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BK Virus-Plasmid Expression Vector That Persists Episomally in Human Cells and Shuttles into *Escherichia coli*

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We describe a novel expression vector, pBK TK-1, that persists episomally in human cells that can be shuttled into bacteria. This vector includes sequences from BK virus (BKV), the thymidine kinase (TK) gene of herpes simplex virus type 1, and plasmid pML-1. TK⁺-transformed HeLa and 143 B cells contained predominantly full-length episomes. There were typically 20 to 40 (HeLa) and 75 to 120 143 B vector copies per cell, although some 143 B transformants contained hundreds. Low-molecular-weight DNA from TK⁺-transformed cells introduced into *Escherichia coli* were recovered as plasmids that were indistinguishable from the input vector. Removal of selective pressure had no apparent effect upon the episomal status of pBK TK-1 molecules in TK⁺-transformed cells. BKV T antigen may play a role in episomal replication of pBK TK-1 since this viral protein was expressed in TK⁺ transformants and since a plasmid that contained only the BKV origin of replication was highly amplified in BKV-transformed human cells that synthesize BKV T antigen.

Development of eucaryotic vectors has provided a direct and convenient means of introducing additional and novel genetic information into cultured cells. Essentially, portions of all eucaryotic vectors include sequences derived from animal viruses (for extensive reviews, see references 12, 39, and 40). Expression systems vary widely in the way they are used. For example, infection of permissive cells occurs with particular vectors of simian virus 40 (SV40) (13, 17, 34), adenovirus (47, 53), herpes simplex virus (50), vaccinia virus (37), and retroviruses (46, 57). However, short-term or transient expression, which is noninfectious, has been described with SV40 vectors that become highly amplified in COS cells (11), e.g., 200,000 episomal copies per cell within 48 h of posttransfection (31). In contrast, permanent cell lines which integrate the vector DNA have been obtained in two ways by using selectable marker genes. The first has been by "per-force" selection, using the thymidine kinase (TK) gene of herpes simplex virus type 1 (HSV-1) in TK-deficient mutant cells (44, 58). The second has been by dominant selection in which expression of a dominant marker gene (e.g., *Escherichia coli* xanthine-guanine phosphoribosyltransferase) is extended to nonmutated cells (33, 55). In addition, permanent mouse cell lines have been obtained by transformation with bovine papillomavirus vectors which do not integrate into host cell DNA but replicate episomally and which can be shuttled into bacteria (6, 43).

We report here the development of a virus-plasmid expression vector, pBK TK-1, that has the unusual feature of being able to persist episomally in human cells. This expression vector contains DNA sequences which originate from the human papovavirus, BK virus (BKV). BKV was isolated from the urine of a renal transplant recipient on immunosuppressive therapy (9) and is ubiquitous in the human population (2, 8). BKV efficiently infects human cells and transforms hamster, mouse, rat, rabbit, and monkey cells in tissue culture (36). The genomic organization of the circular double-stranded DNA of BKV is remarkably similar to that of SV40, even though these viruses are infectious for different hosts. In fact, an homology of more than 80% is found in the nucleotide sequences of the two viruses (45, 59). As in SV40, both large T and small t antigens of BKV are translated from mRNAs which initiate their transcription on one strand near the putative origin of replication (20). In parallel to SV40, capsid proteins of BKV (VP1, VP2, and VP3) are produced after DNA synthesis from late mRNAs which initiate transcription proximal to the 5' end of early mRNAs on the opposite strand (20). One notable distinction between the genomes of SV40 and BKV is the extensive divergence in the DNA sequences of their tandem repeats which constitute transcriptional enhancer regions (41).

Human cells persistently infected with BKV as well as transformed human cells have been isolated (16, 35). In some of these viral host cell relationships, the BKV DNA resides as apparent full-length episomes, whereas in others, the BKV DNA may be free but may contain deletions or be integrated (16, 35). Although the mechanisms for episomal persistence of BKV DNA are not understood, we have exploited this basic feature to produce a recombinant DNA vector which will persist as an amplified episomal resident in human cells. The potential usefulness of this vector for analyzing the expression of artificially acquired genes in human cells is discussed.

MATERIALS AND METHODS

Plasmids, virus, and recombinant DNA constructions. Plasmid pML-1 (referred to here as pML) is a deletion derivative of pBR322 (25). Plasmid pHSV106 (30) was used as the source of the HSV-1 TK gene, inserted at the *Bam*HI site. Propagation of BKV (Gardner strain) and extraction of viral DNA were by the procedure of Meneguzzi et al. (32). Details of the construction of the viral plasmids pBK TK-1 and pBODE are presented in the text. The source of restriction enzymes was New England Biolaboratories and Bethesda Research Laboratories, and T4 DNA ligase was from P-L Biochemicals. DNA fragments were isolated by the method of Vogelstein and Gillespie (56). The above recombinant

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molecules were introduced by transformation and screened and propagated in *E. coli* Hb101, C600, and MC-1061 (10, 28), followed by standard isolation procedures (21, 38).

Cell cultures, DNA transfer, and selection of transformants. Human HeLa TK-deficient (TK⁻) cells and human 143 B TK⁻ cells were used in these experiments. HeLa cells are derived from a human carcinoma, whereas 143 B cells are from a human osteosarcoma (4). TK⁻ cells were maintained in minimal essential medium (MEM) with 8% calf serum containing 30 μg of bromodeoxyuridine per ml. Before transfection, the HeLa TK⁻ and 143 B TK⁻ cells were grown for one passage in the same medium without bromo-deoxyuridine. The BKV-transformed human embryonic fi-broblast cell line L603 (16) was maintained in MEM with 10% fetal bovine serum at all times. Cells at ca. 60% confluency were supplied with fresh medium 4 h before transfection with supercoiled plasmid DNA (20 μg for about 10^7 cells, unless otherwise indicated, and in the absence of carrier DNA) by the calcium phosphate precipitation proce-dure of Graham and van der Eb (14) as modified by Wigler et al. (58). In some instances, cells were shocked by 20% glycerol (7) 4 h after transfection; otherwise, the transfection medium was replaced directly with fresh nonselective medi-um. To select for TK⁺ HeLa and TK⁺ 143B transformants, cells were split 1:2 after 20 h and maintained in MEM with 8% calf serum and HAT medium (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine; 24). Resulting cell colonies were either individually cloned and expanded or pooled and grown as mass cultures.

Preparation and analysis of cellular DNA. Total cellular DNA was extracted as previously described (3, 15). Separa-tion of DNA into supernatant (nonchromosomal) and pellet (chromosomal) fractions was by the procedure of Hirt (18). Extraction of DNA from both fractions was by standard methods, in which proteinase K (Boehringer Mannheim Biochemicals) treatment (50 μg/ml, overnight at 37°C) was followed by phenol extraction and ethanol precipitation. The drained pellets were dissolved in 10 mM Tris-hydrochloride (pH 7.8) and treated with RNase (25 μg/ml at room tempera-ture for 1 h), followed by two extractions in phenol and two in chloroform-isoamyl alcohol (24:1, vol/vol) and precip-itation in ethanol. The pellets were finally dissolved in 15 mM NaCl–1.5 mM sodium citrate.

Cellular DNA was fractionated in agarose gels and trans-ferred to nitrocellulose filters by the Southern procedure (48). Probe DNA was labeled by nick translation (29) with [α-³²P]dXTP (Amersham Corp.) and DNA polymerase I (Boehringer Mannheim Biochemicals). The hybridization reactions were performed either (i) in 50% formamide with 1× Denhardt solution (5), 4× SSC (0.6 M NaCl plus 0.06 M sodium citrate), and 4% sodium dodecyl sulfate at 37°C for 36 h, followed by a series of aqueous washes at 68°C with the last in 0.2× SCC–0.2% sodium dodecyl sulfate, or (ii) according to conditions described by Chenciner et al. (3). Filters were air dried and exposed to XRP-film (usually for 2 to 24 h). The amount of free vector DNA in TK⁺ transform-ants was determined by reconstruction experiments, in which serial dilutions of cellular DNA were electrophoresed adjacent to known amounts of pBK TK-1 DNA, blotted, and probed with vector DNA. The copy number was calculated from densitometric analysis of the autoradiograms carried out with a Corning 750 scanner.

Transformation of bacteria with cellular DNA and analysis. *E. coli* Hb101 or C600 was transformed with cellular DNAs by the calcium chloride procedure (27). *E. coli* MC-1061 was transformed by the calcium chloride-rubidium chloride pro-cedure (23). Plasmid DNA from transformants was isolated by a rapid small-scale extraction procedure (19).

Immunoprecipitation and immunofluorescence. Labeling, immunoprecipitation, and fluorescent antibody staining of BKV T antigen in TK⁺-transformed human cell lines were performed as described by Grossi et al. (16) with serum from hamsters bearing tumors induced by BKV-transformed ham-ster cells.

RESULTS

Vector construction. Vector pBK TK-1 includes DNA sequences originating from plasmid pML, BKV, and the TK gene of HSV-1. The location of these sequences within this vector and its construction are depicted in Fig. 1. The pBK TK-1 vector was derived by digesting both plasmid pML DNA and BKV DNA with *Eco*RI and *Bam*HI (Fig. 1A). The larger of the respective *Eco*RI-*Bam*HI fragments of pML DNA (2,617 base pairs [bp]) and BKV DNA (5,089 bp) were ligated to produce pBK-1. A *Bam*HI fragment of HSV-1 DNA (3,600 bp) which contains the TK gene was introduced into the single *Bam*HI site of pBK-1 to generate vector pBK TK-1 (Fig. 1A). The pBK TK-1 molecule thus includes almost the entire BKV genome (5,196 bp), except for 107 bp eliminated by digestion of BKV DNA with *Bam*HI and *Eco*RI. This 107-bp fragment contained sequences from each of the late viral capsid transcripts Vp1, Vp2, and Vp3 (54). Since BKV DNA is infectious for human cells, deletion of these late viral sequences was intended to obviate potential recombination and release of the complete viral genome from a vector containing full-length BKV DNA.

The topographical arrangement of the transcripts and pertinent coding regions of the genes contained within pBK TK-1 are depicted in Fig. 1B. The essential features of pBK TK-1, in addition to encoding the ampicillin resistance gene of pML and the TK gene of HSV-1, are that it contains the entire early region sequences which encode BKV large T and small t antigens (45, 59). In addition, pBK TK-1 also includes the BKV origin of replication based upon related sequence homology to SV40 and the transcriptional en-hancer sequences (45, 59). The late genes of BKV in pBK TK-1 are not only missing 107 bp as described above but are also disrupted by ligation of pML DNA at the *Eco*RI site and of TK DNA at the *Bam*HI site. Plasmid pBODE, shown in Fig. 1A, is discussed later.

pBK TK-1 vector persists episomally in human cells. Hu-man TK⁻ HeLa cells or 143 B cells were transfected with pBK TK-1 vector DNA and subjected to selection in HAT medium. Individually cloned or mass culture cell lines grown in HAT medium were then analyzed. Either total cellular DNA was extracted from TK⁺-transformed cells, or the DNA was separated into Hirt supernatant (HS) and Hirt pellet (HP) fractions. Essentially, HS DNA contains almost entirely free, low-molecular-weight DNA, whereas HP DNA contains high-molecular-weight DNA that is most often contaminated with free DNA.

The DNA from several TK⁺ clones of HeLa cells was extracted and examined by Southern blot analysis (48) for the presence of pBK TK-1 molecules. Analysis of HS DNA from two HeLa clones H-303 and H-384 (Fig. 2A, lanes 1 and 2, respectively) revealed sequences that comigrated with the supercoiled (form I), circular (form II), or linear (form III) structures of pBK TK-1. When this same DNA from clone H-303 was digested with *Sal*I (Fig. 2B, lane 2), which cuts pBK TK-1 once, a single band comigrated with *Sal*I-linear-ized pBK TK-1 DNA (Fig. 2B, lane 1). These results indicated that full-length pBK TK-1 molecules persist as free
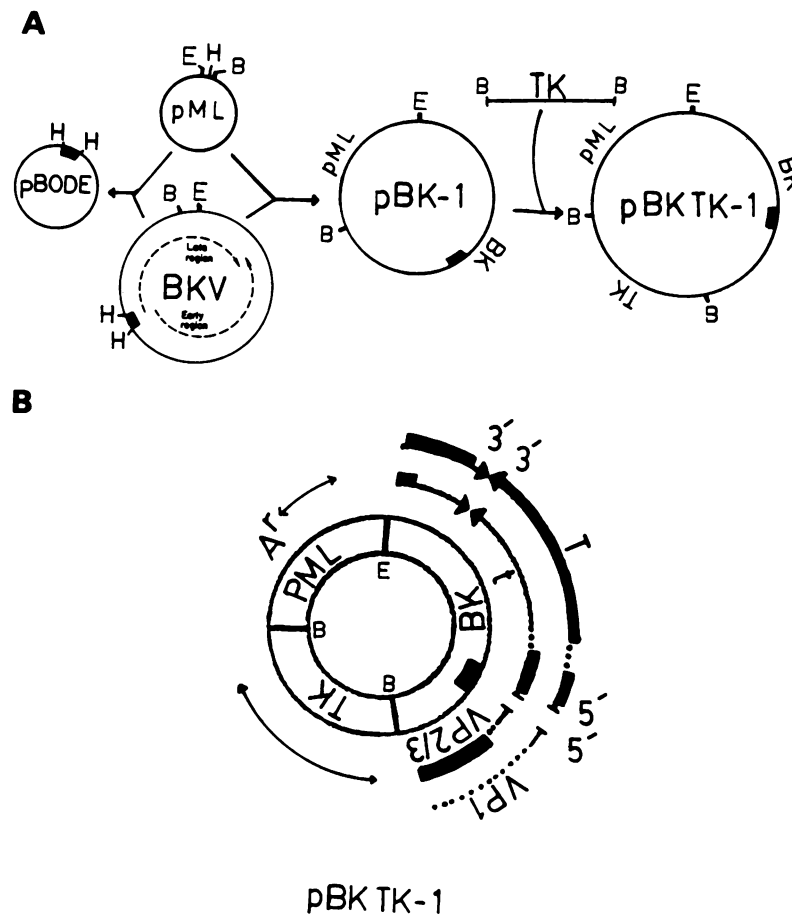
A

B

pBK TK-1

FIG. 1. Construction of vectors. (A) pBK TK-1 was constructed from BKV, the TK gene of HSV-1, and plasmid pML. pBODE was constructed from BKV and pML. The putative replication origin of BKV (0.67 map units) is contained in the HindIII C fragment designated by the black box. The early and late transcription regions of BKV are designated by the dotted lines. The restriction sites are EcoRI (E), BamHI (B), and HindIII (H). (B) Genomic landmarks in pBK TK-1. Locations of DNAs derived from pML, BKV, and TK (described above) in pBK TK-1 are represented in the double circle. Their respective transcripts are indicated by the thin lines, of which the dotted portion represents introns. Where pertinent, the orientation of the transcripts is shown 5' to 3'. Coding regions (thick black lines) contained within specific BKV transcripts indicate early proteins, large T and small t antigens, and late capsid proteins VP1 and VP2/3. The putative BKV replication origin is represented by the black box within the circles. The bacterial ampicillin resistance gene of pML is designated by A^r. The represented restriction sites are EcoRI (E) and BamHI (B). Details are discussed in the text.

molecules in HeLa cells. To further characterize the state of vector DNA in HeLa cells, total DNA from six different TK$^+$ cloned lines was digested with BamHI, which cuts pBK TK-1 DNA twice to generate 3,600- and 7,706-bp fragments. Five of the HeLa clones (Fig. 2C, lanes 1, 2, 3, 5, and 6) contained only two bands which comigrated with the 3,600 and 7,706-bp marker fragments (Fig. 2C, lane 7), which was expected if pBK TK-1 was episomal. Only one HeLa cell clone (Fig. 2C, lane 4) contained an additional band which may have originated from either integrated or rearranged episomal molecules of pBK TK-1 DNA; the additional bands in lane 3 represent incompletely digested free pBK TK-1 DNA, since they comigrated with undigested pBK TK-1 DNA (lane 8). It is noted that although the autoradiogram shown in Fig. 2C had been overexposed, minor bands of pBK TK-1 which could represent integrated sequences are not detected. These results again suggested that in HeLa cells pBK TK-1 molecules exist as full-length episomes.

The transfected 143 B TK$^+$ cell clones were also analyzed for the presence of pBK TK-1 molecules. Undigested HS

DNA from each of several clones (Fig. 3A, lanes 2 to 5) revealed molecules which comigrated with pBK TK-1 marker DNA (Fig. 3A, lane 1). The HS DNA from two of these cell clones, after digestion with SalI which cuts pBK TK-1 once, yielded single bands (Fig. 3B, lanes 2 and 3), which comigrated with SalI-linearized pBK TK-1 DNA (Fig. 3B, lane 1). Free forms of pBK TK-1 (50 to 100 genome equivalents per diploid cell genome) were similarly observed when total cellular DNA from 143 B TK$^+$ cell clones was digested with HpaI, a noncutter for pBK TK-1 (data not shown). Total cellular DNA from nine 143 B clones was digested with BamHI and analyzed for sequences of the expected 7,706- and 3,600-bp fragments that would be generated from nonintegrated molecules of pBK TK-1. Indeed, as seen in Fig. 3C (lanes 1 to 9), each 143 B clone contained both expected BamHI fragments. However, some 143 B cell clones revealed novel bands which hybridized to the pBK TK-1 probe (Fig. 3C, lanes 1, 3, 4, and 8); the band below the 7,706-bp fragment of clone B4 (lane 2) is probably undigested vector DNA, since it comigrates with form I pBK TK-1
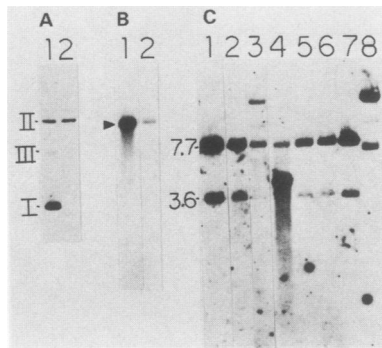
FIG. 2. Southern blots of DNA from TK⁺-transformed HeLa cell clones hybridized with a pBK TK-1 DNA probe. HS DNA or total cellular DNA was extracted from various individual TK⁺ HeLa cell clones that had been transformed by pBK TK-1. HS DNAs (corresponding to 10^5 cells) and total cellular DNAs (10 µg) were digested with restriction endonucleases as indicated, electrophoresed through an 0.8% agarose gel, blotted onto nitrocellulose, and probed with nick-translated pBK TK-1 DNA. (A) Undigested HS DNA from HeLa cell clones H-303 (lane 1) and H-384 (lane 2). The positions of forms I, II, and III of pBK TK-1 marker DNA are indicated. (B) HS DNA of HeLa clone H-303 digested with SalI (lane 2), and SalI-digested pBK TK-1 DNA (4.8 ng) as marker (lane 1). (C) Total cellular DNA was digested with BamHI. Lanes 1 to 6: HeLa clones H-311; H-303; H-383; H-301; H-385; H-386, respectively. pBK TK-1 DNA marker (50 genome equivalents): digested with BamHI (lane 7) and undigested (lane 8).

DNA (lane 10). These novel sequences likely represent polymeric and deleted episomal forms of pBK TK-1 or possibly integrated forms of the vector.

We examined whether the property of pBK TK-1 molecules to persist as episomes in individual cell clones would also extend to a mass culture prepared by pooling hundreds of individual TK⁺ cell colonies. The DNA extracted from such a mass culture of 143 B cells was separated into HS and HP fractions, which, respectively, were digested with five

restriction endonucleases that cut pBK TK-1 DNA twice (BamHI, XbaI, and KpnI) or three times (EcoRI and BglII) (Fig. 4A). As seen in Fig. 4B, in all instances, sequences of pBK TK-1 found in both the HS (lanes 1, 4, 7, 10, and 13) and HP (lanes 2, 5, 8, 11, and 14) fractions after digestion by these enzymes were those which comigrated with the corresponding digestions of marker pBK TK-1 DNA (lanes 3, 6, 9, 12, and 15). The observation that most of the hybridized sequences from each HP fraction comigrated with the respective restriction fragments of marker pBK TK-1 DNA indicated that most of these molecules originated from pBK TK-1 episomes and that only negligible amounts, if any, of this vector DNA integate into chromosomal DNA. Additional bands, particularly in the HS fraction, could represent incompletely digested pBK TK-1 DNA molecules, free molecules containing deletions, or degraded forms of pBK TK-1 DNA. Since these results were obtained with a mass culture, they are representative of hundreds of individually transfected cells. This finding demonstrates clearly that in the majority of independently transfected human cells pBK TK-1 molecules persist in the free state.

The average number of free pBK TK-1 copies maintained per cell was between 75 and 120 in 143 B cells and 20 and 40 in HeLa cells. It is important to note that in some 143 B clones (e.g., clones B-23 and B-36; Fig. 3C, lanes 7 and 9), many times this number of copies persisted.

pBK TK-1 DNA can be shuttled from human cells into E. coli. To further substantiate that pBK TK-1 DNA molecules persist as stable replicating episomes in human cells, HS DNAs from both HeLa and 143 B cell clones were used to transform E. coli to an ampicillin-resistant phenotype. In one experiment, half of a sample of the HS DNA from two clones of HeLa cells and six clones of 143 B cells was directly used to transform E. coli MC-1061, whereas the other half was treated with the restriction endonuclease MboI (which has 17 restriction sites within pBK TK-1 DNA) before transformation. DNA is not cut when the adenine within the recognition sequence of MboI (GATC) is methylated, as occurs in most E. coli strains including Hb101 and MC-1061.
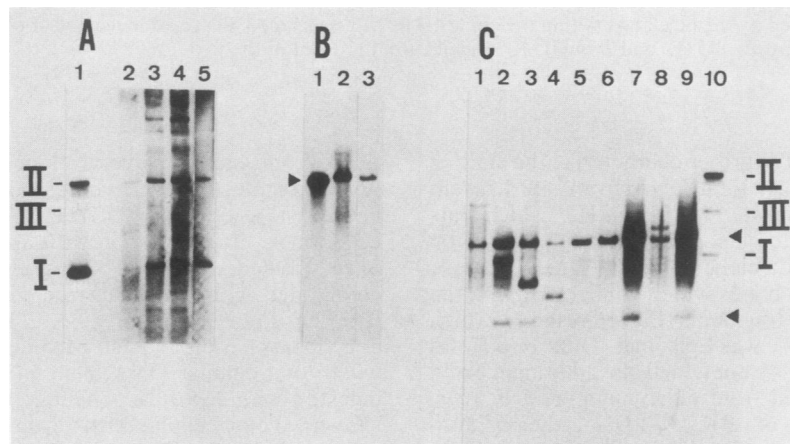

FIG. 3. Southern blots of DNA from TK⁺-transformed 143 B cell clones hybridized with pBK TK-1 DNA. HS DNA or total cellular DNA was extracted from various individual TK⁺ 143 B cell clones that had been transformed by pBK TK-1 DNA. HS DNAs (corresponding to 10^5 cells) and total cellular DNAs (10 µg) were digested with the indicated restriction endonucleases, electrophoresed through a 0.6% agarose gel, blotted onto nitrocellulose, and probed with nick-translated pBK TK-1 DNA. (A) HS DNA from 143 B clones B-6, B-27, B-31, and B-20 (lanes 2 to 5, respectively); lane 1, 4.8 ng of pBK TK-1 DNA marker. (B) SalI digestion of HS DNA of 143 B clones B-31 (lane 2) and B-27 (lane 3); lane 1, 4.8 ng of SalI-digested pBK TK-1 marker DNA (arrow). (C) Total cellular DNA from 143 B clones digested with BamHI; lanes 1 to 9, 143 B clones B-1, B-4, B-6, B-8, B-18, B-20, B-23, B-31, and B-36, respectively; lane 10, undigested pBK TK-1 DNA marker (100 genome equivalents). The upper and lower arrows indicate the positions of the 7,706 and 3,600-bp fragments, respectively, generated by BamHI digestion of pBK TK-1 DNA.
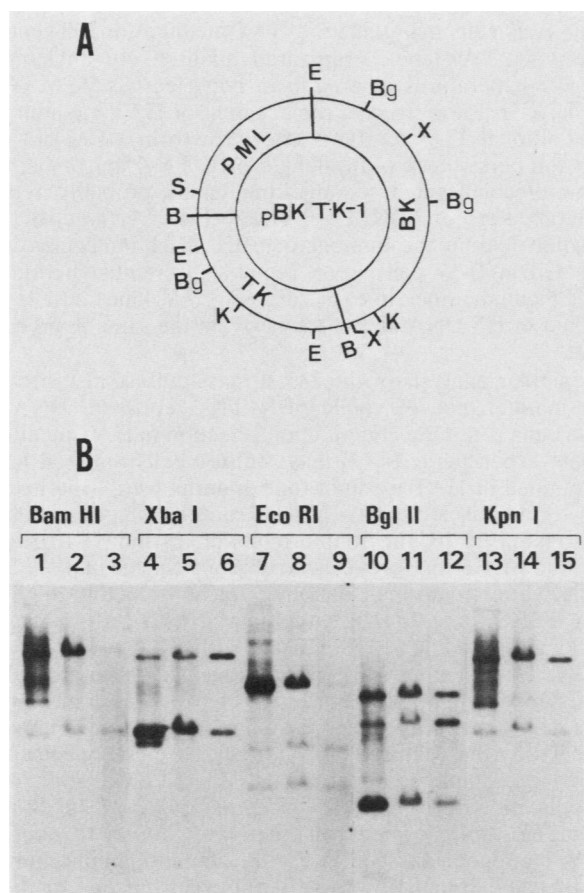
FIG. 4. (A) Positions of *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Kpn*I (K), and *Xba*I (X) restriction sites in pBK TK-1. The unique *Sal*I (S) site is suitable for insertion of additional genes. (B) Southern blot of DNA from TK⁺-transformed 143 B mass culture cells hybridized to pBK TK-1 DNA. HS and HP DNAs were extracted from TK⁺ 143 B mass culture cells, digested with restriction endonucleases *Bam*HI, *Xba*I, *Eco*RI, *Bgl*II, and *Kpn*I, electrophoresed through a 1.0% agarose gel, blotted onto nitrocellulose, and probed with nick-translated pBK TK-1 DNA. Lanes 1, 4, 7, 10, and 13 show HS DNA from 10⁵ cells; lanes 2, 5, 8, 11, and 14 show HP DNA (10 μg); lanes 3, 6, 9, 12, and 15 show pBK TK-1 marker DNA (1 ng or 50 genome equivalents).

TABLE 1. Transformation of *E. coli* MC-1061 with HS DNAs from HeLa and 143 B cell clones transformed to the TK⁺ phenotype by pBK TK-1

Cell clones	No. of Amp^r bacterial colonies[a]	
	Untreated	Treated with *Mbo*I
HeLa H-303	28	ND
HeLa H-384	114	ND
143 B 656	95	0
143 B 657	233	0
143 B F6-D4	30	0
143 B 633	28	0
143 B B-27	25	0
143 B B-3	150	0
pBK TK-1 DNA (control, 10 ng)	26,400	21,000

[a] Amp^r bacterial colonies were produced by transformation of *E. coli* with samples of HSs obtained from 10⁵ cells. ND, Not done.

which cuts pBK TK-1 three times (Fig. 4A), and the fragments thus obtained were analyzed in agarose gels. As seen in Fig. 6A (lanes 1 to 12), plasmid DNA from each of 12 ampicillin-resistant colonies transformed by the HS DNA of HeLa cell line H-303 contained three *Eco*RI restriction fragments that comigrated with those of pBK TK-1 DNA (lane 15). No other DNA bands were seen; the higher-molecular-weight bands seen in lanes 6 and 12 (Fig. 6A) probably represent incomplete digestions. A similar analysis with the HS of an individual 143 B cell clone revealed that the plasmid DNAs from 11 of 12 bacterial transformants were indistinguishable from that of the input vector (data not shown). In addition, the HS DNA from a mass culture of 143 B cells was also used to transform *E. coli* C600. Plasmids extracted from 18 ampicillin-resistant colonies were ana-
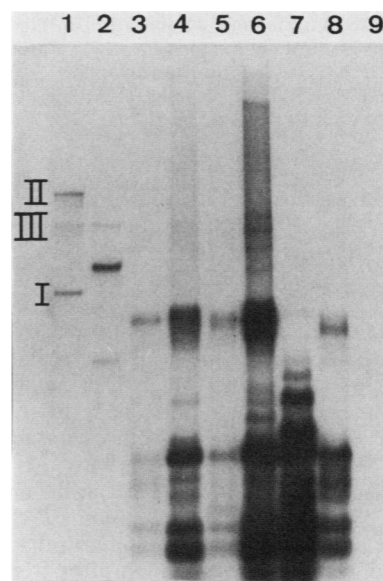
However, the DNA becomes sensitive to *Mbo*I digestion after replication in eucaryotic cells, where the adenine is not methylated. The untreated HS DNA from each HeLa and 143 B cell clone did transform *E. coli* MC-1061, whereas digestion by *Mbo*I rendered these same DNAs incapable of transformation (Table 1). The transforming ability of pBK TK-1 DNA directly isolated from *E. coli* Hb101 was unaffected by *Mbo*I treatment. These results indicate that pBK TK-1 does replicate as a free molecule in human cells and that the gene for ampicillin resistance remains functional.

Replication of pBK TK-1 in TK⁺ transformants was further supported by blot hybridization analysis. The HS DNA from each of seven 143 B TK⁺ individual clones was completely digested by *Mbo*I (lanes 3 to 9), whereas the control pBK TK-1 was not (lanes 1 and 2) (Fig. 5).

In another experiment, plasmids recovered from *E. coli* MC-1061 transformed by HS DNAs from either HeLa or 143 B TK⁺ cells were compared to input DNA. The plasmids from ampicillin-resistant colonies were digested with *Eco*RI,

FIG. 5. Digestion with *Mbo*I and blot hybridization analysis of 143 B cell clones transformed to the TK⁺ phenotype by pBK TK-1. HS DNA from 10⁵ cells was restricted with *Mbo*I, migrated in a 1% agarose gel, transferred to nitrocellulose, and hybridized to a pBK TK-1 probe. Lanes 3 to 9, 143 B clones B-18, B-28, B-6, B-23, B-36, B-31, and B-2, respectively; lane 1, pBK TK-1 marker digested with *Mbo*I (2.4 ng); lane 2, pBK TK-1 digested with *Bam*HI and *Mbo*I (2.4 ng). Forms I, II, and III of marker PBK TK-1 are indicated.
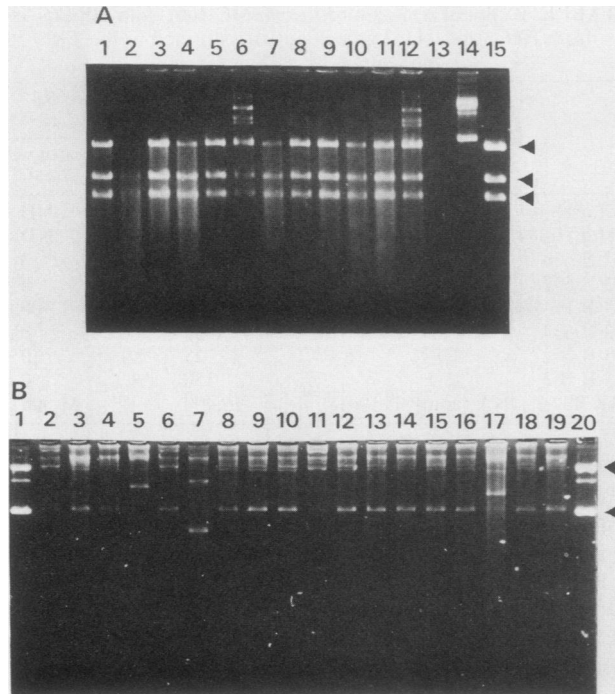
FIG. 6. pBK TK-1 from TK$^+$-transformed HeLa and 143 B cells is shuttled into *E. coli* and reestablished as plasmids. HS DNA from TK$^+$ HeLa and 143 B cells that had been transformed by pBK TK-1 was used to transform *E. coli* MC-1061 (A) or C600 (B). Plasmid DNAs were prepared from *E. coli* ampicillin-resistant (Amp^r) colonies, electrophoresed in 1% agarose gels, and visualized by ethidium bromide staining at 280 nm. (A) *Eco*RI digestion of plasmids from Amp^r colonies, transformed by HS DNA of HeLa cell clone H-303 (lanes 1 to 12). Marker pBK TK-1 was uncut (lane 14) or digested with *Eco*RI (lane 15). The arrows point to the three fragments generated by *Eco*RI digestion (Fig. 4A). Lane 13 is blank. (B) Plasmids (undigested) from Amp^r colonies transformed by HS DNA from 143 B mass culture cells (lanes 2 to 19). Marker pBK TK-1 DNA is in lanes 1 and 20. The lower arrow indicates the position of supercoiled (form I) pBK TK-1 plasmid DNA, and the upper arrow marks its relaxed circular form (form II).

lyzed directly in agarose gels. As seen in Fig. 6B, 15 of 18 colonies (lanes 2 to 19) contained supercoiled and relaxed circular molecules that comigrated with form I and form II DNA of pBK TK-1 (lanes 1 and 20). Two plasmids contained an insertion (Fig. 6B, lanes 5 and 17) and a third plasmid contained a deletion (lane 7), as confirmed by restriction-enzyme analysis (data not shown). These results show that in human cell lines originating either from a single transfected cell or from a mixture of hundreds of independently transfected cells, pBK TK-1 DNA persists as stable full-length episomes for many cell generations.

pBK TK-1 vector continues to persist in the episomal state after removal of selective growth medium. In all the experiments thus far described, the TK$^-$ human cells were placed under conditions of selective growth after transfection by pBK TK-1 DNA. Since only TK$^+$ cells are able to survive in HAT medium, it was not apparent whether selective pressure was required for maintenance of stable episomes. Therefore, an individual clone of 143 B cells (B-27) and a mass culture of 143 B cells which contained pBK TK-1 DNA were removed from the selective HAT medium and placed onto nonselective Eagle MEM.

Surprisingly, pBK TK-1 DNA molecules from the HS of

clone B-27 cells maintained in HAT medium for 200 generations (Fig. 7A, lane 2) appeared qualitatively unchanged after 25 generations of growth in nonselective MEM (Fig. 7A, lane 3). Furthermore, replacement of HAT medium for an additional 25 generations after growth in MEM did not alter the persistence of full-length pBK TK-1 episomes; the high-molecular-weight comigrating bands probably represent polymers of pBK TK-1 DNA (Fig. 7A, lane 4). No apparent loss in the number of pBK TK-1 molecules was detected in B-27 cells upon removal or reintroduction of HAT medium, since in each case (Fig. 7A, lanes 2 to 4) the amount of HS DNA analyzed was from the same number of cells.

A similar analysis of the 143 B mass culture also proved that maintenance of stable pBK TK-1 episomal DNA in these cells is not dependent upon selection in HAT medium. In this experiment, 143 B mass culture cells that had been maintained in HAT medium for 5 months were switched to growth in nonselective MEM for 3 months (48 generations). As seen in Fig. 7B, the Southern blot of HS and HP fractions digested with *Bam*HI reveals no obvious change in pBK TK-1 DNA after many cell doublings in nonselective medium (lane 1, HS; lane 2, HP) as compared with cells grown in HAT medium (lane 3, HS; lane 4, HP).

pBK TK-1 synthesizes BKV T antigen. It is possible that persistence of amplified episomes of pBK TK-1 in human cells may depend upon expression of BKV large T antigen encoded by the vector. This hypothesis seemed reasonable, since large T antigen of the closely related SV40 stimulates its replication (for review, see reference 54). Both 143 B- and HeLa TK$^+$-transformed cell lines were found to express BKV T antigen. As seen in Fig. 8, immunoprecipitation of the 95,000-dalton BKV large T antigen from one of these lines occurs with a specific hamster serum to BKV T antigen
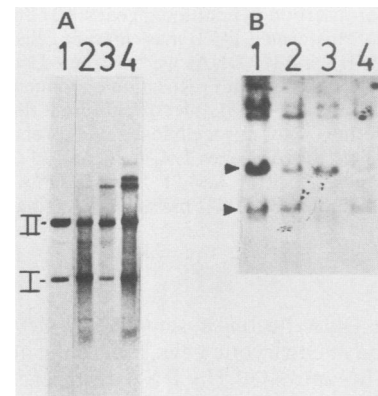

FIG. 7. Blot hybridization of HS DNA from TK$^+$-transformed 143 B cells maintained with and without selective HAT medium. Undigested or *Bam*HI-restricted DNA was electrophoresed through a 0.6% agarose gel (A) or a 1% agarose gel (B), blotted onto nitrocellulose, and probed with nick-translated pBK TK-1 DNA. (A) HS DNA (undigested and corresponding to 10^5 cells) from 143 B cell clone B-27, maintained first in HAT medium for more than 200 generations (lane 2), then for 25 generations in nonselective MEM (lane 3), and then replaced into HAT medium for 25 generations (lane 4). pBK TK-1 marker DNA (2.4 ng), indicating forms I and II, is shown in lane 1. (B) *Bam*HI-digested DNA from 143 B mass culture cells that had been maintained for 5 months in HAT medium: HS DNA (lane 3) and HP DNA (lane 4). DNA from the same cells after 3 months in nonselective MEM: HS DNA (lane 1) and HP DNA (lane 2). Uppermost bands represent partial DNA digests. Positions of the two fragments generated by *Bam*HI digestion of pBK TK-1 are shown by the arrows.
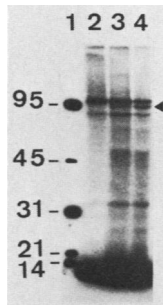
FIG. 8. Immunoprecipitation of BKV T antigen from 143 B cells transformed by pBK TK-1. TK$^+$ 143 B cells (clone B-633) were labeled with $^{32}P_i$, and anti-T hamster serum was used to immunoprecipitate BKV T antigen as described in the text. The immunoprecipitated products were fractioned in a 10% polyacrylamide gel. Labeled cell extracts were reacted with normal hamster serum (lane 2), 15 μl of anti-T serum (lane 3), or 30 μl of anti-T serum (lane 4). Protein molecular weight standards in kilodaltons are in lane 1: phosphorylase *b*, 95; ovalbumin, 45; carbonic anhydrase, 31; trypsin inhibitor, 21; lysozyme, 14. The arrow indicates the position of the 95,000 molecular-weight BKV large T antigen.

(lanes 3 and 4), but not with a normal serum (lane 2). In addition, BKV T antigen was also detected by immunofluorescence in 143 B cells stably transformed to the TK$^+$ phenotype by pBK TK-1 (Fig. 9).

pBK TK-1 contains an active origin of replication. As a corollary to these findings, it was important to demonstrate directly that pBK TK-1 DNA contains viral sequences responsible for replication in the presence of BKV T antigen. The BKV origin of replication, in fact, is predicted to be located at 0.67 map units, based upon extensive sequence homology to the known origin of replication in SV40 (45, 59). Therefore, the HindIII C fragment (555 bp) of BKV which contains sequences including the origin of replication was cloned into pML (Fig. 1A). The resulting recombinant,

referred to as pBODE (Fig. 1A), was transfected into L603 cells. These cells are human embryonic fibroblasts that had been transformed by a BKV DNA fragment containing the entire early region and are 100% positive for BKV T antigen (16). The intent was to see whether the pBODE molecule, containing the predicted BKV origin of replication but void of any complete viral genes, would be amplified in cells that constitutively produce BKV T antigen. The HS DNA was extracted at 16, 50, and 81 h posttransfection, linearized with BamHI, and analyzed by Southern blotting, using pBODE as the labeled probe. The pBODE molecule is greatly amplified, since densitometric analysis of the autoradiograms showed that the amount of pBODE DNA increases from 0.04 ng at 16 h posttransfection (Fig. 10, lane 1) to 29 ng at 50 h (lane 2) and then decreases to 3.7 ng at 81 h (lane 3). Therefore, reduction in copy number at 81 h posttransfection defines this amplification as transient. The pBODE molecules were not amplified in 143 B cells (data not shown). These results indicate that the 0.67-map-unit region of BKV present in the pBK TK-1 molecule serves as an active replication origin which is stimulated by BKV T antigen.

DISCUSSION

We describe an expression vector, pBK TK-1, which is able to persist episomally in human cells. This vector includes sequences derived from BKV, a human papovavirus; pML, a deletion derivative of plasmid pBR322 (25); and the TK gene of HSV-1, which transforms TK$^-$ mutant cells to the TK$^+$ phenotype (58). Free molecules of pBK TK-1 were detected in the low-molecular-weight DNA fraction of established TK$^+$-transformed cells. Enzymatic expression of the TK gene of HSV-1 was also detected in the TK$^+$ transformants (unpublished data). The origin of the human cell into which pBK TK-1 was introduced influenced to a certain extent its episomal status both quantitatively and qualitatively. In HeLa and 143 B TK$^+$ cell transformants, the numbers of extrachromosomal copies of pBK TK-1 per
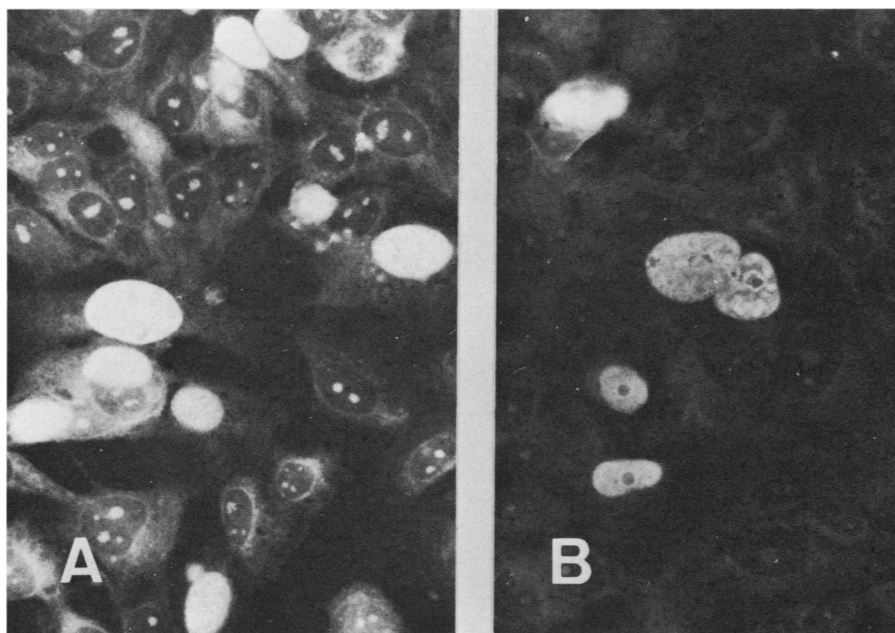

FIG. 9. Fluorescent antibody staining for BKV T antigen in 143 B TK$^+$ cells transformed by pBK TK-1. Cells were first reacted with a specific hamster serum to BKV T antigen and then with fluorescein-conjugated goat antibodies to hamster immunoglobulin G. (A) Clone B-28 and (B) clone B-31.
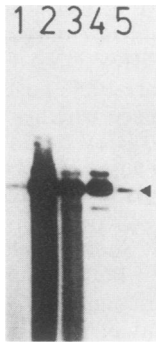
FIG. 10. Amplification of pBODE, a BKV origin recombinant plasmid, in BKV-transformed human cells positive for BKV T antigen, as analyzed by Southern blot hybridization. The BKV-transformed human cells, L603, in which the BKV T-antigen gene is integrated and expressed, were transfected with pBODE (10 μg of DNA per 10^7 cells). At indicated times after transfection, cells were rigorously washed, and HS DNA was prepared. Samples of HS DNA corresponding to 10^5 cells were digested with BamHI, electrophoresed through a 0.6% agarose gel, blotted onto nitrocellulose, and probed with nick-translated pBODE DNA. Lane 1, 16 h posttransfection; lane 2, 50 h posttransfection; lane 3, 81 h posttransfection. Marker pBODE DNA cut with BamHI is shown in lane 4 (3.5 ng) and lane 5 (0.07 ng). The arrow points to linear form III of pBODE DNA.

cell were generally 20 to 40 and 75 to 120, respectively. In HeLa cells, pBK TK-1 molecules almost exclusively appeared as full-length episomes with undetectable rearrangements. Although all 143 B transformants contained full-length episomes, additional free forms that probably included polymeric and deleted molecules were observed in some clones. The nature of these rearranged molecules is unknown. In this connection, it must be pointed out that in BKV-transformed human cells, where BKV DNA replicates episomally, free unintegrated polymeric forms of the viral genome were constantly detected in considerable amounts (16). In all cases, however, full-length pBK TK-1 molecules were the predominant episomal form as specifically demonstrated in 143 B mass culture cells, which represented a pool of hundrds of individual TK$^+$ colonies. In both HeLa and 143 B cells, integration of pBK TK-1 into chromosomal DNA appeared infrequent and certainly negligible in proportion to the number of amplified free molecules.

The pBK TK-1 molecules from TK$^+$-transformed human cells can be readily shuttled into bacteria. Surprisingly, almost 85% of the Amp^r bacterial colonies transformed by DNA from the 143 B TK$^+$ mass culture cells contained plasmid DNA that was indistinguishable from the input pBK TK-1 vector DNA; this value was near 100% when DNA from a TK$^+$ HeLa or 143 B cell clone was used. Furthermore, plasmid DNA could not be rescued in bacteria when these cellular DNAs were digested with the endonuclease MboI, which selectively restricts DNA that has replicated in animal cells. Not only do these results demonstrate definitively that pBK TK-1 persists as stable replicons in human cells, but they also show that a resident bacterial gene, which confers no selective advantage to the eucaryotic cell, retains its capacity to function, i.e., the β-lactamase gene of pML which confers ampicillin resistance in E. coli.

Importantly, episomal maintenance of pBK TK-1 in human cells does not appear to depend upon forced expression of the TK gene under selection for the TK$^+$ phenotype, since the vector is not drastically affected by removal of HAT

medium. This is in contrast to a report which describes the loss of TK-plasmid recombinant episomes in transformed rodent cells after removal of selective medium (51). Our experiments indicate that the sequences inherent in the BK viral moiety of pBK TK-1 dictate the copy level and stability of the vector. This further suggests that any dominant selection marker gene (33) may be substituted for the TK gene of HSV-1 to thereby extend the spectrum of human cell types into which this novel expression vector can be introduced and amplified. The possibility that an origin of replication in HSV-1 TK gene could be responsible for the episomal state of the vector, as suggested by others (51), was ruled out because only 0.2 copies of free plasmid DNA per cell were detected in HS DNA of 143 B cells transformed to the TK$^+$ phenotype by a recombinant molecule containing only pML and the HSV-1 TK gene. In this case, essentially all vector DNA sequences were found to be integrated into high-molecular-weight DNA of the TK$^+$ transformants (G. Barbanti-Brodano, unpublished data).

The features which control the episomal replication function of pBK TK-1 are unknown. It seems unlikely that the late BK viral genes are involved in episomal maintenance of pBK TK-1 since they were partially deleted to circumvent potential infection. However, the early viral large T- and small t-antigen genes are completely encoded in pBK TK-1. It is likely that the immunoreactive BKV large T antigen in the TK$^+$-transformed cells stimulates the BKV origin of replication in pBK TK-1 analogous to SV40 T-antigen-dependent replication (54). The supportive argument for this mechanism is demonstrated by the great amplification of plasmid pBODE, which contains the putative BKV origin of replication (0.67 viral map units; 42, 45, 59), but is void of complete early and late BKV genes, in BKV-transformed human cells that constitutively synthesize BKV T antigen.

This BKV T-antigen-dependent replication of pBODE is similar to transient replication of SV40 vectors in COS cells (31). It is intriguing to consider that in TK$^+$ transformants the copy level of pBK TK-1 may be held constant by autoregulation of BKV T-antigen synthesis, similar to control of SV40 T-antigen production during lytic infection (1, 22, 52). We have begun constructing deletion mutants in the T-antigen gene of pBK TK-1 to define potential elements necessary for episomal replication. Alternatively, maintenance of the episomal state of pBK TK-1 in human cells may depend on specific viral sequences, not necessarily related to the T-antigen coding region, similar to what has been shown for vectors based on bovine papillomavirus DNA (26). A search for such sequences in the BKV genome is under way. Finally, it cannot be ruled out that replication of the vector is also under the control of some host cell components.

The pBK TK-1 molecule represents the first stable expression vector that persists episomally in human cells and that can be shuttled into bacteria. These features are also shared by the bovine papillomavirus-plasmid vectors that function in mouse cells (6, 43). Key advantages and potential uses of the episomal pBK TK-1 expression vector are the following. (i) The inserted gene of interest cannot be interrupted or subjected to regulatory constraints that frequently occur from integration into cellular DNA. (ii) The gene of interest is amplified with the vector. (iii) The vector can be used to study genes which are normally expressed or require expression in human cells. For example, we have produced human cell lines in which the episomal vector contains adenovirus type 12 transforming genes and expresses functional and immunoreactive adenovirus type 12 E1A proteins (R. Vasa-

vada, G. Barbanti-Brodano, and R. P. Ricciardi, unpublished data). The pBK TK-1 vector contains restriction sites convenient for different cloning strategies. (v) Substitution of the TK gene with a dominant selection gene such as Tn5 aminoglycoside phosphotransferase (49) or *E. coli* xanthine-guanine phosphoribosyltransferase (33) in the vector predictably will expand the variety of human cells that can be used. (vi) The system may be applied in transient assays as suggested from this study with pBODE in L603 cells. Indeed, we have already prepared a pBODE-CAT (chloramphenicol acetyltransferase) recombinant that transiently expresses high enzyme activity in 143 B TK$^+$ transformants containing pBK TK-1 (A. Caputo, G. Barbanti-Brodano, and R. P. Ricciardi, unpublished data). (vii) The pBK TK-1 recombinant may be modified for the insertion and expression of cDNAs.

ACKNOWLEDGMENTS

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