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Structure and Function of the Transcriptional Control Region of Nonpassaged BK Virus

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We compared the nucleotide sequence in the transcriptional control region of BK virus isolates cloned directly from human urine (BK-WW) with that of prototype BK virus. BK-WW was found to have a 63-base-pair insertion and only one of the 68-base-pair enhancer repeat elements. In transient expression assays, BK-WW enhancer showed approximately one-half the activity given by the prototype enhancer.

Enhancers are one of a group of DNA elements which regulate transcription of RNA polymerase II in eucaryotic cells (1, 10). They potentiate transcription irrespective of orientation and relatively independent of distance from the 5′ end of the transcriptional unit. Enhancers were first described in simian virus 40 (SV40) (1, 7) and subsequently identified in other viral and cellular transcription units. Typically, they consist of a number of tandemly repeated elements varying both in sequence and in the number of repeats. Most sequenced virus enhancer elements are from viruses passaged in cell culture, a procedure known to predispose to molecular rearrangements (9, 16, 20). If such rearrangements lead to an enhanced growth rate, viruses carrying these rearrangements would outgrow the parental strain.

BK virus is a papovavirus originally isolated from the urine of an immunosuppressed human patient (3), which is similar in many aspects of its genomic organization to SV40. Several BK virus variants have since been isolated, and the genomes of the Dunlop (Dun) and MM strains have been cloned and completely sequenced (16, 20). The origin of replication and transcriptional control elements have been sequenced in a number of other variants (17, 21). Recently, we identified and cloned the DNA of a BK virus strain, BK-WW, directly from human urine (2) and have subsequently cloned a second isolate with the identical restriction pattern (R. Rubinstein, manuscript in preparation). BK-WW is a strain which has been shown to differ in its restriction map from described strains. Evidence particularly from *Nco*I digests localizes extensive changes to the region around the origin of replication. Here we report the sequence and biological activity of the enhancer region of BK-WW to provide a comparison with cell culture-passaged isolates.

BK-WW DNA cloned at the *Bam*HI site in pBR322 and prototype BK virus DNA cloned in the same way and obtained from P. Howley were used as source materials for subcloning the enhancer region (Fig. 1). The *Hha*I-*Sst*I fragment, between BK-prototype (BK-proto) map positions 0.72 and 0.63, was C-tailed and inserted into G-tailed pBR322 at the *Pst*I site. This step facilitated transfer of a *Pst*I-*Sst*I fragment into M13mp10 and M13mp11 for sequencing by use of the dideoxynucleotide chain termination method (15). For functional studies, the *Pst*I fragment from pHS-Pst was blunt-ended by use of the Klenow fragment of DNA polymerase I and inserted by use of *Bam*HI linkers into the *Bam*HI site of pBR322. This procedure allowed amplification of the enhancer fragments with *Bam*HI termini for subsequent ligation into the *Bgl*II site of $pA_{10}CAT_2$. This formed the final construct pBK-CAT with the enhancer appropriately placed to influence expression of the CAT gene. The orientations of the inserts were determined from the fragment sizes given by double digestion with *Bam*HI and *Sst*I, and *Nco*I digestions done in all constructs confirmed that no rearrangements or deletions had occurred during the subcloning procedures.

A comparison of the published sequence of BK-proto DNA (21), our sequence of a BK-proto clone (hereafter referred to as BK-proto-2), and the sequence of BK-WW is

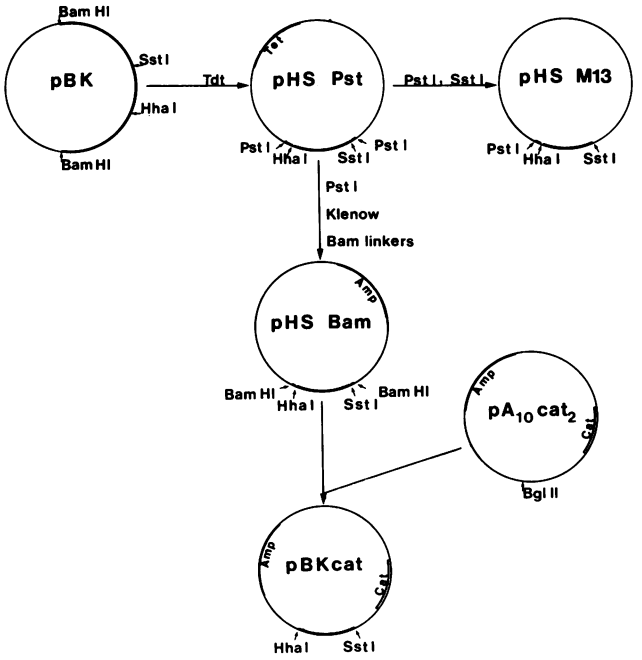

FIG. 1. Protocol for plasmid constructs and subcloning strategy of the BK virus enhancer region. pBK represents the entire genome of either BK-proto or BK-WW (thick line) cloned into the *Bam*HI site of pBR322. Abbreviations: TdT, terminal deoxynucleotidyl transferase; Cat, chloramphenicol acetyltransferase gene; Amp, beta-lactamase gene; Tet, tetracycline resistance gene.

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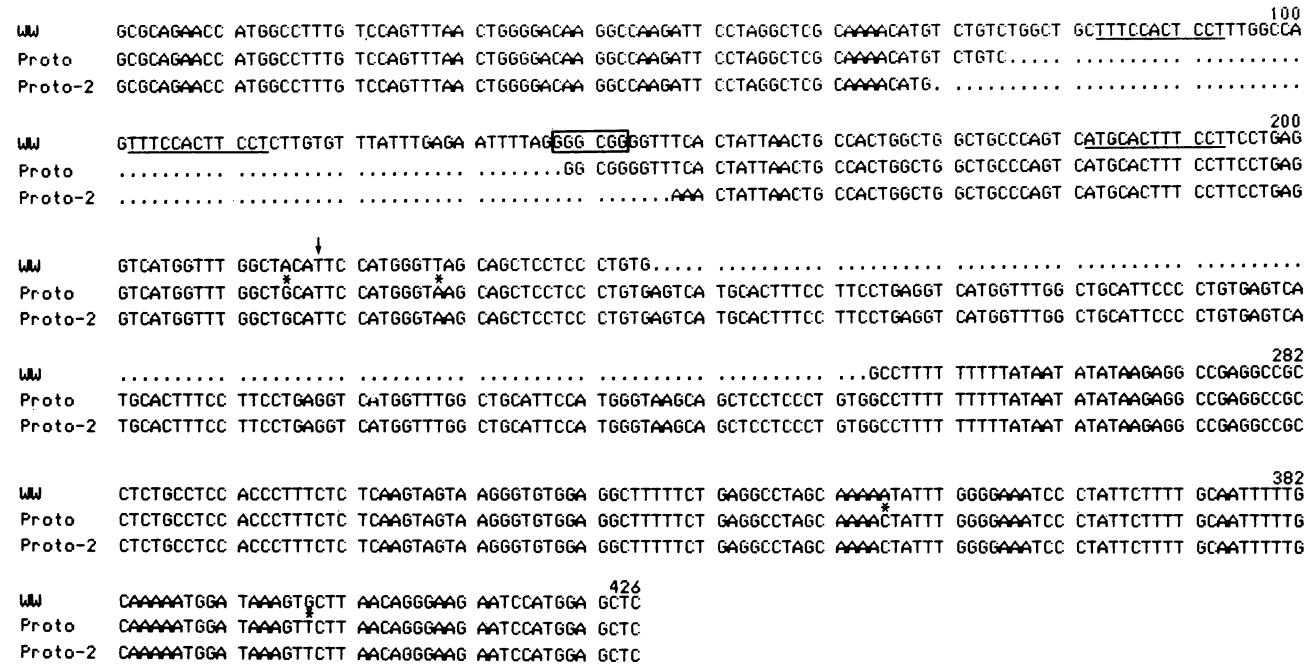

FIG. 2. Comparison of BK-proto and BK-WW sequences between the *Hha*I and the *Sst*I sites. Abbreviations: Proto, the published sequence; Proto-2, our sequence of the cloned prototype. Homologies to the E1a or GT motifs are underlined in the WW sequence, and the GC box is enclosed in solid lines. Asterisks mark single-base differences between the WW and prototype sequences, and the arrow marks the position of a single-base difference between WW and BK-Dik. (A in Dik replaces T in WW and proto.)

shown in Fig. 2. BK-proto-2 contains a deletion of 17 base pairs (bp) relative to BK-proto with an insertion of two adenines situated 30 bp upstream from the start of the repeat elements. There are two major differences between the WW sequence and prototype: first, in WW there is only one of the 68-bp enhancer repeat elements characteristic of the prototype sequence, and second, there is a 63-bp insert in WW on the *Hha*I side of the enhancer. In addition to these major differences, there are four single-base changes between the WW and prototype sequences, three of which are transversions.

The structural features of the sequenced regions of BK-WW are significantly different from earlier described isolates of BK virus, but are similar to those of BK-Dik (17), which also has a single enhancer element. This structure was interpreted at the time as the result of the deletion of two units of the prototype 68-bp triplication. BK-WW differs from BK-Dik by a single-base change at position 218. There is no reason to conclude that this structure arose by deletion of repeated elements, since both of our isolates of BK-WW were cloned from DNA prepared directly from urine. The alternative conclusion is that BK strains with repeated elements arose as a consequence of amplification of an enhancer element in cell culture. A comparison of the structural features of a number of sequenced BK strains is shown in Fig. 3 and illustrates how the repeat elements in each case could have evolved by amplification from a WW archetype. There is no simple way in which any of the other arrangements could have provided an archetypal structure. The different arrangements define a region of 107 bp (13) which encompasses all characterized examples of repeat elements in BK virus DNA. A smaller region of 29 bp, depicted by the shaded area in Fig. 3, defines a sequence included at least in duplicate in all the repeat elements illustrated here. BK-RF represents a special case in which there are two structural forms, R2 and R1a, each defective on its own but able to propagate in culture in the presence of the other (13).

The 63-bp element on the *Hha*I side of the enhancer region in BK-WW is absent in prototype, GS (13), and R1a. Except for a few base pairs at one end, it is also absent in MM. IR contains the *Hha*I-proximal 47 bp of the 63-bp element and lacks 30 bp relative to WW (12). The fully sequenced BK-Dun is identical to prototype in the enhancer region except for the deletion of an additional 43 bp adjoining the 63-bp region (16). Both BK-Dik and R2 contain the complete 63-bp element, which is present in duplicate in R2.

The function of this 63-bp region is not known, but comparison of the structures of the various sequenced BK strains shows that the absence of all or part of this region is generally associated with amplification of the downstream sequence elements. It has been noted (8, 14) that the sequence ATGCACTTTCCT starting at position 193 in the WW sequence, and present in all of the 68-bp repeats of BK-proto, shows homology to the adenovirus type 5 TT TCACTTCCT E1a enhancer core sequence (8). There are two sequences in the 63-bp element, TTTCCACTTCCT at position 102 and TTTCCACTCCTTT at position 83, which show closer homology to E1a, and all three show close homology to the GT motif which in paired form constitutes one of the two domains of the SV40 enhancer (22). These provide BK-WW with three of these homologous sequences, the same number as is contained in prototype, MM, and GS. The latter isolates lack the two elements in the 63-bp segment but gain two from triplication of the repeat region. A GC box consisting of the sequence GGGCGG, which constitutes the principle component of the binding site for the Sp1 transcription factor (4), is present 138 bp down-

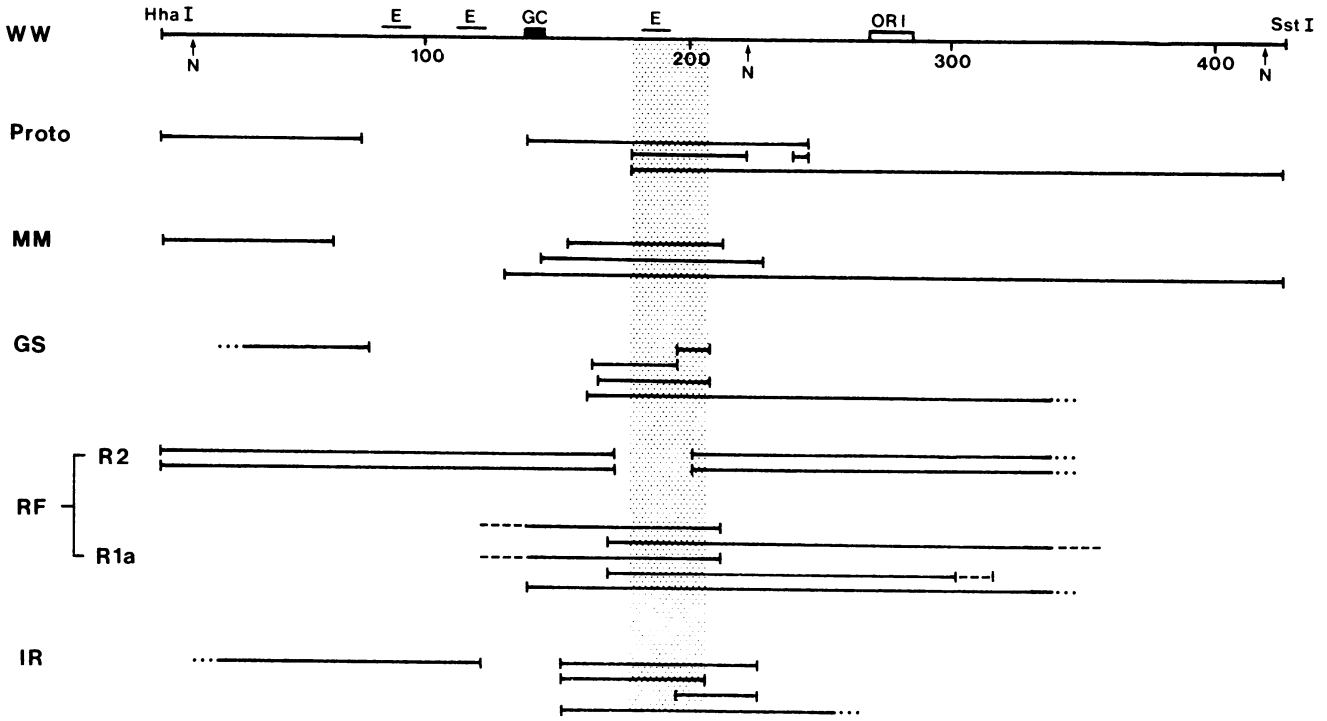

FIG. 3. Diagrammatic representation of the molecular structure of sequenced BK virus isolates. The scale is shown in WW and is in base pairs counterclockwise from the *Hha*I site. Abbreviations: ORI, a GC-rich symmetrical region, bp 268-286 in the above sequence, in the vicinity of the origin of replication; E, sequences showing homology to adenovirus 5 E1a and to SV40 enhancer core sequences; GC, GC box; N, *Nco*I sites. The other isolates are drawn so as to illustrate the gaps relative to the WW sequence. On reading from left to right, when a repeat is encountered the linear representation is displaced to the line below and to the position corresponding to the alignment with the WW sequence. Dotted regions (. . .) flank the limits of the sequenced regions of GS and RF, and dashed segments (– – –) in R1a represent variably sized displaced sequences from the mid-VP1 coding regions. ▭, a 29-bp region present at least in duplicate in the repeats illustrated here.

stream from the *Hha*I site in the WW sequence. It is present only in WW and Dik both in its entirety and in *cis* to the 29-bp enhancer region shaded in Fig. 3.

To measure the effect of these structural features in BK-WW on the enhancement of gene expression, the *Hha*I-*Sst*I fragments containing the sequenced regions of BK-WW and BK-proto 2 shown in Fig. 2 were subcloned into the *Bgl*II site of the expression vector $pA_{10}CAT_2$ (14). This vector contains the bacterial gene for chloramphenicol acetyltransferase (CAT) which is situated downstream from the SV40 21-bp repeat promoter region but shows little expression after transfection into eucaryotic cells unless an enhancer is also present in the construct. Prototype constructs in both orientations and WW constructs in the antisense orientation, which positions the 63-bp element between the enhancer region and the CAT gene, were used to transfect CV-1 cells for transient expression assays (Table 1). The prototype constructs in both orientations gave values similar to those reported for BK-Dun (14) in CV-1 cells. The WW constructs gave values about one-half that of the corresponding prototype construct and more than 20-fold higher than the control enhancerless parent plasmid $pA_{10}CAT_2$. At 24 h after the seeding of 100-mm-diameter dishes with 2×10^6 cells, 15 μg of DNA was transfected in duplicate by the calcium phosphate precipitation method (6). After 48 h, extracts were prepared, and CAT activity was measured as previously described (5) by using incubations of 30 and 60 min at 37°C. Percent acetylation of [^{14}C]chloramphenicol was determined by thin-layer chromatography followed by liquid scintillation spectrometry of acetylated species.

TABLE 1. Assay of CAT activity

Construct[a]	CAT activity at[b]: 30 min		CAT activity at[b]: 60 min	
pSV_2CAT	100 ± 13.2		100 ± 22.0	
pBK proto-sCAT	23.9 ± 5.4		25.7 ± 4.5	
pBK proto-aCAT	5.5 ± 1.3	$P < 0.01$	5.9 ± 2.1	$P < 0.03$
pBK WW-A-aCAT	2.7 ± 0.33		2.7 ± 0.49	
pBK WW-B-aCAT	3.1 ± 0.59	$P < 0.02$	2.6 ± 0.40	$P < 0.04$

[a] A and B refer to the two isolates of BK-WW; s and a refer to sense and antisense orientations (14), respectively, of the enhancer element with respect to the CAT gene.

[b] CAT activities in CV-1 cells transfected with *Hha*I-*Sst*I fragments of BK virus isolates cloned into $pA_{10}CAT_2$. Values are the means ± standard deviation of four assay results and are expressed as a percentage of the activity given by pSV_2CAT (5), which converted 11.7 and 22.6% of the [^{14}C]chloramphenicol-acetylated derivatives at 30 and 60 min, respectively. $pA_{10}CAT_2$ gave values of <0.1% of those given by pSV_2CAT at each time period.

The presence of sequences homologous to known enhancer sequence motifs in the 63-bp region, together with a GC box, raises the question of whether this region can act as an enhancer in its own right. The degree of enhancement given by the BK-WW CAT constructs containing the whole of the sequenced region is about half that of the corresponding prototype constructs. A similar construct containing a single 68-bp prototype repeat unit but lacking the 63-bp region was recently reported to show only 3% of the prototype activity in CAT assays (19). It is therefore probable that the 63-bp region does possess enhancer activity. However, to confirm this, examination of constructs containing each component of this region in transient CAT expression assays will be necessary.

BK virus strains with multiple repeat regions grow well in human cell culture but transform rat and hamster cells inefficiently (18). Laboratory constructs which retain only one repeat element transform more efficiently but grow more slowly and form smaller plaques in human cell cultures (19). It is significant that BK-Dik, which retains a structure similar to BK-WW, although propagated in cell culture, was not plaque purified. BK virus strains with tandemly repeated enhancer regions have in all likelihood acquired this structure during passage in cell culture, with specific structural arrangements being selected for by plaque purification. Since BK-WW has only a single repeat element and since early attempts at propagation of this isolate in cell culture were unsuccessful under conditions in which BK-MM grew well (11), it can be concluded that efficient growth in cell culture is not a criterion for effective maintenance of BK virus in the human population and may even be a contraindication. This conclusion highlights two unanswered questions about the biology of BK virus. What is the normal pattern of virus-cell interaction of BK virus in vivo, and what selects for the structural integrity of the enhancer and 63-bp regions in vivo when there is evidence that it changes so profoundly and in a generally reproducible manner in cell culture?

A more general question is whether in vivo isolates of other viruses which have been studied predominantly in cell culture show analogous differences.

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