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Induction of Malignant Subcutaneous Sarcomas in Hamsters by a Recombinant DNA Containing BK Virus Early Region and the Activated Human c-Harvey-*ras* Oncogene[1]

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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-*ras*A) 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-*ras*A 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[4] 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).

BKV DNA, RNA, and T-Ag have been detected in human brain tumors and tumors of pancreatic islets (17–19). Since BKV DNA is free in the neoplastic tissue, BKV was recovered from tumors (6 brain tumors and 2 tumors of pancreatic islets) by transfection of total tumor DNA into human embryonic fibroblasts (17–19). Restriction endonuclease mapping of viruses isolated from tumors showed that they are all similar to each other and different from wild-type BKV in that they bear an insertion and a deletion in the early region of the genome (17–19). Nucleotide sequence analysis of one of these variants (BKV-IR) showed that the inserted nucleotides can form an insertion sequence-like structure (18). This transposable element might be involved in oncogenesis by excising from viral DNA and inserting 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 insertion sequence contains in its loop 2 of the 3 viral transcriptional enhancers (18). Therefore, we sought an experimental model to evaluate a possible interaction in tumorigenicity between BKV and human oncogenes. To this purpose we constructed recombinant DNA molecules containing BKV early region and the human c-Ha-*ras* oncogene and assayed their oncogenic potential in hamsters.

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.

Cells and Virus. Cell lines derived from tumors and other transformed and 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 semisolid 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% CO_2 in air. Colony number and size were determined after 14 days at ×100. Wild-type BKV (Gardner strain) was grown in Vero cells and purified by CsCl density gradient centrifugation (22). BKV DNA was extracted from purified virions as described (4).

Recombinant DNA Techniques. Standard cloning methods were used

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[4] The abbreviations used are: BKV, BK virus; c-Ha-*ras*, cellular Harvey *ras* oncogene; MEM, Eagle's minimum essential medium; FBS, fetal bovine serum; T-Ag, large tumor antigen; t-Ag, small tumor antigen; kb, kilobase; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate buffered saline (140 mM NaCl:3 mM KCl:0.15 mM KH_2PO_4:8 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.2); p21, a protein with a molecular weight of 21,000; MAb, monoclonal antibody; v-*ras*, viral *ras*; Ha-MuSV, Harvey murine sarcoma virus.

(23). Restriction endonucleases, phage T4 DNA ligase, *Escherichia coli* DNA polymerase I, and calf intestinal alkaline phosphatase were purchased 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 Meneguzzi *et al.* (4). Samples of 20 μg of genomic DNA were digested with an excess (60 units) of *Bam*HI, *Eco*RI, *Hind*III, or *Cla*I 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). ^{32}P-labeled probes of the complete recombinant DNA, with a specific activity of 3.8 to 6 × 10^8 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 ^{32}P-labeled probes (specific activity, 2 to 3.6 × 10^9 cpm/μg) of the gel-purified 6.6-kb c-Ha-*ras* DNA fragment or of the BKV DNA 3.2-kb *Pvu*II fragment containing the entire early region. Probes were labeled by incorporation of [^{32}P]-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; Amersham International, Amersham, England). Hybridization was carried out according to Vasavada *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 with decreasing standard saline citrate (0.15 M sodium chloride: 0.015 M sodium citrate) concentrations from 2× to a final stringency of 0.1× and 0.1% $NaDodSO_4$. 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, acetone-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 chloridrate 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, Glastrup, Denmark). Glial fibrillary acidic protein, S-100 protein, and cytokeratin were analyzed 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 $MgCl_2$: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 × *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% $NaDodSO_4$: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-NEN) for 3 h at 37°C to remove bound $NaDodSO_4$ and to block the residual sites on the cellulose. Then they were sequentially incubated in Tris-NEN 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 × 10 cpm/ml ^{125}I-labeled staphylococcal Protein A (specific activity, 30 mCi/mg; Amersham) for 1 h in an ice water bath. Y13-259 MAb is directed to the p21 encoded by 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 Ultroscan 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 *Bam*HI/*Eco*RI fragments of BKV genome and of pML, a deletion derivative of pBR322 (41). The deletion of the small *Bam*HI/*Eco*RI 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-*ras*A and pBK/c-*ras*N were constructed by inserting the 6.6-kb *Bam*HI fragment, containing the activated c-Ha-*ras* oncogene (c-*ras*A) or the 6.4-kb *Bam*HI fragment, containing the normal c-Ha-*ras* protooncogene (c-*ras*N), into the unique *Bam*HI site of pBK. c-*ras*A and c-*ras*N are genomic clones derived from T24 human bladder carcinoma or normal human fetal liver, respectively. They were first cloned in λ Charon 4A phage (42), then subcloned into the unique *Bam*HI site of plasmid pBR322 (pBR/c-*ras*A and pBR/c-*ras*N). c-*ras*A and c-*ras*N DNA fragments are essentially identical in their restriction map and nucleotide sequence, except that c-*ras*A has a substitution, responsible for its activation, in the 12th codon of the first exon and c-*ras*N 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-*ras*A and c-*ras*N 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-*ras*A, pBK/c-*ras*N, pBR/c-*ras*A, or pBR/c-*ras*N. Eleven of the animals inoculated with pBK/c-*ras*A (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

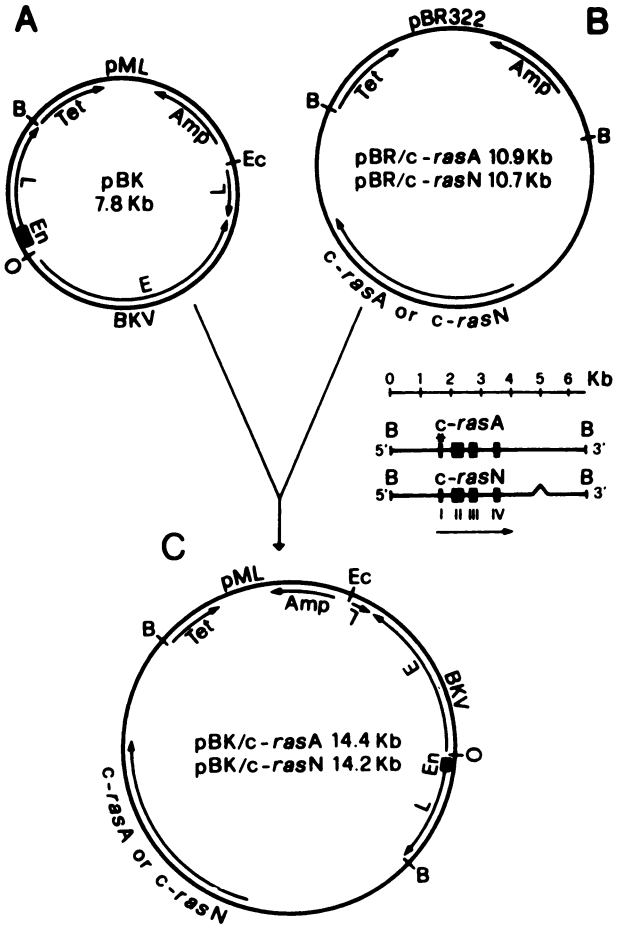

Fig. 1. Construction of recombinant DNAs. *A*, vector pBK was constructed by ligating together the large *Bam*HI/*Eco*RI fragments of wild-type BKV DNA (containing the entire early region) and of pML, a deletion derivative of pBR322. *B*, pBR/c-*ras*A and pBR/c-*ras*N were constructed by inserting the *Bam*HI fragment of the genomic clone of c-*ras*A or c-*ras*N, previously cloned in Charon 4A λ phage, into the unique *Bam*HI site of pBR322. *C*, pBK/c-*ras*A and pBK/c-*ras*N were constructed by inserting the *Bam*HI c-*ras*A or c-*ras*N fragment, excised from pBR/c-*ras*A or pBR/c-*ras*N, into the unique *Bam*HI site of pBK. *E* and *L*, BKV DNA early and late regions; *En*, BKV transcriptional enhancer elements; *O*, origin of DNA replication. The 4 *black boxes* subscribed by roman numerals on c-*ras*A and c-*ras*N genes in *B* represent the 4 exons; * on exon I of c-*ras*A, point mutation responsible for the activation of the oncogene. C-*ras*N has a 200-nucleotide deletion (#) comprising cellular sequence 3′ of c-*ras* sequences. *Arrows*, direction of transcription; *B* and *Ec*, *Bam*HI and *Eco*RI cleavage sites; *Amp* and *Tet*, genes for ampicillin and tetracycline resistance in pML and pBR322.

1 year. In a second experiment which is under way (observation period of only 1 month), 5 of 18 (27.7%) pBK/c-*ras*A 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. 2*A*). 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 dependency, a better colony formation both on plastic and in agar, and a more disorganized and fragmented pattern of actin cables than L702, L703, and its clones (Table 1).

Presence and State of pBK/c-*ras*A DNA in Tumors and Tumor Cell Lines. Total cellular DNA from primary tumors and tumor-derived cell lines was hybridized to a ^{32}P-labeled pBK/c-*ras*A DNA probe. A very similar hybridization pattern was detected in tumors 434, 435, and 436 and in tumor cell lines L702, L704, L703 clone 4B, and clone 6B. Digestion of DNA with *Eco*RI, which has one cleavage site on pBK/c-*ras*A (Fig. 1), produced a prominent band migrating slightly ahead of control 14.4-kb full length linear pBK/c-*ras*A. A small number of other bands were detected, generally of much lower intensity and probably representing junctions of the recombinant DNA sequences to cellular sequences (Fig. 3, *lanes 1* to *5*, *8*, and *9*). Digestion with *Bam*HI, which has 2 cleavage sites on pBK/c-*ras*A (Fig. 1), yielded a band of 7.8 kb, corresponding in size to pBK, and another band of 6.6 kb, corresponding to the c-*ras*A DNA fragment (data not shown). Mapping with *Hind*III and *Cla*I showed that insertions of pBK/c-*ras*A into cellular DNA occurred in pML sequences. By hybridization of uncut cellular DNA, a small amount of free recombinant DNA was detected in some tumors and tumor cell lines (data not shown). Densitometric scanning, by comparison with known amounts of control DNA, showed that the hybridization band of linear forms represents 150 to 200 copies of pBK/c-*ras*A DNA per diploid cell genome, consistent with a large number of tandem insertions of the recombinant DNA into cellular DNA. Tumor cell line L703 and its derived clone 3B showed a weaker band of linear molecules and numerous other hybridization bands (Fig. 3, *lanes 6* and *7*), suggesting a more dispersed integration pattern where a few tandem insertions coexisted with several single insertions.

Analysis of c-Ha-*ras* and BKV Transcripts. Fig. 4 shows the results of a northern blot analysis of total cytoplasmic RNA from tumor cell lines hybridized to the ^{32}P-labeled 6.6-kb c-*ras*A DNA fragment. A hybridization band of 1.2 kb, corresponding in size to the c-Ha-*ras* transcript (44), was detected in lines L702, 703, and 704 (Fig. 4*A*, *lane 1*; Fig. 4*B*, *lanes 1* and *2*). This band was absent in control cell lines (Fig. 4*A*, *lanes 2* and *3*; Fig. 4*B*, *lanes 3* to *6*), suggesting that the exogenous c-*ras*A gene was transcribed in tumor cell lines. Densitometric scanning of autoradiographic bands showed that the c-*ras* transcript was 3 to 6 times more abundant in L704 than in L702 and L703. Hybridization of L702 and L704 total cytoplasmic RNA to ^{32}P-labeled BKV DNA 3.2-kb *Pvu*II fragment, containing the entire early region, produced two major bands of 2.3 and 2.6 kb (Fig. 5, *lanes 1* and *2*), comigrating with the transcripts of BKV-transformed cells (Fig. 5, *lanes 4* and *6*) and corresponding in size to T-Ag 19S and t-Ag 20S mRNA (45), whereas control BKV cells and hamster embryo fibroblasts were negative (Fig. 5, *lanes 3* and *5*). Since the BKV DNA probe overlaps the late region of about 250 nucleotides, where the major 5′ end for late mRNA is located (45), the band of 1.5 to 1.6 kb may represent hybridization to a class of late BKV RNA initiated at the late viral promoter and terminated at a pML or a cellular stop signal. On the whole, the transcription pattern of c-*ras*A and BKV early region is consistent with the tandem insertions of pBK/c-*ras*A in tumor cells and with initiation and termination of transcription at the endogenous internal signals of the 2 transforming genes.

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-*ras*A-induced tumors and tumor cell lines, whereas nor-

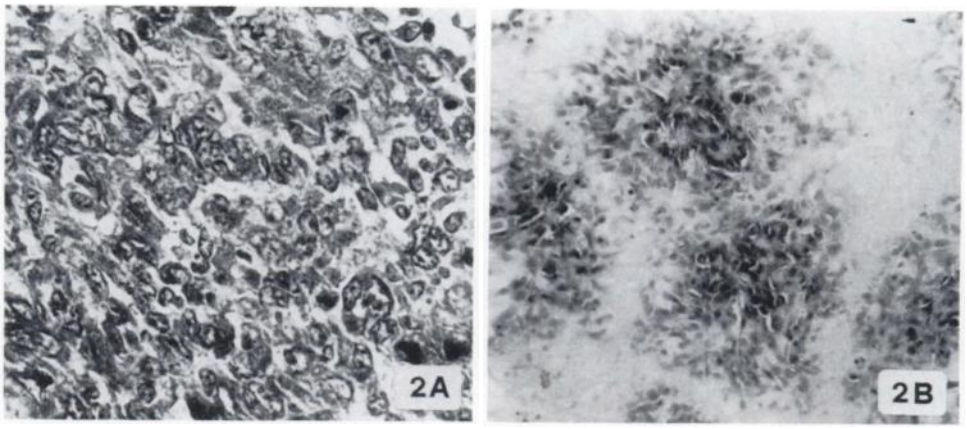

Fig. 2. *A*, undifferentiated s.c. sarcoma 435 induced in a hamster by pBK/c-*ras*A, showing highly atypical cells with polymorphic nuclei. *B*, tumor-derived cell line L704. Immunoperoxidase reaction (*A* and *B*) with anti-c-Ha-*ras*A p21 RAP-5 MAb shows positive staining of cytoplasm and plasma membrane 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*

Cells were cultured in 60-mm plastic Petri dishes. For the determination of serum dependency of growth, 10^3 cells/cm^2 were seeded with MEM containing 2 or 10% FBS. Cells were counted every 2 days. Saturation density and doubling time were calculated from the exponential growth curves. For colony formation on plastic, 500 cells were plated with MEM supplemented with 10% FBS and colonies were counted after 10 days. For growth in agar, 10^4 cells were suspended in semisolid medium supplemented with 10% FBS. Colony counts were performed 14 days later. Actin cables were visualized by specific binding of rhodamine-conjugated phalloidin.

Cell lines[a]	Saturation density[b] (cells/cm^2 × 10^4)		Doubling time[b] (h)		Colony formation[b] (colonies/100 cells)		Disorganization and fragmentation of actin cables
	2% FBS	10% FBS	2% FBS	10% FBS	On plastic	In agar	
L702	0.1	12.3	∞	39	29.8	1.5	++
L703	13.3	27.4	26	25	11.5	8.7	++
L703 clone 3B	ND[c]	14.1	ND	ND	15.8	0.1	++
L703 clone 4B	ND	22.4	ND	ND	3.6	0.1	++
L703 clone 6B	18.7	38.9	44	26	11.9	10.5	++
L704	26.4	53.0	21	20	45.5	16.4	++++
L578	14.8	29.7	35	32	13.4	0.3	+
HEF	0.1	7.2	∞	36	0.3	0	−

[a] L702, L703, and L704, cell lines derived from pBK/c-*ras*A-induced hamster s.c. sarcomas; L578, hamster kidney cells transformed by BKV DNA; HEF, secondary hamster embryo fibroblasts.
[b] Average of 2 Petri dishes.
[c] ND, not determined.

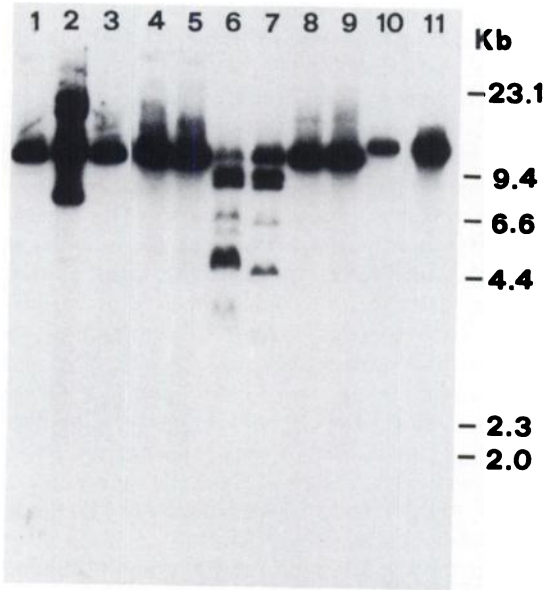

Fig. 3. Southern blot hybridization of total cellular DNA from pBK/c-*ras*A-induced hamster tumors and tumor-derived cell lines to a ^{32}P-labeled pBK/c-*ras*A DNA probe. DNAs (20 μg/lane) were digested with *Eco*RI. The samples examined were s.c. sarcoma 434 (*lane 1*), s.c. sarcoma 435 (*lane 2*), s.c. sarcoma 436 (*lane 3*), L702 (*lane 4*), L704 (*lane 5*), L703 (*lane 6*), L703 clone 3B (*lane 7*), L703 clone 4B (*lane 8*), L703 clone 6B (*lane 9*). pBK/c-*ras*A DNA control, cut with *Eco*RI, is in *lanes 10* and *11* (20 and 100 genome equivalents/diploid cell genome). The position of marker *Hind*III λ DNA fragments is shown on the *right*.

mal hamster skin and 66 BKV-induced tumors (22 ependymomas, 12 s.c. sarcomas, 13 tumors of pancreatic islets, 9 intestinal lymphomas, 5 osteosarcomas, and 5 subendocardial sarcomas from previous experiments; see Refs. 8–10) as well as 8 cultures of BKV or spontaneously transformed and normal cells were negative. Four control tumors and one BKV-transformed hamster cell line (L578) showed a low p21 expression. The immunoperoxidase reaction was localized to the cytoplasm and cytoplasmic membrane of virtually all tumor and tumor-derived cells (Fig. 2, *A and B*) and was concentrated on cultured cells growing in foci (Fig. 2*B*), suggesting that the malignant phenotype was specifically associated to the increased expression of c-Ha-*ras*A p21.

A prominent band of M_r 21,000 was detected by immunoblotting when anti-c-Ha-*ras* p21 Y13-259 MAb was reacted with lysates of tumors and tumor cell lines (Fig. 6, *lanes 1*, *2*, and *4* to *8*). Normal hamster skin as well as BKV- or spontaneously transformed and normal cells showed only a slight band of M_r 21,000 (Fig. 6, *lanes 3* and *9* to *11*), suggesting that in tumor cells p21 is expressed mostly by the acquired c-Ha-*ras*A oncogene. Quantitative analysis indicated that tumors and tumor cell lines produced 12 to 40 times more p21 than controls, L704 showing the highest expression among tumor cell lines. Bands of M_r 50,000 and 25,000 (Fig. 6, *lanes 1* to *3*) are the heavy and light chains of immunoglobulins, probably bound to tumor cells and circulating in blood vessels of tumors and normal skin. BKV 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

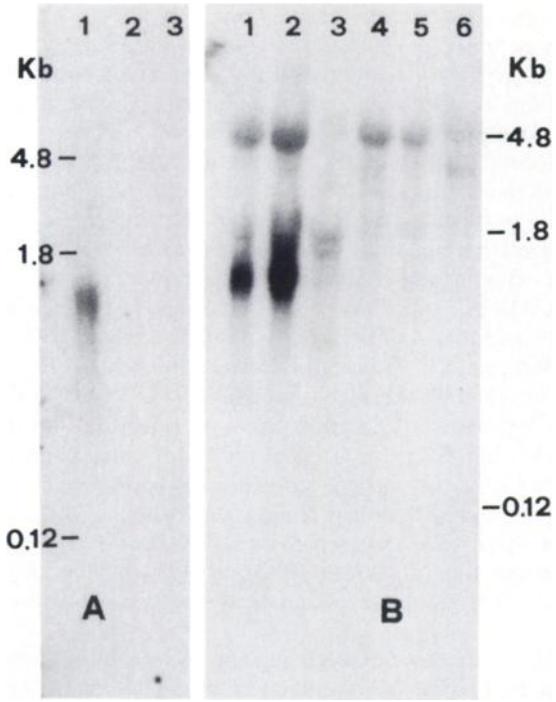

Fig. 4. Northern blot analysis of tumor-derived cell lines to detect c-Ha-*ras* transcripts. Samples of total cytoplasmic RNA (20 μg/lane) were analyzed by agarose gel electrophoresis and hybridization to the ^{32}P-labeled 6.6-kb c-*ras*A DNA fragment as probe. *A*, *lane 1*, L703; *lane 2*, L578, hamster 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 5S ribosomal RNA) are indicated *alongside*.

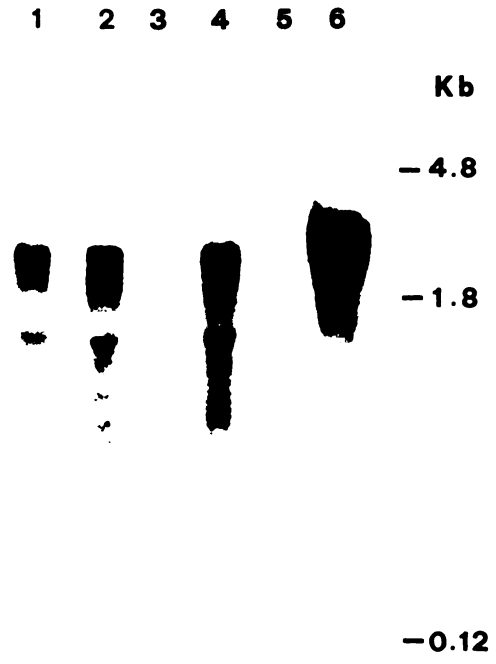

Fig. 5. Northern blot analysis of tumor-derived cell lines to characterize BKV transcripts. Total cytoplasmic RNA (20 μg/lane) was migrated in a 1% agarose gel, transferred to nitrocellulose, and hybridized to BKV DNA 3.2-kb *Pvu*II fragment containing the entire early region. *Lane 1*, L702; *lane 2*,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

[5] A. Corallini, unpublished observations.

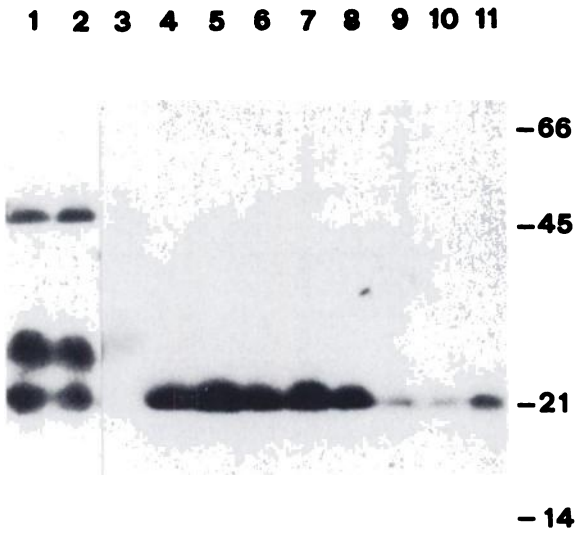

Fig. 6. Detection of c-Ha-*ras* p21 in pBK/c-*ras*A-induced s.c. hamster tumors and tumor cell lines by immunoblotting. Proteins in cell lysates were separated in 0.1% NaDodSO$_4$:12% polyacrylamide slab gels, electrophoretically transferred to nitrocellulose, and reacted with Y13-259 MAb to *ras* p21. Immune complexes were labeled with ^{125}I-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; *lane 7*, L703 clone 4B; *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_r 66,000; ovalbumin, M_r 45,000; soybean trypsin inhibitor, M_r 21,000; lysozyme, M_r 14,000; Bio-Rad, Richmond, CA) are indicated on the *right*. See legend to Fig. 4 for explanation of cell lines.

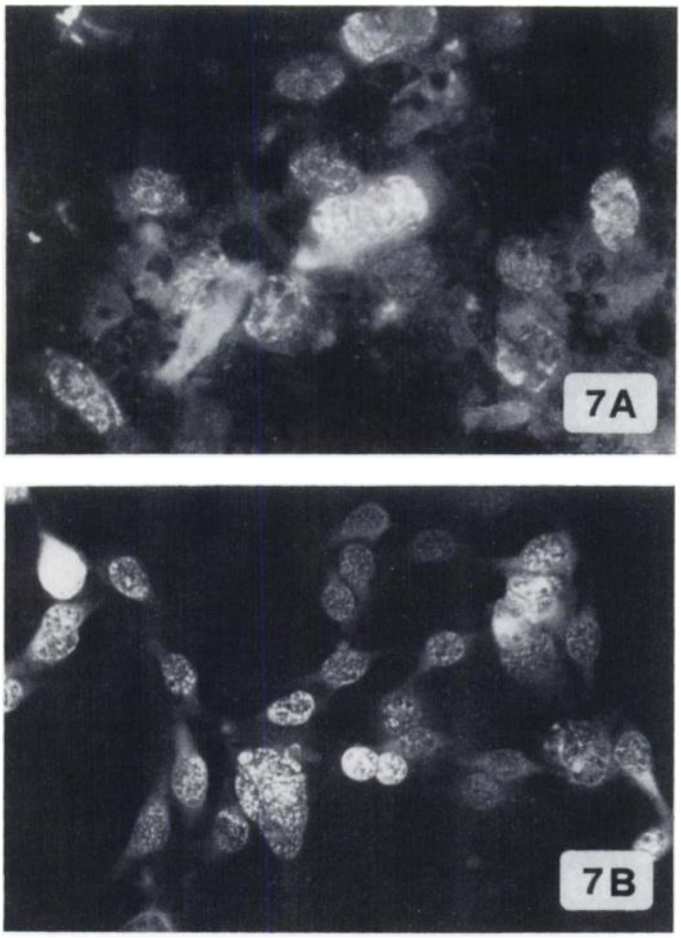

Fig. 7. Detection of BKV T-Ag in primary s.c. hamster tumors induced by pBK/c-*ras*A DNA and tumor-derived cell lines. Tumor imprints on glass slides or cell monolayers grown on coverslips were fixed in acetone for 10 min at room temperature and stained by indirect immunofluorescence with hamster serum to BKV T-Ag followed by fluorescein-conjugated rabbit antibodies to hamster immunoglobulins. *A*, primary tumor 434; *B*, tumor cell line L704. Both specimens show granular and reticular staining of nuclei with exclusion of nucleoli, typical of T-Ag. *A*, × 480; *B*, × 400.

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-*ras*A 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 indemnity 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-*ras*A are specifically transcribed and expressed in tumors and tumor cell lines that contain BKV and c-Ha-*ras*A mRNAs as well as BKV T-Ag and c-Ha-*ras*A 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 transformed phenotype, as determined by growth characteristics *in vitro* and cytoskeletal alterations, correlates with levels of c-Ha-*ras*A transcription and p21 synthesis, suggesting that transformation is modulated by c-Ha-*ras*A effector functions.

The nature and mechanism of the interaction between the 2 transforming genes are at present unclear. Since c-Ha-*ras*A 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-*ras*A 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-*ras*A could clarify whether in the recombinant DNA BKV early region and c-Ha-*ras*A express individually cooperating functions or *cis*-acting DNA sequences, such as BKV transcriptional enhancers, stimulate c-Ha-*ras*A 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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