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Rearrangement patterns of JC virus noncoding control region from different biological samples

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The JC virus (JCV) is generally considered the etiological agent of progressive multifocal leukoencephalopathy (PML), a demyelinating brain illness, often associated with immunosuppression and significantly frequent in acquired immunodeficiency syndrome (AIDS) patients. The primary infection by JCV is usually asymptomatic and the virus can remain in a latent status in the kidney. As a consequence of immunological alterations of the host, the virus can show a genetic variability in the noncoding control region (NCCR) due to deletions, duplications, and insertions as compared with the archetype. The NCCR of the archetype strain can be divided into six regions, named boxes A to F. In this study, the authors evaluated the presence of the JCV genome in different biological samples, such as urine, peripheral blood mononuclear cells (PBMCs) and cerebral spinal fluid (CSF) by means of polymerase chain reaction (PCR). After sequencing of the PCR fragments, the NCCR structure of isolated JCV strains was analyzed in order to verify the presence of different viral variants. An analysis of the homology and of the multiple alignment of the obtained sequences in comparison with the archetype strain has been carried out. The results indicated the presence of different rearrangements among the analyzed samples. Whereas in the urine, the NCCR structure always appeared very similar to that of the archetype, in the PBMCs and CSF, the NCCR sequences showed specific and characteristic rearrangements as compared to the archetype. These different rearrangements could be correlated with the emerging of an NCCR organization more suitable for the development of PML. *Journal of NeuroVirology* (2003) **9,** 603–611.

Keywords: JCV; NCCR; nucleotide sequence; rearrangement patterns

Introduction

The human polyomavirus JC (JCV) is the etiological agent of progressive multifocal leukoencephalopathy (PML), a central nervous system (CNS) demyelinating disease, occurring as a late and fatal complication of persistent or primary infection with JCV in the course of a basic immunosuppressive disease (Weber and Major, 1997). JCV is an ubiquitous virus that infects at least 70% of the human population and could persist lifelong in humans (Dörries *et al*, 2003). Other studies have also provided results suggesting a relatively high prevalence of JCV viruria in an immunocompetent person (Markowitz *et al*, 1993). Immunodeficiencies that occur in conjunction with leukemia, human immunodeficiency virus (HIV) infection, and organ transplantation can contribute to JCV activation, leading to the fatal demyelination observed in cases of PML (Bagnato *et al*, 2001).

Prior to the acquired immunodeficiency syndrome (AIDS) epidemic, PML cases were uncommon. Afterwards, PML affected approximately 3% to 8% of HIV-positive patients, with a mortality rate of 2% to 4%. With the development of highly active antiretroviral therapies (HAART), the incidence of PML and of the correlated mortality appears to be in decline (Berger, 2000).

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In addition to the CNS and urine, JCV has been detected in peripheral blood lymphocytes (PBLs), kidney, tonsil, lung, liver, spleen, lymph node, bone marrow, and gastrointestinal tissues (Elsner and Dörries, 1998; Newman and Frisque, 1999; Ricciardello *et al*, 2001).

The organs that harbor the virus during latency remain controversial, though the kidney, brain, and PBLs seem to be involved. The relationship between JCV infection in the kidney and the brain is unclear, and the mechanism for reactivation remains virtually unknown.

As a consequence of immunological alterations of the host, the JCV genome undergoes an adaptation process in which the noncoding control region (NCCR) of the archetype strain is rearranged by deletion and duplication events that give rise to more active variants with altered tissue tropism (Flaegstad *et al*, 1991; Ault and Stoner, 1993). The hypervariable JCV NCCR is an approximately 300-base pair (bp) noncoding sequence positioned between the early and late protein-coding sequences in the circular, supercoiled, double-stranded viral DNA genome and can be divided into six regions, named boxes A (36 bp), B (23 bp), C (55 bp), D (66 bp), E (18 bp), and F (69 bp), each one containing binding sites for cellular factors involved in viral transcription (Figure 1). Circulating JCV might persist in the kidney with an archetypal NCCR structure and in other tissues with a different rearranged NCCR structure. In fact, the NCCR structure isolated from urine samples is highly conserved in healthy and immunocompromised individuals and appears always very similar to that of the archetype (Agostini *et al*, 1996; Elsner and Dörries, 1998).

In our study, we evaluated by means of polymerase chain reaction (PCR) and sequencing the presence of the JCV genome in different biological samples, such as urine, peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid (CSF) from HIV-positive and -negative subjects with or without PML. We also analyzed the NCCR structure of isolated JCV strains in order to verify the presence of different viral variants. An analysis of the homology and of the multiple alignment of the sequences obtained in comparison with the archetype strain has been carried out.

Results

We have analyzed 47 urine samples of hospitalized patients aged from 33 to 75 years, including 32 HIV-negative and 15 HIV-positive patients. The presence of JCV NCCR was evaluated by means of nested-PCR. Results obtained showed that the JCV genome was present in 33.3% (5/15) of HIV-positive patients, whereas the viral DNA was present only in 6.2% (2/32) ($P = .026$) of HIV-negative subjects.

The PCR fragments from urine samples were sequenced and analyzed by Blast and Clustal W, comparing the JCV NCCR structure found in urine to that of the archetypal structure. The sequences of the NCCR found in the urine samples of both HIV-positive and HIV-negative subjects were identical to the archetype except for some point mutations

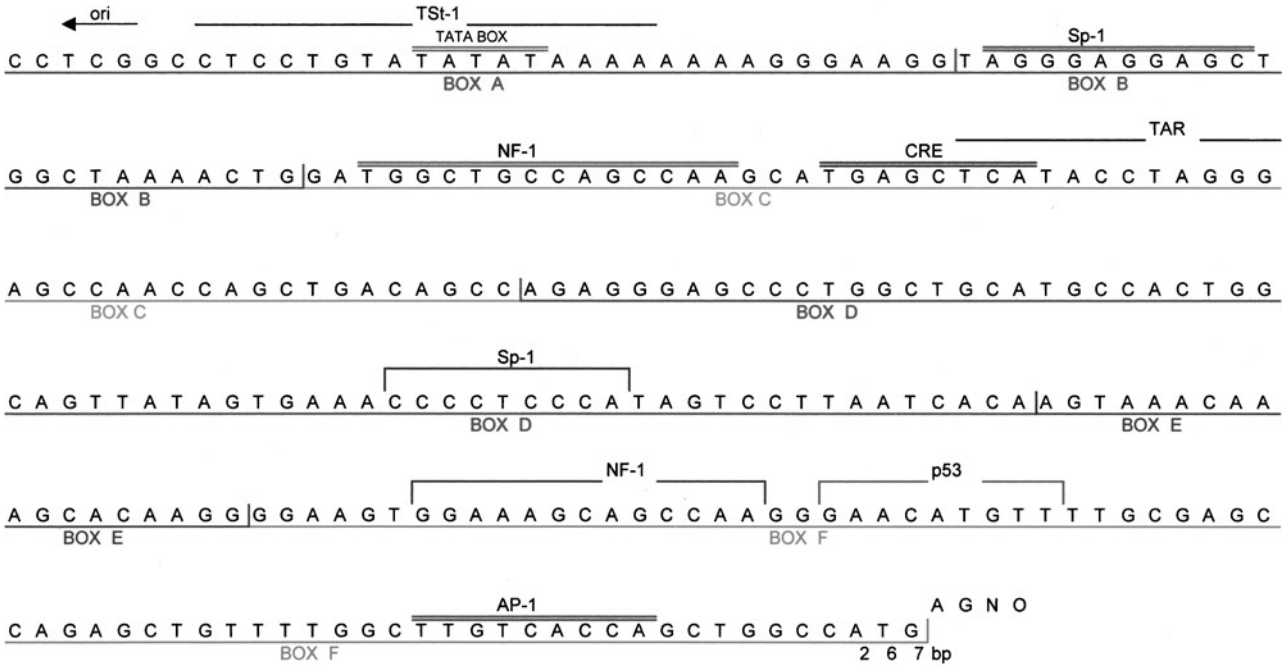

Figure 1 Schematic structure of JCV archetypal NCCR with boxes A to F, the main promoter binding sites are depicted.

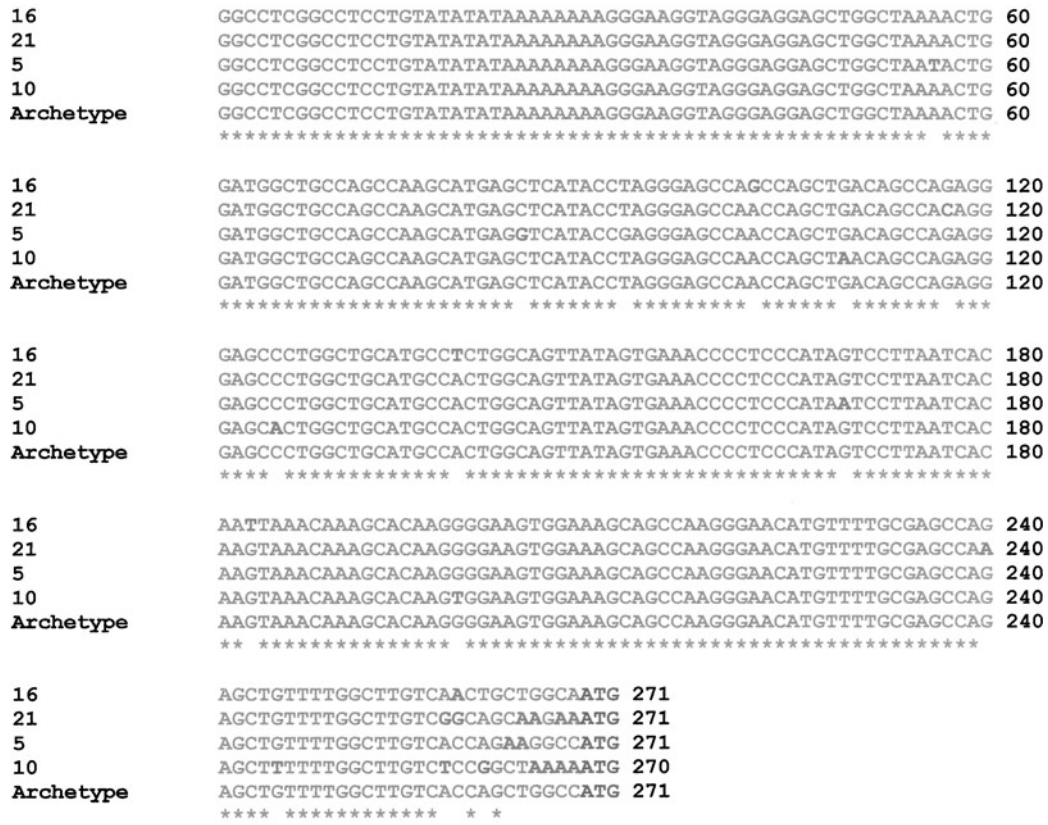

Figure 2 Multiple alignment with Clustal W of NCCR-JCV sequences of urine samples from HIV-positive (5 and 10) and HIV-negative (16 and 21) subjects with archetype. The figure shows an example of sequences with point mutations representative of all urine samples. (Point mutations are indicated in red.)

(Figure 2). With regard to PBMCs, we have analyzed 63 samples from hospitalized patients (33 HIV-negative and 30 HIV-positive) by means of nested-PCR for NCCR. In HIV-positive subjects, the JCV genome is present in 33.3% (10/30) of analyzed samples (5/10 with PML), whereas in HIV-negative subjects, the viral DNA is present only in 15.1% (5/33) (2/5 with PML). Although it is not a statistically significant data ($P > .05$), the presence of JCV DNA was higher in HIV-positive subjects (33.3% in HIV-positive and 15.1% in HIV-negative). The sequences of the NCCR found in PBMC samples of HIV-positive and -negative subjects with and without PML showed many deletions and duplications as compared with the archetype (Figure 3). In HIV-positive and -negative subjects without PML, the sequences found in the PBMCs showed the deletion of boxes B and D, which contain an Sp-1 binding site, and partial repetitions of box C, which contains several enhancer elements, including an NF1 binding site, a CRE-like element, and a TAR site (Figure 3, lane 1). In particular, in HIV-positive subjects with PML, we observed partial repetitions of box C and the loss of box D. In one isolate, partial duplication of box B, which contains an Sp-1 binding site, and box F, which contains NF-1, p53, and AP-1 binding sites, was found (Figure 3, lane 2).

The mutations found in sequences isolated from PBMCs of HIV-negative patients with PML mainly showed duplications of box C. Region A, containing the TATA box, was consistently retained (Figure 3, lanes 4 and 5).

Finally, we have analyzed 38 samples of CSF from hospitalized patients (15 HIV-negative and 23 HIV-positive) by means of nested-PCR for the NCCR. In HIV-positive subjects, the JCV genome is present in 34.7% (8/23) of the analyzed samples (5/8 with PML), whereas in HIV-negative subjects, the viral DNA is present only in 20% (3/15) of the analysed samples (2/3 with PML).

The sequences of the NCCR found in CSF samples of HIV-positive and -negative subjects, without and with PML, showed many deletions and duplications, as compared with archetype (Figure 4). In HIV-negative subjects without PML, the sequence found in the CSF showed characteristic rearrangements: box C was duplicated, box F was partially repeated, and box D was deleted (Figure 4, lane 1). In HIV-positive subjects without PML, we observed that box C was duplicated and box D was not conserved; only in one subject were duplications of boxes A and B found (Figure 4, lanes 2 and 3).

In HIV-positive subjects with PML, we found many duplications of box C and the deletion of box B

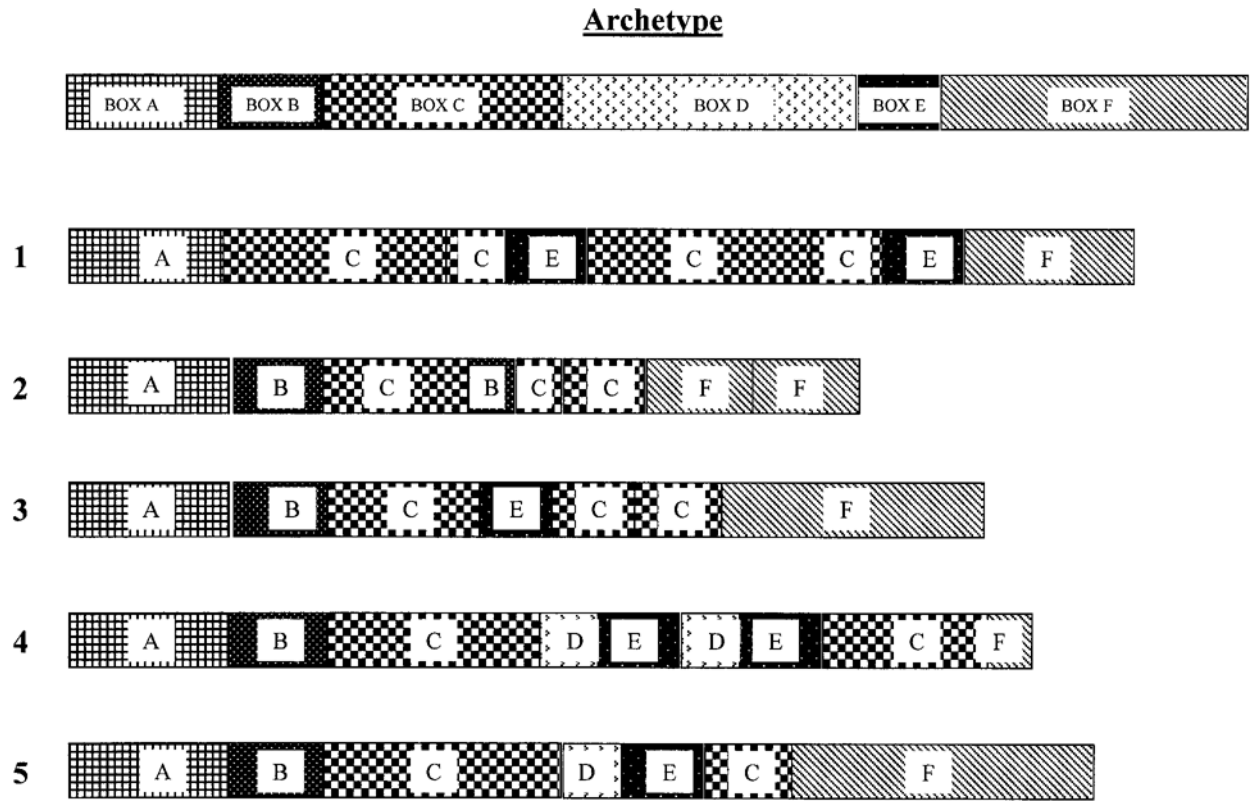

Figure 3 Comparison of archetypal NCCR structure to consensus sequences isolated from four PBMC samples of HIV-positive and -negative subjects without PML (lane 1), to a sequence from a HIV-positive patient with PML (lane 2), to the consensus sequence obtained from four PBMC samples of HIV-positive subjects with PML (lane 3), and to sequences from HIV-negative individuals with PML (lanes 4 and 5). Boxes are depicted maintaining the proportion of nucleotide sequence length.

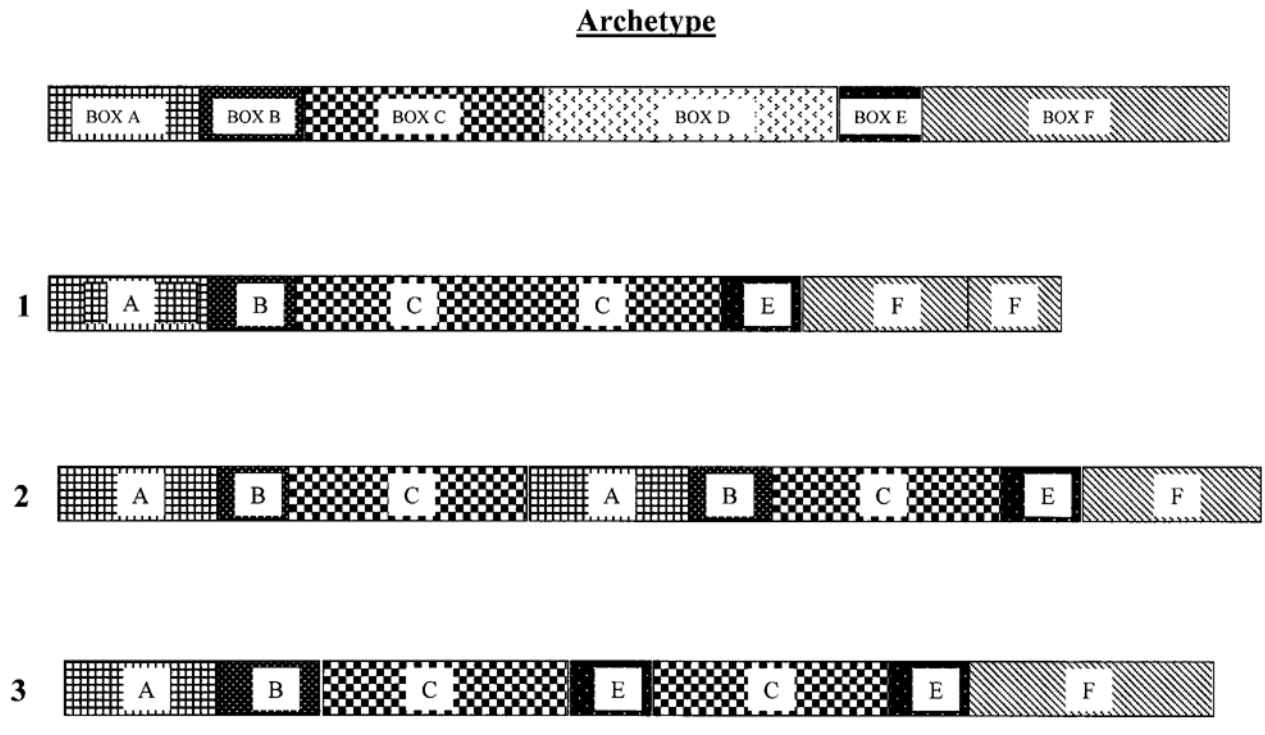

Figure 4 Comparison of archetypal NCCR structure to sequences isolated from CSF samples of an HIV-negative subject without PML (lane 1) and of an HIV-positive subject without PML (lane 2). Lane 3 reports the consensus sequences obtained from CSF samples of two HIV-positive subjects without PML. Boxes are depicted maintaining the proportion of nucleotide sequence length.

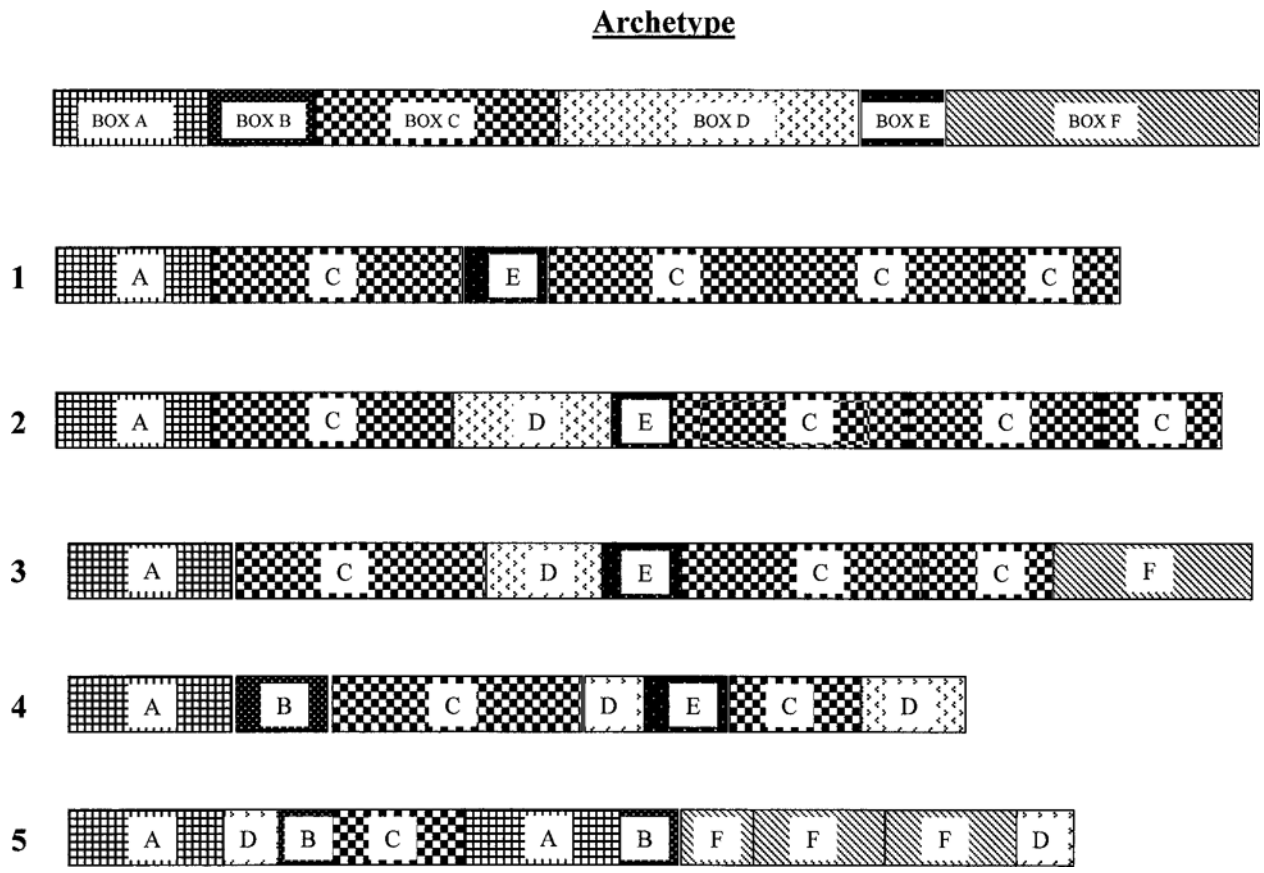

Figure 5 Comparison of archetypal NCCR structure to sequences from CSF sample of an HIV-positive subject (lane 1), to the consensus sequences obtained from each of two HIV-positive subjects (lanes 2 and 3), and to the sequences from HIV-negative subjects with PML (lanes 4 and 5). Boxes are depicted maintaining the proportion of nucleotide sequence length.

(Figure 5, lane 1), and in the majority of the analyzed sequences, the comparative analysis allowed us to form two "consensus" sequences from four samples, with the deletion or the presence of box F (Figure 5, lanes 2 and 3).

Finally, in HIV-negative subjects with PML, we found particular rearrangements within the analyzed sequences, with the exception of box D, constantly repeated (Figure 5, lanes 4 and 5). Moreover, only in one subject were duplications of box A and partial repetitions of box F found (Figure 5, lane 5).

Interestingly, the analysis of JCV NCCR sequences found in the same subjects from different type of samples showed the presence of more than one NCCR structure. In fact, the NCCR structures found in urine samples were identical to the archetype except for some point mutations (see Figure 2), whereas different NCCR structures were found in PBMC and CSF samples. As shown in Figure 6, the sequences obtained from the PBMCs and CSF from the same subject (patients a, b, c and d) were rearranged in a completely different way, independently from HIV-1 seropositivity or PML disease. In particular, the duplication of box C was the only characteristic common to all reported sequences, irrespective of the type of samples.

Discussion

As reported above, after immunological alterations of the host, the JCV genome undergoes an adaptation process in which the NCCR of the archetype strain is rearranged by deletion and duplication events, generating variants that could be more active and could lead to a new tissue tropism (Ault and Stoner, 1993).

Our results indicate that JCV DNA has been found in different tissues, with a higher prevalence in immunocompromised subjects. Moreover, the NCCR structure changes in relation to the anatomical sites and to the type of analyzed samples.

As also reported by other authors (Ciappi *et al*, 1999), the analysis of NCCR sequences showed the presence of more than one NCCR structure within the same subject. In fact, the NCCR structure found in urine samples is identical to the archetype except for some point mutations, whereas different NCCR structures can be found in PBMC and CSF samples. As far as the single patient is concerned, it may be worthwhile to underline that the three samples (urine, PBMC, and CSF) have been taken contemporaneously from the same patient, thus our results give an exact picture of the presence of JCV DNA at that moment.

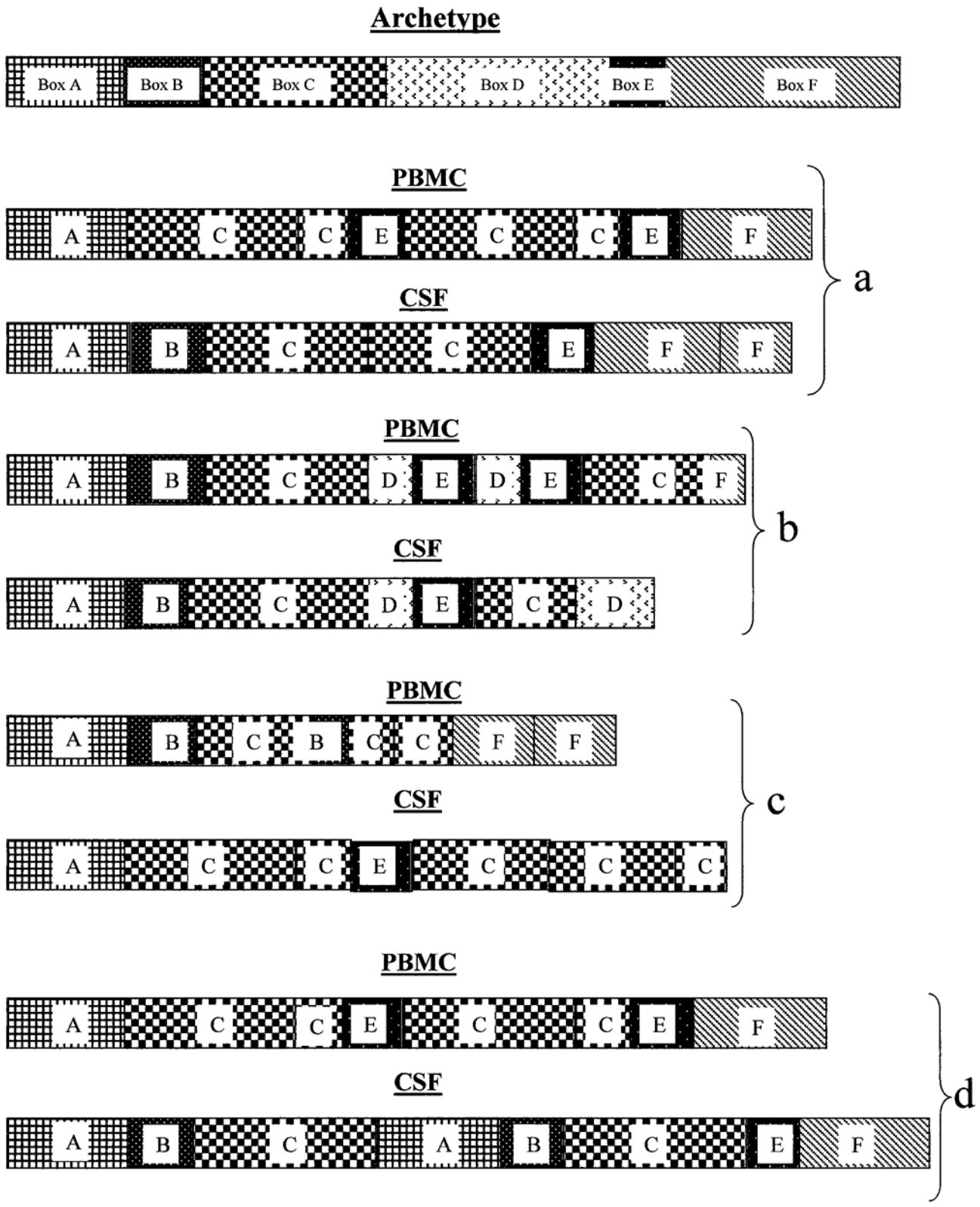

Figure 6 Comparison of archetypal NCCR structure to sequence rearrangements found in PBMCs and CSF from the same patient (a, b, c and d). (a) HIV-negative patient, without PML; (b) HIV-negative patient, with PML; (c) HIV-positive patient, with PML; (d) HIV-positive patient, without PML. Boxes are depicted maintaining the proportion of nucleotide sequence length.

With regard to PBMCs of all patients without PML (HIV-positive and -negative), the comparative analysis of our sequences allowed us to form a "consensus" sequence of JCV NCCR, whereas in PBMCs of PML patients, we observed different rearrangements, characterized by the repetition of box C, especially in the HIV-positive patients. The multiple duplications of box C have been found also in CSF of HIV-positive subjects, with PML.

It is known that the box C contains NF1 binding site, CRE-like element, and upTAR site. CRE and NF-1 are coactivators for GTF and TAF proteins and have been associated with JCV expression in glial cells (Kumar *et al*, 1996; Gronostajski, 2000). NF-1 has been associated with JCV's ability to infect human fetal glial cells, the cells in which JCV replicates most efficiently (Summer *et al,* 1996). Moreover, transfection of an expression clone of NF-1 class D, in HeLa cells, was able to activate the JCV early promoter in cells that are normally nonpermissive to infection (Shinohara *et al*, 1997; Monaco *et al*, 2001).

Although some studies (Sala *et al*, 2001) do not support the hypothesis that particular NCCR variants actually represent additional risk factors for the occurrence of PML in immunocompromised individuals, the higher incidence of PML among individuals with AIDS compared with other immunocompromised patients implies that the presence of HIV type 1 (HIV-1) in the brains of infected individuals may directly contribute to the pathogenesis of this disease. In support of this model, earlier *in vitro* studies have indicated direct intercommunication between HIV-1 and JCV through the HIV-encoded regulatory protein Tat (Daniel *et al*, 2001). An upstream Tat-responsive DNA element (upTAR) of JCV has been shown to be important for HIV-1 Tat stimulation of the JCV late promoter. Specifically, Tat enhances the ability of Purα to bind the upTAR element and thus synergistically activates transcription (Krachmarov *et al*, 1996; Wortman *et al*, 2000).

Moreover, HIV Tat was detected in various infected cells as well as in uninfected oligodendrocytes in tissue from HIV-1–positive patients, supporting the earlier *in vitro* findings that secreted Tat from the infected cells can be localized in neighboring uninfected cells. The presence of Tat in oligodendrocytes is particularly interesting as this protein can up-modulate JCV gene transcription. Furthermore, JCV T antigen has been demonstrated in oligodendrocytes where Tat has also been seen (Valle *et al*, 2000).

These data provide *in vivo* evidence for the role of HIV-1 Tat in the pathogenesis of AIDS/PML by acting as a positive regulatory protein that affects the expression of JCV. In support of these data, our results show the presence of partial or total duplications of box C in HIV-positive subjects with PML, generating especially the duplication of the upTAR element. In particular, it could contribute to the possible synergism between HIV-1 and JCV in the CNS through the HIV-encoded regulatory protein Tat (Daniel *et al*, 2001).

Finally, our data reported that in all CSF samples of HIV-negative subjects with PML, box B was always retained. In fact the presence of the binding site for the cellular transcriptional factor Sp-1 is essential for activating the JCV early promoter in both glial and nonglial cells (Henson, 1994). Interestingly, Sp-1 is associated with Purα and regulates the myelin basic protein gene promoter (Tretiakova *et al*, 1999b). One of the factors contributing to demyelination in PML may be the down-regulation of myelin basic protein transcription by the JCV T antigen (Tretiakova *et al*, 1999a; Richardson-Burns *et al*, 2002). In fact, T antigen may quench the Purα factor and inhibit its activation potential for the myelin basic protein gene promoter. A possible explanation of our results is that the presence of box B could allow JCV to complete its replication cycle, in absence of HIV-1 Tat.

We can conclude that the rearrangements are generated in the host from a basic archetypal sequence and the sites of strand breakage are not random; probably these NCCR rearrangements relate to the immunocompromised state of the subject and the tissue's microenvironment. Therefore, our data support the hypothesis that the JCV genome undergoes an adaptation process, which occurs during an immunocompromising event contributing to the reactivation of a latent kidney infection, and that the severe impairment of immunocompetence plays a crucial role in the development of PML.

Materials and methods

Samples

The analyzed samples included (a) 47 urine samples of hospitalized patients aged from 33 to 75 years (32 HIV-negative and 15 HIV-positive); (b) 63 samples of PBMCs of hospitalized patients aged from 25 to 67 years (33 HIV-negative and 30 HIV-positive), and (c) 38 samples of CSF of hospitalized patients aged from 30 to 75 years (15 HIV-negative and 23 HIV-positive).

All the specimens were collected from patients according to the attending physician and in some cases, it was possible to obtain all the three different samples from the same patient.

Sample collection

Cerebrospinal fluid: CSF was obtained by lumbar puncture and immediately treated. CSF samples were heated at 95°C for 5 min and microcentrifuged for 10 s. The supernatant was collected and stored at −20°C until PCR amplification.

Urine specimen: One milliliter of urine was microcentrifuged for 2 min. The sediment was washed with phosphate-buffered saline (PBS) and resuspended in 100 μl of distilled water. After heating at 95°C for

5 min and spinning in a microcentrifuge for 10 s, the supernatant was collected and stored at −20°C until PCR amplification (Jin *et al,* 1995).

Peripheral blood mononuclear cells: PBMCs were isolated from whole heparinized blood samples by gradient centrifugation (Boyum, 1968), washed twice in PBS, and stored at −20°C.

DNA preparation and PCR conditions

All the samples were incubated in lysis buffer (0.5 M TRIS, 0.02 M EDTA, 0.01 M NaCl, 1% SDS) and 200 μg/ml proteinase K at 55°C for 24 h. Digestion was followed by pheno-lchloroform extraction and ethanol precipitation.

DNA yield was determined by measuring its concentration in the eluate by absorbance at 260 nm and then 0.1 to 1 μg of total DNA was directly used in PCR amplification.

β-Globin PCR was performed on extracted DNA to assess the efficacy of nucleid acid extraction (Saiki *et al,* 1985). General precautions, conditions for PCR analysis, and nested-PCR procedures were performed as published (Pietropaolo *et al,* 1998; Degener *et al,* 1999). β-Globin–positive samples were amplified in a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Emeryville, CA), and all assays included positive (purified recombinant plasmid DNA) and negative (all the PCR components except the template) controls to exclude false-positive and false-negative results (Kwok and Higuchi, 1989). Nested-PCR employed two pairs of primers that anneal to the invariant regions flanking the NCCR of JCV (Pietropaolo *et al,* 1998; Degener *et al*, 1999). Primers BKTT1 (5′-AAG GTC CAT GAG CTC CAT GGA TTC TTC C-3′) and BKTT2 (5′-CTA GGT CCC CCA AAA GTG CTA GAG CAG C-3′) amplified a 724-bp DNA fragment in JCV (Mad-1) (Flaegstad *et al,* 1991). The second pair, JC1 (5′-CCT CCA CGC CCT TAC TAC TTC TGA G-3′) and JC2 (5′-AGC CTG GTG ACA AGC CAA AAC AGC TCT-3′) amplified a portion of the first round PCR product, generating a fragment of 308 bp (Markowitz *et al,* 1993). Two nanograms of recombinant pGem-1 plasmid DNA containing the complete JCV genome, cloned as EcoR1 fragments, were used as positive controls. The PCR products were analyzed on 2% agarose gels by ethidium bromide staining.

Sequencing of the JCV NCCR

The PCR product corresponding to JCV NCCR was purified prior to sequencing to remove the excess of primers with QIAquick PCR purification kit, according to QIAGEN protocol (Degener *et al*, 1999). DNA sequencing was performed by automatic DNA sequencer (Applied Biosystem, model 370 A), according to the manufacturer's specifications (Amplicycle Kit, Applied Biosystem). Sequences were organized and analyzed using the Genetic Computer Group sequence analysis software package.

Data analysis

Statistical analysis was performed utilizing the Chi-Square test. *P* values of less than .05 were considered statistically significant.

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