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Evolutionary Changes of Transcriptional Control Region in a Minute-Plaque Viable Deletion Mutant of BK Virus

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Two plaque morphology BK virus (BKV) mutants (*pm*526 and *pm*527) rescued from a hamster pineocytoma cell line Pc13 were characterized and compared with a similarly rescued and previously characterized mutant (*pm*522), its derivatives (*tr*530, *tr*531, and *tr*532), and the wild type (501) for their biological activities and for the structures of their transcriptional control regions. The two mutants grew somewhat more slowly in human embryonic kidney cells but transformed rat 3Y1 cells more efficiently than did the wild-type BKV. BKV *pm*526 formed minute plaques and had the shortest transcriptional control region, having only one 68-base-pair element, which is triplicated in the wild-type BKV. BKV *pm*526 was unstable during repeated replication in human embryonic kidney cells and yielded large-plaque viruses with longer *Hin*dIII C segments encompassing the BKV DNA replication origin. Comparison of nucleotide sequences of the transcriptional control regions among the mutants and the wild type showed that *pm*526 is a parent virus, from which all the other mutants had evolved, and that the evolutionary changes of the plaque size, from minute to small to large, were due to duplications of a certain segment containing the adenovirus E1A enhancer core or the simian virus 40 enhancer core found in the BKV 68-base-pair element. The activities to enhance early transcription, as measured by the ability to direct the synthesis of chloramphenicol acetyltransferase, approximately paralleled the plaque size. The duplication containing the adenovirus E1A enhancer core did not affect the transforming capacity of the parent virus, but the duplication including the simian virus 40 enhancer core significantly lowered the transforming capacity for rat cells.

The transcriptional control region (enhancer-promoter) of human polyomavirus BK virus (BKV) Gardner (28) or Dun (14) strain contains a triplication of 68-base-pair (bp) elements which seem to be necessary for BKV to grow efficiently in human embryonic kidney (HEK) cells, because decreasing the number of the elements reduces the plaque size (23). The fragment containing the three 68-bp elements and without a TATA box has been shown to have an enhancing effect on the gene expression from the heterologous simian virus 40 (SV40) promoter in an expression vector (13). However, anatomy of the functional domains has not as yet been completed. The structure with the three 68-bp elements, although it is efficient in HEK cells, has not always been found among different BKV isolates (12, 14, 16, 28) and BKV mutants (20). Therefore, the enhancer-promoter structure of BKV seems to be variable and flexible without loss of function, if certain essential elements are retained, during adaptation and evolution to produce effective viral transcriptional elements.

In the present study, a minute-plaque-forming mutant (*pm*526) of BKV rescued from a hamster pineocytoma cell line was characterized and compared with a group of similar mutants isolated and characterized previously (19–22, 24). Comparison of the structures of the control regions among the mutants shows that minute-plaque-forming *pm*526 was the parental virus, from which all the other mutants forming larger plaques had evolved. The evolutionary changes from minute to small to large plaques were found to be accompanied by the DNA rearrangements, which sometimes affected the transforming capacity for rat cells, in the transcriptional control region.

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MATERIALS AND METHODS

Virus. BKV 501 (22, 24), used as a wild type, was a plaque isolate from a stock of Gardner strain. Plaque morphology mutant *pm*522 (22, 24) was used for comparison. Propagation and purification of virus were done as described previously (22, 24).

Cells. Pc13 is a transplantable hamster pineocytoma cell line (18), from which *pm*522 and the mutants characterized in this study (*pm*526 and *pm*527) were rescued. HEK cells were used for virus propagation, preparation of viral DNA by the method of Hirt (7), plaque assays, and immunofluorescence assays for T and V antigens as described previously (25). The rat 3Y1 cell line was used for an assay of transformation by virions and DNA, as described previously (22). HEK cells were used for a transient assay for the enhancer-promoter activity.

Enzymes. Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. (*Bam*HI, *Dde*I, and *Hin*dIII); New England BioLabs, Beverly, Mass. (*Nco*I); and Takara Shuzo Co., Ltd., Kyoto, Japan (*Bgl*II, *Hae*III, *Hin*fI, and *Stu*I). Bacterial alkaline phosphatase was obtained from Worthington Diagnostics, Freehold, N.J. T4 polynucleotide kinase was from Boehringer Mannheim Biochemicals, Indianapolis, Ind., or Bethesda Research Laboratories. T4 DNA ligase and *Escherichia coli* DNA polymerase I were from Bethesda Research Laboratories. Enzymes were used as recommended by the suppliers.

Rescue of virus. Cultured Pc13 cells were fused with HEK cells by the aid of UV-irradiated Sendai virus (17).

Transfection. The DEAE-dextran method (11) was used for an infectivity assay and for production of virus from

TABLE 1. Biological characteristics of BKV mutants

Virus[a]	Growth in human cells		Transformation of rat 3Y1 cells: relative focus-forming activity[b]
	Plaque type[c]	T/V ratio[d]	
Wild type (purified sample)	Large, clear	2.7	0 (3)
*pm*522 (purified sample)	Small, turbid	11.0	517 ± 63 (6)
*pm*526			
Seed stock	Minute (occasional small and large)	31.0	
Purified sample	Mixture of minute, small, and large	8.1	220 ± 53 (6)
*pm*527			
Seed stock	Small, turbid	11.0	
Purified sample	Small, turbid	14.0	14 ± 16 (6)

[a] Purified samples were prepared from HEK cell cultures infected with seed stocks at a low MOI.

[b] Values indicate the ratio of foci of transformed cells to the T-antigen-producing capacity of the inoculum, and are expressed as [(number of foci of transformed cells)/(number of cell-infecting units)] $\times 10^6$. Each value is given as the mean plus or minus the standard deviation. Numbers in parentheses indicate the number of culture dishes for calculation. The transforming activities were determined with threefold dilutions of the inocula and the input MOIs ranged from 5 to 50 cell-infecting units (for T antigen) per cell.

[c] Plaques formed in HEK cell cultures on days 17 to 18 after infection. Large, 2 to 7 mm in diameter; small, 1 to 5 mm in diameter; minute, pinpoint to 2 mm in diameter.

[d] Ratio of T-antigen-positive cells to V-antigen-positive cells, as determined by an immunofluorescence test on day 4 after a low-multiplicity infection.

cloned BKV DNA in HEK cells. The calcium phosphate method (5) was used for a transformation assay with rat 3Y1 cells and a transient assay with HEK cells.

Molecular cloning. *Bam*HI-cleaved whole BKV DNAs and *Hin*dIII C fragments were inserted into pBR322 at *Bam*HI and *Hin*dIII sites, respectively, and the recombinants were grown in *E. coli* HB101 and purified as described previously (22). For cloning of *pm*526 *Hin*dIII-C, the mixture of *Hin*dIII-C and -D was isolated from an electrophoresis gel, digested with *Bgl*II (cleaving *Hin*dIII-D), and cloned in pBR322.

DNA sequencing. Nucleotide sequence was determined by the chemical method of Maxam and Gilbert (9) with [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol; Amersham International plc, Buckinghamshire, England). For sequencing the *Hin*dIII C segment of *pm*526, *Nco*I fragments were used. For sequencing the *Hin*dIII-*Stu*I segment of *pm*527 *Hin*dIII-C, subfragments were prepared by digestion with *Nco*I and then with *Hae*III. The region containing mutations was sequenced for both DNA strands, but the other regions were sequenced partly for both strands and partly for a single strand.

Transient assay for enhancer-promoter activity. The activity was determined by the method of Gorman et al. (4). The pSV0-cat plasmid was prepared by removing the SV40 enhancer-promoter segment from pSV2-cat plasmid, which can induce bacterial chloramphenicol acetyltransferase (CAT) in mammalian cells upon transfection. A series of plasmids containing BKV control regions was constructed by insertion of appropriate BKV DNA fragments into pSV0-cat. HEK cells were transfected with 5 to 20 μg of plasmid DNA per 100-mm dish for determination of the dose-response relation and the time of CAT reaction. DNA concentration of 15 μg per 100-mm dish was used for the most of the assays. Four hours after the addition of DNA, cells were exposed to 15% glycerol at 37°C for 2 min. Cells were collected 48 h after transfection, and extracts were prepared by freezing, thawing, and centrifugation. Conversion of [^{14}C]chloramphenicol to acetylated forms was determined by thin-layer chromatography.

RESULTS

BKV mutants rescued from a BKV-induced hamster pineocytoma. Hamster pineocytoma cell line Pc13, which was induced by Gardner strain, yields infectious virus upon cell fusion with permissive HEK cells (18). The rescued viruses from different experiments were different from the wild-type BKV and had common characteristics, although they are believed to have originated from Gardner strain because the tumor was induced by that strain. The plaques formed in HEK cell cultures by the rescued viruses were smaller and more heterogeneous in size (pin-point size to 4 mm in diameter) than those of the wild type 501 (2 to 7 mm), a plaque isolate of Gardner strain. Digestion of DNA of the rescued viruses with *Hin*dIII, which cleaves the wild-type BKV DNA at four sites (generating *Hin*dIII A, B, C, and D fragments [24, 28]), revealed the presence of a heterogeneous shorter *Hin*dIII C fragment (data not shown). The results suggest that the rescued virus stocks contained a heterogeneous population of BKV mutants differing in plaque size (growth in HEK cells) and in the size of the *Hin*dIII C segment containing the BKV DNA replication origin. One plaque isolate, *pm*522, obtained by three cycles of plaque isolation from a rescued virus stock, has been extensively characterized and compared with the wild-type BKV (501) (24), but some of the plaques produced by the rescued virus stock were minute and smaller than those produced by *pm*522. In the present study, we attempted to isolate such minute-plaque variants.

Two clones (*pm*526 and *pm*527) were isolated from two minute plaques (with a diameter of ca. 2 mm on day 18, smaller than those of *pm*522) selected from the plaques formed by one of the rescued virus stocks (fluid of coculture of HEK and Pc13), and were subjected further to two cycles of plaque isolation. The diameter of plaques formed by *pm*526 was less than 2 mm throughout the three plaque isolation procedures, but the *pm*527 plaques were larger (1 to 5 mm) at the second and third plaque isolations. BKVs *pm*526 and *pm*527 from the third plaque isolation were each inoculated into HEK cell cultures at a multiplicity of infection (MOI) of less than 2×10^{-4} PFU per cell. The viruses obtained after the development of complete cytopathic ef-

TABLE 2. Transformation of rat 3Y1 cells with BKV DNA

Viral DNA[a]	No. of foci per μg of DNA in expt[b]:			
	1	2	3	4
Wild type	0	0	2.8	0
*pm*522	22	36	134	166
*pm*526	36	45	153	92

[a] Linear viral DNA was prepared from cloned BKV DNA in pBR322 at *Bam*HI site. Recombinant plasmids were digested with *Bam*HI and *Hpa*II before assay.

[b] Rat 3Y1 cells were transfected with DNA by the calcium phosphate method (5). Foci of transformed cells were stained and counted 25 days after infection. The number of foci per microgram of DNA was the average of three to eight dishes. In experiments 3 and 4, viral DNA was purified by electrophoresis. A few colonies from those transformed by *pm*526 were isolated and examined by indirect immunofluorescence. All the cell clones were positive for T antigen.

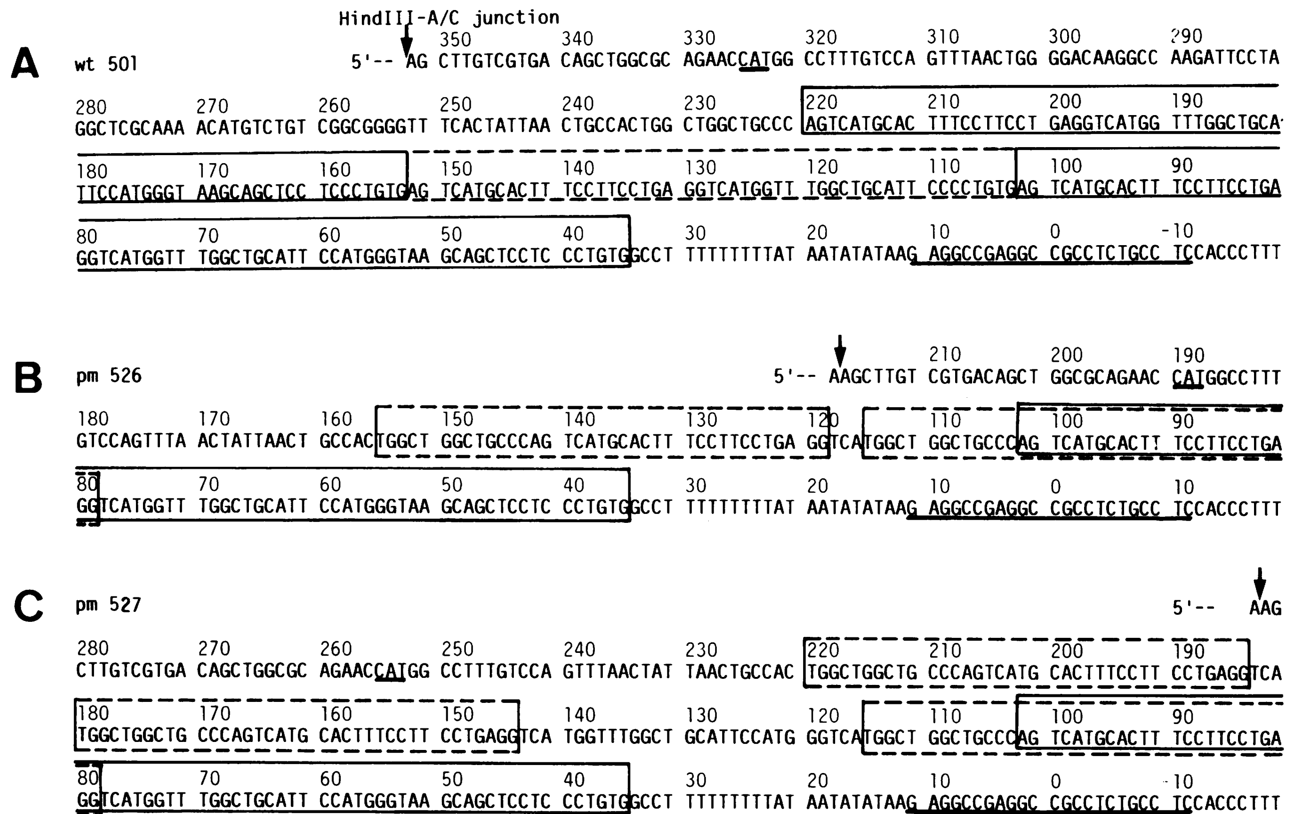

FIG. 1. Nucleotide sequence of minute-plaque-forming BKV DNA: L strands of the control regions. The sequence for the wild type (wt 501) (A) from reference 20 is shown for comparison. The sequences for *pm*526 (B) and *pm*527 (C) have the same polarity as the early mRNA in the 5′ to 3′ direction. The cytosine in the center of the symmetrical structure of DNA replication origin (underlined) was taken arbitrarily as zero for numbering nucleotides, and the number increases toward the late genome. Underlined CAT may be for the initiation codon for VPx (14). The solid-line boxes indicate 68-bp elements. The dashed-line boxes indicate shorter repeats containing part of the 68-bp element. Although the sequences were determined for the entire *pm*526 *Hin*dIII-C and for the *Hin*dIII-*Stu*I segment of *pm*527 *Hin*dIII-C, those downstream of the DNA replication origin (those toward the junction of *Hin*dIII C and D fragments) are omitted here for simplicity. The sequences of the omitted areas were identical to each other and to those of Dun (14) and Gardner (28) strains.

fects gave titers of 10^8 and 2×10^8 PFU/ml for *pm*526 and *pm*527, respectively, and were used as the seed stocks for further characterization.

Characterization of BKV mutants *pm*526 and *pm*527. To examine the properties of the mutants, we purified virions from HEK cells infected with the seed stock of each mutant at an MOI of 0.1 PFU per cell. The yield of purified virions was ca. 8×10^{12} virions per 150-cm^2 culture for the two mutants, *pm*526 and *pm*527, and for the *pm*522 and wild-type strains used for comparison. Table 1 summarizes the biological properties of the BKV mutants.

Plaque morphology was characteristic of each mutant. The seed stock of *pm*526 produced mostly minute plaques with diameters of pin-point size to 2 mm and occasionally larger ones. However, the proportion of larger plaques with diameters of 3 to 6 mm markedly increased when the purified sample of *pm*526 (prepared by inoculation of the seed stock) was plated for plaque formation. On the other hand, both the seed stock and the purified sample of *pm*527 formed turbid small plaques with diameters of 1 to 5 mm on day 17 (on later days, the turbid areas of the plaques became clear).

The ratio of T-antigen-positive cells to V-antigen-positive cells, which reflects the intrinsic property of BKV that cannot switch from early to late stage of infection (24), was higher in infection with *pm*526 or *pm*527 than with the wild-type BKV (Table 1). The ratio obtained with *pm*526 seed stock was 10 times as high as that obtained with the wild type and higher than that of *pm*522, but the ratio with a purified sample of *pm*526 decreased markedly to the level of *pm*522. The ratios obtained with *pm*527 seed stock and the purified sample were higher than that of the wild type and similar to that of *pm*522.

The transforming capacities of *pm*526 and *pm*527 for rat 3Y1 cells were examined with the purified samples, in comparison with the samples of wild type and *pm*522 previously tested (Table 1). The transforming capacity of *pm*522 was the highest among the virus clones tested, and that of 501 was undetectable. Mutant *pm*526 transformed rat cells half as efficiently as did *pm*522. The transforming capacity of pm527 was about 1/40 that of *pm*522.

Characterization of DNAs from BKV mutants *pm*526 and *pm*527. Upon digestion with *Hin*dIII, *pm*526 and *pm*527 were found to have abnormal *Hin*dIII C segments. Two DNA preparations, one extracted from purified virion sample used in Table 1 and the other extracted directly from HEK cells infected at a high MOI (5 PFU per cell) with the seed stock of *pm*526 or *pm*527, were digested with *Hin*dIII and subjected to electrophoresis in 1.6% agarose gels (data not shown). The mobilities of *Hin*dIII-A, -B, and -D from the two mutants were identical to those of the wild type. The greater proportion of *Hin*dIII-C of *pm*526 moved together with *Hin*dIII-D in the agarose gel, but there were some

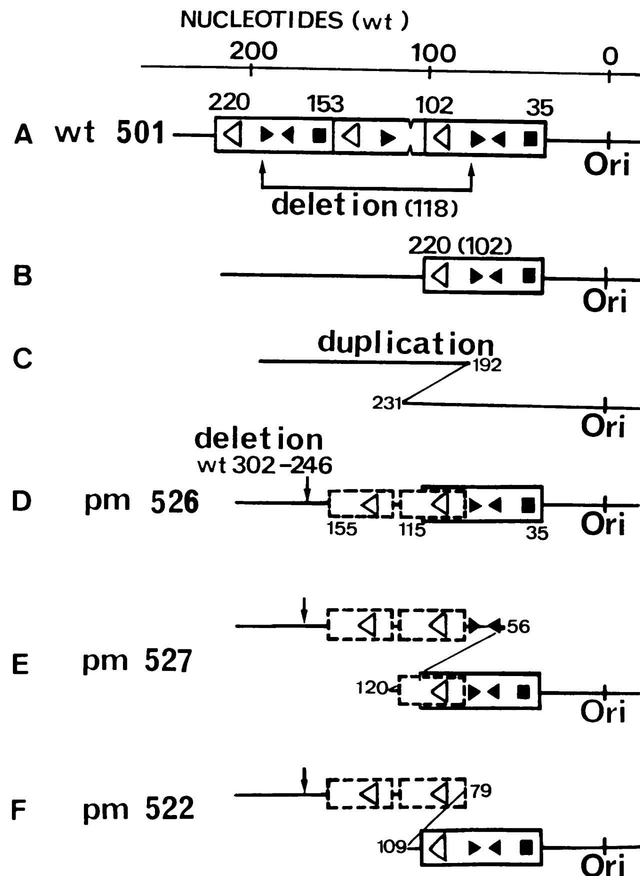

FIG. 2. DNA rearrangement in BKV *pm*526 and *pm*527 DNAs. If *pm*526 control region had evolved from that of BKV wild type (wt 501), the process of evolution can be deduced from comparison of the nucleotide sequences (Fig. 1A and B). First, a deletion of 118 bp had occurred from recombination (homology dependent) between the two sets of 68-bp element (A) and had generated the second molecule from the top (B), with a single 68-bp element. This molecule (B) is identical with artificially made *dl*504 (23). Here, the two original 68-bp repeats, wild-type nt 220 to 153 and 102 to 35, overlap each other. Second, duplication had occurred, as shown in the third molecule from the top (C). Here, the linkage resulting from duplication is from wild-type nt 192 (or 74) to 231. Third, another deletion (wild-type nt 302 to 246) at the place indicated by the vertical arrow had yielded *pm*526 (D). From *pm*526, *pm*527 was generated by duplication of a segment from *pm*526 nt 56 to 120 (E) and *pm*522 (20) was generated by duplication of a segment from *pm*526 nt 79 to 109 (F). Boxes with solid lines indicate 68-bp elements. Incisions indicate deletion of the 68-bp element. Boxes with dashed lines indicate shorter repeats. Small filled triangles represent the potential core nucleotides 5'TGGA(T)A(T)A(T)3' in the polyomavirus enhancer (27). The larger unfilled triangles represent the sequence 5'ATGCACTTTCCT3' resembling the adenovirus E1A enhancer cores (6). Small filled rectangles represent the sequence 5'CCTCCC3', the putative promoter core. The center of DNA replication origin is indicated by Ori at nt 0.

fragments moving more slowly than *Hin*dIII-D. The heterogeneity of *Hin*dIII-C was more prominent with *pm*526 DNA grown at a low MOI than with that grown at a high MOI. The *Hin*dIII-C of *pm*527 grown at either a low or a high MOI moved faster than the wild-type *Hin*dIII-C (555 bp) and more slowly than *Hin*dIII-D.

Because the *pm*526 sample was found to be heterogeneous in size of *Hin*dIII-C and plaque size, 10 larger plaques formed by the *pm*526 purified sample (grown at a low MOI) were isolated, grown separately in HEK cells, and subjected to selective DNA extraction (7). *Hin*dIII cleavage of DNAs from the isolated plaques (data not shown) showed that all the clones had longer *Hin*dIII C fragments, which could be classified into three size classes (ca. 510, 530, and 560 bp long).

Because *pm*526 virion samples always seemed to contain several variants, *pm*526 DNA was molecularly cloned in *E. coli* pBR322 at the *Bam*HI site and characterized for growth in HEK cells and transforming capacity for rat cells. Cloned *pm*526 DNA had a single class of *Hin*dIII C fragments that migrated together with *Hin*dIII D fragments in an agarose gel. The *Bam*HI-cleaved recombinant DNA yielded infectious virus (the seed stock) upon transfection to HEK cells. The seed stock formed the mixed plaques of minute and large ones. The DNA from cells infected with the seed stock contained longer *Hin*dIII C fragments, in addition to the original shorter ones. These changes appeared more readily than those observed with infection with virions, probably because of DNA transfection (2).

*pm*526 DNA purified from *Bam*HI-cleaved recombinant DNA was examined for transforming capacity for rat 3Y1 cells (Table 2). The *pm*526 DNA transformed rat cells as efficiently as did the similarly prepared *pm*522 DNA.

Structures of the transcriptional control regions of BKV mutants *pm*526 and *pm*527. As the *Hin*dIII C segments of *pm*526 and *pm*527 were shorter than that of the wild type, they were molecularly cloned in pBR322, and their primary structures were determined (Fig. 1B and C). The *Hin*dIII C segments of *pm*526 and *pm*527 were 420 and 485 bp long, respectively. Comparison of these sequences with those of 501 and of *pm*522 (Fig. 1 of reference 20) showed that the changes of mutants were limited to the putative transcriptional control region in the noncoding region to the late side of the DNA replication origin. The mutants and wild-type BKV were related to one another through deletions and duplications (Fig. 2).

Compared with the sequence of the wild type, *pm*526 had complex deletions of 135 bp in total in the control region. Since *pm*526 had probably occurred from Gardner strain (*pm*526 was rescued from a tumor induced by Gardner strain), the complex deletions of *pm*526 seem to be best accounted for by three steps involving the first deletion by the homology-dependent recombination between the two complete 68-bp elements, followed by duplication including part of the 68-bp element, and the second deletion of 57 bp (wild-type nucleotides [nt] 302 through 246) between *pm*526 nt 167 and 168 (Fig. 1B), from the wild-type sequence (Fig. 2). Thus, *pm*526 had a single 68-bp element and two shorter repeats sharing the origin-distal area of the 68-bp element, whereas the wild-type had a triplication of the 68-bp element (the central element had a deletion of 18 bp).

Comparison of the sequences between *pm*526 and *pm*527 clearly indicated that *pm*527 was made by duplication of a segment (*pm*526 nt 56 to 120 in Fig. 1B) in the *pm*526 DNA (Fig. 2). The 57-bp deletion of *pm*526 was shared by *pm*527 and the previously characterized *pm*522 (20). Furthermore, comparison of the sequences between *pm*526 and *pm*522 showed that *pm*522 must have evolved from *pm*526 by a single duplication (*pm*526 nt 79 to 109). It is now clear that *pm*526 is a parent virus to the *pm*527 strain characterized in this study and the *pm*522 described previously (20) and is an ancestral virus to *tr*530, *tr*531, and *tr*532, which are partial revertants of *pm*522 and are believed to have evolved by

duplication from *pm*522 during transfection of HEK cells (20).

Function of the transcriptional control regions of BKV mutants in a transient assay. The function of the certain sequence from the putative control region in the viral genome can be measured by using a transient expression vector (4). The SV40 enhancer-promoter segment coupled to the bacterial CAT gene (pSV2-cat) directs synthesis of the enzyme in the cultured mammalian cells after transfection with the recombinant plasmid (4). If the SV40 enhancer-promoter segment of pSV2-cat was replaced by another segment, the function of that segment could be measured in the same assay system as described previously (4).

In the present study, the BKV segments from various different mutants were inserted into the *Hin*dIII site of plasmid pSV0-cat, which was made from pSV2-cat by removing the SV40 enhancer-promoter (4) (Fig. 3). Recombinant plasmids were made to contain the segments from BKV putative control region, so that the functions measured in the transient assay reflect the early transcriptional control in vivo. The function of the inserted BKV sequences were measured in HEK cells.

The enhancing activities of various BKV segments for CAT expression are shown in Table 3. The wild-type BKV sequence presumably controlling early transcription was as effective in HEK cells as pSV2-cat. Thus, this BKV sequence acted on the foreign gene like SV40 enhancer-promoter. Removal of two 68-bp elements and their upstream noncoding region suppressed expression of CAT activity, suggesting that a single 68-bp element and DNA replication origin was not sufficient for enhancement of early transcription. The activities of the sequences from various BKV mutants apparently paralleled the sizes of plaques. The minute-plaque-forming *pm*526 was least effective among the strains tested, and the large-plaque-forming revertants *tr*530, *tr*531, and *tr*532 were as effective as or more effective than the wild type.

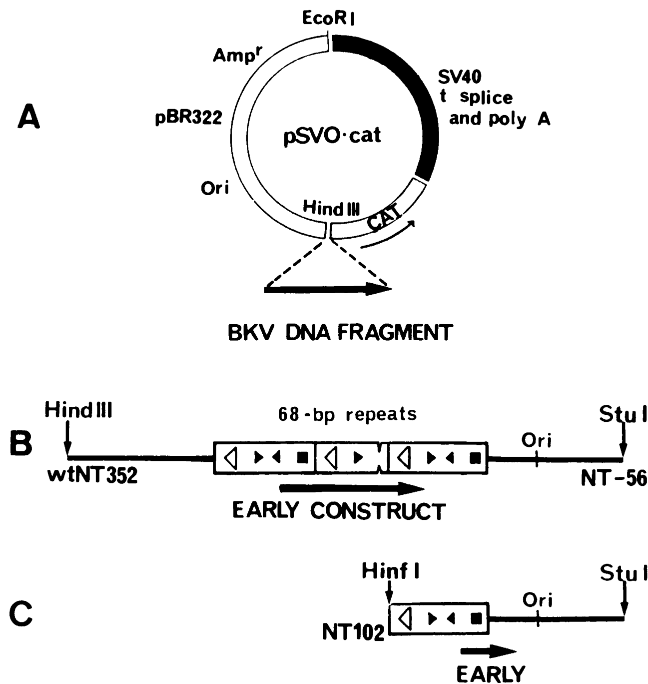

FIG. 3. Construction of pBK-cat vectors. pSV0-cat (A) was made by removal of the SV40 enhancer-promoter from CAT expression vector pSV2-cat (4). For construction of pBK-cat vectors, appropriate DNA fragments from BKV mutants were ligated to *Hin*dIII linkers (Bethesda Research Laboratories) and inserted into pSV0-cat at the *Hin*dIII site in the orientation indicated by the arrow. DNA fragment from the wild-type (wt) control region between *Hin*dIII and *Stu*I sites (B) was used in the orientation indicated by the arrow for examination of the enhancer-promoter activity for early transcription (early construct). Early constructs of various BKV mutants were made similarly with the *Hin*dIII-*Stu*I fragments. The early construct with a single 68-bp element was made by insertion of the *Hin*fI-*Stu*I fragment from the wild type shown in panel C into pSV0-cat in the orientation indicated by the arrow. Symbols are as described in the legend to Fig. 2.

TABLE 3. Promoter-enhancer activity of the BKV DNA control region

Plasmid	Origin of BKV DNA fragment	Relative CAT activity of early construct in HEK cells[a]
pSV0-cat[b]		0.026 ± 0.009 (6)
pSV2-cat[c]		1.07 ± 0.19 (2)
pBK501E-cat	Wild type	1.00 (6)
pBK526E-cat	*pm*526	0.32 ± 0.18 (5)
pBK527E-cat	*pm*527	0.38 ± 0.18 (2)
pBK522E-cat	*pm*522	0.74 ± 0.15 (5)
pBK530E-cat	*tr*530	1.10 ± 0.34 (3)
pBK531E-cat	*tr*531	1.69 ± 0.46 (3)
pBK532E-cat	*tr*532	1.29 ± 0.16 (3)
pBK68E-cat	Single 68-bp element[d]	0.03 ± 0.012 (3)

[a] CAT activity, the conversion of [^{14}C]chloramphenicol to acetylated forms, was measured with the cell extracts prepared 48 h after transfection with the recombinant vectors (4) and expressed as relative to that of the pBK-cat vector containing the wild-type BKV fragment. The proportion of chloramphenicol acetylated with the wild-type BKV recombinant vectors was 8 to 20%. Each value is the mean plus or minus the standard deviation for the number of determinations indicated in the parentheses. BKV DNA fragment was inserted into pSVO-cat so that CAT activity reflects the early transcription in vivo. The range and orientation of the BKV DNA insert are shown in Fig. 3B.

[b] CAT expression vector without promoter-enhancer (4), as shown in Fig. 3A.

[c] CAT expression vector with SV40 enhancer-promoter (4).

[d] BKV DNA insert with a single 68-bp element, as shown in Fig. 3C.

DISCUSSION

We have characterized the two plaque morphology mutants (*pm*526 and *pm*527) rescued from a BKV (Gardner strain)-induced hamster pineocytoma and determined the structures of their transcriptional control regions. Comparison of their structures with each other (Fig. 1) and also with those published earlier (Fig. 1 of reference 20) made the relation among these mutant BKVs clear. We previously showed that large-plaque-forming revertants *tr*530, *tr*531, and *tr*532 had arisen from the small, turbid-plaque-forming *pm*522 by a small duplication in the transcriptional control region (20). The data in this study show that *pm*522 (20) and *pm*527 must have evolved from *pm*526 by a duplication (Fig. 2). We have recently constructed and characterized the mutants having deletions in their transcriptional control regions (23). The constructed mutants are similar to the naturally occurring mutant in their biological characteristics. We also compared the structures of the control regions of those artificial and natural mutants (Fig. 4).

The common stretch of nucleotide sequence among these mutants was a 68-bp element, which is triplicated in the large-plaque-forming wild-type BKV (501) (Fig. 1 and 2).

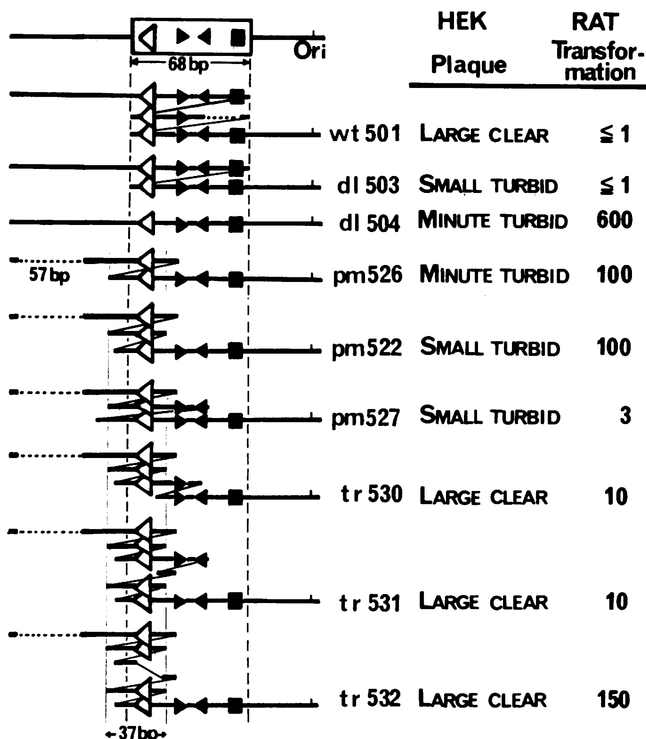

FIG. 4. Duplications in the control region and biological characteristics of constructed and naturally occurring BKV mutants. Duplications are indicated as relative to a BKV with a single 68-bp element (like a constructed deletion mutant *dl*504). The structures and biological characteristics of *dl*503, *dl*504, *pm*522, *tr*530, *tr*531, and *tr*532 are from the published data (20, 23). The biological activities of *dl*503, *dl*504, and *pm*526 were determined with cloned DNA, those of the wild type (wt 501) and *pm*522 were determined with cloned DNA and purified virions, and those of the others were determined with purified virions. Transforming capacity was expressed as relative activity of virions or DNA. Symbols are as described in the legend to Fig. 2.

The 68-bp element contains the signal resembling the sequence in the SV40 promoter (5'CCTCCC3' of BKV [14]; 5'CCGCCC3' of SV40 [1]), the SV40 enhancer core (5'TGGTTT3' and 5'ATTCCA3' of BKV; 5'TGGAAA3' of SV40 [27]), and the adenovirus type 5 E1A enhancer core (5'ATGCACTTTCCT3' of BKV; 5'AGGAAGTGAAA3' of adenovirus [6]), which are distributed as shown in Fig. 1 and 2. The single 68-bp element alone did not function efficiently in HEK cells, as assayed by the CAT activity, and some mutants with a short control region containing a single 68-bp element (*dl*504 and *pm*526 in Fig. 4) grew slowly and formed minute plaques in HEK cell cultures. Clearly, the wild-type control region containing three 68-bp elements was efficient in HEK cells, as seen from the CAT activity and clear large plaques in HEK cell cultures. However, triplication of the 68-bp elements was not always needed in human cells, because some large-plaque-forming mutants (*tr*530, *tr*531, and *tr*532) have only one 68-bp element in their control region (Fig. 4).

The evolutionary changes from minute to small to large plaques in naturally occurring mutants with a single 68-bp element were accompanied by repeated duplications of a certain short segment in the control region. The frequently duplicated segments consisted of an origin-distal part of the 68-bp element containing adenovirus E1A enhancer core and the short upstream region outside the 68-bp element. The duplication sometimes extended downstream to include the SV40 enhancer cores in the middle part of the 68-bp element (*pm*527, *tr*530, and *tr*531) (Fig. 4). The data suggest that BKV needs not only a single 68-bp element but multiple copies of the origin-distal segment, including the adenovirus E1A enhancer core, to be fully viable in HEK cells.

The evolutionary changes of BKV mutants in HEK cells seem to have affected their transforming capacity for rat cells (Fig. 4). The highly transforming mutants (*pm*526, *pm*522, and *tr*532) had only one set of SV40 enhancer cores originally present in the middle of the 68-bp element. The mutants with significantly lowered transforming capacity (*pm*527, *tr*530, and *tr*531) had duplications including the SV40 enhancer cores. The data suggest that the number of SV40 enhancer cores (and possibly their location) is an important factor for transformation of rat cells by BKV. The data are consistent with the results obtained with the constructed deletion mutants (23) (Fig. 4). The transforming capacity seemed to be unaffected by the increased adenovirus enhancer cores (*pm*526, *pm*522, and *tr*532). Although the expression of the wild-type BKV and *pm*522 in rat cells has been compared (21), their difference in transforming capacity has yet to be fully elucidated.

The BKV DNA fragments inserted into an expression vector pSV0-cat functioned as an enhancer-promoter in inducing the CAT activity in HEK cells (Table 3). The evolutionary changes which altered the plaque size from minute to small to large in the control region also improved the activity of BKV DNA fragments to enhance the CAT activity when they were examined for early transcription. The data suggest that the multiple copies of the area including the adenovirus enhancer core are important for efficient early transcription. The BKV segments examined for CAT assay in HEK cells were similarly tested in rat 3Y1 cells. The induced CAT activities were much lower than those in the HEK cells (data not shown).

Probably, the DNA rearrangement by deletion and duplication in the enhancer-promoter is also occurring in polyomaviruses in nature. Among the BKV isolates, Dik strain (16) has the richest variety of oligonucleotides but no tandem repeats, like 68-bp elements of Gardner (28) or Dun (14) strain, in its control region. Comparison of the published sequences of various BKV isolates Gardner, Dun, MM, Dik, JL, and GS (12, 14, 16, 28) indicates that all of the natural variants must have evolved from a hypothetical prototype resembling Dik strain by deletion, duplication, and some point mutations. Naturally occurring JC virus variants have differences in their regulatory sequences (3, 8, 10). Nonviable enhancerless mutants of SV40 can regain their infectivity by DNA rearrangement in the control region (15, 26). The change of DNA found in this study seem to be reminiscent of what had been proceeding in nature to produce polyomavirus variants and during evolution and divergence of polyomaviruses from a common ancestor. Perhaps deletion and duplication described here, sometimes translocation of viral sequences (10), and incorporation of the host sequences (13) in the transcriptional control region had been important steps in changing the host range of virus and in generation of new viruses during evolution.

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LITERATURE CITED

1. **Byrne, B. J., M. S. Davis, J. Yamaguchi, D. J. Bergsma, and K. N. Subramanian.** 1983. Definition of the simian virus 40 early promoter region and demonstration of a host range bias in the enhancement effect of the simian virus 40 72-base-pair repeat. Proc. Natl. Acad. Sci. USA **80**:721–725.
2. **Calos, M. P., J. S. Lebkowski, and M. R. Botchan.** 1983. High mutation frequency in DNA transfected into mammalian cells. Proc. Natl. Acad. Sci. USA **80**:3015–3019.
3. **Frisque, R. J., G. L. Bream, and M. T. Cannella.** 1984. Human polyomavirus JC virus genome. J. Virol. **51**:458–469.
4. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
5. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
6. **Hearing, P., and T. Shenk.** 1983. The adenovirus type 5 E1A transcriptional control region contains a duplicated enhancer element. Cell **33**:695–703.
7. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
8. **Martin, J. D., D. M. King, J. M. Slauch, and R. J. Frisque.** 1985. Differences in regulatory sequences of naturally occurring JC virus variants. J. Virol. **53**:306–311.
9. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
10. **Miyamura, T., A. Furuno, and K. Yoshiike.** 1985. DNA rearrangement in the control region for early transcription in a human polyomavirus JC host range mutant capable of growing in human embryonic kidney cells. J. Virol. **54**:750–756.
11. **McCutchan, J. H., and J. S. Pagano.** 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. J. Natl. Cancer Inst. **41**:351–357.
12. **Pater, A., M. M. Pater, L.-S. Chang, K. Slawin, and G. di Mayorca.** 1983. Multiple origins of the complementary defective genomes of RF and origin proximal sequences of GS, two human papovavirus isolates. Virology **131**:426–436.
13. **Rosenthal, N., M. Kress, P. Gruss, and G. Khoury.** 1983. BK viral enhancer element and a human cellular homolog. Science **222**:749–755.
14. **Seif, I., G. Khoury, and R. Dhar.** 1979. The genome of human papovavirus BKV. Cell **18**:963–977.
15. **Swimmer, C., and T. Shenk.** 1984. A viable simian virus 40 variant that carries a newly generated sequence reiteration in place of the normal duplicated enhancer element. Proc. Natl. Acad. Sci. USA **81**:6652–6656.
16. **ter Schegget, J., C. J. A. Sol, E. W. Baan, J. van der Noordaa, and H. van Ormondt.** 1985. Naturally occurring BK virus variants (JL and Dik) with deletions in the putative early enhancer-promoter sequences. J. Virol. **53**:302–305.
17. **Uchida, S., and S. Watanabe.** 1969. Transformation of mouse 3T3 cells by T antigen-forming defective SV40 virions (T particles). Virology **39**:721–728.
18. **Uchida, S., S. Watanabe, T. Aizawa, A. Furuno, and T. Muto.** 1979. Polyoncogenicity and insulinoma-inducing ability of BK virus, a human papovavirus, in Syrian golden hamsters. J. Natl. Cancer Inst. **63**:119–126.
19. **Watanabe, S., S. Kotake, A. Nozawa, T. Muto, and S. Uchida.** 1982. Tumorigenicity of human BK papovavirus plaque isolates, wild-type and plaque morphology mutant, in hamsters. Int. J. Cancer **29**:583–586.
20. **Watanabe, S., E. Soeda, S. Uchida, and K. Yoshiike.** 1984. DNA rearrangement affecting expression of the BK virus transforming gene. J. Virol. **51**:1–6.
21. **Watanabe, S., Y. Yogo, and K. Yoshiike.** 1984. Expression of viral early functions in rat 3Y1 cells infected with human papovavirus BK. J. Virol. **49**:78–85.
22. **Watanabe, S., and K. Yoshiike.** 1982. Change of DNA near the origin of replication enhances the transforming capacity of human papovavirus BK. J. Virol. **42**:978–985.
23. **Watanabe, S., and K. Yoshiike.** 1985. Decreasing the number of 68-base-pair tandem repeats in the BK virus transcriptional control region reduces plaque size and enhances transforming capacity. J. Virol. **55**:823–825.
24. **Watanabe, S., K. Yoshiike, A. Nozawa, Y. Yuasa, and S. Uchida.** 1979. Viable deletion mutant of human papovavirus BK that induces insulinomas in hamsters. J. Virol. **32**:934–942.
25. **Watanabe, S., K. Yoshiike, Y. Yuasa, and S. Uchida.** 1981. Natural occurrence of deletion mutant of human papovavirus BK capable of inducing T antigen. J. Gen. Virol. **54**:431–435.
26. **Weber, F., J. de Villiers, and W. Schaffner.** 1984. An SV40 "enhancer trap" incorporates exogenous enhancers or generates enhancers from its own sequences. Cell **36**:983–992.
27. **Weiher, H., M. König, and P. Gruss.** 1983. Multiple point mutations affecting the simian virus 40 enhancer. Science **219**:626–631.
28. **Yang, R. C. A., and R. Wu.** 1979. BK virus DNA: complete nucleotide sequence of a human tumor virus. Science **206**:456–462.