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A classification scheme for human polyomavirus JCV variants based on the nucleotide sequence of the noncoding regulatory region

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The human polyomavirus JCV is responsible for the central nervous system (CNS) demyelination observed in cases of progressive multifocal leukoencephalopathy (PML). Lytic infection of oligodendrocytes, the cells that constitute the basis of myelin in the CNS, is established by JCV in conjunction with immunosuppressive conditions. Beyond this, however, many questions related to JCV pathogenesis remain unanswered. The JCV regulatory region is a hypervariable noncoding sequence positioned between the early and late protein-coding regions. The particular nucleotide sequence of a JCV regulatory region affects levels of viral transcription and replication. Modifications to this promoter/enhancer structure can alter the cellular host range and may be responsible for switching JCV between states of lytic and latent infection. The regulatory region structure has, therefore, been used to distinguish JCV variants. Nucleotide sequencing studies have uncovered numerous variations of regulatory region structure. Until now, however, no inclusive nomenclature existed that linked variants by regulatory region structure and/or activity. We have arranged all known variant JCV regulatory regions into quadrants according to the integration of particular sequence sections and repetition of sequence section groups. This arrangement of regulatory regions results in an updated nomenclature that is well-suited for describing the relationships between JCV variants. Four distinct structural forms (I-S, I-R, II-S, and II-R) are defined along with tissue tropisms. This design provides logical connections between the variant regulatory regions and may be useful for elucidating crucial steps in JCV pathogenesis. *Journal of NeuroVirology* (2001) **7,** 280–287.

Keywords: JCV; regulatory region; nucleotide sequence; classification scheme; pathogenesis; progressive multifocal leukoencephalopathy (PML)

Introduction

The human polyomavirus JCV is responsible for neurological disorders as the result of targeted viral lysis of oligodendrocytes, the cells that constitute the basis of myelin in the central nervous system (CNS). With seroconversion occurring before or during adolescence, asymptomatic JCV infection is estimated at 80% worldwide in the adult population. Immunodeficiencies that occur in conjunction with leukemia, HIV infection, and organ transplantation can contribute to JCV activation, leading to the fatal demyelination observed in cases of progressive multifocal leukoencephalopathy (PML).

In addition to the CNS and urine, JCV has been detected in peripheral blood lymphocytes (PBL), kidney, tonsil, lung, liver, spleen, lymph node, bone marrow, and gastrointestinal tissues (Sugimoto *et al*, 1998; Elsner and Dörries, 1998; D Chang *et al*, 1999; Ciappi *et al*, 1999; Laghi *et al*, 1999; Newman

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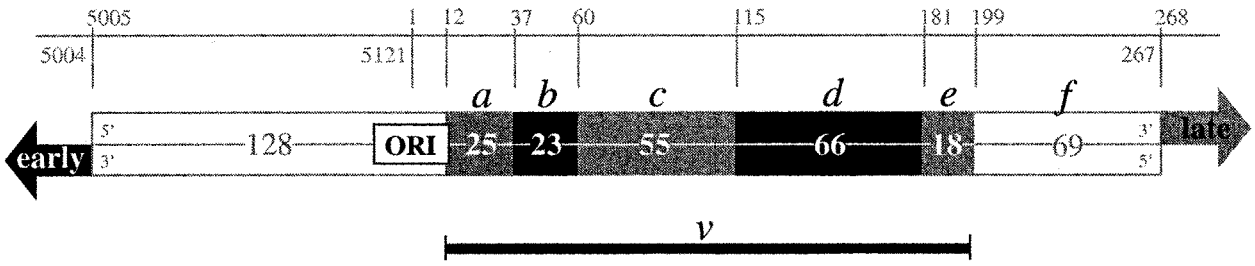

Figure 1 Scaled linear representation of JC virus archetype regulatory region sequence. Modified nucleotide-numbering system adapted from the prototype JCV genome, Mad-1 (Frisque *et al*, 1984). The regulatory region is a hypervariable, noncoding sequence positioned between the early (dark arrow to left) and late (lightly shaded arrow to right) protein coding sequences within the circular, supercoiled, double-stranded JC virus DNA genome. The archetype regulatory region sequence contains a single copy of all sequence sections observed in all other variant forms of JC virus regulatory region. From the early side, the initial regulatory region sequence section contains the origin of DNA replication (ORI) followed by sequence sections designated *a*, *b*, *c*, *d*, *e*, and *f*. The base-pair length of each sequence section is noted. From variant to variant, the dark bar, *v*, denotes sequence sections most likely to present deletions, replications, and/or unique arrangements, e.g., deletion of the dark sequence sections *b* and *d* leaves *ace*, a lightly shaded 98-base pair sequence unit. Also, tandem *ace* repeats constitute *v* in the regulatory region of the prototype JCV sequence, Mad-1 (*v* of archetype is 9 base pairs shorter than *v* of Mad-1, hence the modification of the nucleotide numbering system for all base pairs, excluding 1–36). Many other *v* sequences have been identified. The *v* sequence, therefore, is the basis of a classification scheme for variants of JC virus (see Figure 2).

and Frisque, 1999; Kato *et al*, 2000; Vaz *et al*, 2000; Ricciardiello *et al*, 2001; reviewed in Jensen and Major, 1999). Lymphocytes harboring virus acquired in peripheral sites of initial infection are thought to traffic JCV into the CNS. B-lymphocytes, specifically, possess the machinery necessary for the production and modification of antibodies. The complex processes of genomic rearrangement involved in antibody production, known as somatic recombination and hypermutation, may facilitate the modification of the JCV genome (Ault and Stoner, 1993). The hypervariable JCV regulatory region is an approximately 400-base pair (bp) noncoding sequence positioned between the early and late protein-coding sequences (Figure 1) in the circular, supercoiled, double-stranded viral DNA genome (Osborn *et al*, 1974; Firsque *et al*, 1984). Sequence modification in this promoter/enhancer structure can alter the cellular host range (Vacante *et al*, 1989) and may allow JCV to switch between states of lytic and latent infection.

The cellular host range of JCV could depend upon a number of factors, three of which are cellular characteristics. Initially, a JC virion must come in contact and bind with a candidate-cell that has appropriate cellular membrane receptors (Liu *et al*, 1998), such as sialyloligosaccharides [specifically, glycoproteins with terminal α(2-6)-linked sialic acid]. In addition, the candidate-cell must have uptake mechanisms (Pho *et al*, 2000), such as endocytic clathrin-coated pits, that the JC virion can exploit for passage to the nucleus. Ultimately, however, effectual JCV transcription and replication relies on the presence of suitable cellular DNA binding proteins (C-F Chang *et al*, 1996; Safak *et al*, 1999a), such as those of the nuclear factor-1 (NF-1) family (reviewed in Gronostajski, 2000). The NF-1 proteins bind multiple sites within the viral regulatory region, and growing evidence suggests that cell types capable of supporting JCV infection have upregulated levels of the NF-1 class D (Sumner *et al*, 1996; Shinohara *et al*, 1997). Host DNA binding proteins that are affected by extracellular stimuli may in turn alter viral activity (Safak *et al*, 1999b). Of note, inherent cellular protein polymorphisms (Krynska *et al*, 2000) could also affect all of the JCV/candidate-cell interactions described previously, and pre-existing infections with other viruses could supply trans-activators of JCV transcription and replication (Feigenbaum *et al*, 1987; Hara *et al*, 1998; Okada *et al*, 2000; Winklhofer *et al*, 2000).

The other factors affecting JCV cellular tropism would involve the viral genome itself. Our knowledge of viral gene products would suggest that mutations in the early JCV protein-coding sequences (T, t, and T' proteins) could influence levels of viral DNA replication and late viral transcription (Mandl *et al*, 1987). Mutations in the late protein-coding sequences (Agno, Vp1, Vp2, and Vp3) could alter aspects of virion assembly in addition to changing sites on the JCV capsid proteins involved in cellular receptor binding and nuclear localization signaling (Shishido-Hara *et al*, 2000). Undoubtedly, however, the definitive modulator of viral activity is the JCV regulatory region. The particular nucleotide sequence structure of this hypervariable region within a JCV genome markedly affects the levels of viral transcription and replication (Martin *et al*, 1985; Ault, 1997), and in this way may govern JCV cellular tropism. For this reason, the viral regulatory region can be used to define JCV variants.

In this paper we attempt to categorize current sequence structures of the JCV regulatory region to further address the affects of genomic variations on viral pathogenesis. Our grouping of regulatory regions results in an updated nomenclature that is well-suited for the description of the relationships between variant JCV forms. Caveats and lingering points of contention are addressed. Discussion of possible

routes of JCV genomic diversity are based on the known biological functions, tissue tropisms, and nucleotide sequences of the variant regulatory region structures.

A classification scheme: The JCV regulatory region compass

The JCV regulatory region can be divided into 7 distinct sequence sections (Figure 1). These sections all contain promoter and/or enhancer elements (reviewed in Vaz *et al*, 2000). The 128-bp section that immediately precedes the early coding sequences includes the origin of viral DNA replication. Generally, this section is highly conserved and is not represented in repeat structures that are commonly formed by the other regulatory region sequence sections. Opposite the direction of early transcription, starting 12 bp downstream from the center of the origin of DNA replication, sequence sections designated *a* (25 bp), *b* (23 bp), *c* (55 bp), *d* (66 bp), *e* (18 bp), and *f* (69 bp) constitute what is called archetype sequence. Repeats and/or deletions from archetype sequence account for all other known regulatory region variants (Yogo *et al*, 1990). Along with the preceding sequence section containing the origin of replication, a grouping of sequence sections *a*, *c*, *e*, and *f*, in this order, conveys functional viral activity (Daniel *et al*, 1996; Mayreddy *et al*, 1996; Ault, 1997). When present, however, the interspersing sections *b* and *d* have been shown to inhibit efficient transcription and replication of JCV (Daniel *et al*, 1996; Mayreddy *et al*, 1996; Ault, 1997). Duplication of any singular grouping of sections *a* through *e* in tandem sequences is thought to enhance levels of viral activity (Daniel *et al*, 1996), even if the tandem sequences experience substantial nucleotide deletions. A single sequence section, *f*, generally follows any singular or repeated groupings of sections *a* through *e* and immediately precedes the late coding regions. Sequence section *f*, however, can experience nucleotide deletions. Section *f* is also occasionally incorporated into repeat structures with portions of the *f* sequence appearing between groupings of other repeated sections. Additionally, repeated sections of sequence *f* can appear in tandem, sometimes grouped along with late coding sequence. Regardless of these variations, inclusion of section *f* does not change our scheme. For this reason and purposes of simplification, our categorization of JCV regulatory regions will rely on the 5 sequence sections *a* through *e* only.

We have arranged all known variant JCV regulatory regions into quadrants according to two criteria: sequence section *b* and *d* integration; and tandem repetition of sequence section groups. The placement of regulatory regions into this design, or Compass (Figure 2), allows for a spatial comparison of the diverse structures, with forms sharing one of the aforementioned criteria occupying adjacent quadrants. This scheme defines 4 distinct structural forms (I-S, I-R, II-S, and II-R) along with the cellular and/or tissue tropism of each. As established in the previous literature, 2 major regulatory region types exist. The Compass preserves this division in respect to sequence section *b* and *d* integration. Sequences occupying the upper quadrants (type I) have no inserts. Sequences occupying the lower quadrants (type II) have inserts of at least a portion of the sequence from one of the sections *b* or *d*. The Compass, however, further divides each type into singular (S) and repeat (R) forms positioned in the left and right quadrants, respectively.

In comparison with the "descriptive" terms used in the previous literature, I-S structure (upper left quadrant) is identical to the most recently observed singular "ace" form that consists of sequence sections *a*, *c* and *e*, but is devoid of *b* and *d* (Elsner and Dörries, 1998; Ciappi *et al*, 1999; Jensen and Major, 1999; Ricciardiello *et al*, 2001). I-R structure (upper right quadrant) is similar, but represents the tandem repeats indicative of the "prototype" Mad-1 sequence (first JCV genomic sequence published, Frisque *et al*, 1984) and other closely related forms including Mad-4 (previously excluded from type I classification). II-S structure (lower left quadrant) illustrates the other singular forms (Yogo *et al*, 1990), commonly referred to as archetype (or "archelike" if nucleotide deletions occur), that feature integrated sequence sections *b* and *d*. II-R structure (lower right quadrant) describes forms that also feature some level of sequence section *b* and *d* integration, but also contain repeated sequence. II-R structures have generally been termed rearranged forms. The widespread variation within II-R structure is due to the possibility of numerous sequence section repeats and extensive nucleotide deletions, mainly from the repeated sequences. Previously, I-R and II-R structural forms (R-forms, right quadrants) have also been defined as PML types. *In vitro* transfection studies (Daniel *et al*, 1996; Mayreddy *et al*, 1996; Ault, 1997) have shown that I-R structures have enhanced levels of viral transcription and replication whereas complete II-S structure (archetype) is relatively inactive. The viral activity of I-S and incomplete II-S variants is more similar to that of complete II-S structure (archetype), but may vary greatly among the II-R variants.

Substantially more JCV regulatory region sequence data may be necessary to make a truly accurate Compass. However, this design provides useful bearings, including a working nomenclature, for the analysis of JCV regulatory region diversity. The Compass is a composite, in that it was arranged from all known sequences without regard for host status. In the future, as more sequence is available, it may be useful to construct separate Compasses for individual populations according to factors such as age, gender, genetic mutation, geographical location, immunocompetence, PML status, and incidence of other viral infections or disorders. Temporally arranged Compasses (e.g., pediatric, adolescent, adult) constructed from a

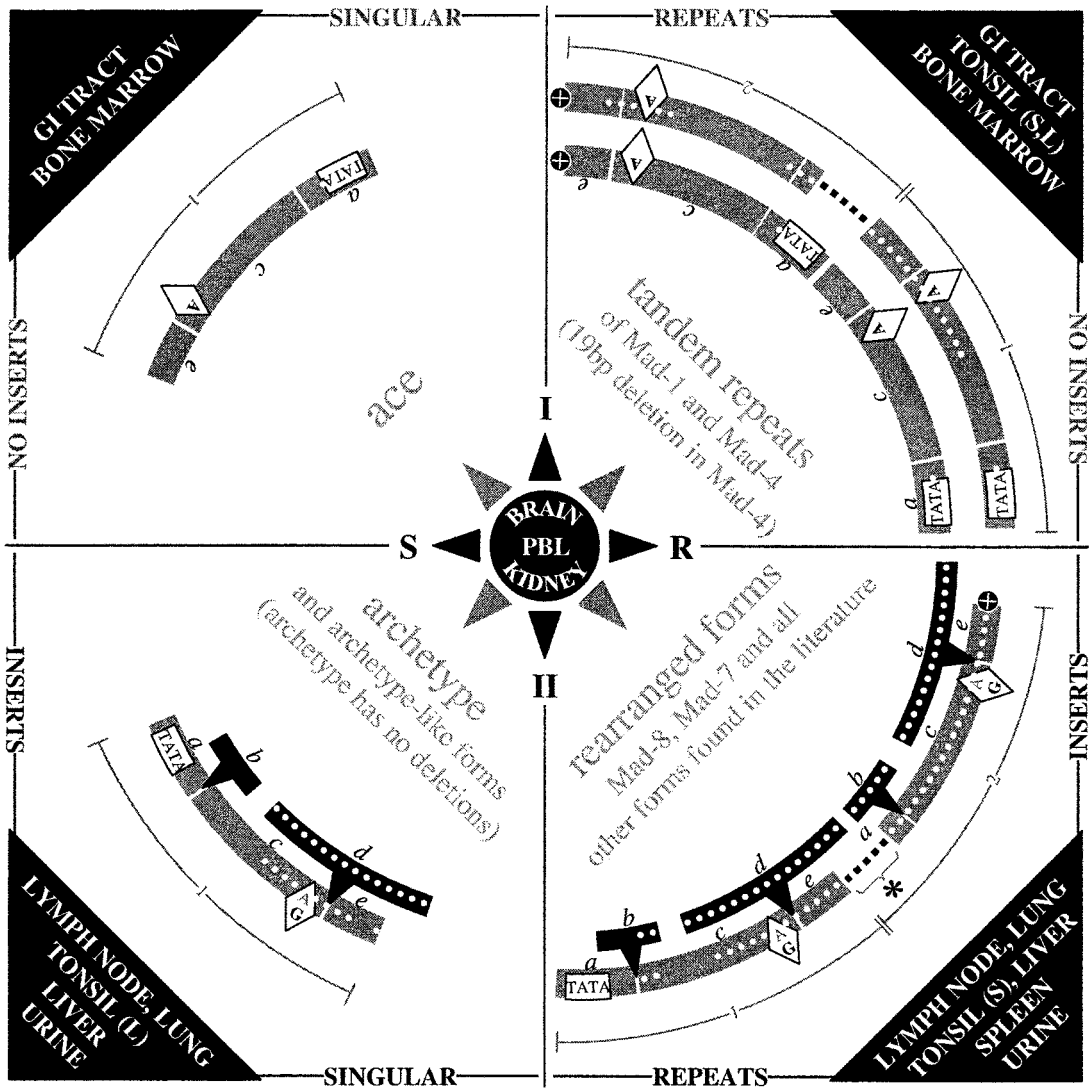

Figure 2 The Compass: A schematic diagram of the relationships between JC virus regulatory region sequences published worldwide. JC virus variant regulatory regions grouped into quadrants (I-S, I-R, II-S, and II-R) with *ace* sequence-units lightly shaded. Upper quadrant variant types (I) have no additional sequence integrated into the *ace* units (no inserts). Lower quadrant variant types (II) have dark integrated sequence sections (inserts), *b* (23 base pairs) and *d* (66 base pairs). Both types I and II are divided into singular (S) and repeat (R) forms by the left and right quadrants, respectively. Unshaded boxes are TATA boxes. Dots represent sites of possible base-pair deletions. Unshaded diamonds contain the base that occupies the 49th nucleotide of sequence section *c* (nucleotide number 85 of I-S, or 108 of II-S), which is adenine (A) in type I variants, but predominantly guanine (G) in type II variants. Right quadrants (R-forms) have dark dashes where sequence is deleted and ⊕ where additional repeats may occur. The * in lower right quadrant (II-R) identifies one reported sequence that retains the second TATA box (Ciappi *et al*, 1999). JCV tropism common to all variant regulatory region forms is contained in dark central circle. Specific JCV tropisms are contained in dark corner triangles. Cells from tonsil are either (L) lymphocytes, or (S) stromal cells (Monaco *et al*, 1998). Cells in bone marrow that contain JC virus have been identified as B-lymphocytes (Houff *et al*, 1988).

particular population (or comparative populations) could also be useful in elucidating aspects of JCV pathogenesis.

Observations from the JCV regulatory region compass

All forms have been found in the brain, PBL, and kidney (Elsner and Dörries, 1998; Sugimoto *et al*, 1998, reviewed in Jensen and Major, 1999; Ciappi *et al*, 1999; Newman and Frisque, 1999; Kato *et al*, 2000; Vaz *et al*, 2000). Only type II has been found in urine (D Chang *et al*, 1999; reviewed in Jensen and Major, 1999; Vaz *et al*, 2000), liver, lung, and lymph nodes (Newman and Frisque, 1999). Only II-R has been found in spleen (Newman and Frisque, 1999). Wherein tonsillar stromal cells only R-forms have been found, tonsillar lymphocytes have presented both I-R (Mad-4) and II-S (archetype) variants (Monaco *et al*, 1998). Only type I structures (usually I-R) have been found in bone marrow (Jensen and Major, 1999), and the mucosa (Laghi *et al*, 1999) and epithelial cells (Ricciardiello *et al*, 2001) of the gastrointestinal tract.

Irrespective of the variant, the JCV regulatory region is highly conserved in sequence sections *a*, *c*, and *e*. If repeat structures exist, sequence sections proximal to the origin of replication are the sequences most highly conserved. The proximal sequence section *a* is represented in every known variant. It is very rare to see deletions in sequence section *a*, although we have sequenced type I structures (S and R) from bone marrow of a PML patient that have the identical deletion of the final TATA box nucleotide (unpublished data). The early side of the proximal sequence section *c* is also represented in all variants. The late side of the proximal sequence section *e* is present in all but a few II-R variants in which this segment is represented by distal repeats. Clearly, the least-conserved sequence section is *d*, which is generally deleted, or at best incomplete, except for in whole II-S structure (archetype). An entire proximal sequence section *b* is commonly found in type II structures but is only guaranteed complete in II-S variants. All of these observations seem to be in agreement with the data that suggest sequence sections *a*, *c*, and *e* convey viral activity, and sequence sections *b* and *d*, to some extent, repress viral activity (Daniel *et al*, 1996; Mayreddy *et al*, 1996; Ault, 1997).

Importantly, if regulatory region variations define crucial steps in JCV pathogenesis, the Compass provides a framework to examine two basic questions: Do conversions between type I and type II regulatory regions occur; and in what direction do regulatory region conversions occur? After examining the Compass, it appears that conversions between I-S and I-R are likely to occur by either duplication of sequence sections, or deletion of these same sequence section repeats. Conversions between II-S and II-R are likely to follow a similar scenario. Both type I and type II conversions between S and R forms could proceed in either direction and do not appear restricted from reverting, or being subsequently altered into variants contained in previously occupied quadrants.

More complex would be the integration of sequence sections *b* and *d* into type I structures to meet type II criteria. Such conversions would involve crossover events, possibly with other portions of the JCV genome, DNA of other co-infecting viruses, or sites within the host genome (a complete sequence section *d* can be constructed from no more than four segments of the human genome). To convert in the opposite direction (II to I) would require the deletion of sequence sections *b* and *d*, and in all but a few variants, the modification of a nucleotide in sequence section *c* (nucleotide number 85 of I-S, or 108 of II-S; numbering adapted from Frisque *et al*, 1984) from guanine (G) to adenine (A). The few type II sequences with A at this nucleotide position could be considered bridge sequences between II-R and I-R (Mad-11; Martin *et al*, 1985) and between II-S and I-S (Yogo *et al*, 1990; Ault and Stoner, 1993). Another II-R sequence (Mad-1 with proximal section *b*; Ciappi *et al*, 1999) is an interesting example of a possible link, requiring only the deletion of sequence to fit I-R criteria.

However, there may be little crossover between type I and type II structures. If type I and type II are not directly related, this division may represent two separate circulating JCV strains. If there is more than one circulating strain, then it is conceivable that multiple start sites exist on the Compass due to co-infections. However, if type I to type II crossovers occur, the Compass poses no obvious endpoints and successive conversions could continue full circle, from one quadrant to the next, even overlapping previously occupied quadrants. Conversions that span 2 criteria (II-S to I-R/I-S to II-R) at once, however, seem less likely. Also, if a unique variant infects diverse cell types within an individual, it may be possible that numerous conversion routes occur within the same time frame, conceivably in opposite directions on the Compass.

Caveats and points of contention

The advent of PCR technology has greatly increased the ability to detect JCV DNA in human cells, tissues, and fluids. This sensitive amplification process has helped to expose a cellular host range more extensive than once thought. In turn, an expanded host range has provided logical explanations for the dissemination of JCV from peripheral sites of initial infection to target cells sequestered in the CNS. Understanding the definitive role of the JCV regulatory region in viral activity has led to numerous nucleotide-sequencing studies on this hypervariable component of the genome. Most current studies employ PCR amplification to furnish the DNA used for sequencing reactions. Although this technique has supplied an ever-increasing variety of regulatory region structures, it is unclear whether many of these nucleotide sequences represent successfully propagating virion, nonviable products of spurious viral replication, or adaptations geared for changes in tropism and/or latency. Without the isolation of intact virion as the source of template DNA, it is unknown if a PCR-generated regulatory region sequence was ever a genomic element incorporated into a JCV capsid.

Another approach, the propagation of biopsy-derived virion *in vitro*, is not only difficult but may also foster the selective growth of variants best suited for the given culture system (Frisque *et al*, 1984). From another viewpoint, culture systems undoubtedly lack the complexity of *in vivo* environments that may provide a number of critical variables for the propagation of particular variants. Also, many cultured cell lines are transformed by the constitutive expression of viral proteins (Hara *et al*, 1998) that can convey activity to otherwise inactive forms of the JCV regulatory region. In addition to these concerns surrounding *in vitro* viral expansion, propagation of JCV in culture systems is a slow process. Even in target human glial cells (HFGC), the most robust of variants

require 5 to 7 days before viral DNA replication is detectable.

It is not clearly discerned from the Compass that although all forms of JCV regulatory region are found in the brain, PBL, and kidney, the R-forms seem to be more prevalent in the brain, whereas type II structure (especially II-S) seems to be more prevalent in the kidney. For this reason, the R-forms were previously defined as 'PML types.' S-forms, however, are also commonly isolated from the CNS of PML patients, but not exclusively. Additionally, I-S structure has always been found in conjunction with I-R. As with our unpublished data mentioned previously and other similar reports (Elsner and Dörries, 1998; Ricciardiello *et al*, 2001), the co-isolated I-S and I-R forms can have identical nucleotide anomalies indicative of directly related structures. Type I variants are the only forms that have been isolated from human tumors (Rencic *et al*, 1996; Ricciardiello *et al*, 2001), suggesting that enhanced levels of JCV transcription and/or replication can translate into the disruption of normal cellular growth cycles.

Another point not made clear by the Compass is that lymphocytes are present in all the tissues examined. Many tissues may be positive for JCV only due to the infiltration, or persistence of infected lymphocytes. Therefore, there is clearly a need for the phenotypic identification of infected cells in JCV-positive tissues to get an accurate perspective of JCV tropism (Houff *et al*, 1988). Lymphocytes, thought to be ideal vehicles of JCV dissemination may also be responsible for regulatory region modifications. Specifically, B-lymphocytes are capable of performing complex genomic rearrangements required for the production and alteration of antibodies (Ault and Stoner, 1993). Interestingly, in one report (Vacante *et al*, 1989), a simian vacuolating virus 40 (SV40) regulatory region was distally fused into the regulatory region of a JCV I-R variant (Mad-1) genome. This chimera was transfected into HFGC that subsequently produced JC virions with enhanced growth characteristics, including a doubling in the rate of virion production as compared with Mad-1. Examination of the regulatory region within these enhanced virions revealed a 294-bp deletion of the medial portion that included the late side of the Mad-1 sequence and the early side of the SV40. This modified regulatory region now contained only the proximal Mad-1 sequence sections *a* and *c*, followed by the late 33 bp of the proximal SV40 72-bp enhancer repeat and the entire distal SV40 72-bp enhancer repeat. Some implications drawn from this report are that a JCV regulatory region may be capable of mutating to complement the nuclear environment of the host cell and that such changes may alter JCV activity.

Considering the points addressed previously, our classification scheme provides a tool for navigating current questions about JCV pathogenesis as related to regulatory region variation: Are all variants derived from one; which variant(s) initiate infection; and after initial infection in which direction(s) do modifications proceed? In one report where entire JCV genomes were sequenced from PML brain tissue, variants with diverse regulatory regions showed no modification of Vp1 sequence, suggesting that these variants were directly related (Kato *et al*, 2000). Much of the literature states that all JCV variants are derived from complete II-S structure (archetype). Whereas complete II-S (archetype) is excreted in the urine (Yogo *et al*, 1990; reviewed in Jensen and Major, 1999) and found in sewage (Bofill-Mas *et al*, 2000), it does not show infectious activity *in vitro* (Ault, 1997; Daniel *et al*, 1996; Mayreddy *et al*, 1996). The latter characteristic may exclude complete II-S (archetype) from establishing initial infections unless it uses activators transcribed by other co-infecting viruses. II-R structures have been found in urine and can show infectious activity *in vitro*. Screening of a pediatric PML case (Newman and Frisque, 1999) revealed a unique II-R variant in all tissues tested that was devoid of sequence section *d*. Plausibly, this II-R form may have been the variant that established the initial infection, but in this case did not experience any modifications. If deletion of the repeated sequence group, or acquisition of section *d* had occurred, this variant may have persisted latently. Type I variants are not found in excretions but show enhanced infectious activity *in vitro*. Type I variants, therefore, must be considered transmissible/infectious forms when there is more than casual contact between humans.

Most questions surrounding the relationships between JCV regulatory region variation and viral pathogenesis remain unanswered. The Compass format, however, provides a useful classification scheme that may facilitate greater understanding of past and future data. The nomenclature established here provides logical connections between the variant regulatory regions and may be useful for elucidating crucial steps in JCV pathogenesis.

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