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Short communication

Amplification of the complete polyomavirus JC genome from brain, cerebrospinal fluid and urine using pre-PCR restriction enzyme digestion

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A method is described for amplification of the complete genome of the human polyomavirus JC (JCV) from progressive multifocal leukoencephalopathy (PML) brain, cerebrospinal fluid (CSF) of PML patients, and from the urine of controls without neurological disease. Efficient amplification with the 'long PCR' method is achieved with primers overlapping the single BamHI or EcoRI site following digestion of the DNA sample with the restriction enzyme BamHI or EcoRI, respectively. Cutting of the supercoiled JCV genome allows full separation of the strands during the denaturation step, and permits primer annealing to compete successfully with reassociation of the genomic DNA. With this method a single PCR amplification allows restriction fragment length polymorphism (RFLP) analysis and direct cycle sequencing of any part of the viral genome. RFLP analysis of JCV amplified from CSF has identified a new mutant sequence. Sequencing of clinical samples is useful for typing of JC virus isolates as Type 1 or Type 2 and for characterization of the JCV regulatory region as archetypal or rearranged.

Keywords: DNA restriction enzyme BamHI; EcoRI; JC virus; long PCR; supercoiled DNA

The human polyomavirus JC (JCV) is a 5 kb double-stranded polyomavirus with a circular, supercoiled DNA genome which is closely related to BK virus and SV40. The distribution is worldwide and two different genotypes have been identified to date (Yogo *et al*, 1991; Ault and Stoner, 1992). The essential elements for the initiation of replication are localized within the regulatory region from which early and late coding regions are transcribed divergently. Most of the adult population (70–90%) show a humoral immune response to JCV. The primary infection is not known to be associated with clinical symptoms. However, the virus can be reactivated during immunosuppression in oligodendrocytes and astrocytes (Walker, 1985) causing the central nervous system disease progressive multifocal leukoencephalopathy (PML) which is usually fatal within 3–9 months.

The first sequence of JCV (strain Mad-1) was obtained from viral DNA molecularly cloned using recombinant DNA technology (Frisque *et al*, 1984). This method has remained the best source of complete JCV DNA since, in contrast to SV40 or BKV, the virus replicates very slowly even in permissive cell cultures such as primary human fetal glial cells. An additional problem of *in vitro* cultivation is the possible generation of defective viral DNA genomes after even a single passage (Martin *et al*, 1983). Application of the polymerase chain reaction (PCR), based on the amplification of short fragments of the viral genome, has greatly improved the detection of JCV in clinical samples (Telenti *et al*, 1990; Flaegstad *et al*, 1991; Henson *et al*, 1991). Recently, the effective range of PCR has been dramatically increased by combining heat resistant DNA polymerases with and without 3'–5' exonuclease activity and optimizing buffer conditions to protect the enzymes and promote strand separation of the target sequence ('long PCR') (Ponce and Micol, 1992; Cohen, 1994; Cheng *et al*, 1994). We therefore developed a method for the PCR amplification of the complete JCV genome from brain, cerebrospinal fluid (CSF) and urine using primers overlapping at the BamHI or EcoRI site.

Brain biopsy samples (15–20 mg) were lysed overnight at 55°C in 100 µl buffer containing 0.2 mg

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ml^{-1} proteinase K (BRL, Gaithersburg, MD) and 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 2.5 mM $MgCl_2$, 10% (w/v) gelatin, 0.45% (v/v) NP40 and 0.45% (v/v) Tween20. DNA from CSF was extracted by adding NP40 at a final concentration of 0.05% (v/v) and incubating at 56°C for 30 min. Brain and CSF samples were obtained as previously described (Stoner and Ryschkewitsch, 1995). 50 ml of fresh urine was centrifuged, the pellet washed with PBS and lysed with proteinase K in a total volume of 150 µl. Urine samples were obtained from unselected adult donors in a general medical clinic laboratory (Agostini *et al,* manuscript in preparation). The DNA extract (urine and brain: 6–15 µl, CSF: 24 µl) was digested with 2–8 U of BamHI or EcoRI (Promega, Madison, WI) in standard buffers in a total volume of 30 µl for 30–120 min at 37°C followed by 20 min at 65°C for partial heat inactivation of the enzyme. Longer incubation times or higher concentrations of BamHI or EcoRI for predigestion did not result in a higher yield of PCR products. JCV specific primers, overlapping at the unique BamHI site, were BAM-1 (5'- GGGATCCTGTGTTTTCATCATCACTGGC-3') and BAM-2 (5'-AGGATCCCAACACTCTACCCCACC-3'. The primer pair for the EcoRI site was ECO-7 (5'AGAATTCCACTACCCAATCTAAATGAGGAT-3') and ECO-12 (5'-TGGAATTCTGGCCACACTGTAACAAG-3'). The reaction mix contained 3 U of rTth Polymerase XL plus Vent Polymerase in the appropriate buffer (GeneAmp XL PCR, Perkin Elmer Cetus, Norwalk, CT), 0.7 mM Mg $(OAc)_2$ and 20 pmoles of each primer. The thermostable DNA polymerase was added at 80°C to a total of 100 µl through a layer of mineral oil. Initial denaturation at 94°C for 1 min was followed by 15 cycles of 94°C for 40 s and 6 min at 64°C (BAM-1 and 2) or 65°C (ECO-7 and 12). The second step was prolonged by 2 min every six cycles for a total of 39 cycles using the thermal cycler PTC-100 (MJ Research Inc, Watertown, MA). After 10 min at 72°C the reaction was stopped at 4°C. Products were detected by electrophoresis in 0.9% agarose ethidium bromide gels and purified by the Qiaex gel extraction procedure (Qiagen, Chatsworth, CA). For PCR amplification of a 129-bp product from the VP1 coding region with primers JLP-1 and JLP-4 see Stoner and Ryschkewitsch (1995).

In contrast to short-range PCR, virus-specific products above 5 kb amplified from brain and CSF were only seen following enzymatic digestion of the sample DNA at the BamHI or EcoRI site prior to PCR. In urine a PCR product of the expected length was sometimes detected without digestion, but the yield increased significantly with predigestion. The complete length and identity of the PCR product was verified by HincII digestion (Figure 1). A deviation from the expected five band pattern was found in one of the CSF samples in which a shift between two bands of about 130 bp suggests two mutations or a rearrangement within the VP-2/3 gene (Figure 1). This strain has not yet been sequenced, and the exact nature of this alteration is unknown. Deletions in the VP1 gene of JCV amplified from the CSF have been described (Stoner and Ryschkewitsch, 1995), and the present finding suggests further instability within the JCV coding region.

The unexpected requirement for predigestion with a restriction enzyme is probably related to the supercoiled structure of the circular viral genome. Denaturation of supercoiled circular DNA yields intertwined single-stranded circles which can rapidly reassociate when the temperature is lowered, thereby preventing primer annealing.

Restriction enzyme cleavage of the amplified genome with other restriction enzymes will allow rapid determination of restriction fragment length polymorphisms and preliminary genotyping (Yogo *et al,* 1991). In addition, direct cycle sequencing of any part of the genome is now possible with the product of a single PCR reaction as shown for the

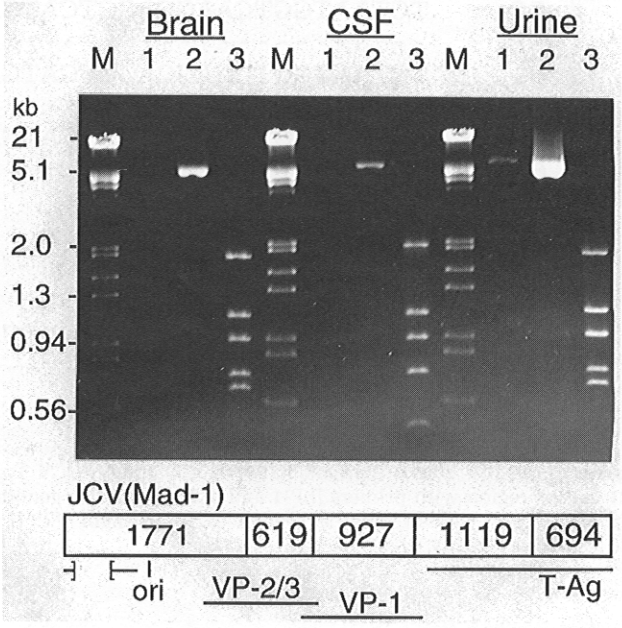

Figure 1 The complete genome of JC virus amplified by PCR from PML brain (patient 28), PML CSF (patient 13) and urine from a patient (2) without neurological disease. Lanes 1 without, lanes 2 with BamHI in the predigestion mix. Lanes 3, HincII digestion of the PCR product results in a five band pattern (graphically shown for the linearized JCV (Mad-1) sequence, GenBank no. J02227). The BamHI site lies within the large T-antigen coding region (T-ag). *VP-1-3*: capsid proteins, *ori*: origin of replication, *M (marker)*: HindIII and EcoRI digested lambda DNA.

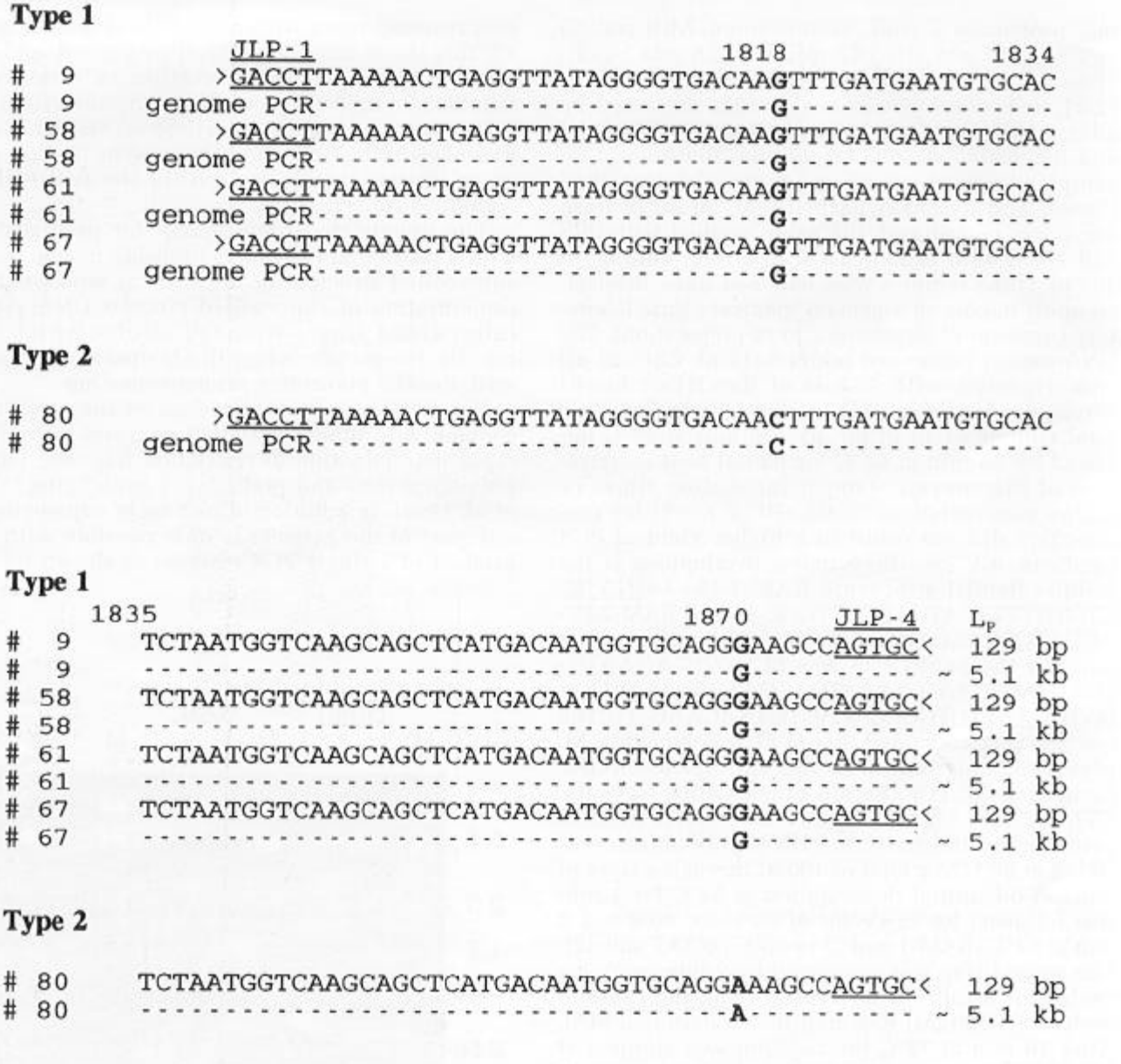

Figure 2 Four JCV Type 1 (patients 9, 58, 61, 67) and one JCV Type 2 (patient 80) strains were amplified with conventional PCR of the VP1 coding region with primers JLP-1 and 4 and complete genome PCR. Cycle sequencing results of both methods were compared for each sample and yielded identical sequences. L_p = Length of PCR product. Type determining nucleotides are in bold print. Dashes indicate sequence identity.

VP1 coding region (Figure 2) and the regulatory region (Figure 3). Cycle sequencing of different parts of the viral genome like the V-T intergenic region is useful to definitively determine the virus genotype (Type 1 or 2) (Ault and Stoner, 1992), as well as the regulatory region arrangement (Loeber and Dörries, 1988; Yogo *et al*, 1990; Flaegstad *et al*, 1991; Markowitz *et al*, 1991; Ault and Stoner, 1993). To evaluate the fidelity of the method, part of the VP1 coding region including JCV type determining positions was cycle sequenced from the long range PCR products and compared to the 'short range' sequences amplified from the same samples with primers JLP-1 and 4. The results were identical in all four Type 1 strains and one Type 2 strain (Figure 2). In addition the regulatory region of one complete genome amplification product was analyzed and compared to the archetypal sequence of the Japanese strain CY (Yogo *et al*, 1990) (Figure 3). The only difference found at position 217 was previously described (Yogo *et al*, 1990; Flaegstad *et al*, 1991) and is known to be strain specific.

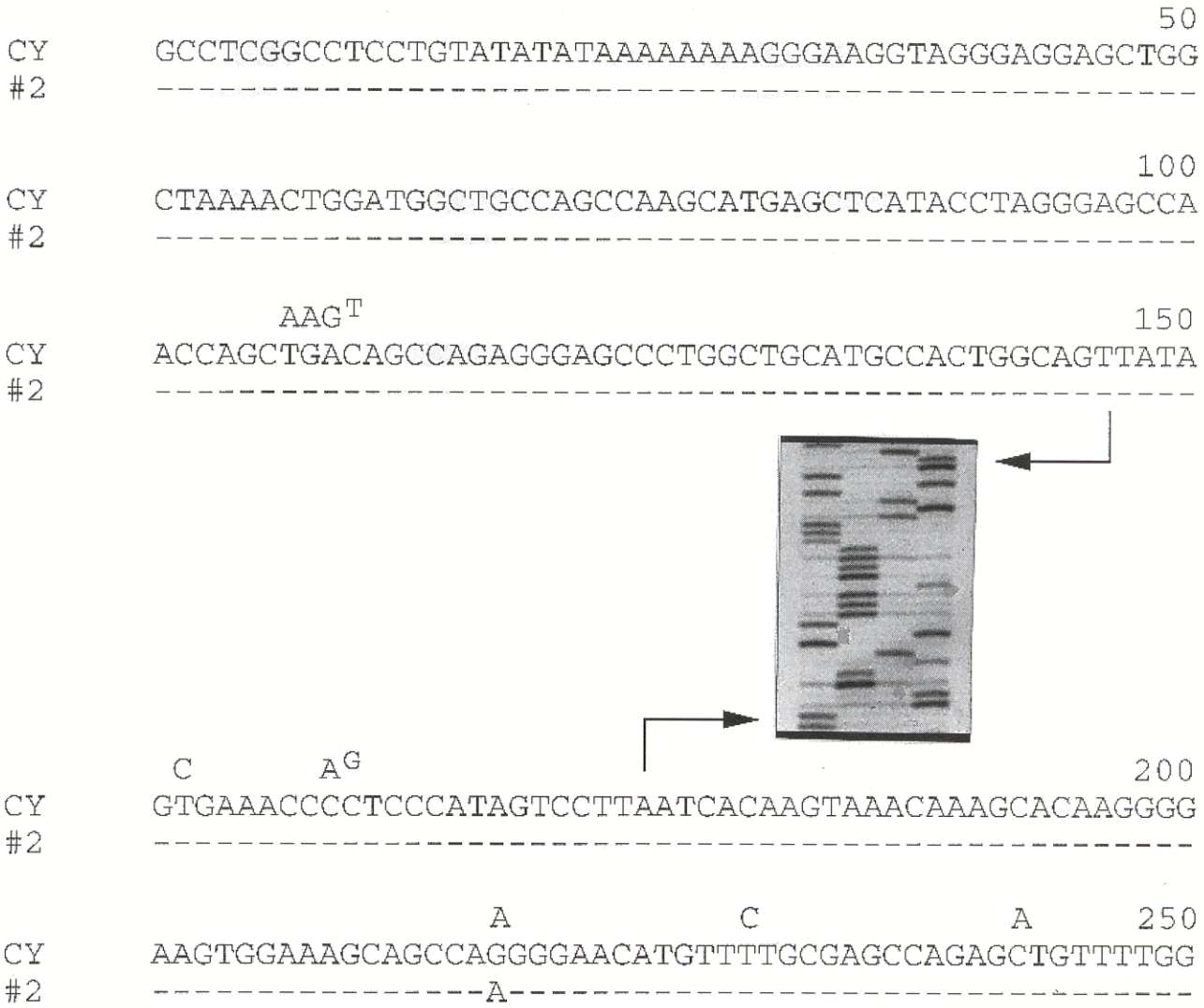

Figure 3 Direct cycle sequencing of the JCV regulatory region using 6% of the PCR-amplified complete viral genome from the predigested urine sample (patient 2). The sequence shown (left to right: A, C, G, T) contains the second recognition site for the transcription factor SP-1 which is usually deleted in rearranged forms of the regulatory region found in PML brain. Compared to the archetypal sequence of the Japanese strain CY (Yogo *et al*, 1990, GenBank no. 35834), #2 differs only at position 217 which is a polymorphic site as indicated by the nucleotides above the line. After standard unidirectional cycle sequencing of the PCR product #2 with a ^{33}P-labeled primer, products were separated on a 6% polyacrylamide gel. The dried gel was exposed to X-ray film for 20 h.

Since the unique BamHI or EcoRI cleavage site is included in the 5'-end of both primers, the complete genome PCR product can be cloned in a suitable vector (data not shown). This will be important when only small volumes of clinical samples or samples with a low viral copy number are available and cloning by routine methods is precluded. This general method may prove useful for the complete PCR amplification of other viruses with circular genomes such as BK virus or SV40, each of which has a unique BamHI and EcoRI restriction site.

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