Transformation of Primary Human Embryonic Kidney Cells to Anchorage Independence by a Combination of BK Virus DNA and the Harvey-*ras* Oncogene

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Primary human embryonic kidney (HEK) cells were transformed by a focus assay with BK virus (BKV) DNA molecularly cloned at its unique *EcoRI* site. Both viral DNA sequences and viral tumor antigens were present and expressed in all the foci that we examined. However, cells isolated from foci were incapable of growth in soft agar. We then examined the transformation of HEK cells after their transfection with a combination of BKV DNA and either the normal or the activated form of the human *Ha-ras* oncogene (EJ c-*Ha-ras*-1). Only the cells transfected with a combination of BKV DNA and the activated form of *Ha-ras* were capable of growth in soft agar. Both BKV and *Ha-ras* DNAs were present in the transformed colonies. BKV tumor antigens and the *Ha-ras* p21 protein were also expressed.

The human papovavirus BK virus (BKV) was first isolated from the urine of a transplant patient in 1971 (3). This virus is common, and 75 to 80% of the human population has antibody against the virus by age 5 (1, 2, 18). The fact that BKV is oncogenic in rodent systems is certain. BKV is both highly oncogenic and polyoncogenic when inoculated either intravenously or intracerebrally but weakly oncogenic when injected subcutaneously into rodents. In vitro transformation of rodent cells has also been observed by several groups (for reviews, see references 5 and 22). Immortalization of primary human embryonic kidney (HEK) cells by BKV has been reported (8, 15). HEK cells surviving infection or infected with UV-inactivated virus grew in media containing low concentrations of serum and reached a higher saturation density than did uninfected cells. However, none of the surviving cells were capable of growth in soft agar or of forming tumors in nude mice. We have also observed the transformation of HEK cells by BKV DNA (molecularly cloned at its unique *EcoRI* site, disrupting the coding sequences for viral capsid protein VP1 and thus preventing lytic infection by this virus) as selected by a focus assay. However, none of the selected foci yielded cells capable of growth in soft agar. In the present report, we provide evidence for the transformation of HEK cells to anchorage independence by a combination of BKV DNA and the activated form of the human *ras* oncogene.

BKV DNA was mixed with an equal amount of bacterial plasmid pML (7), cleaved with the restriction endonuclease *EcoRI*, ligated, and then used for the transformation of *Escherichia coli* HB101 cells as described previously (10). Positive clones were identified by colony hybridization, and the appropriate plasmid was isolated preparatively (10). HEK cells were plated in 60-mm dishes (5×10^5 cells per plate) and then transfected with 10 μ g of each plasmid per plate as described previously (9). The cells were passaged in 100-mm dishes, repassaged two to three more times, and then incubated to form foci for 2 to 3 weeks. To assess the role of the intact early region of BKV in transformation, we also used the DNA of the R2 species of RF (a variant of

BKV), which has a deletion in the coding sequences for large T antigen (9). Examination of the plates revealed the presence of foci in the plates which were transfected with BKV-pML plasmids (Table 1). We examined five of the foci for the presence of BKV DNA. Total cellular DNA was isolated, and the status of plasmid DNA in the transformed foci was examined by Southern blot hybridization after cleavage of cell DNA with a combination of restriction endonucleases *HindIII* and *EcoRI*. Viral DNA was cleaved into five fragments (B, A₁, A₂, C, and D) after digestion with these enzymes (Fig. 1). All the foci contained the intact early region of BKV (fragments B, C, and D) (Fig. 1). Moreover, all the foci contained additional bands, indicating integration of the viral DNA into the host chromosome. We also examined the expression of BKV large T and small t antigens in the transformed foci. Both large T and small t antigens were expressed in all five foci (an example for one focus is shown in Fig. 2). We next examined the capacity of cells in each focus for anchorage-independent growth. Cells (10^6) were plated in soft agar and incubated. No colonies were detected after 6 weeks of incubation.

We examined the growth of HEK cells in soft agar after

TABLE 1. Transformation of HEK cells by various plasmids^a

Treatment	Formation of foci	Colony formation in soft agar
Calcium phosphate	—	—
BKV-pML	+	—
R2-pML	—	—
EJ c- <i>Ha-ras</i> -1	—	—
c- <i>Ha-ras</i> -1	—	—
BKV-pML + EJ c- <i>Ha-ras</i> -1	+	+
BKV-pML + c- <i>Ha-ras</i> -1	+	—
R2-pML + EJ-c- <i>Ha-ras</i> -1	—	—

^a Primary HEK cells (5×10^5 per 60-mm dish) were transfected with 10 μ g of the indicated plasmids as described previously (9). The cells were then passaged three to four times and incubated further for the formation of foci or growth in agar after each passage. Foci were detected after 2 to 3 weeks. The cells in control plates (treated with calcium phosphate) and in plates treated with R2-pML did not survive after passage 3 or 4. The same results were obtained when the experiment was repeated twice.

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FIG. 1. Status of BKV DNA in transformed foci. Total cellular DNA (5 μ g) was cleaved with a combination of the restriction endonucleases *Hind*III and *Eco*RI. Viral DNA was then detected by Southern blotting with BKV DNA as a probe (11). The control lane (lane BK) contained 100 pg of BKV-pML and 5 μ g of HEK cellular DNA.

FIG. 3. Status of BKV and Ha-*ras* DNAs in transformed colonies. (A) Cellular DNA (5 μ g) was cleaved with a combination of the restriction endonucleases *Hind*III and *Eco*RI and hybridized with a BKV probe after blotting. (B) Cellular DNA (5 μ g) was cleaved with the restriction endonuclease *Bam*HI and hybridized with an Ha-*ras* probe after blotting. Control lanes contained 100 pg of either BKV-pML or Ha-*ras* DNA and 5 μ g of HEK cellular DNA.

their transfection with a combination of BKV DNA and either the normal or the activated form of the human Ha-*ras* oncogene (4, 14, 19). Only HEK cells tranfected with a combination of BKV DNA and EJ c-Ha-*ras*-1 (the activated form) were capable of growth in soft agar (Table 1). Note also that HEK cells transfected with a combination of R2-pML and EJ c-Ha-*ras*-1 were not capable of growth in soft agar, indicating that the intact large T antigen of BKV was necessary in combination with EJ c-Ha-*ras*-1 for transformation to anchorage independence. We examined four selected colonies for the presence of BKV and EJ c-Ha-*ras*-1 DNAs. High-molecular-weight DNA from each colony was isolated and digested with a combination of the restriction endonucleases *Eco*RI and *Hind*III. The results of blot hybridization with BKV DNA as a probe showed (Fig. 3A) that each of the colonies contained all the BKV DNA fragments. We also digested DNA from each colony with the restriction endonuclease *Bam*HI, which cleaves the Ha-*ras* gene into a

FIG. 2. Detection of BKV tumor antigens in transformed foci and colonies. [³⁵S]methionine-labeled cell extracts were incubated in the presence of normal serum (lanes C) and serum from a tumor-bearing hamster (lanes T) and then precipitated by *Staphylococcus aureus* (12). BKV-transformed LSH (12) was used as a control. The extract from the transformed focus (BK 1) belongs to focus 1 in Fig. 1. Numbers at the right indicate molecular weights in thousands.

single fragment. Blot hybridization with Ha-*ras* DNA as a probe showed that all four colonies contained Ha-*ras* integrated DNA (Fig. 3B). Note that the band patterns in colonies 1 and 4 are identical and that the band patterns in colonies 2 and 3 are identical. Thus, each of these two colonies were probably from the same parental colony. Note also that clones 1 and 4 are lacking the band corresponding to the endogenous *ras* gene. This is probably due to DNA rearrangements during the integration of the incoming plasmids. We also examined the expression of BKV tumor antigens and the p21 protein (the gene product of Ha-*ras*) in clones 1, 2, and 4 by immunoprecipitation. The results (Fig. 2) showed that both large and small tumor antigens of BKV were expressed in these cells. The results of immunoprecipitation with a monoclonal antibody to Ha-*ras* p21 showed (Fig. 4) that the BKV DNA-transformed HEK focus contained only two bands corresponding to the normal *ras* gene product (20). As expected (20), two additional bands with higher apparent molecular weights were expressed in the colonies transformed with a combination of BKV and the activated form of the Ha-*ras* oncogene.

FIG. 4. Immunoprecipitation of Ha-*ras* p21 proteins from transformed colonies. Ha-*ras* p21 protein was immunoprecipitated from [³⁵S]methionine-labeled extracts (12) by fivefold dilution in the buffer PBSTDs described by Oncogene Science, the supplier of monoclonal antibody. Fluorography was used for the 12% polyacrylamide gels run with sodium dodecyl sulfate. Numbers at the right indicate molecular weights in thousands; + and -, presence or absence, respectively, of rat monoclonal antibody against *ras* p21 protein. The monoclonal antibody-p21 complex was pelleted and washed with PBSTDs after complexing with rabbit anti-rat immunoglobulin G (Cappel Laboratories) bound to Sepharose-protein A (Pharmacia).

A quantitative estimation of the frequency of transformation by either a focus assay or growth in soft agar was not possible, since HEK cells had to be passaged three to four times before any focus or colony could be detected. However, it is useful to analyze the band patterns of Southern blots of DNA from cells transformed to either produce foci or grow in soft agar. Of the five and four randomly selected foci and colonies, respectively, only two different band patterns were seen for each. From this result we estimated the frequency of transformation to be about $10^{-6}/\mu\text{g}$ of plasmid DNA. This low frequency of transformation could be typical of primary human cells requiring several molecular events before successful transformation can occur. Another possibility is that although the BKV DNA used in these experiments was disrupted in its late region, recombination events during transfection may have resulted in the production of intact viral DNA and therefore may have caused cell killing due to lytic infection. We should, however, mention that we have not noticed any evidence of infection during cell passage after transfection.

The data presented in this report reveal that transformed HEK cells selected by the focus assay are not capable of growth in soft agar. A similar observation has been reported for the Ha-*ras* oncogene isolated from T24/EJ human bladder carcinoma cells (4, 14, 19). The transforming activity of this gene was first tested in established mouse cell lines, and it was shown subsequently that transformation by this gene is due to a single point mutation resulting in a change of one amino acid (16, 20, 21). However, Ha-*ras* was unable to transform primary cells (6, 17) to anchorage independence. Transformation was observed when Ha-*ras* was used in combination with the *myc* gene of avian MC29 retrovirus, polyomavirus large T antigen, or early region E1a of adenovirus (17, 19). We also observed growth in soft agar when primary HEK cells were transfected with a combination of BKV and EJ c-Ha-*ras*-1 DNAs. These studies indicate that the transformation of primary cells is a multistep process and that more than one gene is involved. The necessity of multiple genes for transformation may be related to the cellular locations and functions of the products of these genes.

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