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Nucleotide Sequence of the Region Encompassing the JC Virus Origin of DNA Replication

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The region of the JC virus (JCV) genome from 0.58 to 0.73 map units was sequenced by the Maxam-Gilbert technique. This segment of DNA specifies several regulatory elements and the amino-terminal portion of the early viral proteins. Comparisons with the analogous regions in the polyomaviruses simian virus 40 (SV40) and BK virus (BKV) confirm the close evolutionary relationship of these three viruses. Similarities include palindromic and symmetrical sequences near their origins of DNA replication, binding sites for their large T proteins, an AT-rich region (the Goldberg-Hogness, or TATA, box), and a large tandem duplication or triplication to the late side of their replication origins (however, these sequences differ). Homology between the sequences coding for the early proteins is also evident (79 and 93 of the first 110 amino acids are shared with SV40 and BKV, respectively). Of greater interest are features of the JCV genome which differ from those of other polyomaviruses. Absent in JCV and BKV are sequences which resemble the third T-antigen binding site of SV40. In addition, a set of sequences present in JCV and BKV DNAs (33 nucleotides in JCV and 22 nucleotides in BKV) and located near a 17-base-pair palindrome shared by all three viruses is missing in SV40 DNA. Another sequence, GGGXGGAG, which is repeated several times in many polyomaviruses and adenoviruses and which is thought to play a role in DNA replication or transcription or both, is not found near the JCV origin of replication. Finally, the tandem repeat of JCV, unlike those of BKV and SV40, includes the Goldberg-Hogness sequence.

JC virus (JCV) is a human polyomavirus which infects most people early in life (23). It is the probable causative agent of progressive multifocal leukoencephalopathy, a fatal demyelinating brain disease which occurs in a small number of immunodeficient individuals (25). JCV is also an oncogenic virus; it induces a variety of tumors in hamsters (26, 37) and is the only human virus known to cause brain tumors in primates (16, 29).

Several obstacles have been encountered during experimentation with JCV; however, two major problems have recently been solved by recombinant DNA technology. Because of the restricted host range of JCV, it had been difficult to obtain adequate amounts of DNA for molecular characterization (22). Furthermore, the heterogeneity of the viral DNA precluded any attempts to sequence the DNA or to identify potential biological differences among the variants in the population (5). Through molecular cloning, unlimited supplies of viral DNA, representing full-length and deleted species, are now available. An additional advantage of this technology is the ability to isolate JCV DNA directly from human tissue without isolating and propagating the virus in tissue culture, a procedure which appears to generate alterations in the viral DNA (5, 17, 19; B. W. Grinnell, J. D. Martin, B. L. Padgett, and D. L. Walker, *in* J. Sever and D. L. Madden, ed., *Polyomaviruses and Human Neurological Disease*, in press).

A major goal of the experiments outlined here was to identify potential JCV regulatory signals through sequence analysis. The physical maps of JCV, BK virus (BKV), and simian virus 40 (SV40) DNAs have been aligned through cross-hybridization studies, indicating that they share a similar genetic organization (14). These results and those from other laboratories (19; R. J. Frisque, *in* J. Sever and D. L. Madden, ed., *Polyomaviruses and Human Neurological Disease*, in press) suggest that the origin of DNA replication of JCV, as with other polyomaviruses, is near 0.67 map unit. This region is especially interesting since the greatest degree of nonhomology among the genomes of JCV, BKV, and SV40 occurs here (14). Therefore, detection of differences in the noncoding region (i.e., regulatory sequences rather than coding

sequences for proteins) might begin to explain the diverse biological properties (tissue tropism, pathogenicity, and tumorigenicity) exhibited by these viruses. Findings with other polyomaviruses have already emphasized the influence of the noncoding region on the properties of host range and tumorigenicity (7, 12, 13, 39, 40).

In this report the nucleotide sequence of the regulatory region of two cloned species of JCV DNA, pMad1-TC and pMad1-Br, is presented. These clones were chosen for several reasons: (i) Mad 1 virus is the prototype JCV first isolated in 1971 by Padgett and co-workers (24), and this strain is frequently used in JCV studies; (ii) both clones appear to contain full-length JCV DNA (~5,100 nucleotides); (iii) the pMad1-TC clone transforms primary hamster brain cells in culture and therefore is biologically active (Frisque, in press); and (iv) both DNAs represent isolates from the same brain tissue but have had different passage histories (direct isolation of DNA from brain [pMad1-Br] versus extraction of DNA from virus propagated in tissue culture [pMad1-TC]). Therefore, it was possible to determine whether passage-induced alterations had occurred by comparing the nucleotide sequences of these cloned DNAs.

MATERIALS AND METHODS

Molecular cloning. DNA extracted from prototype JCV or the progressive multifocal leukoencephalopathy tissue from which this virus was derived was used to construct the pMad1-TC and pMad1-Br clones, respectively. The DNA was cleaved with *Eco*RI and ligated to pBR322 digested with the same enzyme (Frisque, in press). Both recombinant molecules represent full-length JCV DNA (~5,100 nucleotides).

DNA sequence analysis. Restriction endonuclease fragments generated by digestion with *Hin*dIII or *Nco*1 (cuts JCV DNA two times) were end labeled using the large fragment of *Escherichia coli* DNA polymerase I (Klenow reagent) and the appropriate α-^{32}P-deoxynucleoside triphosphate. These DNAs were cleaved with a second restriction enzyme, and fragments labeled at only one end were isolated from low-melting-agarose gels, purified, and chemically cleaved by the method of Maxam and Gilbert (18). Electrophoresis on polyacrylamide gels (8 to 12%) was carried out for various lengths of time. Gels were frozen and autoradiographed without intensifier screens for 17 to 72 h. Approximately 70% of the nucleotide sequence was determined for both DNA strands. All determinations were repeated at least once.

RESULTS

The assignment of nucleotide numbers in this manuscript follows that used by Seif et al. (30) for BKV. Numbering proceeds away from the presumed initiation codon for large T and small t proteins, through the replication origin, and toward the late gene region. The first position of the ATG is given a value of $^{-}1$ since it represents the final nucleotide in the numbering system.

Origin of DNA replication. The replication origins of BKV and SV40 appear to include a true palindrome of 17 nucleotides and two sets of twofold symmetries (15 and 27 nucleotides long in SV40 and 23 and 27 nucleotides long in BKV [4, 28, 30, 41]). Inspection of the JCV DNA sequence (both clones were identical [Fig. 1]) revealed an organization similar to the other two viral DNAs; again, a 17-nucleotide palindrome (nucleotides 61 to 77; 16 and 15 nucleotides shared with SV40 and BKV, respectively) and two sets of symmetries (nucleotides 83 to 101 and 105 to 129) were found. Considerable relatedness is apparent between these symmetries in all three viral DNAs. The second symmetry, which is the most highly conserved, may be the center of the replication origin and includes the second T-antigen binding site of SV40 (36; see below).

To the early (counterclockwise) side of the palindrome is a third shared symmetry (nucleotides 7 to 21 for JCV [4, 28, 30, 41]). Between this sequence and the true palindrome, JCV and BKV have an additional set of nucleotides (33 and 22 nucleotides, respectively) not found in the SV40 sequence. Within this stretch of both DNAs is an additional dyad symmetry (nucleotides 44 to 60 for JCV [30, 41]).

To the late side of the origin region of SV40 and BKV, the sequence GGGXGGAG is found repeated several times. It has also been detected in polyomavirus and some adenovirus DNAs and is thought to play a role in DNA replication or transcription or both (2; reviewed in reference 30). This sequence is absent from the JCV origin region and is represented only one time in the sequence presented (within the early coding region, nucleotides $^{-}128$ to $^{-}121$).

T-antigen binding sites. The large T protein of SV40 and the closely related D2T protein of the adenovirus-SV40 hybrid Ad2D2 sequentially bind to three sites around the SV40 origin of DNA replication (32, 36), and the latter protein also binds to the *Hin*dIII C fragment of JCV DNA (Frisque, in press). Analysis of this JCV sequence revealed a stretch of 22 nucleotides (nucleotides 56 to 77), 21 of which are found in the first binding site of SV40 (36). A potential second binding site in JCV was located immediately upstream from the *Hin*dIII C fragment (nucleotides 105 to 131). If the nucleotide at each end of the site is ignored, then the sequence is identical in JCV, BKV, and SV40 DNAs, except at a single position (nucleotide 111 in JCV). Sequences resembling the third SV40 T-antigen binding site proposed by Tjian (36) have not been identified in JCV or BKV.

Promoter elements. There appear to be at least

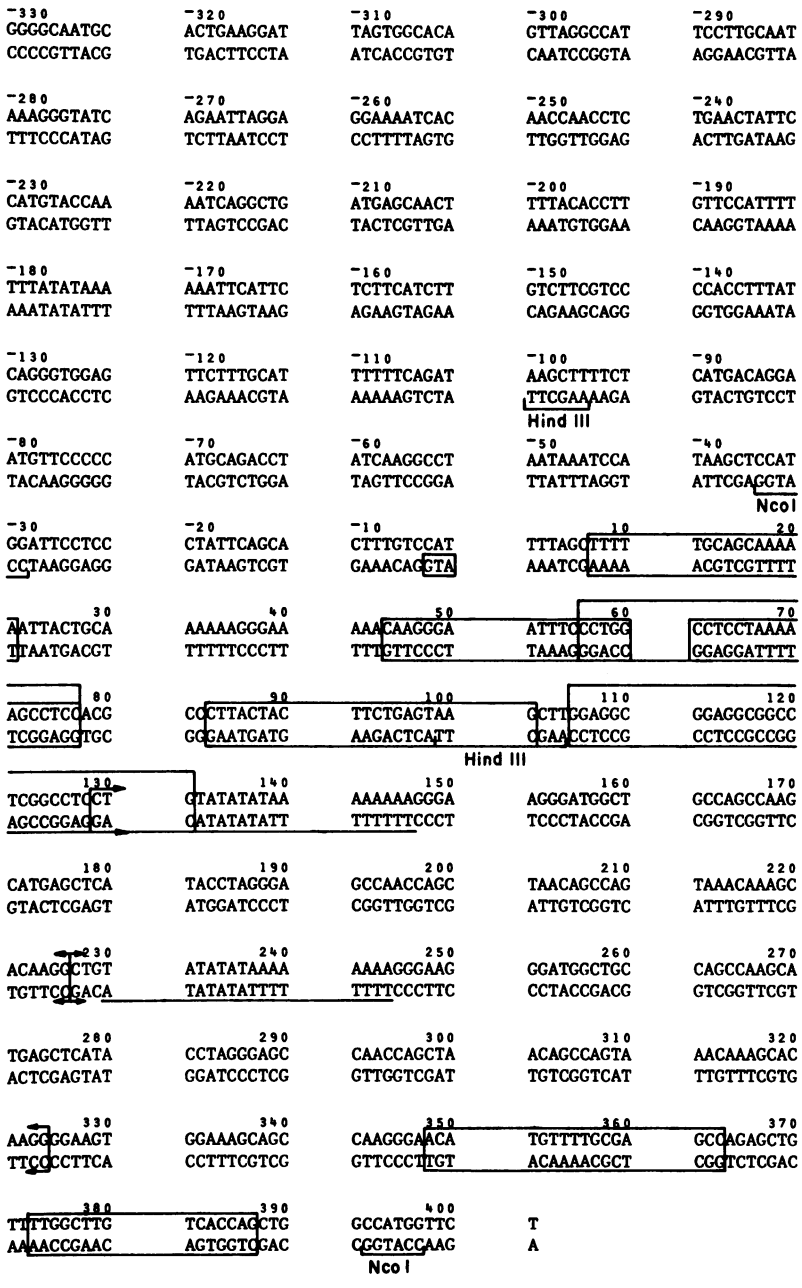

FIG. 1. Nucleotide sequence of the region near the JCV origin of DNA replication (0.58 to 0.73 map units). This sequence was determined for both pMad1-TC and pMad1-Br and was identical for both DNAs. The upper strand, read left to right, has the polarity and sequence of the late mRNAs and begins at 0.58 map unit. The lower strand, read right to left, represents the polarity and sequence of early JCV mRNAs. Relevant features include the presumed initiation codon for large T and small t proteins (nucleotides $^{-}1$ to $^{-}3$), a true palindrome shared by JCV, BKV, and SV40 DNAs (nucleotides 61 to 77), and three sets of twofold symmetries (nucleotides 7 to 21, 44 to 60, and 83 to 101). Of the 22 nucleotides from 56 to 77, 21 are identical to those found in the first T-antigen binding site of SV40 DNA. JCV, BKV, and SV40 DNAs all share the symmetrical sequence from nucleotides 105 to 129. This set of nucleotides is thought to be part of the second T-antigen binding site in SV40, as well as the center of the DNA replication origin (nucleotides 105 to 131). A tandem duplication of 98 nucleotides (nucleotides 129 to 324) is denoted by brackets with arrows, and unlike SV40 and BKV, this repeat includes the Goldberg-Hogness sequence (underlined, nucleotides 132 to 146 and 230 to 244). Also indicated are two stretches of DNA found in a similar position in BKV DNA (nucleotides 348 to 363 and 373 to 387).

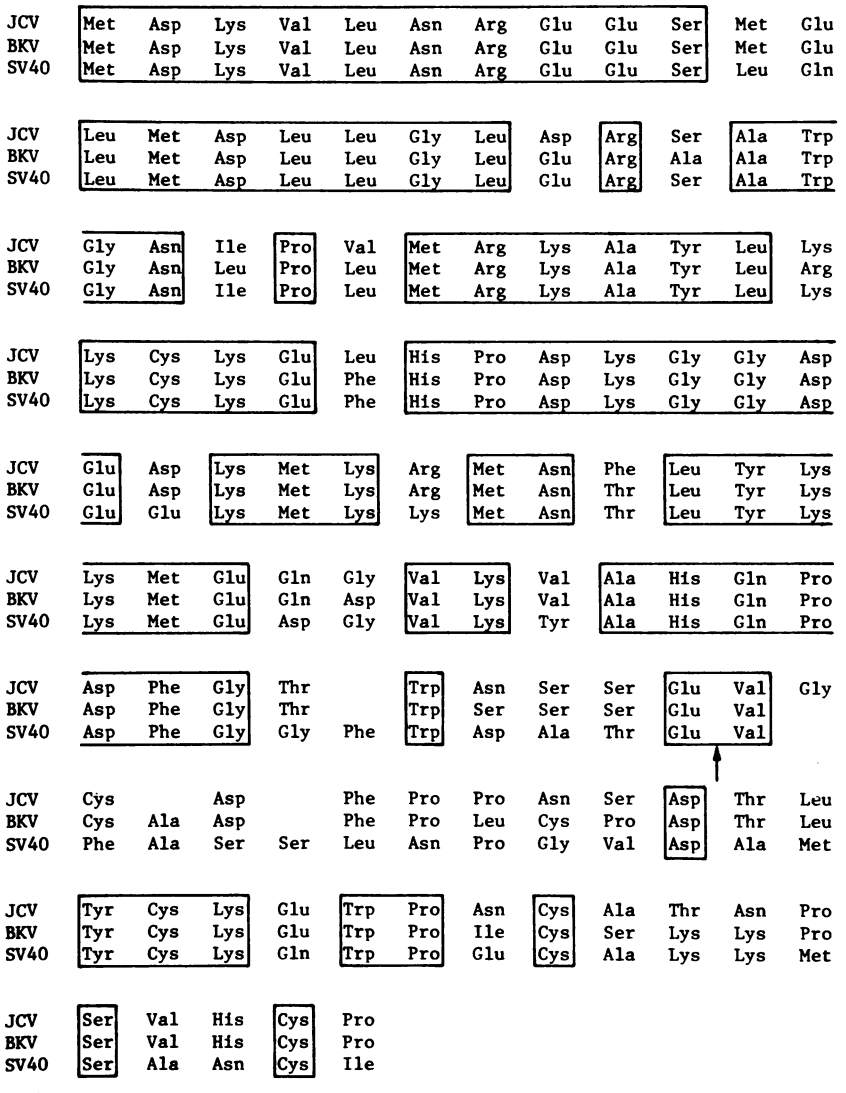

FIG. 2. Amino acid sequence of the amino-terminal portion of the large T and small t proteins of JCV, BKV, and SV40 extending 330 nucleotides downstream (counterclockwise) from the presumed initiation codons at approximately 0.65 map unit. Sequences unique to small t begin at the arrow, which marks the donor splice site for large T message.

two components of the early SV40 promoter: the Goldberg-Hogness sequence (also called the AT-rich region, or TATA box) and the 72-nucleotide tandem repeat (or enhancer). Similar sequences are found in JCV and BKV.

The Goldberg-Hogness sequence is located approximately 25 nucleotides upstream from the 5′ ends of the early viral mRNAs and is thought to play a role in their positioning (8, 9). In all three viral DNAs this signal is longer than the consensus sequence TATAAT (15, 17, and 20 nucleotides for JCV, SV40, and BKV, respectively), and it is located within a GC-rich stretch of DNA.

The second component of the SV40 early promoter appears to be required for the initiation of transcription in vivo (1, 10) and is located 78 nucleotides upstream from the AT-rich region (4, 28). An almost perfect 68-nucleotide tandem triplication is located 4 nucleotides upstream from the corresponding position in BKV DNA (30, 41). Although similar in location and structure, these tandem repeats do not demonstrate any obvious sequence homology. In JCV a tandem repeat of 98 nucleotides (nucleotides 129 to 324) is observed immediately adjacent to the second T-antigen binding site, and again, homology with the other viral repeats is not evident. Furthermore, this repeat, unlike those of SV40 and BKV, includes the Goldberg-Hogness box.

5′ ends of early mRNAs. Analysis of the 5′ ends of the early SV40 mRNAs suggested at least two major start sites located 22 and 28 nucleotides downstream from the Goldberg-Hogness sequence (27). The sequence, beginning at the first site, reads GCCTCTGAGCTATTCCA.

Seif et al. (30) indicated two potential 5′ ends for the early BKV messages located 19 and 25 nucleotides from the TATA box. Beginning at the first site, the DNA sequence is GCCTCCACCCTTTCTC.

Inspection of the same region of the JCV sequence also suggests two possible termini, one at a 22- and the other at a 28-nucleotide distance from the same landmark. The sequence reads GCCTCCAAGCTTACTCA.

The locations of the 5′ termini in JCV and BKV DNAs remain to be verified by sequencing techniques and cap structure determination.

Early viral proteins. The probable amino acid sequence of the amino termini of the JCV large T and small t proteins is shown in Fig. 2. Of the first 110 predicted amino acids, 79 and 93 are shared with SV40 and BKV, respectively. Assignment of this primary structure to the JCV proteins was based mainly on a comparison with the SV40 and BKV sequences; however, it was also consistent with data derived from immunoprecipitation of viral proteins (6) and S1 analysis of viral mRNAs (Frisque, in press).

A donor splice site for the SV40 and BKV large T messages occurs between nucleotides specifying a glutamine and valine residue (Fig. 2, arrow). These same residues occur at an identical position in the JCV sequence, and localizing the upstream splice here would be consistent with S1 mapping data.

The large degree of homology evident between the amino-terminal ends of both early proteins for all three viruses is significantly reduced beyond the large T donor splice site (Table 1). These latter sequences are unique to small t antigen, a protein which is not required for growth of SV40 or BKV in vitro (33, 35).

TABLE 1. Comparisons among JCV, BKV, and SV40 of the amino acid sequences of the early viral proteins[a]

Comparison between:	T protein[b]		t protein[c]	
	No. of aa[d] shared/ total no. of aa	% Homology	No. of aa shared/ total no. of aa	% Homology
JCV × BKV	72/81	89	21/29	72
JCV × SV40	67/81	83	12/29	41
BKV × SV40	67/81	83	13/29	45
JCV × BKV × SV40	63/81	78	10/29	34

[a] The first 110 amino acids of the early coding regions of these viruses are compared.
[b] Includes amino acids shared by large T and small t proteins.
[c] Includes amino acids unique to small t protein (beyond the donor splice site for large T message).
[d] aa, Amino acids.

DISCUSSION

Hybridization and serological studies (14, 21, 31, 37) have predicted a close evolutionary relationship among JCV, BKV, and SV40; nucleotide sequence analysis confirms these predictions. Sequences specifying the amino-terminal portions of the early proteins, the replication origins, and the TATA boxes are strongly conserved; however, sequences upstream from the AT-rich region (including the long tandem repeats) have diverged in these three viruses.

Before these studies, pMad1-TC was shown to transform primary hamster brain cells in culture (Frisque, in press). In agreement with previous results obtained with JCV and its DNA (6, 11), transformation was inefficient; frequency was low, and the latent period was prolonged. Unlike the earlier experiments, however, a small t message and protein were detected in the pMad1-TC transformants, indicating that the poor transforming ability of JCV was not due to the absence of a small t protein (as shown for *dl*54/59 mutants of SV40 [34]). From the sequence data presented here, 78% of those amino acids held in common by the large T and small t antigens were found to be shared by JCV, BKV, and SV40. Beyond the large T splice (unique small t coding sequences), this value dropped to 34%, and differences were especially apparent when the monkey virus (SV40) was compared with the human viruses (JCV and BKV). One might speculate that since small t is dispensable for the lytic growth of these viruses in vitro, alterations in its coding sequence might be better tolerated than changes in sequences encoding the multifunctional large T protein. Alternatively, small t might contribute to the host range phenotype of the polyomaviruses, and differences in its coding sequence might reflect a functional requirement in the various cells permissive for each virus.

JCV grows well only in primary human fetal glial (PHFG) cells, a heterogeneous population of cells which are difficult to obtain and grow. Because of the difficulties posed by this cell system, the availability of JCV and its DNA has been limited. Even in these cells the replication cycle of JCV appears to be prolonged and inefficient, as judged by immunofluorescence and pulse-labeling studies (22; J. Martin, personal communication). In light of this inefficiency, the

absence of the sequence GGGXGGAG from the origin region of JCV is noteworthy. This sequence is repeated several times near the replication origins of many papovaviruses and adenoviruses and is thought to play a role in replication or transcription or both (2; reviewed in reference 30). Also relevant to a discussion of replication is the apparent lack of interaction between D2T protein and the potential second T-antigen binding site of JCV DNA (Frisque, in press). Rather than reflecting an inability of the protein to bind to its recognition sequence, however, this finding probably results from a lack of cooperativity of binding (20) since the first and second sites were on different restriction fragments in these experiments.

Although the replication of JCV in PHFG cells is inefficient, in other cells it rarely occurs at all. Unlike SV40, which is expressed in a variety of eucaryotic cells, most cells do not even express T antigen after JCV infection (22). This restriction does not appear to involve the early stages of adsorption, penetration, and uncoating (5); one possible explanation is that JCV has a weak or defective early promoter. The strength of different promoters has been shown to vary (3, 38), and substitution of one promoter for another can increase or decrease the expression of the associated gene (15; M. Botchan, personal communication; unpublished data). Furthermore, recent studies suggest that polyomavirus enhancer sequences may affect the host range of these viruses (W. Schaffner, personal communication). Inspection of potential JCV promoter sequences revealed one striking difference with similar sequences in BKV and SV40. In addition to being nonhomologous, the tandem duplication of JCV includes the TATA box. Assessment of any effect that this might have on RNA polymerase function will await a more detailed analysis of this region.

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ADDENDUM IN PROOF

Miyamura et al. (J. Virol. 45:73–79, 1983) have recently published the sequence of a portion of the JCV genome which overlaps with part of the sequence reported here. The 511 nucleotides held in common are identical.

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