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Naturally Occurring BK Virus Variants (JL and Dik) with Deletions in the Putative Early Enhancer-Promoter Sequences

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The genomes of two independently isolated BK virus (BKV) variants (JL and Dik) were compared with prototype BKV DNA by restriction endonuclease mapping and sequence analysis. Differences were mainly detected in two regions: the BKV (JL) and BKV (Dik) putative early enhancer-promoter regions and the middle of the T-antigen-coding regions. Base sequence analysis of these two regions showed the following. (i) The putative enhancer-promoter regions of BKV (Dik) and BKV (JL) contained only one 68-base-pair (bp) unit of the 68-bp triplication (the central copy of which is missing 18 bp) present in prototype BKV. (ii) In the same region, BKV (JL) and BKV (Dik) contained unique stretches of DNA 33 and 63 bp long, respectively. In these 63 bp, a sequence which was very similar to the proposed simian virus 40 enhancer core sequence (GGAGTGGAAAG) was present. (iii) The altered restriction endonuclease recognition sites in the sequenced part of the T-antigen-coding region of BKV (JL) and BKV (Dik) were due to base sequence changes, leaving the amino acid sequence unchanged.

Since the initial isolation of BK virus (BKV) from the urine of a renal allograft recipient undergoing immunosuppressive therapy (3), a number of variants of BKV have been isolated (see reference 8). Some of these naturally occurring variants (RF, GS, and MM) have base sequence differences near the origin of DNA replication (12, 13, 21) when compared with prototype BKV [BKV (pt)]. The genome of BKV (MM) contains deletions in two of the three tandem repeats located just clockwise from the origin of DNA replication. Another viable BKV variant, pm 522, rescued from a BKV-induced pinocytoma, has small deletions and duplications in the noncoding region near the origin of DNA replication that are possibly responsible for an enhanced transforming capacity (18–20). In this study, the genomes of two other naturally occurring BKV variants, JL and Dik, are compared with the genome of BKV (pt).

BKV (JL) and BKV (Dik) were isolated from the urine of a patient who had undergone a bone marrow transplantation after a relapse of acute myeloid leukemia (14) and the urine of an immunocompetent 2-year-old child with acute tonsillitis (5), respectively. BKV (pt) (3) and BKV (JL) were plaque purified; BKV (Dik) was not plaque purified. The various viral DNAs were extracted from infected (multiplicity of infection, ca. 1:10,000) primary cultures of human embryonic cells by the Hirt procedure (7) and purified by equilibrium centrifugation in cesium chloride containing ethidium bromide as described previously (17). The genomes of BKV (JL) and BKV (Dik) were compared with the genome of BKV (pt) by cleavage with *Hae*III, *Mbo*I, *Hin*dIII, *Mst*II, *Pst*I, *Eco*RI, *Bam*HI, *Pvu*II, *Xba*I, *Taq*I, *Msp*I, and *Hin*dII and agarose gel electrophoresis.

Only the electropherograms of *Hae*III-, *Mbo*I-, *Hin*dIII-, *Mst*II-, *Pst*I-, and *Eco*RI-digested BKV (JL), BKV (Dik), and BKV (pt) DNAs showed differences; these differences were mainly in two regions: the enhancer-promoter region and the middle of the T-antigen-coding region, except the, missing *Mbo*I and *Hin*dIII sites (at 0.113 and 0.618 map units, respectively) (Table 1 and Fig. 1). Base sequence analysis of these two regions was then carried out. To avoid any ambiguity in sequence analysis, both genomes were molecularly cloned. BKV (JL) and BKV (Dik) DNAs were cleaved with *Bam*HI, ligated to pBR322 DNA digested with the same enzyme, and transfected into *Escherichia coli* K-12 HB101. Ampicillin-resistant and tetracycline-sensitive transformants were selected, and plasmid DNA was purified from these transformants. Plasmid DNA containing an insert of BKV (JL) DNA (pJL) or BKV (Dik) DNA (pDik) was selected by restriction enzyme cleavage and analysis of the cleavage products in agarose gels. The appropriate *Hin*dIII fragments of pJL and pDik were purified and labeled with ^{32}P at the 5′ end with polynucleotide kinase or at the 3′ end with DNA polymerase I (Klenow fragment). Base sequence analysis was performed after cleavage with a second restriction endonuclease was done or after strand separation as described by Maxam and Gilbert (10) was done.

The results of this sequence analysis (Fig. 2) confirmed the results of the restriction endonuclease mapping. The missing *Mst*II recognition sites in the BKV (Dik) and (JL) genomes (Table 1) appeared to result from the deletion of two units of

TABLE 1. Alterations in the restriction endonuclease cleavage patterns of BKV (JL) DNA and BKV (Dik) DNA in comparison with the BKV (pt) DNA pattern

BKV variant	Restriction endonuclease site (map units)	
	Extra	Missing
Dik and JL	*Pst*I (0.260), *Hae*III (0.267)	*Mbo*I (0.113), *Mst*II (0.673 and 0683), *Hin*dIII (0.618)
JL	*Eco*RI (0.242), *Hae*III (0.711), *Hin*dIII (0.306)	
Dik	*Hae*III (0.703)	*Hae*III (0.254)

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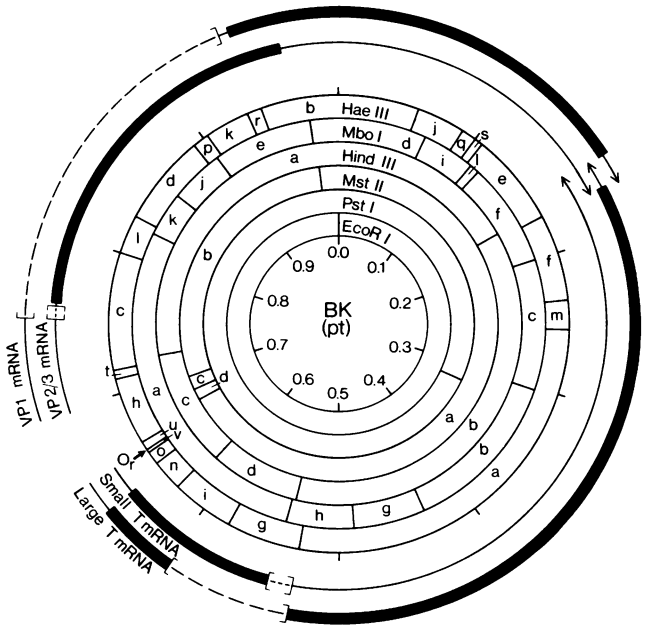

FIG. 1. Restriction endonuclease map of BKV (pt) oriented with the *Eco*RI site at 0.0 map units The restriction endonuclease recognition sites of BKV (pt) were determined from the complete nucleotide sequence of BKV DNA (16, 21) by computer-assisted analysis. The recognition sites of *Bam*HI (0.979 map units), *Pvu*II (0.723 and 0.098 map units), and *Xba*I (0.967 and 0.227 map units) are not indicated. *Taq*I, *Msp*I, and *Hin*dII do not cleave BKV (pt) DNA. The spliced early mRNAs (small T mRNA and large T mRNA) and late mRNAs (VP1 mRNA and VP2/3 mRNA) are indicated outside the circular genome, with intervening sequences indicated by dashes (16). The origin of DNA replication (O_r) is indicated by an arrow. Restriction endonuclease fragments are indicated by lowercase letters.

the 68-base-pair (bp) triplication, leaving only one 68-bp unit unimpaired. The extra *Hae*III site in BKV (Dik) DNA (bp 237 in Fig. 2B) appeared to be part of unique stretch of 63 bp. In this unique part of the promoter-enhancer region of BKV (Dik), we observed a sequence very similar to the putative enhancer core sequence of simian virus 40: GGAGTGGAAAG (bp 188 to 198 in Fig. 2) (15). It is very interesting that the same 63 bp are present in the nucleotide sequence of BKV RF (R2) described by Pater et al. (12), suggesting an evolutionary relationship between these viruses. In this region, BKV (JL) contained a unique stretch of 33 bp, 32 bp of which formed a subset of the unique stretch of 63 bp present in the BKV (Dik) genome. The extra *Hae*III site at 0.711 map units in the BKV (JL) genome resulted from a point mutation at bp 222 (Fig. 2B). The sequence GAAGTGGAAG, also similar to the above-mentioned core sequence, is part of the JC virus (JCV) genome just outside the tandem duplication of 98 bp described by Frisque (2). The sequence AGTGGAAAG was part of a stretch of 36 bp present in the BKV (Dik) genome, 31 bp of which are shared with a stretch of DNA in a similar position in the JCV genome. This observation shows that the sequences in the enhancer-promoter region diverged less in JCV and BKV (Dik) than in JCV and BKV (pt) (Fig. 2).

Our observation that BKV (JL) and BKV (Dik) contained only one 68-bp unit in the promoter-enhancer region warranted the conclusion that one such unit is sufficient for early gene expression. This is in agreement with reports that only one entire simian virus 40 72-bp "repeat" is required for efficient early gene expression in vivo (1, 4, 6). We think it unlikely that the deletion of two of the three repeats in the BKV (JL) and BKV (Dik) genomes developed during the period in which these viruses were cultured in vitro; when we analyzed two other plaque variants isolated from our BKV stock, we observed small deletions of ca. 15 and 35 bp between the *Mst*II site at 0.696 map units and the *Hae*III site at 0.714 map units (data not shown), leaving unimpaired the putative enhancer core sequence within the third repeat.

It is remarkable that only one alteration was detected in the late region of the BKV (JL) and BKV (Dik) genomes: the absence of an *Mbo*I site at 0.113 map units. The other alterations in both genomes were all localized in the early region (Table 1) and the promoter-enhancer region. The loss of the *Hin*dIII site at 0.618 map units was shared by the genomes of BKV (JL), BKV (Dik), BKV (MM), and BKV (GS). The part of the early region in which BKV (Dik) and BKV (JL) differed most from BKV (pt) was also further analyzed by base sequence analysis. For that purpose, pJL and pDik DNAs were cleaved with *Acc*I at 0.218 and 0.463 map units. The fragments containing the above-mentioned parts of the genomes were isolated, labeled at the 5′ and 3′ ends, and subsequently cleaved with *Xmn*I at 0.427 map units. The sequence determined stretched from bp 2876 (0.220 map units) to bp 3201 (0.283 map units). The five and four point mutations detected in this region in BKV (Dik) and BKV (JL), respectively (Table 2), were all neutral, in agreement with the hypothesis that this region of BKV is under strong evolutionary constraint (8). This number of point mutations was surprisingly low in view of the number of altered restriction endonuclease sites. This probably indicates that the clustering of these altered restriction endonuclease sites in this part of the T-antigen-coding region was fortuitous.

A recent comparison of BKV and simian-virus 40 by Yasunaga and Miyata showed a high rate of synonymous substitutions (substitutions not leading to an amino acid change) which were approximately uniformly distributed

TABLE 2. Differences observed in the sequenced region (bp 2876 to bp 3201) of BKV (Dik) and BKV (JL) in comparison with the genome of BKV (Dun)

BKV variant	bp[a]	Mutation	Codon alteration	Amino acid alteration	Restriction endonuclease site (map units)
Dik and JL	2926	T → A	ACU → ACA	Thr → Thr	None
JL	2991	T → C	UUA → CUA	Leu → Leu	Extra *Eco*RI site (0.242)
Dik	3010	C → T	UUC → UUU	Phe → Phe	None
Dik	3052	G → A	AGG → AGA	Arg → Arg	no *Hae*III site (0.254)
Dik and JL	3082	A → G	CAA → CAG	Glu → Glu	Extra *Pst*I site (0.260)
Dik and JL	3123	T → C	UUG → CUG	Leu → Leu	Extra *Hae*III site (0.267)

[a] BKV (Dun) numbering is from Seif et al. (16).

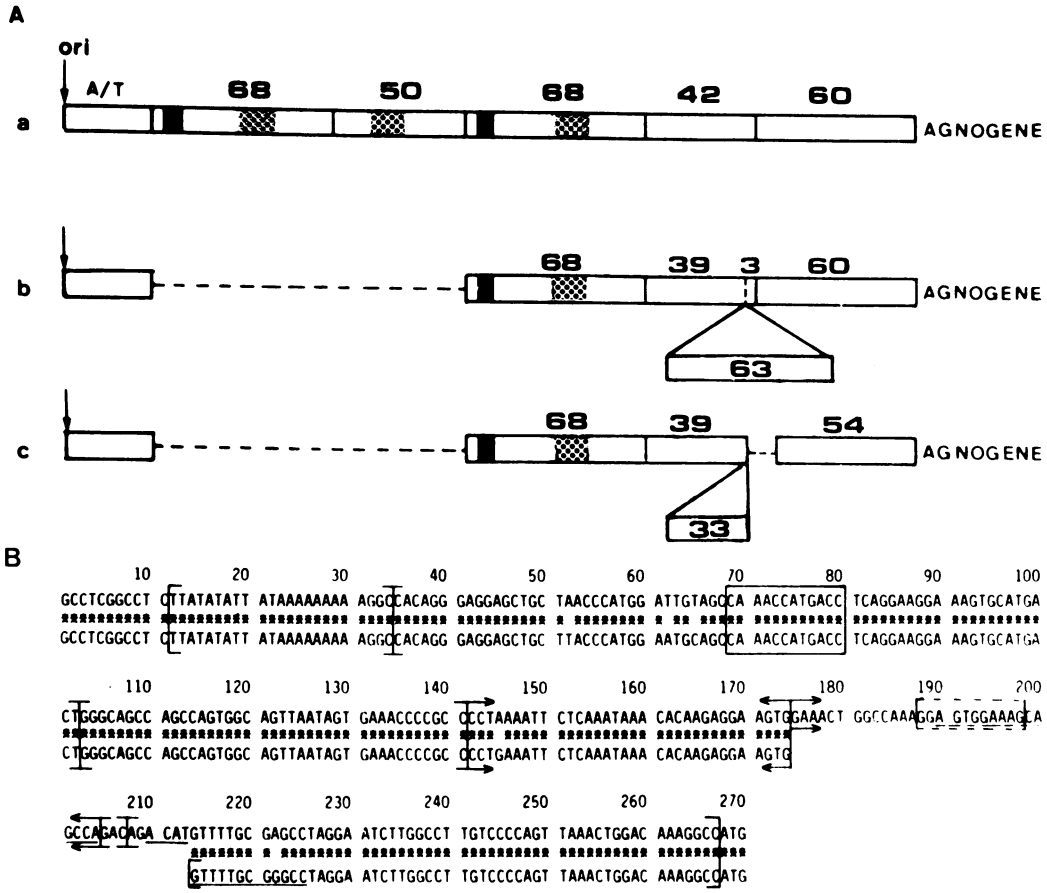

FIG. 2. (A) Structure of the enhancer-promoter regions of the BKV (pt) (a), BKV (Dik) (b), and BKV (JL) (c) genomes (0.648 to 0.720 map units) (16). The 42 bp missing from BKV (Dun) but present in BKV (pt) (21) are indicated. Relevant features include an AT-rich region (A/T), the 68-bp triplication, the central copy of which is missing 18 bp, and the origin of DNA replication (ori). Dotted regions indicate the putative enhancer core sequence, and black boxes indicate a putative promoter sequence (CCTCCC) (15). Numbers indicate the numbers of bp. Dotted lines indicate deletions. (B) Nucleotide sequences of the BKV (Dik) (upper strand) and BKV (JL) (lower strand) enhancer-promoter regions. Homologous bp are indicated by asterisks; bp 52, 62, 65, and 145 are mutated in BKV (Dik), in comparison with BKV (pt) and BKV (JL), and bp 222 is mutated in BKV (JL), in comparison with BKV (pt) and BKV (Dik), creating a new *Hae*III site in BKV (JL). A *Hae*III site is present at bp 182 in the BKV (Dik) unique stretch of 63 bp. The strands, read left to right, have the polarity of the late mRNAs. The regions, as shown in (A), are indicated by brackets. The BKV (JL) and BKV (Dik) unique regions of 33 and 63 bp, respectively, are indicated by brackets with arrows. The bp homologous to JCV are underlined. The putative enhancer core in the BKV (Dik) unique DNA region (GGAGTGGAAAG) is indicated by a dotted box, and the putative enhancer sequence in the 68-bp region is indicated by a solid box.

among the different genes (22). These authors estimated the rate of evolution at the synonymous sites (*Vs*) for BKV and simian virus 40 to be 3.8×10^{-8} per site per year. If this rate of evolution also applies to BKV (JL) and BKV (Dik), we can calculate the time (T) elapsed since BKV (JL) and BKV (Dik) diverged from BKV (pt). (i) The numbers of synonymous substitutions (M_s) in the sequenced part of the T-antigen-coding regions of BKV (JL) and BKV (Dik) were four and five, respectively (Table 2). (ii) A total of 21% of the 325 bp (68 bp) in the corresponding part of the BKV (pt) T-antigen-coding region turned out to be synonymous sites (N_s). (iii) The synonymous differences (K_s) were 4/68 = 0.059 for BKV (JL) and 5/68 = 0.074 for BKV (Dik). (iv) From $V_s = K_s/2T = 3.8 \times 10^{-8}$ per site per year (10) and a formula given in reference 9 for computing the standard error, we were able to calculate T[BKV/(JL)] to be $(8 \pm 2) \times 10^5$ years and T[BKV (Dik)] to be $(10 \pm 2) \times 10^5$ years.

We conclude that BKV (JL) and BKV (Dik) are stable variants of BKV (pt), mainly differing from BKV (pt) in the putative enhancer-promoter region. It will be interesting to see whether the observed differences in the putative enhancer-promoter region affect the biological properties of these viruses.

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