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BK Virus and JC Virus Shed during Pregnancy Have Predominantly Archetypal Regulatory Regions

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Twenty-three BK virus and JC virus DNA samples obtained from urine of pregnant women had almost exclusively archetypal transcriptional control regions. Rearrangements characteristic of laboratory strains are apparently not required for reactivation in humans. Unexpectedly, alignment shows that many elements identified previously in the BK virus enhancer are conserved in the JC virus archetype.

The human polyomaviruses BK virus (BKV) and JC virus (JCV) are endemic worldwide (for reviews, see references 15 and 32). Infection occurs during childhood, and virus persists latently within the renal system (3, 9, 10). Reactivation occurs during pregnancy (5, 12, 28) or as a consequence of immunodeficiency (15, 32). JCV infection of the brain causes the fatal brain disease progressive multifocal leukoencephalopathy (reference 32 and references therein).

BKV and JCV each contain a noncoding regulatory region located between divergent early and late transcription units (15, 32). Isolates vary widely in the number and boundaries of direct DNA sequence repeats in this region (15, 32), and in the laboratory rearrangements occur spontaneously and relatively frequently (14, 33). Recently, it has been proposed that regulatory regions of laboratory strains derive from a common archetype (27, 35). Archetypelike DNA, which lacks sequence repeats in the regulatory region and contains additional sequences not commonly found in laboratory strains, has been isolated by molecular cloning from several sources (18, 27, 29, 30, 31, 34). Unlike the regulatory region of laboratory strains, that of the BKV archetype directs little or no early gene expression in transfection assays (21). The JCV archetype has not yet been tested.

It is not yet clear whether sequence rearrangements are relevant to infection or reactivation in the natural host. Direct DNA analysis and molecular cloning showed that archetypal BKV was predominant in a renal allograft recipient (27). By contrast, a patient with lupus erythematosus (29) and pediatric oncology patients (30) had mixtures of archetypelike and rearranged DNAs. In a number of patients, JCV DNA from kidney and urine was archetypal (18, 34), whereas DNA from urine and brain tissue of other patients was rearranged (18, 24).

We characterized BKV and JCV regulatory region DNA present in the urine of pregnant women. Pregnancy is among the most common conditions that have been linked to viral reactivation, and viral DNA obtained from pregnant women is therefore likely to be more representative of that in the general population than viral DNA from patients with rare disorders. In addition, reactivation during pregnancy has been extensively characterized in earlier serologic and cytologic studies (4, 5, 12).

Urine samples were collected from 129 patients receiving routine prenatal care at the Obstetrics and Gynecology Clinic at the University of Colorado Health Sciences Center in Denver. Samples were centrifuged for 90 min at 142,000 × *g* at 4°C, and the pellets were resuspended in water. An aliquot was taken, adjusted to 10 μg of salmon sperm DNA per ml, boiled for 10 minutes, and subjected to polymerase chain reaction (PCR) amplification. The methods used will be described in more detail elsewhere (18a). PCR primers anneal to sequences that flank the regulatory regions of BKV and JCV and are not usually affected by rearrangements, as diagrammed in Fig. 3.

Representative products obtained by using BKV-specific primers are shown in Fig. 1. Eighteen clinical samples were positive for BKV regulatory region DNA (for example, see lanes 4 through 6 and 15 in the upper panel). Each showed a prominent band corresponding in size to the unrearranged archetypal BKV regulatory region contained in the BK(WW) strain (27) (lanes 2 and 18 in the upper panel). Many samples also showed minor bands that migrated above the archetype (for example, lanes 5 and 6). These are evidently PCR artifacts, since the same products were seen with cloned input DNA and with clinical samples. The most slowly migrating of these species was full-length single-stranded DNA, as judged by resistance to restriction enzyme digestion and comigration with the single-stranded product of asymmetric PCR (data not shown). We estimate that most positive samples contained about 10 pg of viral DNA, corresponding to about 1,000,000 copies of viral regulatory region DNA per ml of voided urine.

In addition to the 18 samples that were clearly positive, another 14 showed a faint trace of product when gels were exposed approximately six times as long as in Fig. 1 (data not shown). These signals were often difficult to distinguish from the diffuse low-level background present throughout each lane. These samples were therefore not listed as positive in Table 1, although the possibility remains that some of the patients from whom these samples were obtained were undergoing low-level reactivation.

The presence of residual substances from urine in PCRs can produce false-negative results (7). To control for this possibility, we performed reactions in which each sample was spiked with 10 pg of DNA of a plasmid containing the genome of BK(MM), a rearranged strain with a regulatory region significantly larger than that of the BKV archetype. Representative results are shown in Fig. 1 (bottom panel). Of 129 samples tested, 12 did not support the amplification of

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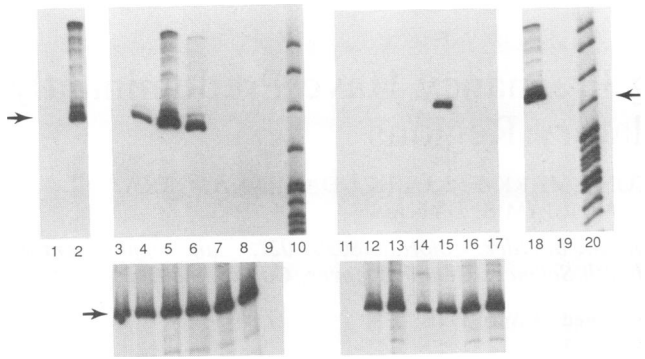

FIG. 1. PCR amplification of BKV DNA. Reaction mixtures contained 0.12 μM primer BK1 (5′-GGCCTCAGAAAAAGCTTCC ACACCCTTACTACTTGA-3′ [nt −59 to −24]), including 5×10^6 cpm of radiolabeled primer BK1; 0.12 μM primer BK2 (5′-CTTGTC GTGACAGCTGGCGCAGAAC-3′ [nt +295 to +271]); 25 mM Tris-HCl (pH 8.0); 5 mM $MgCl_2$; 50 μM NaCl; 100 μg of bovine serum albumin per ml; 250 μM each deoxynucleoside triphosphate; and 2 U of AmpliTaq polymerase (Perkin Elmer Cetus) in a final volume of 100 μl. Samples were subjected to 29 cycles of amplification, fractionated by electrophoresis, and visualized by autoradiography overnight with an intensifying screen. Lanes 1 and 19, negative control reaction mixtures containing no BKV DNA. Lanes 2 and 18, positive control reaction mixtures containing 10 pg of pBK(WW) (archetype) plasmid DNA. The arrows in the upper panel mark the position of the BK(WW) amplified product. Lanes 3 to 9 and 11 to 17 (top panel), reaction mixtures containing material from clinical samples 177 to 183, 241 to 244, and 147 to 149, respectively. The corresponding lanes in the lower panel are from reaction mixtures containing material from clinical samples spiked with 10 pg of pBK(MM) (see text). The arrow in the lower panel marks the position of the BK(MM) amplified product. Lanes 10 and 20, marker DNA consisting of pBR322 cut with *Hpa*II; the top four bands are 622, 527, 404, and 309 bp long, starting at the top.

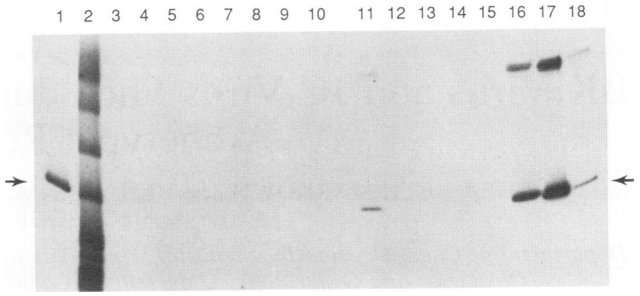

FIG. 2. PCR amplification of JCV DNA. Samples were analyzed as described in the legend to Fig. 1, except that the PCR mixture contained 0.12 μM primer JC1 (5′-CCTCCACGCCCTTACTACTT CTGAG-3′ [nt −45 to −21]); 0.12 μM primer JC2 (5′-AGCTGGTG ACAAGCCAAAACAGCTCT-3′ [nt +263 to +238]), including 5×10^6 cpm of radiolabeled primer JC2; 20 mM Tris-HCl (pH 8.8); 2.0 mM $MgCl_2$; 50 mM KCl; 100 μg of bovine serum albumin per ml; 400 μM each deoxynucleoside triphosphate; and 2 U of AmpliTaq DNA polymerase. Samples were subjected to 34 amplification cycles and analyzed as described in the legend to Fig. 1. Lane 1, positive control reaction mixture containing 100 pg of pM1TCR1A plasmid DNA [cloned JC(Mad-1)]. The arrows mark the position of the JC(Mad-1) amplified product. Lane 2, marker DNA consisting of pBR322 cut with *Hpa*II; the top four bands are 622, 527, 404, and 309 bp long, starting at the top. Lane 3, negative control reaction mixture containing no JCV DNA. Lanes 4 through 18, reaction mixtures containing material from clinical samples 121 to 123, 126 to 129, 131, 139 to 142, 164, 174, and 186, respectively.

control DNA (for example, lanes 9 and 11). Results from these samples were excluded when the percentage of patients who were positive for virus excretion was calculated.

Controls containing no added virus or viral DNA were also amplified and analyzed in each set of reactions. These were uniformly negative at the standard level of exposure exemplified by Fig. 1 and 2, although on very rare occasions a faint trace of product was seen upon prolonged exposure of the films.

Positive samples were further characterized by restriction enzyme analysis (data not shown). All samples which tested positive in the initial PCR analysis showed a diagnostic 140-nucleotide (nt) fragment following *Bsu*36 I digestion, confirming that the DNA was of BKV origin.

Samples from the same set that was analyzed for BKV DNA were also analyzed for JCV DNA. The subset that was assayed for JCV was randomly chosen, except that the 12 samples previously shown to contain inhibitors of the PCR reaction were excluded. Representative products obtained by using JCV-specific primers are shown in Fig. 2. Of 76 clinical samples that were assayed, 5 were positive for JCV regulatory region DNA in two or more screenings (for example, lanes 11 and 16 through 18). Each showed a prominent band of approximately 310 bp. This size is similar to that of the JC(Mad-1) amplification product (316 bp; lane 1) but also does not differ significantly from that of the product predicted for the JCV archetype (308 bp). As with BKV, most samples, including the cloned JC(Mad-1) DNA, yielded an additional low-mobility product that is believed to be a PCR artifact corresponding to full-length single-stranded DNA. One sample had an additional minor band of approximately 320 bp (not shown). It is likely that the patient from whom this sample was obtained was shedding two forms of JCV, whereas all other patients appeared to be shedding only one each.

Amplified JCV DNA was further characterized by restriction analysis (data not shown). *Sac*I digestion produced a radiolabeled fragment of 129 bp, which is characteristic of the JCV archetype but differs from the band of 104 bp that would have been produced from JC(Mad-1). *Sph*I digestion produced a radiolabeled fragment of 175 bp, which is characteristic of the archetype, whereas most rearranged strains lack this site.

The combined results of BKV and JCV screening are summarized in Table 1. Only one sample was positive for both viruses, which is approximately what would be expected by chance if reactivation of each virus were independent.

Definitive identification of BKV and JCV DNA was accomplished by DNA sequencing. DNA was amplified by asymmetric PCR and sequenced directly. Unambiguous sequence was obtained at most positions, indicating that the DNA sample was relatively homogeneous. The sequence data are summarized in Fig. 3. Sequence was obtained for 13 of the 18 positive BKV samples and for 4 of the 5 positive JCV samples. In some cases, sequence was obtained for both strands, as shown in Fig. 3.

The BKV samples showed a divergence on the order of 2% from each other and from BK(WW), the archetypal strain cloned in South Africa. Several of the polymorphisms (at nt 52, 65, 145, and 222) matched those reported for BK(WWT), a recent Norwegian isolate (30). Two of the BKV polymorphisms lie within the central, variable portions of nuclear factor 1 (NF-1)-binding sites (2, 19). The others lie

TABLE 1. Summary of results

Virus and result	No. of samples	%
BKV[a]		
Positive	18	15
Negative	99	85
Subtotal (assayable samples)	117	100
Inhibitory to assay	12	
Total	129	
JCV[b]		
Positive	5	7
Negative	71	93
Total	76	100

[a] Samples were assayed for BKV with primers BK1 and BK2. Samples were scored as positive if a distinct band was seen after overnight autoradiography. Samples were scored as inhibitory if no signal was seen when the sample was spiked with 10 pg of a control plasmid containing BKV sequences. The remaining samples were scored as negative.

[b] Samples were assayed for JCV with primers JC1 and JC2. The same criteria as in footnote *a* were used for scoring. Samples previously shown to be inhibitory for BKV amplification were not assayed for JCV.

outside known protein-binding sites and functional elements (1, 2, 8, 19). On this basis, none are predicted to affect the functional properties of the promoter/enhancer region. Cloned BK(WW) DNA was also sequenced and showed one difference, a G-to-A substitution at nt 131, from the published sequence (27) (data not shown). It is possible that this reflects a mutation that occurred during propagation of the cloned DNA. The regulatory regions of all four JCV samples that were sequenced were identical to each other and to the published archetype (34). Cloned JC(Mad-1) DNA was also sequenced and showed no differences from the published sequence (11).

Alignment of the archetypal BKV and JCV regulatory region sequences shows that, unexpectedly, almost all of the protein-binding sites and functional elements that have been defined in recent studies of BKV appear to be conserved in JCV (Fig. 4). These sites occur in the same order, and in many cases with approximately the same spacing, suggesting that the regulatory regions as a whole may share functional properties.

The four sites marked N1, N4, N5, and N6 in Fig. 4 have been shown to bind a protein related to NF-1 in BKV (2, 19, 20). A fifth site, L1, binds the protein related to NF-1 and one or more unknown proteins (19). Four of these NF-1 sites appear to be conserved in JCV. Strikingly, in every instance, it is only the ends of the sites that are identical in BKV and JCV, whereas the central six or seven nucleotides, which are not essential for NF-1 DNA recognition, are variable. This pattern of conservation is evidence that NF-1 binding is an essential function, and moreover the only essential function, for these sequences.

In addition to the NF-1 sites, there is a conserved recognition site for the transcription factor Sp1 (S1 in Fig. 4). Of 10 nucleotides thought to be important for Sp1 DNA recognition, 8 are identical in BKV and JCV. The changes at the other two positions are consistent with sequences present in other known Sp1-binding sites (16, 17). Two other elements (Fig. 4, G1 and CRE-like) have been identified by mutational analysis as important for BKV transcription (1, 6, 8; discussed in reference 30). Although the binding of transcription factors to these sequences has not been demonstrated directly, the conservation of these elements between BKV and JCV suggests that they are likely to be functionally

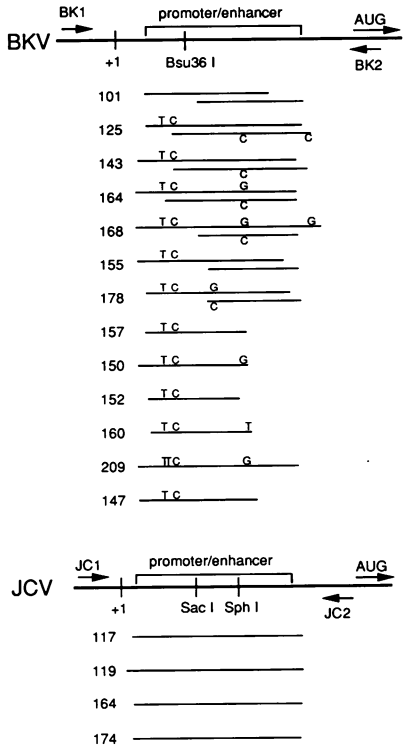

FIG. 3. Sequencing of amplified viral DNA. (Top) Map of BKV archetype. Numbering system is based on the simian virus 40 system, with the first nucleotide to the late side of the origin palindrome assigned as +1. The locations of primers, the promoter/enhancer region, and agnoprotein initiation codons are shown. The lines below the map summarize sequence results for 13 different clinical samples. The sequence was obtained for both strands of seven samples (paired lines) and for the top strand alone of six samples (single lines). The sequence was the same as that published for BK(WW) (27), except for polymorphisms indicated above and below the lines. The sequence data were obtained by asymmetric PCR (13) in a reaction mixture containing 20 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 100 μg of bovine serum albumin per ml, 100 μM each deoxynucleoside triphosphate, 0.5 μM excess primer, 0.01 μM limiting primer, and 2 U of AmpliTaq DNA polymerase, with 39 cycles of amplification. Products were separated from unincorporated nucleotides by ethanol precipitation and subjected to dideoxynucleotide sequencing using a TaqTrack sequencing system (Promega). (Bottom) Map of JCV archetype. The lines below the map summarize sequence results for four different clinical samples. In each case, the top-strand sequence was obtained.

important in both viruses. Finally, there are several extended runs of identical nucleotides for which no function has yet been assigned. It seems likely that these also contain functional elements, but they have not been investigated in detail.

Approximately 15% of the patients tested were positive for BKV and 7% were positive for JCV. This result does not differ materially from expectations based on the results of a prior serologic and cytologic survey of a similar population (5). In that study, 10.2% of the patients showed high or rising antibody titers against BKV, 16.3% showed high or rising antibody titers against JCV, and 3.2% showed evidence of virus-infected cells shed in urine (5). Interestingly, the investigators in that study found that from 121 cytologically positive samples, only 12 viral isolates could be obtained, and even these grew slowly and with minimal cytopathic

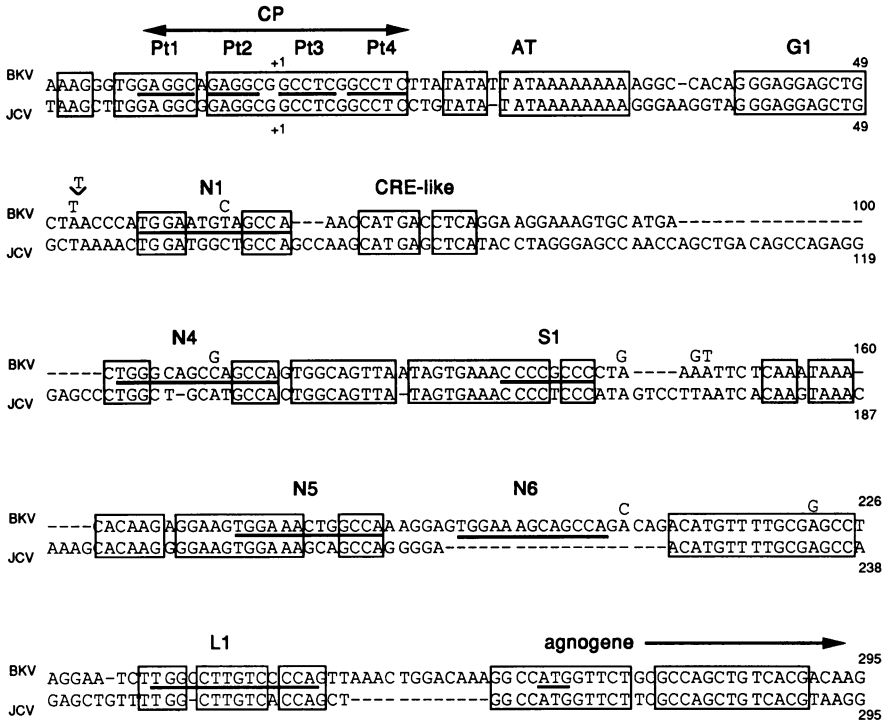

FIG. 4. Alignment of BKV and JCV archetype DNA sequences. Numbering is as in Fig. 3. Boxes indicate identity at four or more contiguous nucleotides or at three contiguous nucleotides separated by one nucleotide from another block of identity. Polymorphisms detected in various BKV isolates are marked by letters above the BKV sequence. N1, N4, S1, N5, N6, and L1 denote binding sites for nuclear proteins, as determined experimentally with BKV (2, 19, 20). Relevant consensus sequences are underlined. G1 and CRE-like denote regions which are believed to function in transcriptional regulation but where protein binding has not been found experimentally (30). CP denotes the central palindrome of the replication origin, Pt1 through Pt4 denote T-antigen recognition pentamers, and AT denotes the adenosine-thymidine-rich tract of the replication origin. The beginning of the agnogene is marked.

effect. In retrospect, these results may reflect the fact that the BKV and JCV shed in urine of pregnant women are primarily archetypal and lack rearrangements that facilitate growth under laboratory conditions.

The present results differ from those obtained with a patient with lupus erythematosus, in whose case approximately 50% of the BKV in urine contained a rearranged regulatory region (29), and from a recent study of samples collected primarily from pediatric oncology patients, in which rearranged forms were detected in addition to the archetype (30). It is possible that the presence of rearranged forms results from long-term immunosuppression, or in the case of the pediatric patients, horizontal transmission of an unusual variant within a geographically clustered population.

Because our results suggest that sequence rearrangements usually do not occur in virus shed during pregnancy, it will be of interest to investigate alternative possible mechanisms of reactivation. The degree of reactivation in pregnancy has been correlated with monocyte function, suggesting that alterations of the immune system during pregnancy may play a role (4). However, in other viruses that are reactivated during pregnancy, hormonal induction of transcription is a major factor. The best-known example is mouse mammary tumor virus (25, 36). Another example is mouse polyomavirus, which is particularly relevant because it is related to BKV and JCV. Mouse polyomavirus is reactivated during pregnancy (22, 23), and in the laboratory, growth is increased in the presence of either an estrogen-progesterone mixture or glucocorticoids (23). Recent studies show that a sequence similar to half of a hormone response element in the enhancer region of mouse polyomavirus is essential for establishment of infection in mice (26). A sequence that is weakly homologous to the glucocorticoid-progesterone response element is found in BKV and JCV, overlapping the agnogene initiation codon (30). Whether these or other sequences in BKV and JCV constitute functional hormone response elements has not yet been determined.

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