

Recurring Theme of Changes in the Transcriptional Control Region of BK Virus during Adaptation to Cell Culture

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BK virus, when cloned directly from human urine, shows no amplification in the transcriptional control region, unlike cell culture-passaged strains, but possesses an additional sequence element. To confirm our proposal that this represents the archetypal in vivo form of the virus, we passaged this BK virus through cell culture. Changes in the transcriptional control region occurred as early as the first passage and were characterized in all cases by a deletion followed by amplification events.

BK virus is a papovavirus originally isolated from the urine of an immunosuppressed patient (5). Many BK virus variants have been isolated, mostly after amplification of the source material in cell culture (11-13, 19, 21, 22), and a feature of these isolates is the marked degree of variability in the structure of the transcriptional control region (TCR). Typically, parts of this region are repeated two or three times in tandem, but there is variation in the size and location of the repeated region in different isolates.

We have described two isolates of BK(WW), namely, BK(WW)A and BK(WW)B, which were cloned directly from urine without passage through cell culture (1, 14). A third isolate, BK(WW)C, was isolated in Cape Town in 1988. This isolate was obtained from the urine of an immunosuppressed bone marrow transplant recipient, and the TCR of this isolate was sequenced after polymerase chain reaction amplification. The structure of the TCR in all three isolates was identical and differed from that of the cell culture-passaged strains in having no repeat elements and in having a sequence which was absent in most other strains. Examination of the published sequences of the TCRs from cell culture-passaged strains showed that all of them could easily be derived from the WW structure by a single deletion together with an amplification event. We therefore proposed that BK(WW) represented the archetypal form found in vivo and that the sequence of the cell culture-derived strains was a consequence of passage in cell culture, with such changes being required before productive growth could occur (15).

Four micrograms each of BK DNA from pBR322-cloned WW (14), prototype, and MM (23) strains was used to transfect human embryo kidney (HEK) cells in order to determine whether the predicted changes occur on passage in cell culture. Cells were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with either 10% or 3% fetal calf serum after transfection. Transfections were performed by calcium phosphate precipitation as previously described (15). The DNA was digested with *Bam*HI, and then the linearized BK DNA was ligated. Both BK-(Proto) and BK(MM) showed a visible cytopathic effect (CPE) by 7 to 10 days after transfection, whereas BK(WW) required 33 days before a CPE was visible. The presence of

progeny virus was confirmed by electron microscopy when a CPE became demonstrable. For subsequent passage, 500 μ l of the medium from infected cells was added to 3×10^6 to 4×10^6 cells. This approach was used since the significance of titer measurements on mixed populations of virus was uncertain.

Total viral DNA was prepared from cells (18) at the first and second passages and examined by gel electrophoresis after digestion with *Hind*III. This restriction enzyme produces a 917-bp fragment in BK(WW) DNA which includes all of the TCR and therefore gives an indication of major structural changes affecting this region (Fig. 1A). Whereas BK(Proto) and BK(MM) show unchanged banding patterns, the results of two separate transfections with BK(WW)B show a number of bands of various degrees of intensity in the vicinity of the original 917-bp fragment. This suggests that multiple novel viral forms containing rearrangements in the TCR have arisen. This effect was seen in three separate experiments with HEK cells (Fig. 1B), two of which involved transfection with the DNA from two separate clones of BK(WW)B and the third of which involved infecting 1.5×10^6 cells with 300 μ l of urine from the original urine viral preparation of BK(WW)C. The same change in the 917-bp fragment was seen with BK(WW)C virus in adult human skin fibroblast (HF) cells. An advantage of using cloned DNA was that this precludes selection of quantitatively minor variants in the initial inoculum as a cause for such a rapid deviation from the original structure (21).

In order to define the nature of the changes in more detail, viral DNA extracted from cells transfected with DNA from BK(WW)B was linearized with *Bam*HI and cloned into the *Bgl*II site of pEcoR252 (M. Zabeau, Plant Genetic Systems, Ghent, Belgium). Individual transformants were digested with *Nco*I, which provided more detailed information because it cut the TCR into two fragments of 211 and 196 bp (Fig. 1A). The 196-bp fragment was unchanged in all clones, whereas fragments smaller or larger than the 211-bp fragment, instead of the 211-bp fragment, were present in most of these clones (Fig. 2). Additional bands were present in one of the passage 1 clones and in all of the passage 2 clones.

The way in which these changes progressed on prolonged passage was monitored in HF cells. These cells were used because of their ready availability and their ability to be maintained through many passages and to determine whether similar changes occur in cells of a different tissue specificity. HF cells were transfected with BK(MM) DNA

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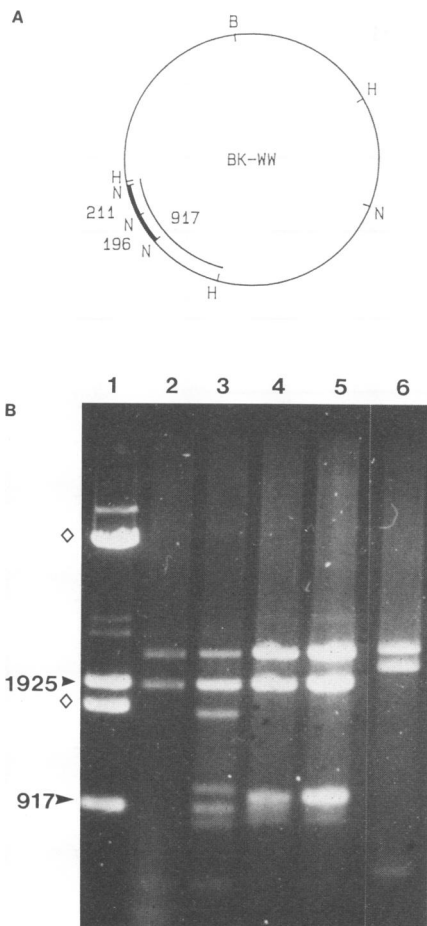


FIG. 1. (A) Restriction map of the 5,142-bp circular BK(WW) DNA, showing relevant restriction sites. The heavy line indicates the TCR bounded by *Nco*I sites at positions 3281 and 3688. This region is also included within the 917-bp *Hind*III fragment. B, *Bam*HI; N, *Nco*I; H, *Hind*III. (B) Agarose gel electrophoresis of *Hind*III digests of total viral DNA extracted after passage in HEK cells. Lane 1, BK(WW) DNA cloned into pBR322. Fragments 1925 and 917 are BK viral fragments; composite plasmid-virus fragments are indicated (\diamond). The faint bands in this lane are partial digest fragments. Lane 2, BK(Proto), passage 1; *Hind*III fragment sizes are 2,300, 1,925, 551, and 416 bp, the latter two corresponding to the BK(WW) 917-bp fragment. Lanes 3 and 4, BK(WW), passage 1 (cells were transfected with DNA from two different plasmid clones). Lane 5, BK(WW) (from the same clone as in lane 4), passage 2. Lane 6, BK(MM), passage 2; the fragment sizes are 2,300, 2,076, and 587 bp. The TCR of BK(MM) is included in the 587- and 2,076-bp fragments.

and BK(WW)B DNA. Cells transfected with BK(MM) DNA developed a CPE within 10 days, whereas no CPE was evident after transfection with BK(WW)B on several occasions. However, when HF cells were infected with urine samples containing virus of the BK(WW)C isolate (the strain for which the original virus was still available), a CPE developed only after 30 days. Thereafter, BK(WW)C was passaged sequentially as soon as a CPE developed or after 30 days if no CPE was visible. The time required for development of a CPE was found to decrease with passage number (Fig. 3).

Viral DNA was cloned at various passages, and changes in the structure of the TCR were identified by using *Nco*I

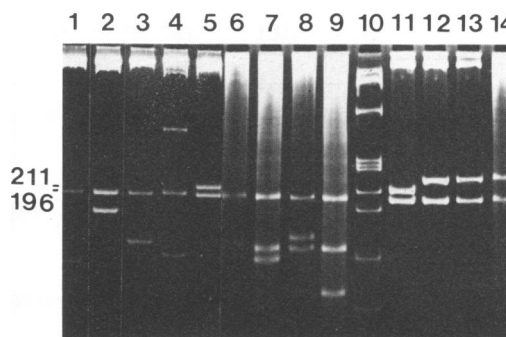


FIG. 2. Gel electrophoresis in 5% polyacrylamide of *Nco*I digests of DNA from individual clones of BK(WW) DNA in pEcoR252. Lanes 1 through 4 and 6 through 9, Passages in HEK cells; lane 5, control nonpassaged BK(WW) DNA; lane 10, *Hae*III digested ϕ X174 molecular weight markers; lanes 11 through 14, passages in HF cells. Lanes 1 through 6, passage 1; lanes 7 through 9, passage 2; lanes 11 and 12, passage 6; lane 13, passage 9; lane 14, passage 16.

restriction digests (Fig. 2). At early passage, the clones showed many different patterns similar to those seen in HEK cells. By passage 6, only two patterns were observed. Of seven clones examined, six showed the pattern seen in Fig. 2, lane 11, and one showed the pattern seen in lane 12. The latter pattern was the only one observed in subsequent passages, which suggests that it represents a structure that is selected to achieve more efficient growth in cell culture.

The TCR was sequenced in representative early- and late-passage clones by using the dideoxy method with Sequenase (United States Biochemical) (16) and synthetic oligonucleotide primers corresponding to either end of the TCR. The organization of this region in the sequenced clones is illustrated in Fig. 4. The sequences are aligned with the archetypal BK(WW) sequence and compared with published sequences.

Two major features of the sequenced passaged clones are evident. The first is the presence in every case of a deletion ranging in size from 38 bp (C3-1) to 91 bp (B1-3) and differing in position, with a region of only 17 bp common to all the BK(WW) deletions. BK(Proto), BK(MM) (23), and BK(GS)

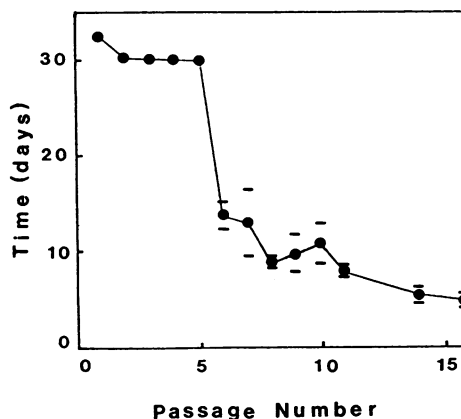


FIG. 3. Correlation of time required for the appearance of a CPE with passage number for BK(WW)C in HF cells. Points are the means of three separate experiments, and bars indicate the standard deviations.

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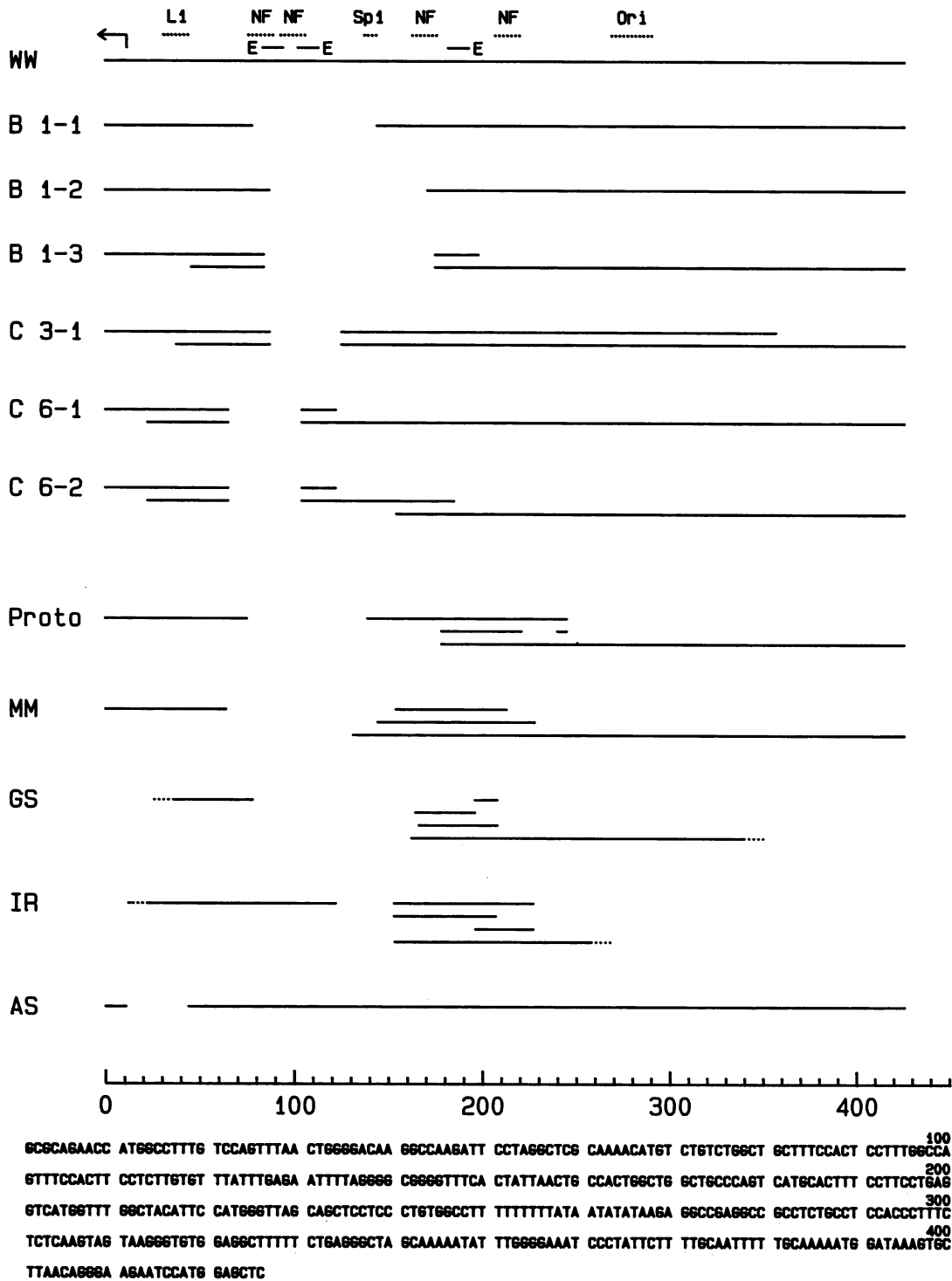


FIG. 4. Diagram of the molecular structure of the TCR of sequenced BK virus isolates. At the top is the reference BK(WW) archetypal structure. The other isolates are drawn so as to illustrate the gaps relative to the WW sequence. From left to right, when a repeat is encountered, the linear representation 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 strains GS and IR. The AUG initiation codon for the agnogene is at the top left (arrow). The L1, NF-1 (NF) (9), and Sp1 sites and sequence showing homology to adenovirus type 5 E1A and simian virus 40 enhancer core sequences (E) are shown. B1-1 through B1-3 are clones of BK(WW)B isolated after one passage in HEK cells, corresponding to lanes 3, 1, and 2, respectively, in Fig. 2. C refers to clones sequenced at different passages after infection with the original virus preparation of BK(WW)C; C3-1 was obtained after three passages in HEK cells, and C6-1 and C6-2 were obtained after six passages in HF cells. Sequence identical to that of C6-2 was found in clones from passages 9 and 16. The sequence was numbered as by Rubinstein et al. (15) and extends from the single *HhaI* site at position 399 in the BK(Dun) sequence (17) to an *SstI* site at position 5115 in the BK(Dun) sequence.

(12) share deletions which include this 17-bp region. In two other sequenced strains, BK(IR) (11) and BK(AS) (21), there is a deletion in this vicinity, but it does not include the 17-bp region shared by the other strains. This demonstrates that the 17-bp sequence is not a sequence whose deletion is specifically required. One possibility is that growth in cell culture requires a conformational change in this region of the TCR which interrupts the interaction between a number of transcriptional regulatory elements, rather than requiring a specific deletion event.

The second characteristic of passaged strains is the presence of one or more amplification events. This occurred in four of our six illustrated sequences (B1-3, C3-1, C6-1, and C6-2), and when amplification has occurred, it has taken place subsequent to the deletion event, since in every case the deletion boundaries are identical in each of the amplified regions. A second amplification event, seen in variant C6-2, has clearly evolved as a later event from the structure illustrated in C6-1. This evolutionary process can be seen in the *Nco*I patterns (Fig. 2), in which the C6-2 arrangement is seen as early as passage 6 and is the pattern found in all the clones from subsequent passages up to passage 16. This was confirmed by sequencing clones from passages 6, 9, and 16, which were all found to be identical.

Since in two of our sequences and in the published sequence of the AS virus (21) there is a deletion but no amplification, the latter cannot be essential for productive infection. Published sequences of BK virus strains showing amplified regions in the TCR all have overlapping components which appear to define a core region necessary for mediating rapid growth. Our variants B1-3 and C3-1 conform in this respect in that the amplification overlaps this core area. However, C6-1 has an amplification in a novel region, on the late side of the region usually amplified, therefore precluding the concept of a single core region.

We observed that the time taken to produce a CPE decreased from more than 30 days at the first passage to 5 days by passage 16. In addition, C6-1 with one amplification was subsequently replaced by C6-2 with an additional amplification, suggesting that the latter is associated with more efficient growth. The rapidity with which rearranged forms come to predominate on passage in cell culture implies that the archetypal form found *in vivo* grows poorly relative to the modified forms. This occurs whether DNA or virus is used as the initial inoculum. This finding would be consistent with previous reports relating the number of enhancer repeat elements to the growth rate in culture (6) and with the finding that constructs containing only one enhancer element, therefore differing from BK(WW) only by the absence of the 63-bp region, grow slowly in culture (3). Our results are therefore consistent with the idea that these rearrangements are a requirement for successful growth in culture, but our cloning protocol precluded easy retrieval of viral DNA for infectivity studies of individual clones.

Table 1 correlates the precise locations of deletions and amplifications in the TCR with the number of transcription factor-binding sites gained or lost by these rearrangements. Protein-binding domains NF-1, Sp1, and L1 (Fig. 4 and Table 1) have recently been located in BK(WW) by DNase I footprinting (9) and are likely to play a role in transcriptional regulation. B1-2 has the least number of these sequence motifs and may therefore represent the minimal structure necessary for growth in culture. Relative to the number of motifs in B1-2, amplification events have increased the number of the E1A core motifs (E) (7, 13) in B1-3, of NF-1 and E in C3-1, of L1 in C6-1, or of both L1 and NF-1 in C6-2.

TABLE 1. Correlation of rearrangements in passaged BK virus with the number of binding sites for DNA transcription factors

BK virus isolate	Rearrangement				No. of binding sites for:				
	Deletion		Amplification		L1	NF-1	Sp1	E	Ap-1
	Starting bp	Ending bp	Starting bp	Ending bp					
WW					1	4	1	3	0
B1-1	79	143			1	2	0	1	0
B1-2	88	170			1	1	0	1	0
B1-3	85	174	45	198	1	1	0	2	0
C3-1	88	124	37	357	1	4	2	2	0
C6-1	66	103	22	122	2	2	1	1	0
C6-2	66	103	22	122	2	3	1	1	0
C6-2			159	185					
Proto	76	138	178	221	1	4	0	3	1
Proto			240	245					
Proto			178	245					
MM	65	130	154	213	1	5	1	3	0
MM			144	228					
GS	79	161	196	208	1	3	0	3	0
GS			164	196					
GS			166	208					
IR	123	152	153	227	1	8	0	5	0
IR			153	207					
IR			196	227					
AS	12	43			0	4	1	3	0

This suggests that amplification of one or another of these sequence motifs is associated with enhanced growth. In the second amplification event, in which C6-1 evolves to the C6-2 structure, there is amplification only of an NF-1 site. There is no consistent pattern to the number of Sp1 sites retained, nor are any new Ap-1 sites generated at the junctions of amplified regions, as has occurred in BK(Proto) and BK(Dun) (10).

A question which needs to be addressed is the biological role of the archetypal form represented by BK(WW). We conclude that this is the form normally present *in vivo*, and we infer that this structure is required both for transmissibility in the human population and for its persistence in latent form *in vivo*. Since there is evidence that rearrangements in the TCR can modify cell-type-specific expression (20), it appears that there is a requirement for the archetypal form to have restricted tissue specificity.

The phenomenon described here emphasizes that the structures of the TCR in the majority of reported BK virus strains arise following passage in cell culture. How general this phenomenon might be is not known, but it is possible that other viruses that have been characterized only after passage through cell culture, such as simian virus 40, may not accurately reflect the structure and function of the form found *in vivo*. It is interesting that a similar phenomenon occurs in JC virus. The TCR of the kidney isolate of JC virus (8) has a close sequence similarity to BK(WW), whereas a brain isolate from the same patient with progressive multifocal leukoencephalopathy has amplification and deletion events analogous to those seen in cell culture-passaged BK virus. Similar changes are also seen in the cell culture-passaged strain JC Mad1 (4).

The question arises as to whether these changes in BK virus have pathological significance. In JC virus, rearranged forms have been found in diseased human brain tissue, whereas in BK virus, rearrangements have been observed only in kidney tumor DNA of transgenic mice (2). It is

proposed that rearranged forms be looked for in tissues other than kidney from chronic disease states and tumors.

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