Induction of Malignant Subcutaneous Sarcomas in Hamsters by a Recombinant DNA Containing BK Virus Early Region and the Activated Human c-Harvey-ras Oncogene

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ABSTRACT

Malignant undifferentiated sarcomas were induced in 11 of 15 (73.3%) newborn Syrian hamsters by s.c. inoculation of a recombinant DNA (pBK/c-rasA) containing BK virus (BKV) early region gene and the activated human c-Harvey-ras (c-Ha-ras) oncogene derived from T24 bladder carcinoma. The two genes inoculated independently as well as a recombinant DNA of BKV early region gene and normal human c-Ha-ras proto-oncogene were not tumorigenic. Tumor-derived cell lines propagated in culture were immortalized and had growth characteristics consistent with a fully transformed phenotype. Tumors and tumor cell lines showed tandem insertions of pBK/c-rasA in high copy number and expressed BKV- and c-Ha-ras-specific transcripts as well as BKV T-antigen and c-Ha-ras protein with a molecular weight of 21,000. We conclude that BKV DNA requires interaction with other oncogenic functions for tumorigenicity. These findings may be relevant to the role of BKV in human neoplasia, where cooperation or synergism between BKV and cellular oncogenes could occur as an aspect of the multifactorial process of carcinogenesis.

INTRODUCTION

BKV* is a human papovavirus ubiquitous in human populations and with a worldwide distribution (1). Primary infection generally occurs during childhood and is followed by a persistent, latent infection which is reactivated under conditions of impaired immunological response (1). BKV and BKV DNA transform hamster, rat, mouse, rabbit, monkey, and human cells in vitro (2, 3). BKV T-Ag and DNA are regularly found in BKV-transformed cells. While BKV DNA sequences are mostly integrated into cellular DNA in transformed rodent cells (4), they are detected only in a free, episomal state in transformed human cells (5–7). BKV is highly oncogenic when inoculated i.c. or i.v. in immunosuppressed or immunocompetent hamsters, causing tumors in 73 to 88% of animals. The virus has a marked tropism for specific organs, since tumors observed in infected animals belong to only 3 histotypes: ependymomas and choroid plexus papillomas, tumors of pancreatic islets, and osteosarcomas (8–13). BKV is very weakly oncogenic when inoculated s.c. (8, 14–16). BKV DNA is not oncogenic after s.c. and i.v. inoculation and it induces tumors at low frequency (5.1%) when inoculated i.c. in hamsters (10).

MATERIALS AND METHODS

Animals. Newborn Syrian golden hamsters less than 24 h old were inoculated s.c. in the back with DNA dissolved in PBS (50 µl). Each animal in the first group received 2 µg of BKV DNA. Recombinant DNAs were inoculated in equimolar proportions referred to this amount of BKV DNA. Hamsters were sacrificed when tumors had a size of 2 to 3 cm in diameter and the animals without tumors were killed 1 year after inoculation. They were necropsied completely and their organs were carefully examined for abnormalities. All tissues were fixed in 10% buffered formalin, processed for histology by conventional methods, and stained with hematoxylin and eosin.

Cell lines. Cells derived from tumors and other transformed normal cells were grown in MEM, supplemented with 10% FBS. For the determination of serum dependency of growth, 10^3 cells/cm^2 were seeded in 60-mm Petri dishes with MEM containing 2 or 10% FBS. Cells were counted every 2 days using the dye exclusion test for viability determination. Saturation density and doubling time were calculated from the exponential growth curves as described by Risser and Pollack (20). For colony formation assays on plastic, 500 cells/60-mm Petri dish were plated with MEM plus 10% FBS and colonies were counted after 10 days. For colony formation in semiolid medium, 10^4 cells were suspended in MEM plus containing 2 or 10% FBS. Cells were counted every 2 days using the dye exclusion test for viability determination. Saturation density and doubling time were calculated from the exponential growth curves as described by Risser and Pollack (20). For colony formation assays on plastic, 500 cells/60-mm Petri dish were plated with MEM plus 10% FBS and colonies were counted after 10 days. For colony formation in semiolid medium, 10^4 cells were suspended in MEM plus 10% FBS containing 0.35% noble agar (Difco Laboratories, Detroit, MI) and were seeded in 60-mm Petri dishes over a basal layer of 0.8% noble agar in MEM (21). The dishes were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Colony number and size were determined after 14 days at x100. Wild-type BKV (Gardner strain) was grown in Vero cells and purified by centrifugation gradient centrifugation (22). BKV DNA was extracted from purified virions as described (4).

Recombinant DNA Techniques. Standard cloning methods were used
Clonal antibodies according to Sternberger (37), using a peroxidase- protein, S-100 protein, and cytokeratin were analyzed with rabbit polyclonal antibodies and peroxidase-conjugated rabbit anti-mouse desmin filaments were visualized by indirect immunoperoxidase stain with hematoxylin and mounted for viewing by light microscopy. Vimentin and Graham and Karnowsky (36). All incubations were at room temperature biotin-peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) was then added for 30 min and the reaction was chased from New England BioLabs (Beverly, MA) or Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany) and used according to the specifications from the manufacturers.

Southern Blot Analysis of DNA. High molecular weight DNA was prepared from tumors or cultured cells as described by Meneguzi et al. (4). Samples of 20 µg of genomic DNA were digested with an excess (60 units) of BamHI, EcoRI, HindIII, or CiaI restriction enzymes. DNA fragments were separated in 0.8% (w/v) agarose gels and blotted to a BA85 nitrocellulose membrane (Schleicher and Schuell, Dassel, Federal Republic of Germany) according to Southern (24). 32P-labeled probes of the complete recombinant DNA, with a specific activity of 3.8 to 6 x 106 cpm/µg, were prepared by nick translation (25). Blots were hybridized, washed, and dried as described (4). The dried membranes were exposed to a Kodak X-Omat SO-282 film at -70°C with an intensifying screen.

RNA Analysis. Total cytoplasmic RNA was extracted as described by Favaloro et al. (26) and treated for 45 min at 37°C with 1 unit/µg of RNase-free DNase I (Worthington, Freehold, NJ), which was further deprived of residual RNase activity by treatment with proteinase K according to Tullis and Rubin (27). Samples of 20 µg of RNA were then denatured at 65°C for 10 min in 50% formamide:6% formaldehyde, run on 6% formaldehyde:1% agarose gels (28), blotted to BA85 nitrocellulose sheets (29), and hybridized to 32P-labeled probes (specific activity, 2 to 3.6 x 106 cpm/µg) of the gel-purified 6.6-kb c-Ha-ras DNA fragment or of the BKV DNA 3.2-kb PvuII fragment containing the entire early region. Probes were labeled by incorporation of [32P]-dCTP using the method of random sequence exanucleotides (30, 31) to prime DNA synthesis on denatured DNA with the Klenow fragment of E. coli DNA polymerase I (Multiprime DNA labeling system; Amerham International, Amersham, England). Hybridization was carried out according to Vassavada et al. (32) at 37°C for 22 h in 50% formamide (Fluka, Buchs, Switzerland), followed by 6 washings of 30 min each at 52°C in standard saline citrate (0.15 M sodium chloride; 0.015 M sodium citrate) concentrations of 2x to a final stringency of 0.1x and 0.1% NaDodSO4. Film exposure was carried out as for Southern blots.

Fluorescence Techniques. For indirect immunofluorescence, tumor imprints on glass slides or nearly confluent cells seeded on glass coverslips were fixed for 10 min in acetone at room temperature, incubated for 1 h at 37°C with hamster serum to BKV T-Ag, washed 3 times in PBS, and incubated for 1 h at 37°C with fluorescein-conjugated rabbit anti-hamster IgG (Antibodies Incorporated, Davis, CA). After washing 3 times in PBS and 2 times in distilled water the preparations were mounted with buffered glycerol. For visualization of actin cables, actin-free fixed cells grown on coverslips were treated with rhodamine-conjugated phalloidin as described by Wulf et al. (33).

Immunoperoxidase Staining. Immunoperoxidase staining was carried out on tumor tissue or cells grown on coverslips after fixation in 10% buffered formalin. Samples of tumor tissue were embedded in paraffin, cut into 4- to 6-µm thick sections, deprived of paraffin with xylol and rehydrated. For detection of c-Ha-ras p21, tissue sections and cell monolayers were incubated with normal horse serum diluted 0.1 for 20 min to saturate aspecific sites, followed by anti-c-Ha-ras p21 RAP5 mouse MAB (34, 35) diluted 1/50 for 60 min and horse anti-mouse biotinylated antibodies diluted 1/50 for 30 min. A preformed avidin-biotin-peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) was then added for 30 min and the reaction was developed for 5 min with diaminobenzidine chloride according to Graham and Karnowsky (36). All incubations were at room temperature and all dilutions and rinses between incubations were performed with PBS. The preparations were then counterstained with Mayer's hematoxylin and mounted for viewing by light microscopy. Vimentin and desmin filaments were visualized by indirect immunoperoxidase staining with mouse MAbs and peroxidase-conjugated rabbit anti-mouse antibodies (Dakopatts, Glostrup, Denmark). Gial fibrillary acidic protein, S-100 protein, and cytokeratin were visualized with rabbit polyclonal antibodies according to Sternberger (37), using a peroxidase-antiperoxidase system (Dakopatts). Immunoblotting. Tumors or cell pellets were pulverized in liquid nitrogen with a Micro-Dismembrator II (Braun AG, Melsungen, Federal Republic of Germany) and homogenized in a Teflon-glass homogenizer with 4 volumes of ice-cold lysis buffer (0.1 M NaCl;5 mM MgCl2;1% Nonidet P-40;0.5% sodium deoxycholate;2 KI units/ml bovine aprotinin;20 mM Tris-HCl, pH 7.4). The homogenates were centrifuged at 750 x g for 20 min at 4°C and the resulting supernatants were used as the lysates. A constant amount (40 µg) of proteins was separated by electrophoresis in 0.1% NaDodSO4;12% polyacrylamide gels and electrophoretically transferred (38) to nitrocellulose membranes which were washed 3 times and first incubated with 3% bovine serum albumin in 50 mM Tris-HCl (pH 7.5):150 mM NaCl:2 mM EDTA:0.1% Nonidet P-40 (Tris-REN) for 3 h at 37°C to remove bound NaDodSO4 and to block the residual sites on the cellulose. Then they were sequentially incubated in Tris-REN containing p21 rat MAB Y13-259 (39) (1 µg/ml) for 16 h at 4°C, rabbit anti-rat IgG (Cappel Laboratories, Philadelphia, PA), diluted 1/500 for 2.5 h in an ice water bath, and 5 x 10/cpm/ml 125I-labeled staphylocoelic Protein A (specific activity, 30 mCi/mg; Amersham) for 1 h in an ice water bath. Y13-259 Mab is directed to the v-ras gene of Ha-MuSV and cross-reacts with the closely related product of c-Ha-ras gene (39). Addition of rabbit anti-rat antibodies was necessary because rat IgG does not bind efficiently to Protein A. The nitrocellulose sheets were air-dried and exposed to Kodak X-Omat SO-282 films for 12 to 36 h at ~20°C.

Densitometric Analysis. Densitometric scanning of autoradiograms for quantification of DNA, RNA, and protein bands was performed with an Ultrascan XL laser densitometer (LKB, Bromma, Sweden).

RESULTS

Construction of Vectors. The construction of recombinant DNA vectors is described in Fig. 1. The vector pBK (40), containing BKV wild-type early region, was constructed by ligating together the large BamHI/EcoRI fragments of BKV genome and of pML, a deletion derivative of pBR322 (41). The deletion of the small BamHI/EcoRI fragment in BKV DNA removed from the late region 107 nucleotides, containing sequences for each of the late viral capsid proteins VP1, VP2, and VP3, thereby ensuring that excision of viral sequences from the recombinant does not yield infectious viral DNA. pBK/c-rasA and pBK/c-rasN were constructed by inserting the 6.6-kb BamHI fragment, containing the activated c-Ha-ras oncogene (c-rasA) or the 6.4-kb BamHI fragment, containing the normal c-Ha-ras protooncogene (c-rasN), into the unique BamHI site of pBK. c-rasA and c-rasN are genomic clones derived from T24 human bladder carcinoma or normal human fetal liver, respectively. They were first cloned in Charon 4A phage (42), then subcloned into the unique BamHI site of plasmid pBR322 (pBR/c-rasA and pBR/c-rasN). c-rasA and c-rasN DNA fragments are essentially identical in their restriction map and nucleotide sequence, 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 sequences related to c-Ha-ras (42). 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.

Tumors and Tumor Cell Lines. In the first experiment, groups of 15 hamsters were inoculated s.c. with BKV DNA or pBK, pBK/c-rasA, pBK/c-rasN, pBR/c-rasA, or pBR/c-rasN. Eleven of the animals inoculated with pBK/c-rasA (73.3%) developed tumors at the site of injection with a latency period of 2 to 8 weeks. Once the tumors had appeared, they grew rapidly with a doubling time of 2.5 to 5 days. In all animals of the other groups no tumors were detected after an observation period of...
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1 year. In a second experiment which is under way (observation period of only 1 month), 5 of 18 (27.7%) pBK/c-rasA inoculated hamsters developed tumors. The tumors were diagnosed as undifferentiated pleomorphic sarcomas. Their mesenchymal origin was confirmed by positive staining with a MAb to vimentin, a typical mesenchymal marker (43). They consisted of highly atypical, polymorphic cells, with wide cytoplasm and large polymorphic nuclei (Fig. 2A). Giant, often plurinucleated, cells were frequently observed. Mitoses, sometimes tripolar or tetrapolar, were common. Metastases were generally not detected, except in 2 animals that showed metastases to genital organs.

Small pieces of tumors were explanted in vitro and cells outgrowing the explants were propagated as cell cultures, three of which, L702, L703, and L704, were studied in detail. The analysis of their morphology, growth characteristics and cytoskeletal alterations indicated that they had a fully transformed phenotype and were immortalized, since all of them were beyond the 100th population doubling. Remarkable differences, however, were detected, L704 showing a lower serum depend-

Expression of c-Ha-ras p21 and BKV T-Ag. c-Ha-ras p21 was detected by immunoperoxidase staining with RAP5 MAb in all pBK/c-rasA-induced tumors and tumor cell lines, whereas nor-
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Fig. 2. A, undifferentiated s.c. sarcoma 435 induced in a hamster by pBK/c-rosA, showing highly atypical cells with polymorphic nuclei. B, tumor-derived cell line L704. Immunoperoxidase reaction (A and B) with anti-c-Ha-rosA p21 RAP-5 MAb shows positive staining of cytoplasm and plasma membrane of tumor and cultured cells. In B, the staining is specifically concentrated on cells growing in foci. Immunoperoxidase and Mayer’s hematoxylin. A, × 400; B, × 250.

Table 1 Characterization of the phenotype of tumor-derived and control cell lines

<table>
<thead>
<tr>
<th>Cell linesa</th>
<th>Saturation densityb (cells/cm² × 10⁴)</th>
<th>Doubling timec (h)</th>
<th>Colony formationd (colonies/100 cells)</th>
<th>Disorganization and fragmentation of actin cables</th>
</tr>
</thead>
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<tr>
<td></td>
<td>2% FBS</td>
<td>10% FBS</td>
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</tr>
<tr>
<td>L702</td>
<td>0.1</td>
<td>12.3</td>
<td>–</td>
<td>39</td>
</tr>
<tr>
<td>L703</td>
<td>13.3</td>
<td>27.4</td>
<td>26</td>
<td>25</td>
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<td>53.0</td>
<td>21</td>
<td>20</td>
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<tr>
<td>L578</td>
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<td>29.7</td>
<td>35</td>
<td>32</td>
</tr>
<tr>
<td>HEF</td>
<td>0.1</td>
<td>7.2</td>
<td>–</td>
<td>36</td>
</tr>
</tbody>
</table>

* L702, L703, and L704, cell lines derived from pBK/c-rosA-induced hamster s.c. sarcomas; L578, hamster kidney cells transformed by BKV DNA; HEF, secondary hamster embryo fibroblasts.

** Average of 2 Petri dishes.

T-Ag was detected by indirect immunofluorescence in cell nuclei of all primary tumors (average, 65% of positive cells) and tumor cell lines (average, 98% of positive cells) (Fig. 7). Sera from 8 tumor-bearing hamsters were tested for the presence of antibodies against BKV T-Ag. All sera were positive for the presence of antibodies against BKV T-Ag, indicating that BKV T-Ag was present in all tumor cells. The results suggest that BKV T-Ag is an important factor in the development of BKV-induced tumors.
Fig. 4. Northern blot analysis of tumor-derived cell lines to detect c-Ha-ras transcripts. Samples of total cytoplasmic RNA (20 µg/lane) were analyzed by agarose gel electrophoresis and hybridization to the 32P-labeled 6.6-kb c-rosA DNA fragment as probe. A, lane 1, L703; lane 2, L578, hamster kidney cells transformed by BKV DNA; lane 3, normal secondary hamster embryo fibroblasts. B, lane 1, L702; lane 2, L704; lane 3, BHK cells, continuous cell line of baby hamster kidney cells; lane 4, T284, hamster cell line derived from a BKV-induced osteosarcoma; lane 5, normal secondary hamster embryo fibroblasts; lane 6, L578. A and B were different experiments. Molecular weight markers (28, 18, and 55 ribosomal RNA) are indicated alongside.

Fig. 5. Northern blot analysis of tumor-derived cell lines to characterize BKV transcripts. Total cytoplasmic RNA (20 µg/lane) was migrated in a 1% agarose gel, transferred to nitrocellulose, and hybridized to BKV DNA 3.2-kb PvuII fragment containing the entire early region. Lane 1, L702; lane 2, L704; lane 3, BHK cells; lane 4, T284; lane 5, normal secondary hamster embryo fibroblasts; lane 6, L578. Molecular weight markers (28, 18, and 55 ribosomal RNA) are shown on the right. See legend to Fig. 4 for explanation of cell lines.

by immunofluorescence and found to contain antibodies to BKV T-Ag.

**DISCUSSION**

Neoplastic conversion is the end result of a multistep, multifactorial process (46) that occurs by different pathways and involves at least 2 sequential events: initiation characterized by immortalization and completion characterized by the acquisition of the transformed phenotype (47, 48). Different agents such as chemical and physical carcinogens, viruses, and cellular oncogenes can participate in both the first and the second phase of oncogenesis (49).

Transformation by papovaviruses is induced and maintained by T-Ag (50). Although human cells transformed by BKV or BKV early region DNA and expressing BKV T-Ag grow as immortalized cell lines, they never show a completely transformed phenotype (5-7). Human embryo kidney cells transfected with a recombinant plasmid containing BKV early region and the adenovirus 12 E1A gene are fully transformed and grow as a continuous cell line (32) suggesting that, at least in human cells, BKV T-Ag is competent to contribute only a partially transformed phenotype and must interact with other oncogenic functions to induce complete transformation.

In the present study we show that BKV early region gene and the activated human c-Ha-ras oncogene, linked together in a recombinant DNA, produce highly undifferentiated, rapidly growing, malignant sarcomas by s.c. inoculation in newborn hamsters, whereas the 2 genes independently or BKV DNA linked to normal c-Ha-ras protooncogene are not tumorigenic.

* A. Corallini, unpublished observations.

**Fig. 6. Detection of c-Ha-ras p21 in pBK/c-rosA-induced s.c. hamster tumors and tumor cell lines by immunoblotting.** Proteins in cell lysates were separated in 0.1% NaDodSO4:12% polyacrylamide slab gels, electrophoretically transferred to nitrocellulose, and reacted with Y13-259 MAb to ras p21. Immune complexes were labeled with 125I-labeled staphylococcal Protein A and recognized by autoradiography. Lane 1, sarcoma 435; lane 2, sarcoma 438; lane 3, normal hamster skin; lane 4, L702; lane 5, L704; lane 6, L703 clone 4B; lane 7, L703 clone 6B; lane 8, L703 clone 6B; lane 9, L578; lane 10, BHK cells; lane 11, secondary hamster embryo fibroblasts. Molecular weights (× 10^3) of marker proteins (bovine serum albumin, M, 66,000; ovalbumin, M, 45,000; soybean trypsin inhibitor, M, 21,000; lysozyme, M, 14,000; Bio-Rad, Richmond, CA) are indicated on the right. See legend to Fig. 4 for explanation of cell lines.
transformed phenotype, as determined by growth characteristics in vitro and cytoskeletal alterations, correlates with levels of c-Ha-rosA transcription and p21 synthesis, suggesting that transformation is modulated by c-Ha-rosA effecter functions. The nature and mechanism of the interaction between the 2 transforming genes are at present unclear. Since c-Ha-rosA confers morphological alteration and anchorage independence to early passage rodent cells and can be complemented by the immortalizing functions of polyoma virus large T-Ag (48, 51), in our experimental model immortalization may be contributed by BKV large T-Ag. This hypothesis can be tested by analyzing transformation in vitro of early passage hamster cells by the recombinant DNA molecules used in these experiments. The in vitro system would establish if BKV and c-Ha-rosA functions can be expressed at different times, if sequential transfection with each of the 2 genes leads to the transformed phenotype in 2 steps. Moreover, cotransfection of early passage hamster cells with pBK and pBR/c-rosA could clarify whether in the recombinant DNA BKV early region and c-Ha-rosA express individually cooperating functions or cis-acting DNA sequences, such as BKV transcriptional enhancers, stimulate c-Ha-rosA expression.

A direct influence of BKV sequences on cellular oncogenes may be exerted by BKV variants associated with human tumors (17–19) that contain in the early region a putative transposable element enclosing in its sequence 2 viral enhancer repeats elements (18). Several cellular oncogenes are involved in different steps of human carcinogenesis (55, 56) and could be deregulated or activated through a mutagenic effect or enhancement of transcription (57) by insertion of this specific transposable sequence. Therefore, it seems advisable to explore further the interaction of wild-type BKV and its variants with other human oncogenes.

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REFERENCES

These findings indicate that interaction of BKV with activated c-Ha-ras is required for tumorigenicity, in agreement with lack of BKV DNA oncogenicity after s.c. inoculation in hamsters (10) and inability of activated c-Ha-ras to induce complete transformation of primary hamster cells (51, 52). Cell lines derived from tumors and propagated in culture are immortalized and show a fully transformed phenotype, indicating that BKV and c-Ha-rosA oncogenic functions are able to maintain stable transformation.

The recombinant DNA is detected in high copy number in tumors and tumor-derived cell lines. It is mostly arranged in tandem insertions of linear molecules as observed previously in BKV-induced hamster tumors (53). Furthermore, integrations occur in plasmid sequences, suggesting that the immortality of both BKV and c-Ha-ras sequences is required for the induction and maintenance of the transformed state. BKV early region and activated c-Ha-rosA are specifically transcribed and expressed in tumors and tumor cell lines that contain BKV and c-Ha-rosA mRNAs as well as BKV T-Ag and c-Ha-rosA p21, not detected in controls. The small amount of p21 immunoreactive product found in normal hamster skin as well as in some control tumors and a cell line is consistent with previous reports (54) and may depend on a low expression of the endogenous c-ras genes. In tumor cell lines the expression of the
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