK virus (BKV) is a human papovavirus ubiquitous in human populations. Primary infection generally occurs during childhood and is followed by a persistent, latent infection which is reactivated under conditions of impaired immunological response. BKV and BKV DNA transform hamster, rat, mouse, rabbit, monkey and human cells in vitro. BKV is highly oncogenic when inoculated intracerebrally (i.c.) or intravenously (i.v.) into immunosuppressed or immunocompetent hamsters, causing tumours in 73 to 88% of animals. The virus has a marked tropism for specific organs, since tumours observed in infected animals belong essentially to only three histotypes: ependymomas and choroid plexus papillomas, tumours of pancreatic islets and osteosarcomas (for review, see Yoshiike & Takemoto, 1986). BKV DNA is not oncogenic when inoculated subcutaneously (s.c.) or i.v., but induces ependymomas at low frequency (5-1%) when injected i.c. in hamsters (Corallini et al., 1982).

BKV DNA, RNA and T antigen have been detected in human brain tumours and tumours of pancreatic islets (Caputo et al., 1983; Pagnani et al., 1986; Corallini et al., 1987). Since BKV DNA in the neoplastic tissue is free, BKV was recovered from tumours by transfection of total tumour DNA into human embryonic fibroblasts. Restriction endonuclease mapping of isolated viruses showed that they are all similar to each other and different from the wild-type in that they bear an insertion and a deletion in the early region of the genome. Nucleotide sequence analysis of one of these variants (Pagnani et al., 1986) showed that the inserted nucleotides can form an insertion sequence (IS)-like structure (Finnegan, 1985). This transposable element might be involved in oncogenesis by excision from viral DNA and insertion into the cell genome, thereby inducing a mutagenic effect or activating the expression of cellular oncogenes. The latter hypothesis is supported by the observation that the IS contains in its loop two of the three viral transcriptional enhancers.
BamHI BamHI
\[ \text{EcoRI} \]
\[ \text{Amp} \]
\[ \text{Tet} \]
\[ pBK \]
7.8 kb
\[ pBR/c-rasA 10.9 kb \]
\[ pBR/c-rasN 10.7 kb \]
\[ \text{BamHI} \]
\[ \text{EcoRI} \]
\[ \text{Amp} \]
\[ \text{Tet} \]
\[ pBK/c-rasA 14.4 kb \]
\[ pBK/c-rasN 14.2 kb \]
\[ \text{BamHI} \]
\[ \text{En, BKV transcriptional enhancer elements; Or, BKV origin of DNA replication; BamHI and EcoRI indicate enzyme cleavage sites. E and L mark the early and late regions, respectively.} \]

Transformation by papovaviruses is induced and maintained by T antigen (Tegtmeyer, 1980). Although human cells transformed by BKV or BKV early region DNA and expressing BKV T antigen grow as immortalized cell lines, they never show a completely transformed phenotype (Purchio & Fareed, 1979; Takemoto et al., 1979; Grossi et al., 1982; A. Corallini, unpublished observations). Human embryo kidney cells transfected with a recombinant plasmid containing the BKV wild-type early region and the adenovirus 12 early region 1A gene are fully transformed and grow as a continuous cell line (Vasavada et al., 1986), which suggests that, at least in human cells, BKV T antigen can cause only a partially transformed phenotype and must interact with other oncogenic functions to induce complete transformation. Therefore we established an experimental model to evaluate the possible cooperation in tumourigenicity between BKV and cellular oncogenes. For this purpose we constructed recombinant DNA molecules containing the BKV early region and either the human activated c-Ha-ras oncogene (c-rasA) or the normal c-Ha-ras proto-oncogene (c-rasN) and assayed their oncogenic potential in hamsters. c-rasA and c-rasN are genomic clones derived from T24 human bladder carcinoma cells or normal human foetal liver respectively. They are essentially identical in their restriction maps and nucleotide sequences, except that c-rasA has a substitution, responsible for its activation, in the 12th codon of the first exon and c-rasN has a deletion of 200 nucleotides in the 3' portion of the DNA segment, outside the coding sequences of c-Ha-ras.

Techniques for DNA and RNA hybridization as well as immunoblotting, immunoperoxidase staining and indirect immunofluorescence have been described (Pagnani et al., 1986; Corallini et al., 1987). Recombinant DNAs (Fig. 1) were prepared according to standard cloning methods.
Fig. 2. Hamster brain tumour induced by pBK/c-rasA. (a) Macroscopic appearance showing a large neoplastic mass projecting from the brain. (b) Histology (Mayer’s haematoxylin) and immunoperoxidase staining of c-ras p21 with RAP-5 monoclonal antibody. The tumour consists of highly atypical, polymorphic cells with frequent giant cells. The p21-reacting product appears as a dark substance concentrated in the cytoplasm and plasma membrane of tumour cells. Bar marker represents 50 μm.

(Maniatis et al., 1982). The vector pBK (Milanesi et al., 1984), containing the BKV wild-type (Gardner strain) early region, was constructed by ligating the large BamHI/EcoRII fragments of the BKV genome and pML, a deletion derivative of pBR322 (Lusky & Botchan, 1981). c-rasA and c-rasN were first cloned in lambda Charon 4A phage (Santos et al., 1982), then subcloned into the unique BamHI site of the plasmid pBR322 (pBR/c-rasA and pBR/c-rasN), pBK/c-rasA and pBK/c-rasN were constructed by inserting the 6.6 kb BamHI fragment, containing c-rasA, or the 6.4 kb BamHI fragment, containing c-rasN, into the unique BamHI site of pBK. In these experiments we used recombinants of c-rasA and c-rasN with the same orientation of transcription and promoter position relative to BKV transcriptional enhancers.

In the first experiment 12 newborn hamsters (less than 24 h old) were inoculated i.c. with 2 μg of pBK/c-rasA DNA dissolved in phosphate-buffered saline (20 μl per animal). Eight of these hamsters (66.6%) developed brain tumours with a latency period of 2 to 8 weeks. In a second confirmatory experiment groups of 21 newborn hamsters were inoculated i.c. with BKV DNA, pBK, pBK/c-rasA, pBK/c-rasN, pBR/c-rasA or pBR/c-rasN. Each hamster in the first group received 1 μg of BKV DNA. Animals in the other groups were injected with equimolar proportions of the recombinants with respect to this amount of BKV DNA. Between 2 and 6 weeks after inoculation 16 (76.2%) of the animals inoculated with pBK/c-rasA developed brain tumours. None of the animals in the other groups had tumours after an observation period of 1 year. Tumours had a rapid exophytic growth, protruding from the brain surface (Fig. 2a). Histologically they were all similar, consisting of highly atypical, polymorphic cells (Fig. 2b). By immunohistochemical analysis they were negative for desmin, glial acidic fibrillary protein, S-100 protein and cytokeratin, whereas they reacted with a monoclonal antibody to vimentin, a typical marker of mesenchymal cells (Osborn & Weber, 1983). They were therefore diagnosed as undifferentiated pleomorphic sarcomas. Cell lines were derived from tumours explanted in vitro and cultured in Dulbecco’s minimum essential medium supplemented with 10% foetal bovine serum. They were propagated beyond 200 population doublings and showed high saturation density, short doubling time, low serum dependence, high cloning efficiency on plastic and in agar and a disorganized and fragmented pattern of actin cables when tested with rhodamine-conjugated phalloidin (Wulf et al., 1979) (data not shown).
Total cellular DNA from brain tumours and tumour-derived cell lines was hybridized to a $^{32}$P-labelled pBK/c-rasA probe. Cellular DNA had been previously digested with EcoRI and BamHI which have one and two cleavage sites respectively in pBK/c-rasA (Fig. 1). Both EcoRI and BamHI hybridization patterns (Fig. 3a and b) showed that all tumours contained pBK/c-rasA integrated sequences, as either single or tandem insertions. The latter are indicated by bands comigrating with full-length linear pBK/c-rasA (14.4 kb) in EcoRI-digested cellular DNAs or with pBK (7.8 kb) and c-Ha-rasA (6.6 kb) in BamHI-cleaved DNAs. Tumours contained one to six genome equivalents of exogenous DNA per diploid cell genome, as determined by densitometric scanning of hybridization bands. The integration pattern was different in each tumour. Moreover, after a short time of autoradiographic exposure no hybridization bands appeared in DNA from early passage normal hamster embryo cells (Fig. 3a and b, lanes 9), indicating that in tumours hybridization occurs primarily with the transfected DNA and not with the endogenous c-ras sequences of the hamster host cells. EcoRI and BamHI hybridization profiles of tumour cell lines were very similar to those of the primary tumours from which each cell line originated (data not shown).

The 1.2 kb c-Ha-ras-specific transcript (Yaswen et al., 1985) was detected in tumour cell lines (Fig. 4a, lanes 2 to 6) by hybridization of total cytoplasmic RNA to the c-Ha-ras cDNA probe (Fasano et al., 1983), whereas BHK cells, a spontaneously transformed hamster cell line, were negative (Fig. 4a, lane 1). Two major bands of 2.3 and 2.6 kb, corresponding in size to large T
antigen 19S and small t antigen 20S mRNAs (Seif et al., 1979), were detected by hybridization of total cytoplasmic RNA from tumour cell lines to the 32P-labelled BKV DNA 3·2 kb PvulI fragment, containing the entire early region (Fig. 4b, lanes 2 to 6). These bands comigrated with the hybridization bands observed in L578 cells transformed by BKV DNA (Fig. 4b, lane 1). Bands that migrated faster may represent transcripts that were initiated at the BKV early promoter and terminated at a cellular stop signal.

c-Ha-ras p21 was detected by immunoperoxidase staining with RAP-5 monoclonal antibody (Thor et al., 1984) in all pBK/c-rasA-induced brain tumours. Normal hamster brain and 43 out of 45 BKV-induced tumours (22 ependymomas, 13 tumours of pancreatic islets, five osteosarcomas and five subendocardial sarcomas from previous experiments) which were used as controls were negative, whereas two control tumours showed a low expression of p21, probably derived from endogenous c-ras genes. The immunoperoxidase reaction was localized in the cytoplasm and cytoplasmic membrane of virtually all tumour cells (Fig. 2b). A prominent band of Mr 21000 was detected by immunoblotting when anti-Ha-ras p21 Y13-259 monoclonal antibody (Furth et al., 1982) was reacted with lysates of tumours and tumour cell lines (Fig. 5, lanes 2 to 6 and 8 to 13). Normal hamster skin, chosen as a control owing to the mesenchymal origin of tumours, was negative (Fig. 5, lane 1) and spontaneously or BKV-transformed hamster
cells showed only a weak p21 expression (Fig. 5, lanes 7 and 14), suggesting that in tumour cells p21 is expressed mostly by the acquired c-Ha-ras oncogene. BKV T antigen was detected by indirect immunofluorescence in cell nuclei of all primary tumours (data not shown) and tumour cell lines (Fig. 6). Eight out of 10 sera from tumour-bearing hamsters, tested by immunofluorescence, were found to contain antibodies to BKV T antigen.

All brain tumours induced by pBK/c-rasA were similar in histotype and showed the histochemical markers of undifferentiated sarcomas, suggesting that pBK/c-rasA displays tissue-specific expression and mesenchymal cells are the primary target for its tumourigenicity. According to the multifactorial concept of carcinogenesis, these findings indicate that in hamsters cooperation of the BKV early region gene with another oncogenic function is required for neoplastic transformation of mesenchymal cells. This conclusion is in agreement with the lack or low degree of BKV DNA oncogenicity after s.c. or i.c. inoculation in hamsters (Corallini et al., 1982) and the inability of activated c-Has-ras to induce complete transformation of primary rodent cells (Land et al., 1983; Ruley, 1983; Newbold & Overell, 1983). Cell lines derived from tumours and propagated in culture were immortalized and showed a fully transformed phenotype, indicating that the combination of BKV and c-Ha-rasA oncogenic functions is able to maintain stable transformation.
As over-expression of the activated c-Ha-ras oncogene induces transformation of early passage rodent cells in vitro (Spandidos & Wilkie, 1984), we cannot exclude the possibility that stimulation of c-Ha-ras expression by cis-acting BKV transcriptional enhancers contributes to the oncogenic effect, although it seems unlikely for the following reasons. Over-expression of c-Ha-ras was obtained by closely linking the very strong promoter–enhancer element of Moloney murine sarcoma virus (Laimins et al., 1982) to the oncogene in sense orientation, whereas BKV enhancer repeats are weak in rodent cells (Rosenthal et al., 1983). In addition, in our recombinants BKV enhancers are positioned about 4.5 kb upstream of the c-Ha-ras promoter and in antisense orientation relative to the c-Ha-ras direction of transcription (Fig. 1), a situation producing a low enhancing effect (Rosenthal et al., 1983).

The mechanism of the interaction between the two transforming genes was clarified by cotransfection of primary hamster embryo cells with BKV DNA and either pBR/c-rasA or pBR/c-rasN (Pagnani et al., 1988). Cotransfected molecules produced a number of foci comparable to that produced by pBK/c-rasA and pBK/c-rasN respectively, demonstrating that the BKV early region gene and c-Ha-ras independently express functions that cooperate in transformation. Moreover, transfection of BKV DNA-transformed hamster embryo cells with pBR/c-rasA and of pBR/c-rasA-transformed cells with BKV DNA induced foci of rapidly growing, morphologically altered cells. These results indicate that the second transfected molecule is able to confer additional phenotypic properties to transformed cells and that a fully transformed phenotype can be caused by the BKV early region gene and c-Ha-ras acting separately in two steps. Cell lines obtained from cotransfected and supertransfected foci expressed high levels of c-ras p21 and produced BKV T antigen. As to the nature of cooperation between the two transforming genes, since c-Ha-rasA confers morphological alteration and anchorage independence to early passage rodent cells and can be complemented by the immortalizing functions of polyoma virus large T antigen (Land et al., 1983), in our experimental model immortalization may involve BKV large T antigen.

Cooperation of wild-type BKV and oncogenes in induction of the neoplastic phenotype may be relevant to the possible role of BKV in natural human oncogenesis. Moreover, BKV variants associated with human tumours contain in the early region a putative transposable element including in its sequence two viral enhancer repeats (Pagnani et al., 1986). Several cellular oncogenes are involved in different steps of human carcinogenesis (Slamon et al., 1984; Bishop,
would seem to be worthwhile to explore further the interaction of wild-type BKV and its variants with other cellular oncogenes, in order to gain more information on the possible cooperation or synergism between viral and oncogene functions in human neoplasia.

We thank A. Bevilacqua and M. Bonazzi for animal care, technical assistance and typing the manuscript. We are grateful to Professor T. Wieland for the gift of rhodamine-conjugated phalloidin and to Dr J. Schiorn for the gift of RAP-5 monoclonal antibody. This study was supported by the Italian National Research Council (Special Projects ‘Oncology’ and ‘Genetic Engineering and Molecular Basis of Hereditary Diseases’) and by the Associazione Italiana per la Ricerca sul Cancro.

REFERENCES
Short communication


(Received 30 March 1988)