Molecular and Biological Properties of BK Virus-IR, a BK Virus Variant Isolated from a Human Tumor

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We describe the molecular and biological properties of BK virus (BKV)-IR, a new BKV variant isolated from a human tumor of pancreatic islets. BKV-IR bears a 253-base-pair (bp) deletion and an 80-bp insertion in the early region of the genome. The deletion abolishes the expression of small-t antigen. The inserted sequences, grouped in four clusters, produce rearrangements in the first and second enhancer elements. They are bound by 12-bp direct repeats and could form a 217-base stem-loop structure suggestive of an insertion sequence. As compared with wild-type BKV, BKV-IR transformed hamster cells with a reduced efficiency and induced ependymomas in hamsters at a lower frequency and with a longer latency period. Tumors induced by BKV-IR, however, showed features of higher malignancy. The possible role of the insertion sequence-like element in transformation by BKV-IR is discussed.

BK virus (BKV) is a human papovavirus first isolated from an immunosuppressed patient (14). BKV infection is common in normal human populations (13, 31, 37), and numerous BKV variants have been isolated in recent years (3, 17, 35, 43). BKV transforms cells from several species (18, 30), including human cells (15, 32, 42), and is highly oncogenic in hamsters (4-6, 45, 46), displaying a remarkable tropism for particular tissues and organs. When BKV is inoculated either intracerebrally or intravenously into immunosuppressed or immunocompetent hamsters, 73 to 88% of the animals develop tumors. Tumors observed belong essentially to only three histotypes: ependymomas and choroid plexus papillomas, tumors of pancreatic islets, and osteosarcomas (4-6, 45, 46). Recently, we have started to analyze by blot hybridization those human tumors corresponding to the histotypes most frequently induced by BKV in laboratory animals. We have detected free BKV DNA in brain tumors as well as in tumors of pancreatic islets and have isolated infectious virus from several of them by transfection of tumor DNA into human embryonic fibroblasts (A. Corallini, G. Milanesi, and G. Barbanti-Brodano, submitted for publication). In this study, we present a molecular and biological characterization of BKV-IR, a BKV variant isolated from a human tumor of pancreatic islets harboring episomal viral DNA (2).

Tumor DNA was extracted as described previously (2). During the extraction procedure, care was taken to avoid ethanol precipitation to save even small amounts of free viral DNA. BKV-IR was isolated by transfection of tumor DNA into WI-38 cells by the calcium phosphate coprecipitation technique (2). About 1 month after transfection a typical cytopathic effect appeared in the cell monolayer. The culture medium showed hemagglutinating activity for human erythrocytes which was inhibited by a serum specific for BKV. BKV-IR was grown to high titers in WI-38 and Vero cells.

Viral DNA was extracted and purified as described previously (2) from CsCl-purified virions.

First, we compared BKV-IR and wild-type BKV (BKV-WT) DNAs by restriction endonuclease mapping. The cleavage sites on BKV-WT DNA of restriction endonucleases used in these experiments are shown in Fig. 1. Digestion of BKV-IR DNA with *Hind*III yielded a normal fragment A, whereas fragments B, C, and D were missing. Instead, two other fragments were observed: B*, which was longer than BKV-WT fragment B, and C*, which was longer than BKV-WT fragment C. Of the three fragments obtained by digestion of BKV-IR DNA with *Sac*I, the largest fragment comigrated with the corresponding fragment in the *Sac*I digest of BKV-WT DNA, whereas the middle fragment was shorter and the smallest fragment was longer than the corresponding *Sac*I fragments of BKV-WT DNA (Fig. 2). This restriction endonuclease pattern is the result of (i) an insertion in the region corresponding to *Hind*III fragment C and to the small *Sac*I fragment and (ii) a deletion which eliminates the *Hind*III site at nucleotide 2797 (0.538 map units [m.u.]) (Fig. 1) extending into *Hind*III fragment D and the nearest portion of *Hind*III fragment B as well as into the middle *Sac*I fragment.

To better characterize the genome of BKV-IR, we carried out a nucleotide sequence analysis of the regions of BKV-IR DNA containing the insertion and the deletion and compared them with the corresponding regions of BKV-WT and BKV-MM, a naturally occurring BKV viable deletion mutant which was isolated from a human brain tumor (43) and which has a restriction endonuclease map similar to that of BKV-IR (19). BKV-IR DNA *Hind*III fragments B* and C* were inserted in the unique *Hind*III site of pML-tet, a deletion derivative of pBR322 (24). After cloning and amplification in bacteria (25), the fragments were excised from the recombinant DNA molecules, purified by agarose gel electrophoresis, and labeled with ³²P at the 5' end with polynucleotide kinase (25). Prior to end labeling, *Hind*III fragment B* was cleaved with *Hin*FI, which cuts BKV DNA at nucleotide 2694 (0.518 m.u.) (Fig. 1), and the nucleotide sequence was determined from this point to the end of the fragment at the *Hind*III site located at nucleotide 3213 (0.618 m.u.) (Fig. 1).

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FIG. 1. Physical map of the BKV-WT genome with the cleavage sites of the enzymes used in these experiments. The unique cleavage site of *Eco*RI is designated as 0.0 m.u., and the cleavage sites of the other restriction endonucleases are given in m.u. from 0.0 to 1. A, B, C, and D represent the four *Hind*III fragments. For *Hinf*I (which has 19 cleavage sites on BKV DNA), only the site at 0.518 m.u., used for sequence analysis, is shown. The spliced early mRNAs (small-t-antigen mRNA and large-T-antigen mRNA) are marked outside the circular genome, with translated sequences indicated by boxes, untranslated portions indicated by solid lines, and introns indicated by dashed lines. The origin of DNA replication (Or) and the three enhancer repeats (ER) of 68, 50, and 68 nucleotides are also indicated.

DNA strands were separated, and sequence analysis was performed on both strands by the chemical cleavage method of Maxam and Gilbert (28).

The nucleotide sequences of BKV-WT, BKV-IR, and BKV-MM *Hind*III C fragments are presented in Fig. 3A. This region contains the three repeats (68, 50, and 68 nucleotides in BKV-WT) that function as transcriptional enhancers for the early viral genes. Although in some areas of this region the nucleotide sequences of the three genomes are colinear, BKV-IR shows unique stretches of nucleotides, shared neither with BKV-WT nor with BKV-MM. BKV-IR has an insertion of 80 nucleotides, as compared with BKV-WT, whereas it has an insertion of 79 nucleotides and a deletion of 31 nucleotides, as compared with BKV-MM. In BKV-IR the inserted nucleotides are grouped in four clusters of 25, 12, 7, and 36 nucleotides, three of which fall into the first and second enhancer repeats, which then contain 75 and 87 nucleotides, respectively, while the fourth immediately follows the third enhancer repeat, which is not affected by insertions.

Several interesting features are notable in the regions unique to BKV-IR. First, a sequence of 12 nucleotides (CCATGACCTCAG) is tandemly repeated from nucleotides 3312 to 3335. This 12-base-pair repeat contains the eight terminal nucleotides (CCATGACC) from the enhancer core sequence (CAAACCATGACC) of the second enhancer element. Second, a 12-base-pair sequence (AGGAAGTG-GAAA) showing a substantial similarity to the simian virus 40 enhancer core sequence (GGTGTGGAAAG) (20) and to

the enhancer core sequence (GGAGTGGAAAG) of BKV-Dik, a recently isolated BKV variant (35), is present at nucleotides 3466 to 3477 and is repeated from nucleotides 3486 to 3497. These characteristics suggest that BKV-IR is better equipped in enhancer-like structures than are BKV-WT and BKV-MM. Third, the dodecanucleotide GCAGC-CAGCCAG present at nucleotides 3269 to 3280 is repeated, with a difference of only one nucleotide (GCAGC-CAGACAG), from nucleotides 3498 to 3509. Nearly perfect inverted repeats (21) are adjacent to these direct repeats and can pair to form a stem and a 200-base loop containing the second and third enhancer elements. This structure resembles an insertion sequence (IS) (1, 21) (Fig. 4) and recalls the transposable elements containing enhancer sequences recently described in yeasts (9, 34). The direct repeats flanking the IS-like structure enclose all the inserted sequences (Fig. 3A), suggesting that the insertion in the BKV-IR genome may have been acquired by transposition.

The nucleotide sequence of BKV-IR *Hind*III fragment B* has a deletion of 253 base pairs between nucleotides 2792 and 3044. No rearrangements are observed in the regions flanking the deletion where the nucleotide sequence appears to be colinear with BKV-WT DNA. Also, the deletion in BKV-IR is different from that in BKV-MM, in which 262 base pairs are missing between nucleotides 2752 and 3013 (Fig. 3B). The deletion in BKV-IR DNA falls within the sequence spliced out of the early region primary transcript (nucleotides 2725 to 3069) to yield the 19S mRNA for large-T antigen, while the sequences coding for the middle and carboxy-terminal portions of small-t antigen and containing the donor splice site for small-t antigen 20S mRNA (nucleotides 2792 to 2793) are removed.

We next analyzed the biological properties of BKV-IR. BKV-IR grew to the same titer as BKV-WT in Vero cells and human embryonic fibroblasts. BKV-IR and BKV-IR

FIG. 2. Agarose gel electrophoresis of BKV-IR (I) and BKV-WT (W) DNAs cleaved with *Hind*III and *Sac*I. A, B, C, and D represent the four *Hind*III fragments of BKV-WT DNA. Owing to technical problems in efficiently contrasting the low-molecular-weight bands, the negative of the photograph, with black bands on a white background, is shown.

FIG. 3. (A) Nucleotide sequence of the region of BKV-IR DNA *Hind*III fragment C* containing the insertion and of the corresponding regions of BKV-WT and BKV-MM. Enumeration of the nucleotides starts clockwise at the *Eco*RI recognition sequence, whose second base is taken as nucleotide 1. The strands, read from left to right, have the polarity of the late mRNAs. Homologous base pairs are indicated by asterisks. R indicates the presence of a nucleotide that could not be determined. The three enhancer repeats are indicated by brackets. The enhancer core sequences are enclosed in dashed boxes, and repeated sequences are underlined once or twice. The sequences enclosed in solid boxes are the direct repeats flanking a possible IS-like structure (see Fig. 4). E and L represent the early and late regions, respectively. (B) Nucleotide sequence of the region of BKV-IR DNA *Hind*III fragment B* containing the deletion and of the corresponding region of BKV-MM. Homologous base pairs are indicated by asterisks.

FIG. 4. Schematic representation of the IS-like structure found in BKV-IR *Hind*III fragment C*. The two dodecanucleotide direct repeats are at nucleotides 3269 to 3280 and 3498 to 3509. They enclose a stem with one mismatch and a 200-base (b) loop containing the second and third enhancer elements. The dots indicate the two nucleotides that differ in the direct repeats.

DNA transformed primary hamster kidney cells, although with an efficiency 3 to 10 times lower than that of BKV-WT and BKV-WT DNA (Table 1). BKV-IR-transformed cells showed a fully transformed morphological phenotype and yielded permanent cell lines. BKV-IR induced tumors upon intracerebral inoculation in newborn hamsters. Of 31 injected animals, 22 (71%) developed tumors, a lower incidence than that obtained by intracerebral inoculation of BKV-WT (88%) (6). Moreover, the latent period before the appearance of tumors was longer (5 to 9 months) for BKV-IR than for BKV-WT (3 to 6 months). As with BKV-WT-induced tumors, all the BKV-IR-induced tumors were ependymomas. Histologic examination of these tumors constantly showed notable features of malignancy, such as invasiveness and infiltration of normal brain tissue, remarkable cell atypia and altered nuclei, and conspicuous and diffuse subependymal and interventricular metastasis. Such pronounced malignant characteristics were never observed in ependymomas induced by BKV-WT in immunocompetent hamsters (5, 6). Both transformed cells and tumors induced by BKV-IR contained integrated viral DNA sequences in two to five copies per cell, as determined by Southern blot hybridization (41).

To ascertain whether the expression of BKV-IR early gene products was in accord with the structure of the viral

early region, we analyzed two BKV-IR-transformed hamster kidney cell lines and one cell line derived from a BKV-IR-induced hamster ependymoma by immunofluorescence and immunoprecipitation (2) after labeling the cells with $^{32}\text{P}_i$. A serum to BKV large-T and small-t antigens (15), obtained from hamsters bearing tumors induced by BKV-transformed hamster cells, was used in both immunofluorescence and immunoprecipitation tests. Large-T antigen was detected by immunofluorescence in the nuclei of 95 to 100% of transformed and tumor-derived cells. When the same cells were analyzed by immunoprecipitation, a prominent band of 94 kilodaltons corresponding to a full-sized large-T antigen (39) was detected. The 56-kilodalton band may represent the p56 cellular tumor antigen (39). No 17-kilodalton or smaller bands were observed (Fig. 5), suggesting that neither the 17-kilodalton small-t antigen (36) nor a truncated equivalent is made in detectable amounts in BKV-IR-transformed hamster cells. The results are in agreement with the arrangement of the BKV-IR early region, which has a coding capacity for a normal large-T antigen but has lost a substantial coding portion of the small-t-antigen gene as well as sequences required for mature small-t antigen mRNA.

This study shows that BKV-IR, isolated from a human tumor of pancreatic islets, is a new variant of BKV. The alterations in the genome of BKV-IR do not affect its biological properties. As with BKV-MM (7, 27), BKV-IR can lytically infect human and monkey cells, stably and fully transform rodent cells, and induce tumors in hamsters. The reduced transforming and oncogenic capacities of BKV-IR could depend on the loss of small-t-protein activities. In fact, although data on the functions of the BKV small-t antigen are not available, the viable simian virus 40 small-t-antigen mutant *d/884*, bearing a deletion very similar to that in BKV-IR (38), was deficient in the transformation of rodent cells in cultures (10, 26, 29, 40). Moreover, like BKV-IR-induced experimental tumors, hamster tumors induced by *d/884* appeared at a reduced frequency and after a longer latency period than tumors produced by wild-type simian virus 40 (8, 23, 44) but had features of higher malignancy, such as the frequent production of metastasis (8). On the other hand, the insertion of new sequences in the first and second enhancer elements may also affect transformation by BKV-IR, since rearrangements in the region of the enhancer repeats change the transformation efficiency of BKV (16, 47, 48).

It is intriguing that the sequences inserted in BKV-IR may form a stem-loop structure bound by direct repeats. This IS-like element may be involved in transformation by BKV-IR, for instance, by excision from viral DNA and insertion into the cell genome, thereby specifically activating the

TABLE 1. Transformation of primary hamster kidney cells by BKV-IR, BKV-WT, and their DNAs

Virus	HAU ^a /10 ⁶ cells	No. of colonies in expt ^b :		Viral DNA	μg of DNA/10 ⁶ cells	No. of colonies in expt ^b :	
		1	2			1	2
BKV-IR	2.5	1		BKV-IR	0.01	0	
BKV-IR	25	4		BKV-IR	0.1	9	
BKV-IR	250	9	0 (4)	BKV-IR	1	1	6.7 ± 2.2 (3)
BKV-IR	2,500	30	4.3 ± 3.1 (6)	BKV-IR	2	3	13.7 ± 4.4 (4)
BKV-WT	2.5	0		BKV-WT	0.01	1	
BKV-WT	25	8		BKV-WT	0.1	7	
BKV-WT	250	41	5.7 ± 3.9 (6)	BKV-WT	1	25	16.8 ± 5.7 (3)
BKV-WT	2,500	289	14.4 ± 5.3 (7)	BKV-WT	2	31	17.6 ± 5.2 (3)

^a HAU, Hemagglutinating units.

^b In experiment 1, only one culture flask was infected with each amount of virus or viral DNA. In experiment 2, each value is the mean ± standard deviation; numbers in parentheses indicate the number of culture flasks used for calculation.

FIG. 5. Immunoprecipitation of hamster kidney cells transformed by BKV-IR (lanes 1 and 2) or BKV-IR DNA (lanes 3 and 4) and of cells cultured from a BKV-IR-induced hamster ependymoma (lanes 5 and 6). Cell homogenates were reacted with normal hamster serum (lanes 1, 3, and 5) or with serum to BKV large-T and small-t antigens (lanes 2, 4, and 6). Molecular mass markers were as follows: phosphorylase *b* (95 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). The relative position of the p56 cellular tumor antigen is indicated (56 kDa).

expression of cellular oncogenes. This hypothesis is supported by the observation that the IS contains in its loop two enhancer repeats. Indeed, the possible involvement of an IS-like element containing enhancer sequences from the long terminal repeat of a retrovirus in the activation of cellular oncogenes has been described previously (22, 33). Another possibility is that the IS acts more generally as a mutagen by integration at random into cellular genes and excision from them imprecisely.

The failure to find integrated viral sequences in the human tumor from which BKV-IR was isolated (2) may be due to the insufficient sensitivity of hybridization. In reconstruction experiments our hybridization assay could detect from 0.5 to 0.1 genome equivalents per cell, according to the specific activity of the probes, while a single integration of the IS-like element corresponds to 0.04 genome equivalents per cell. Moreover, the probe used in the hybridization experiments was prepared with full-length BKV-WT DNA that was not perfectly homologous to the BKV-IR IS-like structure, owing to clusters of inserted nucleotides in this segment of the BKV-IR genome. This would result in regions of mismatch favoring the instability of the hybrid DNA molecules under stringent conditions of hybridization. Alternatively, the IS-like structure could be subjected to a "hit-and-run" mechanism (11). In this connection, it is noteworthy that an IS-like element was detected in the transforming regions of herpes simplex virus type 2 and cytomegalovirus (12). It will be

interesting to determine (i) if the region of BKV-IR DNA containing the IS-like structure is able to transform or mutagenize rodent and human cells or both and (ii) if cellular oncogenes are switched on in BKV-IR-transformed cells and in the human tumor from which BKV-IR was isolated.

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