Isolation and characterization of a type II JC virus from a brain biopsy of a patient with PML

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Brain tissue of a patient with multiple myeloma suffering from neurological disorders similar to those seen in progressive multifocal leukoencephalopathy (PML) patients was evaluated for the presence of the papovavirus, JCV. Results from polymerase chain reaction (PCR) revealed the presence of JCV with structural organization at the control region which is distinct from well-characterized isolates, ie Mad-1 and Mad-4. The control region of the new isolate, named JCVPhila-I, contains a 22 nucleotide insertion which separates the TATA box from the NF-1 regulatory motif. Only 18 nucleotides of the insert are duplicated in the second copy of the enhancer/promoter of the new isolate, which is 84 nucleotides in size. Results from a transcription assay indicate a modest elevated level of JCVPhila-I early promoter activity compared to that of JCVmad-4 in glial cell lines. The basal and T-antigen-induced transcriptional activities of the JCVPhila-I late promoter was lower with respect to Mad-4 late gene activity in glial cells. Of particular interest was the observation that in the cells producing the early protein, T-antigen, JCVPhila-I DNA replicated more efficiently than the Mad-4 DNA. These results suggest that the alterations seen in the JCVPhila-I control region may differentially influence early and late gene expression and facilitate amplification of the viral genome in cells derived from the CNS.

Keywords: neurodegenerative disease; neurotropic polyomavirus

Introduction

The polyomavirus, JCV, is a common neurotropic virus which was first isolated from brain tissue of a patient with the neurodegenerative disease, progressive multifocal leukoencephalopathy (PML) (Padgett et al, 1971). PML is a subacute demyelinating disease occurring almost exclusively in immunocompromised patients (Astrom et al, 1958; Richardson, 1961). The most prominent neurological deficits of PML patients include dementia, hemiparesis, and cortical blindness (Astrom et al, 1958; Richardson, 1961; Padgett et al, 1971; Walker and Padgett, 1983; Walker, 1985). Pathologically the disease is identified by demyelination, loss of oligodendrocytes, astrocytosis with bizarre multi-nucleated giant cells, and macrophage infiltration (ZuRhein, 1969). Characteristically affected oligodendrocytes in the lesions have enlarged nuclei with a loss of normal chromatin and gain of inclu-

sions that have been seen to represent viruses in electron photomicrographs.

JCV shares significant genome homology, in particular in the protein coding regions with other polyomaviruses including SV40 and BKV, however, unlike these viruses, JCV has a narrow host range and tissue tropism which restricts viral replication to glial cells. Several lines of evidence indicate that the restricted tissue specificity of JCV is determined at least in part by the viral control region which exhibits the greatest degree of divergence from those of other polyomaviruses (Martin and Foster, 1984; Martin et al, 1985; Kenney et al, 1984; Loeber and Dorries, 1988; Ault and Stoner, 1993). Analysis of viral gene expression by transient transfection and in vitro transcription assays led to the identification of several tissue-specific and ubiquitous transcription factors which, by binding to the viral regulatory sequences, participate in glial-specific expression of the viral genome (reviewed in Frisque and White, 1992). Comparison of the regulatory sequences from a number of JCV isolates revealed hypervariability in the structural organization of the
viral enhancer/promoter sequences for the viral early and late genes. These variations include deletions and duplications that appear to segregate the viruses into two types or classes (Frisque and White, 1992). The control region of the type I virus is characterized by the presence of a 98 base pair tandem repeat, each containing a TATA box in juxtaposition with a pentanucleotide repeat, AGCGAACGGA, and the NF-1 motif. The Mad-1 strain, the most studied JCV strain, belongs to the type I class. The regulatory region in type II strains differs from that of type I viruses in several aspects: (1) the duplication tends to vary in size in which the distal repeat with respect to the origin of replication lacks the TATA sequence; (2) there tends to be a 23 bp insertion within the proximal repeat usually at nucleotide position 36 (based on the JCV_Mad-1 sequence). JCV_Mad-4, which exhibits oncocytic properties in experimental animals, lacks the second TATA sequence observed in the type II strains and is devoid of the 23 bp insertion found in this type. Although the biological significance of these variations in the tissue-specific expression of viral genes, and replication of the viral DNA in the permissive cells are not well understood, it is generally accepted that both type I and type II classes (including JCV_Mad-1) are derived from the archetype strains of JCV which have been detected only in kidney tissue (Loeber and Dorries, 1988; Yogo et al, 1991, 1990). It is, therefore, postulated that deletions and duplication of an archetype regulatory region may result in a virus with the capacity to replicate in glial cells.

In this study we have isolated a strain of JCV from the brain biopsy of a patient suffering from neurological disorders similar to PML with the underlying immunosuppressive disease, multiple myeloma. Structural analysis of the viral regulatory sequence revealed that this isolate belongs to class II of JCV with distinct variations. Electron photomicrographs demonstrate an accumulation of viral particles in the nuclei of oligodendroglial cells. Examination of viral early and late gene transcription by a transient transfection assay revealed a variation in the transcriptional activity of the early and the late genes of the new isolate, termed JCV_Pilha-1, with reference to the JCV_Mad-4 in glial and non-glial cells. Moreover, results from a replication assay indicated that JCV_Pilha-1 DNA replicates more efficiently than JCV_Mad-4 in the permissive cells. Of particular interest was the notion that the new strain was able to replicate at low but detectable levels in a cell line derived from primate kidney tissue.

Results

Clinical and pathological analyses
A 66-year-old woman with a 2 month progression of short term memory and cognitive deficits exhibited visual apraxia, weakness in the left lower and upper extremities, decreased pain, temperature and vibration sense in the lower extremities, pronator drift, extinction to double simultaneous stimulation, decreased graphesthesia, point localization and two point discrimination all on the left side. Moreover, Babinski’s sign was positive on the left and there was diffuse increase in muscle tone without fasciculations. Of note is that the patient’s past

Figure 1 Magnetic resonance imaging (MRI) scan of the brain. Right parasagittal section of a T2-weighted MRI (left panel). The arrow demonstrates the position of an irregular area of hypointensity in the parietal lobe without mass effect. Axial section of a T2-weighted MRI (middle panel) and a T1-weighted MRI (right panel). A hyperintense signal in the right parietal lobe extends across the splenium of the corpus callosum into the left hemisphere as shown by arrow.
medical history was significant for multiple myeloma with focal vertebral and skull involvement. The magnetic resonance imaging (MRI) scan of the brain revealed a right sided parietal white matter lesion that extended across the splenium of the corpus callosum and into the left parietal lobe (Figure 1). The patient underwent a stereotactic needle biopsy of the involved right parietal lobe. Light microscopic evaluation of the biopsy showed an active demyelinating lesion characterized by a decrease in the number of normal appearing oligodendroglia, scattered macrophages, and reactive hypertrophic astrocytes with hyperchromatic, enlarged and irregular nuclei (Figure 2A). A few nuclei in the remaining oligodendroglia were slightly enlarged with loss of the normal chromatin pattern. No definite intranuclear inclusions were detected on the limited biopsy at the level of light microscopy. Results from ultrastructure analysis of the biopsy revealed a number of abnormalities including the presence of concentric membrane lamellae, lipid containing empty or partly granular vacuoles in the cytoplasm (Figure 2B). Usually the nuclei of oligodendroglia were irregular with loss of the usual spherical or oval shape. Frequently the nuclei were scalloped or indented by cytoplasmic vacuoles or myelin-like lamellae. The nuclear membranes appeared intact. Intranuclear spherical virions measuring 30–35 nm were seen scattered in low number throughout, focally present in dense arrays, or almost completely filling the nuclei. Rarely virions appeared membrane bound. Sometimes similar virus-like structures were seen scattered in the cytoplasm. Infrequently seen were intranuclear filamentous structures measuring 20–25 nm.

**Structural organization of the regulatory region of the JCV isolate**

The clinical and pathological analyses of the brain biopsy implied involvement of a papovavirus, most likely JCV, in inducing neurological disorders seen in this patient. In order to assess the identity of the

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**Figure 2** Morphological analysis of the brain biopsy. (A) Histopathology of the brain biopsy. A hematoxylin-eosin stained section of the paraffin embedded biopsy shows an area of demyelination containing hypertrophic astrocytes, foamy macrophages and decreased numbers of oligodendrocytes. (B) Transmission electron micrograph of the demyelinated lesion. Low numbers of virus particles are uniformly distributed in the nuclei (N) of some glial cells (left panel). Nuclei of oligodendrocyte filled with viral particles (middle panel), glial nucleus contains dense arrays of viral particles (left panel). Magnifications $2 \times 10^4$. 
participant virus, we attempted to define the structural organization of the viral regulatory region. Toward this end, DNA isolated from serial sections of the paraffin block was used as a template in a PCR amplification reaction directed with specific primers derived from a conserved region of the Mad-1 strain of JCV. The amplified product was isolated and its nucleotide composition was determined by direct sequencing. Computer-assisted analysis of the primary structure from the amplified DNA revealed a close relation of the isolate to the control region of Mad-4 and the type II strain of JCV with distinct variations. A 22 nucleotide insertion, identical to that found in archetype strain separates the TATA box from the NF-1 regulatory motif. Furthermore, the new isolate encompasses only one copy of the previously identified pentanucleotide repeat, AGGAAGGGA, in juxtaposition with the TATA box (Figure 3). An 84 bp tandem repeat which initiates from the fourth nucleotide of the 22 bp insertion resides in the late side of the viral control region. With the exception of the sequences spanning the 18 nucleotide insertion, the 84 bp repeat exhibits substantial sequence homology with the enhancer repeats of the Mad-4 and Mad-1 strains (Figure 3). Sequence analysis of the isolate propagated through infection of primary human fetal glial (PHFG) culture with the biopsy tissue revealed no alteration in the structural organization of the viral regulatory regions.

Based on the primary sequence and the architectural organization of the new isolate, we classified this virus, hereafter called JCV_{Phila-1}, within the class II strain of JCV.

Expression of the JCV_{Phil-1} early and late promoters in glial and non-glial cells
To gain insight into the expression profile of the JCV_{Phil-1} genome in glial and non-glial cells, we performed transient transfection experiments utilizing constructs containing the early and late regulatory regions of the viral isolate fused to the reporter chloramphenicol acetyl transferase (CAT) gene. For comparison, similar constructs containing the regulatory regions from Mad-4 were created and used in parallel transfection assays. The transcriptional activity of the β-actin promoter was used to normalize the levels of CAT gene expression by the viral promoters in glial and non-glial cells. Results shown in Figure 4A illustrate the basal activity of

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**Figure 3** Structural organization of regulatory regions from JCV isolates. The positions of the kb enhancer motif, the origin of viral DNA replication (ori), A/T rich region (TATA box), pentanucleotide repeat, AGGAAGGGA, and NF-1 binding sites are depicted. The nucleotide sequence of the JCV_{Phil-1} and JCV_{Mad-1} are compared and presented below the schematic.
the viral promoters in primary human fetal glial (PHFG) cells, a human glioblastoma cell line, and non-glial Hela cells. We observed that the expression of the early promoter of JCV<sub>Phila-1</sub> was modestly higher than the JCV<sub>Mad-4</sub> in PHFG cells and the glial cell line. Conversely, the basal transcriptional activity of the JCV<sub>Phila-1</sub> late promoter was lower than the JCV<sub>Mad-4</sub> late gene expression in PHFG and glial cell lines. Similar to the Mad-4 strain, the activity of the early and late promoters from JCV<sub>Phila-1</sub> were extremely low in Hela cells suggesting that the divergent genomic organization of the isolate may not influence the specificity of the viral gene transcription.

Next, we performed co-transfection experiments to determine the level of viral late gene expression in the presence of the early protein, T-antigen. Since our previous studies demonstrated that T-antigen is capable of elevating the basal level of late gene transcription in glial and non-glial cells (Lashgari et al. 1989) these experiments were performed in PHFG, U-87MG, and non-glial Hela cells. As shown in Figure 4B, the basal level of late gene transcription from both isolates was dramatically increased by T-antigen in glial and non-glial cells. Although the extent of late gene activation from the Phila-1 isolate was lower than Mad-4 in PHFG and U-87MG cells, the Phila-1 late promoter was more responsive than Mad-4 to T-antigen activation in Hela cells. This observation suggests that the altered structural organization of the JCV<sub>Phila-1</sub> control region may facilitate the late events of the viral lytic cycle in non-permissive cells.

Replication of the viral DNA in glial and non-glial cells

Studies on other papovaviruses such as SV40 indicated that, in addition to the origin of DNA replication, sequences located within the enhancer/promoter of the virus influence the rate of viral DNA replication (DePamphilis and Bradley, 1986). Accordingly, recent studies have demonstrated that the pentanucleotide repeat sequence, AGGGAGGGA, located in juxtaposition with the TATA box is important for the efficient replication of the Mad-1 and Mad-4 genome in transiently transfected cells (Chang et al., 1994; Lynch and Frisque, 1990). Comparison of the genomic structure of the Phila-1 isolate with the Mad-4 strain revealed complete sequence identity between the origin of DNA replication from these two strains. However, we observed substantial differences in the enhancer region which is located in close proximity to the origin of DNA replication. In fact, JCV<sub>Phila-1</sub> possesses only one copy of the pentanucleotide repeat within the proximal 84 bp enhancer repeat. This variation in the sequences prompted us to evaluate the rate of viral DNA replication in glial and non-glial cells. This study was performed by transfecting the reporter plasmids containing the
Figure 5 Examination of JCV DNA replication in glial and kidney cells. Glial (A) or CV-1 (B) cells were transfected JCV_Phil-a1 or JCV_Mad-4 replication competent plasmids, P and M, respectively, in the absence or presence of the expresser plasmids producing JCV or SV40 T-antigen. In all studies the control plasmid (4.5 μg) containing JCV_SV40 DNA was included in the transfection mixture. Low molecular weight DNA was isolated 72 h post-transfection by the Hirt procedure (Hirt, 1967) and after digestion with Dpn I and Sac I, DNAs were electrophoresed in a 1.2% agarose gel. Southern blot technique was carried out as described previously (Peigenbaum et al., 1987). The levels of the Mad-1 DNA replication served as an internal control to normalize the values obtained from the replication of the Phil-a1 and Mad-4 isolates.

Discussion

It is well established that JCV is the etiologic agent for the subacute demyelinating disease, progressive multifocal leukoencephalopathy (PML) (for review see Frisque and White, 1992; Major et al., 1992). PML is an opportunistic disease that generally affects patients already exhibiting immunocompromised conditions due to illness, genetic disorders, or therapeutic immunosuppression for transplant (Walker, 1978). In this study, we report the isolation and characterization of a new strain of JCV, named JCV_Phil-a1, from the brain lesion of a patient with neurological symptoms similar to those seen in PML, with the underlying disease of multiple myeloma. Analysis of the Phil-a1 control region and its comparison to well-characterized JCV strains, i.e Mad-1 and Mad-4, revealed substantial variations in the organizational structure of the cis-acting regulatory motifs which are believed to be important for the transcription of the viral genome, and are critical for replication of the viral DNAs. Particularly, the LCE region of the Mad-1 and Mad-4 enhancer/promoter encompassing a pentanucleotide repeat sequence is deleted in the distal 84 bp enhancer/promoter repeat of Phil-a1, and exists only in a single copy in the proximal 84 bp repeat. Similar to the Mad-8 isolate, enhancer/promoter in the Phil-a1 isolate is 84 bp in size in contrast to the 80 bp and 98 bp repeats observed in Mad-4 and Mad-1, respectively (Martin et al., 1985).
The pentanucleotide repeat is shown to negatively regulate JCV late gene transcription, and in the early orientation positively affects transcription of a heterologous promoter (Tada et al., 1989, 1991). Furthermore, this motif has been shown to be required for T-antigen-mediated replication of JCV DNA (Chang et al., 1984; Lynch and Frisque, 1990). The lack of the complete pentanucleotide repeat in the JCV_{Phila-1} control region and the efficient transcription and replication of the viral genome in glial cells suggests that through re-arrangement, the Phila-1 isolate has gained sequences which are functionally equal to the pentanucleotide repeat, AGGGAAGGGGA, motif. In previous studies, Henson and his colleagues (Henson et al., 1992) were able to sequence the early promoter of JCV isolates derived from brain tissue of expired PML patients, and identify a GA-rich region which was located upstream of the TATA box. This region, which is conserved in the Phila-1 strain, was shown to have the capacity to interact with the SP1 transcription factor (Henson et al., 1992). However, the importance of this interaction in regulation of viral early and late gene expression remains to be elucidated. Another interesting feature of the JCV_{Phila-1} regulatory region is the physical separation of the NF-1 motif located in the B-domain from the basal transcriptional element, the TATA box. The B-domain of JCV Mad-1 and Mad-4 isolates, by binding to a 45 kDa protein from brain, stimulate basal transcriptional activity of the JCV early promoter (Ahmed et al., 1990). Furthermore, a DNA-binding protein, termed GF-1, by interacting with the NF-1 motif located within the B-domain, increased transcription of the viral early and late promoter (Kerr and Khalili, 1991) and stimulated T-antigen-mediated viral DNA replication (Kerr et al., in preparation). Results from transient transfection studies suggested that transcriptional activation by GF-1 occurs in the context of the JCV promoter and that a chimeric heterologous promoter containing the B-domain is not responsive to GF-1 (Kerr and Khalili, 1991). Thus, it is likely that proper spacing of the B-domain with the other JCV regulatory motifs which facilitate interactions of GF-1 with the neighboring DNA-bound proteins such as TATA box binding factors is critical for activation of the viral promoter by GF-1. It is likely that 22 nucleotide insertion between the TATA box and the B-domain in JCV_{Phila-1} disrupts such an interaction, while exhibiting no effect on transcription and replication of the viral genome. Therefore, an alternative regulatory mechanism which includes participation of a distinct regulatory factor may regulate expression of viral early and late gene transcription. Experiments are currently in progress to examine the importance of GF-1 and the other previously characterized regulatory proteins in transcription of the Phila-1 promoters, and identify the specific regulatory protein which, by interacting with the unique sequence formed in Phila-1, participates in replication of this isolate in glial cells.

**Materials and methods**

**Morphologic analysis of the biopsy specimen**

For morphological analyses, a stereotactic biopsy was obtained. Most of the specimen was rapidly frozen and cryostat sections were stained with hematoxylin and eosin at the time of surgery with a small aliquot aseptically frozen. The remainder of that specimen and an additional biopsy was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, Luxol fast blue and periodic acid Schiff reagent, and Bielschovsky silver method for axons. For electron microscopy, a separate small aliquot of the biopsy was fixed in 2.5% glutaraldehyde, post-fixed with osmium tetroxide, and embedded in Spurr-Low viscosity resin. Thin sections were stained with uranyl acetate and lead acetate. A Zeiss EM9-S2 electron microscope was used for transmission electron microscopy.

**Isolation, amplification, and sequencing of DNA from the biopsy**

Template DNA for polymerase chain reaction (PCR) amplification was obtained by deparaffinizing 6 to 10 sections from the paraffin block, incubating in 20 mM Tris-HCl (pH 8.0), 20 mM EDTA, 2% SDS (wt/vol.) in the presence of 200 μg ml⁻¹ proteinase K at 37°C for 16-20 h. For PCR, primers spanning nucleotides 4595 to 5007 (5'-CCTCCCTATTCA-GGACTTT-3') and nucleotides 222 to 246 (5'-GGC-TGGAAAACATGTTCC-3') of Mad-1 were used with 1 μg of template DNA in the presence of 2.5 mM MgCl₂ at 94°C, 52°C, and 72°C each for 1 min for a total of 30 cycles, followed by an extension at 72°C for 10 min. The specificity and the integrity of the amplified DNA was initially assessed by agarose gel electrophoresis. The distinct PCR amplified DNA band was isolated from a preparative agarose gel and cloned into the pcRII vector (Invitrogen). The dideoxy methods of chain termination sequencing modified for double-stranded DNA templates was performed using primers flanking the insert and the sequenase enzyme (USB). The nucleotide composition from six independent clones of the isolate was determined.

**Reporter plasmids**

To create the JCV-containing reporter constructs, the 444 EcoRI DNA fragment from the pcRII was terminally modified with T4 DNA polymerase and placed in the Bam HI site of the promoterless reporter plasmid pBLCAT₃ (Luckow and Schultz, 1987). The orientation and the integrity of the recombinants was evaluated by DNA sequencing (Ausubel et al., 1989). The reporter plasmids con-
taining the regulatory region of Mad-4 were generated by cloning a 388 bp PCR product derived from the Mad-4 strain positioned upstream of the CAT gene in the pBLCAT<sub>5</sub> vector. The primers used in the PCR were identical to those used for cloning the insert in the pCRII vector (shown above). The pBJC-T plasmid was constructed by placing the JCV DNA fragment that encodes for viral early region under the control of herpes simplex virus promoter. The plasmid pRSV-T is a recombinant in which SV40 T-antigen is under the control of Kous sarcoma virus long terminal repeat.

**Cell culture and transcription assay**

Primary human fetal glial cells (FHFG) were prepared from 15 to 18 week old abortuses by procedures described previously (Padgett et al. 1977). CV-1 is a continuous line of monkey kidney cells. The Hela cell line was isolated from a carcinoma of the cervix. U-87MG is an established glioblastoma cell line which was obtained from American Type Culture Collection. All cell types were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum and plated in 60 mm dishes for 16 h prior to transfection. Cells were transfected by the calcium phosphate/DNA precipitation method (Graham and van der Eb, 1973). The amount of DNA plasmid in each transfection was kept constant at 15 μg per dish by adding appropriate carrier plasmid DNA with the test plasmids and was co-precipitated with Ca-phosphate in a final volume of 1.0 ml. For CAT assay, all extracts were made 36 h post-transfection and CAT enzyme activity was evaluated as described previously (Gorman et al. 1982) The transcriptional activity was quantitated by scintillation counting of spots cut from the TLC plate. Each transfection was repeated multiple times to ensure the reproducibility of data.

**Replication assay**

For replication, the cells were transfected with origin-containing test plasmid (3 μg) plus a plasmid expressing T-antigen (10 μg) and the internal control plasmid containing the Mad-1 control region blocked by β-globin gene in pBL plasmid (Wildeman, 1989) as described previously. Low molecular weight DNA was isolated by Hirt procedure (Hirt, 1967) and digested with DpnI and SacI endonucleases. The DNA was then resolved by agarose gel electrophoresis and Southern blot analysis was performed using a [32P]-labeled DNA fragment retrieved from the Mad-4 control region as a probe. Hybridization was carried out in 6X SSC, 0.1% SDS, 5X Denhard, 300 μg μl⁻¹ denatured salmon sperm DNA at 68°C overnight. The blot was washed in 3X SSC, 0.1% SDS at 68°C and in 2X SSC, 0.1% SDS at 68°C prior to exposure to film at -70°C.

**Acknowledgements**

We would like to thank Dr R Frisque for his helpful comments and discussion regarding the manuscript prior to publication. We are thankful to members of the Molecular Neurovirology Section of the Jefferson Institute of Molecular Medicine for sharing their reagents and for their support and helpful suggestions during the course of this study. We thank Jennifer Gordon for critical reading of this manuscript and Cynthia Schriver for preparation of this manuscript. This work was supported by grants awarded by the National Institutes of Health to KK.

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