JOURNAL OF VIROLOGY, June 1982, p. 978-985
0022-538X/82/060978-08$02.00/0

Vol. 42, No. 3

Change of DNA near the Origin of Replication Enhances the Transforming Capacity of Human Papovavirus BK

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Received 16 December 1981/Accepted 16 February 1982

A turbid-plaque-forming mutant (*pm*522) of human papovavirus BK, which has a small deletion at about 0.7 map unit and grows somewhat more slowly in human cells than does wild-type BK virus, transformed hamster and rat cells in culture much more efficiently than did wild-type virus. Another plaque morphology mutant, *pm*525, forming turbid plaques larger than those of *pm*522 also had a high transforming capacity. The similar difference in transforming capability between wild-type and plaque morphology viruses was observed with DNAs extracted from virions. Recombinant viruses were constructed from the wild-type DNA fragment lacking *Hin*dIII-C (0.62 to 0.73 map unit) and *pm*522 *Hin*dIII-C (including the origin of replication) by the molecular cloning method. Characterization of the recombinants showed that the change near the origin of DNA replication was responsible both for the altered plaque morphology and for the enhanced transforming capacity of the BK virus mutant.

Human papovavirus BK (BKV) resembles simian virus 40 in its DNA homology, genomic organization, nature of virus-coded proteins, and transforming and tumorigenic capacity (12). Purified BKV has been shown to induce a variety of hamster tumors, from which infectious virus was not readily rescued by the cell fusion with permissive cells (27). One rare, rescued BKV isolate (from a pineocytoma), which formed small, turbid plaques on human embryonic kidney (HEK) cell cultures and was therefore designated plaque morphology mutant 522 (*pm*522), proved to be an interesting mutant having the following properties.

*pm*522 is a viable virus with a small deletion at 0.708 to 0.725 map unit (30), which is probably within the section corresponding to the early promoter region of simian virus 40 (1, 9). *pm*522 grows more slowly in HEK cells than does the wild-type plaque isolate (*wt*501), but is about five times as tumorigenic as *wt*501 in hamsters (30; S. Watanabe, S. Kotake, A. Nozawa, T. Muto, and S. Uchida, Int. J. Cancer, in press). Furthermore, *pm*522 frequently induces insulinomas which have not been induced after inoculation with the three plaque-purified clones of *wt* virus, *wt*500, *wt*501, and *wt*502 (30; Watanabe et al., in press). Whether and how the deletion in the early promoter region is related to the highly turmorigenic and insulinoma-inducing properties of *pm*522 have yet to be investigated.

Although the tumorigenic activity is an important biological parameter of BKV, its test has been hampered by the lengthy period required for observation. In search for an alternative test, we examined the in vitro transforming capability of *pm* mutants and *wt* BKV in this study. We also constructed and characterized the recombinant viruses between *wt*501 and *pm*522 to determine the part of DNA responsible for the altered properties of the mutant. This paper describes the transforming capability of *wt* and *pm* BKV, the molecular cloning procedure for construction of the recombinant viruses, and their biological properties.

MATERIALS AND METHODS

Cells. HEK cell cultures grown in Eagle minimal essential medium supplemented with 0.25% lactalbumin hydrolysate and 10% calf serum were used at passage 4 for propagation of virus and assay for its plaque-forming and antigen-inducing capacities. For assay of transformation, hamster cell cultures from 13-day embryos were used at passages 3 to 4, and the rat 3Y1 cell line (15), which was a gift from N. Yamaguchi, Institute of Medical Science, University of Tokyo, was also used. The medium was Eagle minimal essential medium supplemented with 10% calf serum and 10% fetal bovine serum for hamster and rat cells, respectively.

Viruses. Prototype BKV (Gardner's original strain) was supplied by K. K. Takemoto, National Institutes of Health, Bethesda, Md. The properties of plaque-cloned *wt*501 and *pm*522 have been described previously (30). *pm*522, which was rescued from a hamster pineocytoma and has a small deletion at 0.708 to 0.725 map unit, grows more slowly in HEK cells, but is more tumorigenic in hamsters than is *wt*501. *pm*525, which was also rescued from the pineocytoma, forms larger turbid plaques and has a smaller deletion, but has equal tumorigenicity when compared with those of *pm*522 (Watanabe et al., in press).

Purification of virus and assay for infectivity in HEK cells. Viruses were grown at input multiplicities of infection (MOIs) of 10^{-5} to 10^{-2} PFU per cell and purified from infected cells by banding in CsCl density gradients. The purified samples were measured for absorbance at 258 nm (1 optical density unit corresponds to 6.4×10^{12} virions) and assayed for infectivity by the plaque method (30) and for T- and V-antigen-forming activities by the indirect immunofluorescence method (30, 31). T- and V-antigen-inducing capacities were calculated from the dilution factor and the number of positive cells on day 4 in the presence of anti-BKV serum and expressed as cell-infecting units, CIU(T) and CIU(V), respectively, as described previously (29).

Preparation of viral DNA. Supercoiled form I DNA was extracted from purified virions as previously described (33). Purified DNA was measured for absorbance at 258 nm (1 optical density unit corresponds to 50 μg of DNA), assayed for T- and V-antigen-inducing capacities by the DEAE-dextran method (18) and the indirect immunofluorescence method as described previously (31), and used for transformation. DNAs of the constructed viruses were extracted directly from the infected cells by the method of Hirt (11), and form I DNA was isolated by the dye-buoyant density method (20).

Transformation assay with virions. Monolayer cultures of hamster or rat cells were allowed to adsorb BKV for 24 h at 36°C and then trypsinized (with 0.05% trypsin–0.54 mM EDTA in phosphate-buffered saline without Ca^{2+} and Mg^{2+}). The infected cells were diluted and seeded in soft agar (0.4% agarose in Eagle minimal essential medium with 10% fetal bovine serum) at 10^5 cells per 60-mm dish as described by Macpherson and Montagnier (16), and colonies arising in agar were examined and counted under a low-power microscope.

For a focus assay, 5×10^3 infected cells were plated per 60-mm dish and cultured at 36°C in liquid medium (Eagle minimal essential medium with 10% fetal bovine serum), with a change of medium at 4- to 5-day intervals. After cultivation for 13 to 14 days for hamster cells and 25 to 29 days for rat cells, the dishes were rinsed with phosphate-buffered saline without Ca^{2+} and Mg^{2+}, fixed in methanol, and stained with 5% Giemsa. Densely stained foci were counted and examined under a microscope.

Some of the colonies and the foci were isolated, and the transformed cell lines were established.

Transformation with DNA. Subconfluent cultures of hamster and rat cells were transfected with viral DNA by the calcium phosphate method (8). The mixture of BKV DNA, HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.05), calf thymus DNA (5 μg/ml), and $CaCl_2$ (0.125 M) was kept still at room temperature for 30 min and then inoculated into cells at 0.5 ml per 60-mm dish. After adsorption for 4 h, the infected cells were cultured in Eagle minimal essential medium with 10% fetal bovine serum, which was refreshed at 4- to 5-day intervals thereafter. The dishes were rinsed, fixed with methanol, and stained with Giemsa 3 and 5 weeks after infection for hamster and rat cells, respectively. Densely stained foci were counted.

Enzymes. Restriction endonucleases *Bam*HI, *Hha*I, and *Hin*dIII and T4 ligase were purchased from Bethesda Research Laboratories, Inc., Rockville, Md. Digestion and ligation were carried out according to the supplier's instructions. Bacterial alkaline phosphatase was purchased from Millipore Corp., Freehold, N.J., and was dialyzed against 0.05 M Tris (pH 8.4) before use.

Agarose gel electrophoresis. DNA samples were adjusted to 8% sucrose and 0.02% bromophenol blue and subjected to electrophoresis in horizontal 0.6 or 2% agarose slab gels. The running buffer was either Tris-borate buffer, pH 8.2 (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA), or E buffer, pH 7.9 (40 mM Tris-acetate, 5 mM sodium acetate, 1 mM EDTA). After running, gels were stained in buffer containing 0.5 μg of ethidium bromide per ml for 30 min. DNA bands were photographed on a short-wavelength UV transilluminator. For isolation of DNA fragments, DNA was completely or partially digested with *Hin*dIII and subjected to electrophoresis in horizontal 0.6% low-melting-point agarose (Bethesda Research Laboratories, Inc.) slab gels. DNA bands were visualized and isolated on a long-wavelength UV transilluminator, and agarose was removed by phenol extraction as indicated by the supplier.

Molecular cloning and construction of recombinant viruses. Appropriate linear BKV DNA fragments were prepared by complete or partial digestion with *Hin*dIII, cleaving BKV DNA at four sites (13), subjected to electrophoresis, and isolated from agarose gels. Linear vector DNA, bacterial plasmid pBR322 (2) which was cleaved once by *Hin*dIII digestion (23) or a recombinant plasmid which was constructed from pBR322 and a *wt* BKV DNA fragment lacking *Hin*dIII-C and isolated from agarose gels after partial *Hin*dIII digestion and electrophoresis, was treated with bacterial alkaline phosphatase. The mixture of donor BKV DNA fragments and linear vector DNA was incubated at 12°C for 14 h with T4 ligase in a solution containing 66 mM Tris-hydrochloride (pH 7.6), 6.6 mM $MgCl_2$, 0.4 mM ATP, and 10 mM dithiothreitol. The product of the ligation reaction was used to transform *Escherichia coli* K-12 (strain HB101) bacteria, under the conditions described by Cohen et al. (6), and ampicillin-resistant transformants were selected. Selected colonies were grown in 10 ml of L-broth, and plasmid DNA was isolated by the rapid isolation method (7). Colonies were then selected according to the size of recombinant plasmid DNA constructed. For selection of constructed recombinant plasmids, *Bam*HI, *Hha*I, and *Hin*dIII were used to determine the site and orientation of the insert (*Hin*dIII C fragments). For further characterization, plasmid DNA was prepared from chloramphenicol-amplified bacterial cultures (5). Form I DNA was extracted by a modification of the clear lysate procedure (10) and purified by the dye-buoyant density method (20). The recombinant plasmids containing constructed complete viral genomes were partially digested with *Hin*dIII to transfect HEK cultures.

RESULTS

Transformation of hamster cells with virions. Figure 1 shows the development of colonies after infection with BKV. Mock-infected hamster cells, when seeded in soft agar at 10^5 cells

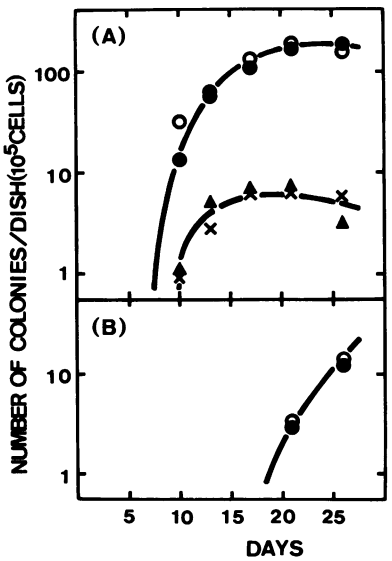

FIG. 1. Colony formation by hamster embryo cells in soft agar after infection with BKV. (A) Small colonies, 0.1 ± 0.05 mm in diameter; (B) large colonies, 0.3 ± 0.15 mm in diameter. Symbols: ●, *pm*522 infected; ○, *pm*525 infected; ▲, *wt*501 infected; ×, mock infected.

per dish, formed small colonies of cells which became detectable on day 10 and increased in number until day 17. After that time, the small colonies did not grow in number or size, and their diameters stayed at about 0.1 ± 0.05 mm. The behavior of the cells infected with *wt*501 [MOI, 30 CIU(T)] was virtually indistinguishable from that of mock-infected cells. They formed about eight small colonies per dish. On the other hand, the hamster cells infected with *pm*522 and *pm*525 [MOIs, 30 CIU(T)] formed about 30 times as many small colonies (about 200 colonies per dish) as the background level. Furthermore, larger colonies, with diameters of about 0.3 ± 0.15 mm, emerged among small ones on day 21 and reached a level of about 15 large colonies per dish upon infection with *pm*522 and *pm*525 on day 26. The cell lines isolated from these large colonies were all positive for T antigen, as assayed by indirect immunofluorescence, and showed the general growth properties of the transformed cells. About two-thirds of the T-antigen-positive cell lines contained a small fraction (0.03 to 0.2%) of V-antigen-positive cells. Small colonies that arose in the *pm* infection had probably resulted from abortive transformation (17).

The transforming capacity of BKV, as assayed by focus formation, is shown in Table 1. Whereas *wt* (*wt*501) and uncloned prototype BKV showed a very low activity and formed only a few foci at MOIs of 20 or more CIU(T) per cell, *pm*522 produced more than 10 times as many foci as did *wt* virus at a comparable MOI. The capacity of *pm*522 seemed to be higher than that of *pm*525. The cell lines isolated from some of these foci were all positive for T antigen, as assayed by indirect immunofluorescence. About one-third of the cell lines contained a small fraction (0.01 to 0.1%) of V-antigen-positive cells. All of the cell lines formed colonies in soft agar. The efficiency (colonies per cells seeded) was 0.03 to 0.18, as scored 3 weeks after the seeding.

It is clear that *pm*522 and *pm*525 can transform hamster cells in culture more efficiently than can *wt* BKV (Fig. 1 and Table 1).

Transformation of rat 3Y1 cells with virions. Unlike hamster cells, rat 3Y1 cells infected with BKV did not form colonies in soft agar, but produced foci on monolayer cultures when seeded in the regular liquid medium. Table 2 summarizes the results of the two sets of experiments with rat cells. In these experiments, *wt*501 did not produce any foci, even at an MOI of as high as 240 CIU(T) per cell. In contrast, rat cells infected with *pm*522 produced foci approximately in proportion to the dose of virus. The transforming activity of *pm*525 seemed to be lower (1/10 to 1/3) than that of *pm*522. Some of the foci were isolated and characterized. The cells contained nuclear T antigen, as revealed by indirect

TABLE 1. Transformation of hamster embryo cells with BKV virions[a]

Virus		MOI [CIU(T)][c]	Foci/60-mm dish (avg [total/no. of dishes])	
Clone	Sample[b]		Expt 1	Expt 2
Prototype	P23	70	0.3 (1/3)	
*wt*501	P17	40	0.3 (2/7)	
		20		0.3 (1/4)
*pm*522	P24	60	4.7 (14/3)	
		30		19.8 (79/4)
		6		5.0 (20/4)
		1.2		5.0 (21/4)
*pm*525	P43	60	1.7 (5/3)	
		30		7.0 (28/4)
		6		1.8 (7/4)

[a] Cultures were stained 2 weeks after infection, and dense foci were counted.

[b] The specific activities of the purified samples are as follows: 3.5 CIU(T), 1.2 CIU(V), and 6.9 PFU per 10^5 virions for P23; 2.0 CIU(T), 1.3 CIU(V), and 5.7 PFU per 10^5 virions for P17; 3.0 CIU(T), 0.5 CIU(V), and 2.4 PFU per 10^5 virions for P24; and 2.9 CIU(T), 1.1 CIU(V), and 5.0 PFU per 10^5 virions for P43.

[c] The activity was calculated from the number of T-antigen-positive cells (after cultivation for 4 days in the presence of anti-BKV serum) and the dilution factor (29).

TABLE 2. Transformation of rat 3Y1 cells with BKV virions[a]

Virus		MOI [CIU(T)][c]	Foci/60-mm dish (avg [total/no. of dishes])	
Clone	Sample[b]		Expt 1	Expt 2
Prototype	P23	60	0 (0/3)	
		15	0 (0/3)	
		4	0 (0/3)	
*wt*501	P17	240	0 (0/3)	
		60	0 (0/3)	0 (0/3)
		15	0 (0/3)	
*pm*522	P24	60	40 (80/2)	
		15	25 (50/2)	8.7 (26/3)
		4	9 (18/2)	
		3		1.3 (4/3)
*pm*525	P43	30		4.0 (12/3)
		20	2 (4/2)	
		5		0.7 (2/3)

[a] Cultures were stained 4 weeks after infection, and dense foci were counted.

[b] The specific activities of the purified samples are given in Table 1, footnote *b*.

[c] The activity was calculated from the number of T-antigen-positive cells and the dilution factor (29).

immunofluorescence. The staining for T antigen was weaker in rat cells than in the transformed hamster cells. Of the 12 cell lines, 2 contained a small fraction (0.03%) of V-antigen-positive cells. The cell lines from foci formed colonies in soft agar, although they grew slowly and became detectable at week 7. The efficiency of colony formation (colonies per cells seeded) was 0.03 to 0.05 at week 9. The results of these experiments indicate that *pm*522 and *pm*525 transform rat cells in culture much more efficiently than does *wt* BKV.

Transformation with viral DNA. Supercoiled form I DNA extracted from virions was tested for its capacity to transform hamster and rat cells in culture (Table 3). Whereas mutant DNAs produced foci of transformed cells (some foci were isolated and characterized), *wt*501 and uncloned prototype BKV produced none when 1.5 or 2 μg of DNA per 60-mm dish was used for transfecting subconfluent cells. The difference in transforming capacity between *pm* and *wt* DNAs seemed to be more than sixfold in hamster cells and ninefold in rat cells. *pm*522 DNA produced about five times as many foci as *pm*525 DNA did. These results are similar to those obtained with virions in the preceding sections, suggesting that the difference between *wt* and *pm* viruses is not ascribable to the possible mutation in the virion protein.

Construction of recombinants between *wt* and *pm* viruses. The results shown above indicate that *pm*522 can transform hamster or rat cells much more efficiently than can *wt*501 and that the difference in this capacity is not related to the virion protein. To examine which portion of the viral genome is responsible for the altered transforming ability, we constructed and characterized recombinant viruses between *wt*501 and *pm*522. Since *pm*522 has a small deletion at 0.7 map unit near the origin of DNA replication, the segment containing this area (0.62 to 0.73 map unit) of *wt*501 DNA was replaced with the equivalent fragment of *pm*522.

Figure 2 shows how the recombinant viruses were constructed. A fragment of *wt*501 DNA lacking *Hin*dIII-C (0.62 to 0.73 map unit), which was prepared by partial digestion with *Hin*dIII, was molecularly cloned into bacterial plasmid pBR322 at the *Hin*dIII site, and recombinant plasmid pW034 was constructed. *Hin*dIII-C of *pm*522, isolated as a mixture with the D fragment from agarose gels after complete digestion with *Hin*dIII, was also ligated to pBR322 DNA, and recombinant plasmid pP024 was constructed. The two recombinant plasmids (pW034 and pP024) were grown in bacteria and purified. Recombinant plasmid pW034 was partially di-

TABLE 3. Transformation with BKV DNA

DNA (form I)		Inoculum/60-mm dish		No. of foci/dish	
Clone	Sample[a]	DNA (μg)	T-antigen-inducing ability (CIU)[b]	Hamster embryo cells[c]	Rat 3Y1 cells[d]
Prototype	P23	1.5	1.1×10^5	0, 0[e]	0, 0
*wt*501	P17	2.0	9.4×10^4	0, 0	0, 0
*pm*522	P24	1.5	1.4×10^5	7, 5	11, 7
*pm*525	P43	1.5	1.1×10^5	1, 0	1, 3

[a] The specific activities of the virion samples from which DNAs were extracted are given in Table 1, footnote *b*.

[b] Expressed as CIU(T) in HEK cells (31). CIU(V) was also determined but not included here.

[c] Infected cultures were stained 20 days later.

[d] Infected cultures were stained 37 days later.

[e] Two values represent two dishes.

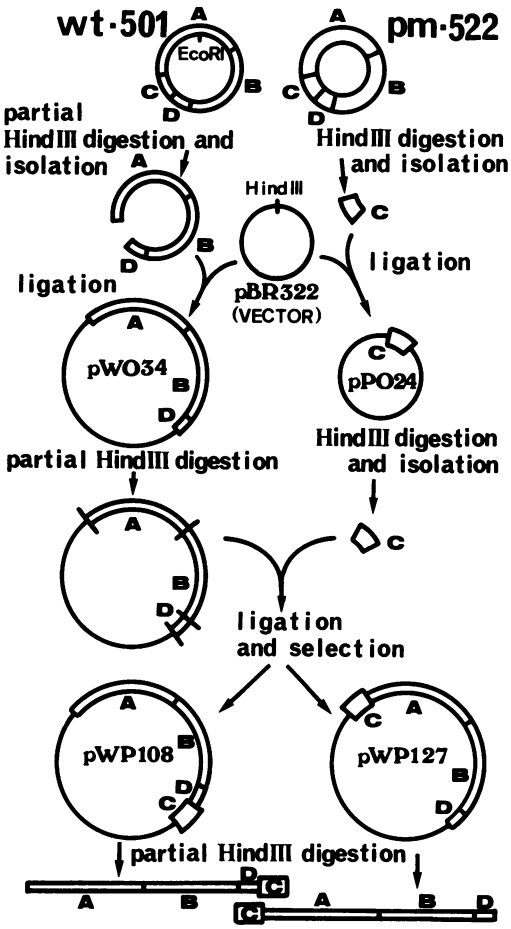

FIG. 2. Construction of recombinant BKV (see text). The *Hin*dIII cleavage map of BKV DNA (5.2 kilobases [kb] long) is from Howley et al. (13). *Hin*dIII generates four BKV fragments: A (2.3 kb), B (1.9 kb), C (0.56 kb), and D (0.42 kb). pBR322 DNA (4.36 kb long) has a single *Hin*dIII site (23). Appropriate fragments were purified from the partial or complete *Hin*dIII digests by isolation from agarose slab gels after electrophoresis. Selection of recombinant plasmids was based on the size of DNA and the cleavage patterns generated with restriction endonucleases. Linear recombinant viral DNA (in the mixtures of partial digests) was used for transfection of HEK cells.

gested with *Hin*dIII, and the fraction containing molecules cleaved at single sites was isolated from agarose gels after electrophoresis. Linear pW034 DNA was ligated with T4 ligase to purified *pm*522 *Hin*dIII-C cleaved out of pP024. The ligation mixture was used for transfection of bacteria. After screening about 60 bacterial colonies, we obtained two recombinant plasmids, pWP108 and pWP127, which contained inserted *Hin*dIII-C (of *pm*522) at the appropriate positions with the right orientation.

*Hin*dIII-C of *pm*522 had been inserted at the *Hin*dIII-D/pBR322 junction in pWP108 and at the *Hin*dIII-A/pBR322 junction in pWP127. The two recombinant plasmids, pWP108 and pWP127, which contain recombinant BKV genomes, were partially digested with *Hin*dIII and used for transfection of HEK cells. The infected HEK cell cultures showed complete cytopathic effects 4 to 5 weeks later, and the recombinant viruses were thus rescued (original stocks WP108H0 and WP127H0). For further characterization, the recombinant viruses were grown twice in HEK cell cultures and purified from the infected cells (stocks WP108H2 and WP127H2). Figure 3 shows the *Hin*dIII cleavage patterns of DNAs from the virions, the recombinant plasmids used for construction of recombinant viruses 108 and 127, and recombinant virus DNAs. The transforming capacity was determined with stocks WP108H2 and WP127H2 as described below.

In the second series of experiments, we constructed two more recombinant viruses, WP318 and WP331, by the same procedure described above. Recombinant plasmids pWP318 and pWP331 were made from pW034 and pP177, another plasmid containing *Hin*dIII-C of *pm*522. *Hin*dIII-C had been inserted at the *Hin*dIII-

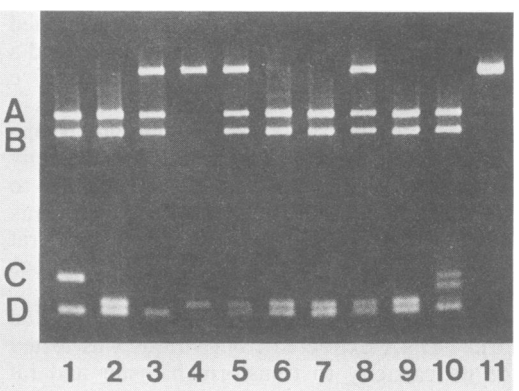

FIG. 3. Cleavage of DNAs from recombinant plasmids and viruses with *Hin*dIII. Electrophoresis was done at 100 V for 2 h in 2% agarose slab gel in E buffer. Lane 1, *wt*501 viral DNA; lane 2, *pm*522 viral DNA; lane 3, recombinant plasmid pW034 (pBR322 plus *wt* *Hin*dIII-ABD); lane 4, recombinant plasmid pP024 (pBR322 plus *pm*522 *Hin*dIII-C); lane 5, recombinant plasmid pWP108 (complete BKV DNA plus pBR322); lane 6, recombinant virus WP108 DNA (rescued by transfection and grown once in HEK cells: stock WP108H1); lane 7, recombinant virus WP108 DNA (grown twice in HEK cells: stock WP108H2); lane 8, recombinant plasmid pWP127 (complete BKV DNA plus pBR322); lane 9, recombinant virus WP127 DNA (rescued and grown once in HEK cells: stock WP127H1); lane 10, recombinant virus WP127 DNA (grown twice in HEK cells: stock WP127H2); lane 11, pBR322 DNA.

A/pBR322 junction in pWP318 and at the *Hin*dIII-D/pBR322 junction in pWP331.

For comparison, we reconstructed *wt* BKV by the same procedure used for constructing recombinant viruses. Linear pWP034 DNA was ligated with *wt* *Hin*dIII-C purified from plasmid pW168 (in which the *wt* C fragment had been cloned). The three reconstructed *wt* viruses, WW349, WW360, and WW361, were included in the test for transforming capacity.

Viruses with longer *Hin*dIII C segments tended to emerge among recombinant viruses and in one case constituted the major proportion of the population after repeated growth in HEK cells. The longer C fragments became detectable with virus 108 DNA digests after the virus was grown twice in HEK cells (Fig. 3, lane 7). The original C fragments of virus 127 found in plasmid pWP127 and the virus DNA grown once in HEK cells (WP127H1) almost disappeared and were replaced by the two kinds of longer C fragments (Fig. 3, lanes 8, 9, and 10) in the virus stock after two passages in HEK cell cultures. Recombinant virus WP318 contained no long C fragments after being grown once in HEK cells (WP318H1). The stock of WP331 grown once in HEK cells (WP331H1) had a small amount of longer C fragments (data not shown). The shorter C fragments were undetectable with the digests of reconstructed *wt* virus DNAs.

Biological properties of recombinant viruses. The plaques formed by the reconstructed *wt* viruses and the constructed recombinant viruses were compared with those formed by the parent *wt* and *pm* viruses. The plaques of reconstructed *wt* viruses were indistinguishable from those of *wt*501. The plaques formed by the recombinant viruses recovered from the initially transfected HEK cultures were small, turbid, and virtually indistinguishable from those of *pm*522. These results indicate that the change of DNA in *Hin*dIII-C is responsible for the altered plaque morphology of *pm*522.

After subsequent passage in HEK cells, larger turbid or clear plaques tended to arise among small turbid plaques. This tendency was the most obvious with stock WP127H2, which had two kinds of longer C fragments (Fig. 3, lane 10). Of 127 plaques, most became large and clear, resembling those of *wt* plaques, with this stock. The yields of virions of all of the reconstructed and constructed viruses were similar to those of parent viruses, which were about 10^5 virions per cell (30).

Table 4 shows the transforming capabilities of reconstructed *wt* and constructed recombinant viruses. Like parental *wt*501 (Table 2), *wt* viruses WW349, WW360, and WW361 had a very low capacity to transform rat cells. In contrast, all of the recombinant viruses transformed rat cells as efficiently as did parental *pm*522. Virus 127, despite its elongated *Hin*dIII C fragments and its ability to form clear large plaques on HEK cells, showed a high transforming capability. These results clearly indicate that the change of DNA within *Hin*dIII-C (0.62 to 0.73 map unit) is responsible for the enhanced transforming capacity of *pm*522.

DISCUSSION

BKV has been shown to transform hamster cells (17, 19, 25) and rat cells (28) in culture. Using these two systems, we compared the transforming capacities of uncloned and plaque-cloned *wt* viruses and highly tumorigenic *pm* mutants. We have found that the uncloned *wt* virus and *wt*501 are very weak transforming agents for hamster and rat cells, both of which were readily transformed by the *pm* mutants. Our results obtained with *wt* BKV are consistent with those described by Takemoto and Martin (24), who failed to transform hamster, mouse, and rat cells in culture with the prototype BKV virions. On the other hand, Seehafer et al. (21) reported that their BKV, which was plaque cloned twice in monkey BSC-1 cells and grown in HEK cells, efficiently transforms hamster cells. The results of our present study suggest that the apparent discrepancy in transforming capacity can be accounted for by the genetic heterogeneity probably existing among various BKV stocks.

The difference in transforming capacity between *pm*522 and *wt*501 was greater than that expected from the in vivo tumorigenicity test. The tumorigenic capacity of *pm*522 was about five times as high as that of *wt*501, as calculated from the response of hamsters inoculated with 10-fold dilutions of purified virions (Watanabe et al., in press). The difference in transforming capacity between *pm*522 and *wt*501 was more than 10-fold with hamster cells (Table 1) and 100-fold with rat cells (Tables 2 and 4). The transforming activity of *pm*522 was always higher than that of *pm*525 in the focus assay (Tables 1, 2, and 3; unpublished data), but these two were indistinguishable in the agar assay (Fig. 1) and in the in vivo tumorigenicity test (Watanabe et al., in press). The significance of the difference between in vivo and in vitro assays of BKV is unclear at present.

All of the cell lines established from the colonies and foci developing after BKV infection contained nuclear T antigen in all cells. Some of them had a small fraction of V-antigen-positive cells. The T-antigen-positive cell lines were found to contain BKV DNA sequences in various quantities, and free, nonintegrated viral DNA was found to occur in the majority of the hamster cell lines and in some of the rat cell lines

TABLE 4. Transformation of rat 3Y1 cells with recombinant viruses between *wt*501 and *pm*522[a]

Virus	Inoculum per dish at 1:1 dilution		Foci/60-mm dish (avg of three dishes)	
	Virions	CIU (T-antigen-forming activity)	1:1 dilution	1:3 dilution
Reconstructed *wt*[b]				
WW349	6.2×10^5	90	0.3	0
WW360	6.9×10^5	70	0.3	0
WW361	1.1×10^6	140	0.3	0
Constructed recombinant[c]				
WP108	8.1×10^5	60	40	19
WP127	4.2×10^5	60	73	35
WP318	6.6×10^5	45	73	26
WP331	5.7×10^5	63	85	23
*pm*522	5.4×10^5	60	78	29

[a] Cultures were stained 4 weeks after infection, and dense foci were counted.
[b] Viral DNA was constructed from *wt* *Hin*dIII-ABD and *wt* *Hin*dIII-C.
[c] Viral DNA was constructed from *wt* *Hin*dIII-ABD and *pm*522 *Hin*dIII-C.

tested (unpublished data). It is possible that the presence of free viral DNA (3, 4, 26, 32) is related to the occasional production of V antigen in transformed cells.

The results obtained with viral DNA (Table 3) suggest that the possible mutation in the virion protein, which can affect the adsorption and uncoating process of BKV, is not responsible for the observed difference between *pm* and *wt* viruses. The question is then raised as to which part of the BKV genome had been changed to affect the transforming capacity. We have shown that the change of DNA that had occurred near the origin (0.66 map unit) of replication, somewhere within *Hin*dIII-C (between 0.62 to 0.73 map unit), is related to the altered plaque morphology and the enhanced transforming capability.

The base sequences of the *Hin*dIII C fragments of both *wt*501 and *pm*522 have been determined (E. Soeda, personal communication). The analyses showed that the two C fragments have common base sequences, except that *pm*522 has a cluster of three minute deletions (total of 100 base pairs) in the noncoding region at a site approximately corresponding to the one (0.708 to 0.725 map unit) previously recognized as a small bubble by the electron microscope heteroduplex method (30).

The minute deletions of *pm*522 lie in the section recently identified with simian virus 40 as the early promoter region in vivo (1, 9). A group of host range mutants of mouse polyoma virus has been shown to have mutations in the same region (14, 22). Probably, *pm*522 is a new type of host range mutant that grows somewhat slowly in permissive cells but can express the early functions to transform efficiently non- or semipermissive rodent cells. How the deletions in the early promoter region affect the regulation of expression of the early functions and eventually enhance the transforming capability remains to be studied.

It appears that new BKV mutants differing from the parental viruses have arisen from the constructed recombinant viruses. Stock WP127H2 formed clear large plaques in HEK cells, had longer *Hin*dIII C segments (Fig. 3, lane 10), and still had a high transforming capacity (Table 4). Apparently, the viruses with longer C segments have had the advantage over the others with shorter C segments while growing in HEK cells. Perhaps the viruses with longer C segments were generated during the long incubation period after transfection of HEK cells with partial digests of recombinant plasmids (for rescue of virus) and became the majority of the population after repeated passages in HEK cells. The two parameters, transformation and plaque morphology (the formation of turbid plaques and decreased growth rate probably result from the genetic properties of mutants causing abortive infection in HEK cells, as discussed previously [30]), which were found to be linked in *pm*522 appear to have been separated in the viruses contained in stock WP127H2. Recently, we isolated by the plaque purification method these new transformation mutants forming clear large plaques. A comparison of these mutants, *pm*522, and *wt* BKV will be interesting and important for the study of the structure and function of the early promoter of BKV in vivo.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture, Japan.

We are indebted to Seijiro Uchida for his generous support and to Nobuo Yamaguchi for review of the manuscript.

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