Journal of NeuroVirology (2000) 6, 398–409

www.jneurovirol.com

Analysis of the transcriptional control region in progressive multifocal leukoencephalopathy

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Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease caused by the human polyomavirus JCV. The hypervariable noncoding transcriptional control region (TCR) largely regulates replication of JCV in glial cells. Two distinct types of the TCR can be distinguished. Type II is derived from the archetype sequence. All type I TCRs, including the prototypical Mad-1 isolate contain a 23 bp deletion at nucleotide position 36. In a prospective study, TCR-DNA could be amplified and sequenced in 16/29 (55%) suspect cases of PML from the cerebrospinal fluid (CSF) and in 14/28 (50%) urine samples. Sequencing of the CSF-TCR identified Mad-1 like sequences in 5/17 (29.5%) instances and a type II TCR in 12/17 (70.5%) of cases. Of 14 urine TCRs, 12 (86%) displayed the archetype sequence, while two showed complex rearrangements. In all type II TCR sequences, the tst-1/oct-6 binding sites present in regions C and E of Mad-1 were missing. In 11/12 type II TCR sequences the pentanucleotide repeat in region A showed a G to T substitution of one nucleotide at position 36 relative to the Mad-1 TCR. All type II TCRs contained an Sp1 binding site at the beginning of region B. Of the 12 TCR type II sequences, 10 (83%) were of the 'D-retaining' pattern. In eight of these (80%) additional juxtapositioned nuclear factor 1, glial factor 1 and/or AP-1 binding motifs were created by duplications and/or insertions in region D. These findings indicate that type II TCRs are frequently present in PML and suggest to use TCR type II constructs for *in vitro* and *in vivo* studies of the evaluation of the functional role of DNA binding motifs. *Journal of NeuroVirology* (2000) **6,** 398–409.

Keywords: JCV; transcriptional control region; PCR; direct sequencing; DNA binding motif; PML

Introduction

The polyomavirus JC (JCV) is the causative agent of progressive multifocal leukoencephalopathy (PML) (Padgett *et al*, 1971; Zu Rhein and Chou, 1965), a demyelinating disease of the central nervous system (CNS), observed primarily in patients with impairment of the immune system (Weber and Major, 1997). Currently, almost 85% of all PML cases are associated with human immunodeficiency virus (HIV) infection, while PML ultimately develops in up to 5% of all patients with AIDS (Berger and Major, 1999).

JCV persists in the kidney, bone marrow, haematopoietic system and in peripheral blood leukocytes (Ciappi *et al*, 1999; Elsner and Dörries, 1992; Houff *et al*, 1988; Koralnik *et al*, 1999b). Reactivation of JCV may occur with an impairment of cellular immunity and appears to be influenced by the regulation of viral gene expression via cytokines (Atwood *et al*, 1995; Chang *et al*, 1996).

Detection of JCV DNA by polymerase chain reaction (PCR) in the cerebrospinal fluid (CSF) has been shown to have a sensitivity of about 80% and a specificity of about 99% for the diagnosis of PML (Sugimoto *et al*, 1998; Weber *et al*, 1996). Comparative sequence analysis of the transcriptional control region (TCR) amplified from CSF and brain tissue has demonstrated identical sequences thus providing direct evidence for the concept that JCV-DNA in CSF is brain-derived (Sugimoto *et al*, 1998). In addition, sequencing of brain derived clones has demonstrated a predominance of a single rearranged promoter (Ault and Stoner, 1993).

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Received 22 December 1999; revised 20 April 2000; accepted 31 May 2000

The TCR is the most variable region of the JCV genome and is responsible for its cellular tropism and infectivity (Dörries, 1998; Newman and Frisque, 1997; Raj and Khalili, 1995). The urinary JCV strains have a non-rearranged regulatory region named archetype sequence (Agostini *et al*, 1996b; Major *et al*, 1992; Yogo *et al*, 1990). In contrast, strains isolated from the brain tissue of patients with PML have highly variable regulatory regions with rearrangements as a result of deletions and/or duplications that may be formed from the archetype sequence (Ault and Stoner, 1993; Iida *et al*, 1993). By comparing the archetype consensus sequence with Mad-1, the archetype sequence can be divided into six regions numbered A to F (Ault and Stoner, 1993; Frisque *et al*, 1984; Yogo *et al*, 1990). Although the sequence analysis of the TCR shows complex hypervariability, two general types of the JCV genome have been identified (Martin *et al*, 1985). The control region termed TCR type I is characterized by the presence of a 98 bp tandem repeat, each containing a TATA box in juxtaposition to a pentanucleotide repeat, 5′-AGGGAAGGGA-3′, and the NF-1 motif (5′-TGGC/A(N_5)G/ACCA-3′) at positions nt 29–38 and nt 127–136 relative to the prototype Mad-1 strain (Amemiya *et al*, 1992; de Vries *et al*, 1985; Frisque and White, 1992; Leegwater *et al*, 1985; Frisque *et al*, 1984; Raj and Khalili, 1995) (Figure 1). Relative to type II TCRs and the archetype, type I TCR including Mad-1 contain a 23 bp deletion at position 36. The type II TCR has one TATA box proximal to the early mRNA initiation site not included in the repeated elements. The tandem repeats of type II TCRs vary in size and nucleic acid sequence, but are related to one another. The most distal repeat with respect to the origin of replication lacks a TATA box (Elsner and Dörries, 1998; Frisque and White, 1992). At least three NF-1 binding motifs have been identified in the JCV-TCR. An additional pseudo-NF-1 site (5′-TGGAAAGCAGCCA-3′) is located at position nt 213–225 (Amemiya *et al*, 1992; Leegwater *et al*, 1985; Raj and Khalili, 1995). A protein related to NF-1, c-jun, binds to the AP-1 motif (5′-TGAGCTCA-3′) adjacent to the NF-1 sites in the tandem repeats (Atwood *et al*, 1995; Deyerle and Subramani, 1988; Henson, 1994; Raj *et al*, 1996). An AP-1-like site (5′-TGTCACCA-3′) is also present juxtapositioned to an NF-1 site (nt 262–269). A tst-1/oct-6 binding site A (5′-TCCTGTATATATAAAAA-3′) maps to nt 10–26, while two tst-1/oct-6 binding sites B (5′-GCCAGTAAACAAAGCA-3′) have been mapped to nt 89–104 and nt 187–202 (Frisque *et al*, 1984; Wegner *et al*, 1993). GF_1 is a glial protein with the consensus sequence 5′-GCCAGCCA-3′ specifically binding to NF-1 sites in the 98 bp repeats (Kerr and Khalili, 1991). Figure 1 shows an alignment of the archetype (type II TCR) with Mad-1 and with a representative sequence (118D2) of the rearranged TCRs reported in this study.

The GA box (5′-AGGGAGGAGC-3′), a site for Sp1, is present in the promoters of many viral isolates from the brains of patients with PML (nt 38–47), all corresponding to a type II TCR. It is not found in the prototype Mad-1 isolate (type I TCR) (Henson *et al*, 1992). Independent of the distinction between type I and type II TCRs, simple rearrangements consisting of either deletions or duplications in the TCR can be distinguished from complex rearrangements showing deletions and duplications and/or insertions (Elsner and Dörries, 1998; Kitamura *et al*, 1994; Yogo *et al*, 1990).

Here we established a PCR to amplify the JCV-TCR in urine and in CSF of 70 patients suspected of PML. The PCR products were direct cycle sequenced. We determined the frequency of archetype and rearranged JCV-DNA in urine and CSF. Furthermore we evaluated the regulatory region configurations and the implication of these altered regulatory sequences for the development of PML.

Results

Twenty-eight urine samples and 69 CSF specimens were tested for the presence of JCV-DNA using a manual hot start PCR and a nested PCR, respectively. JCV-DNA was demonstrated in 14/28 (50%) urine samples and 17/69 (24.6%) CSF samples. Two types of DNA sequences were identified in urine and CSF. Of 14 urine samples, 12 contained TCR type II sequences. In two of six paired urine/CSF specimens, a deviation from the archetype was identified in urine. One sequence showed a duplication in the junction of region D and E and an insertion in region E. The second urine derived sequence had a duplication in region B combined with a deletion at the junction of region B and C.

Of the 17 positive CSF samples, one sequence (137D1) was obtained from a patient with definite multiple sclerosis in whom a second CSF sample showed no JCV-DNA specific signal. This sequence showed a TCR type II (Figure 3; 137D1). Of the remaining 16 patients, 13 were HIV-positive, male, aged from 26 to 61 years. Of the three HIV-negative patients, one suffered from leukaemia, one from breast cancer and one from a biopsy confirmed astrocytoma. Unfortunately, no biopsy or autopsy specimens were available for the diagnosis of definite PML. According to the predefined criteria, 11/13 (84.6%) HIV-positive patients were classified as probable PML, two patients as possible PML. Interestingly, both cases classified as possible had a TCR sequence identical to Mad-1, while only one HIV-positive patient (1/11; 9%) classified as probable PML had a TCR sequence identical to Mad-1. Of the three HIV-negative patients all were classified as probable PML. Of these, two had TCR sequences related to Mad-1 showing simple rearrangements (Figure 2; 127D1 and 138D1), while

the third had a TCR type II sequence (Figure 3; 123D1). Of 14 patients classified as probable PML, 11 (78.5%) had type II TCRs identified and the remaining two probable cases had a TCR type I differing from Mad-1 by either insertions in region C or substitutions in region C, E and F (Figure 2).

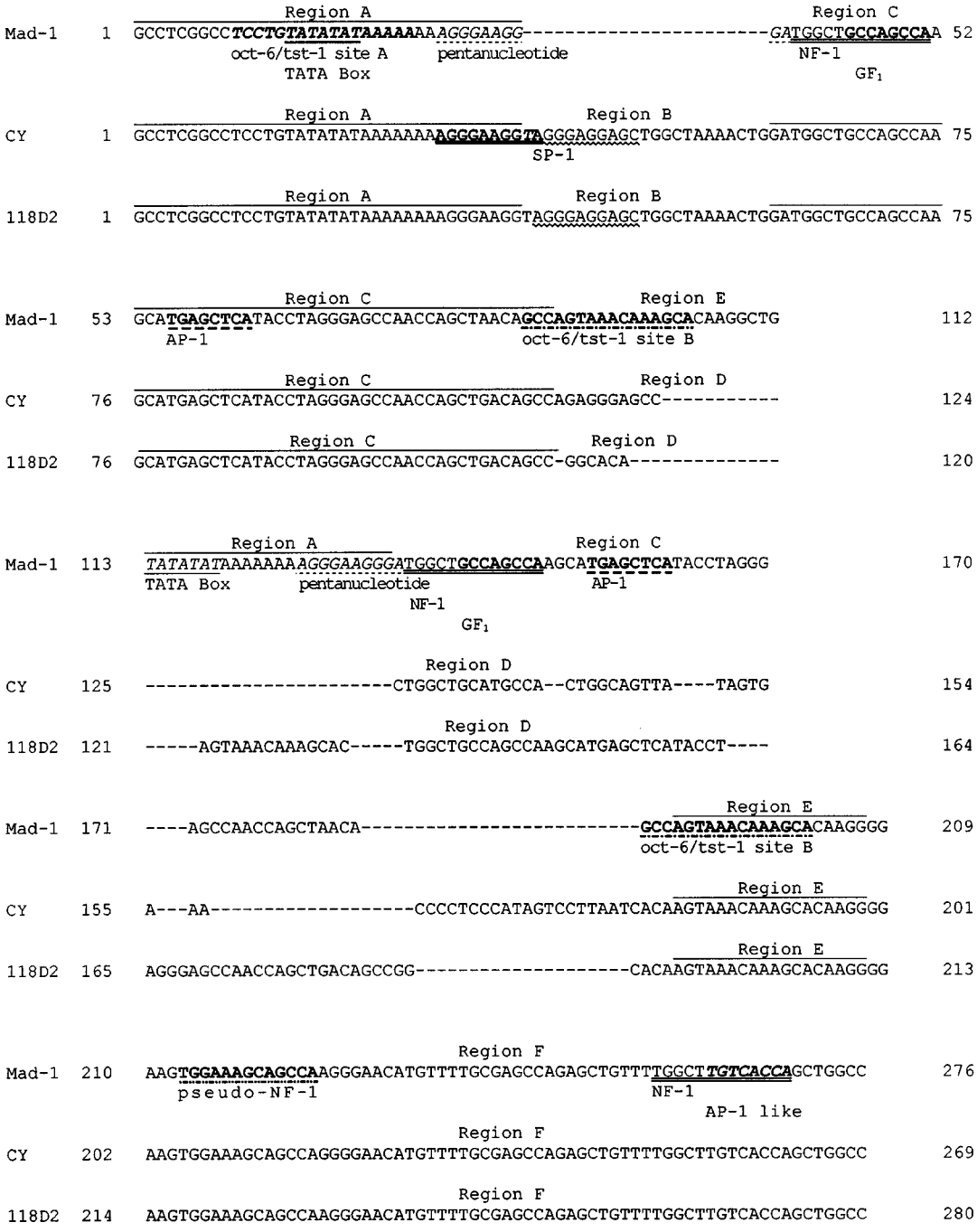

Figure 1 The sequences are numbered according to Frisque *et al* (1984). The sequence of the TCR region of CY (TCR type II), Mad-1 (TCR type I) and a representative rearranged sequence (TCR type II; 118D2) are shown. Regions A, C and E are overlined. The tst-1/oct-6 binding site A (5′-***TCCTGTATATATAAAAA***-3′) is present within region A in Mad-1 (nt 10 to 26). It contains the TATA box (5′-TATATAT-3′). The pentanucleotide sequence (5′-*AGGGAAGGGA*-3′) is present twice in Mad-1 at positions 29 to 38 and 127 to 136. The mutated sequence pentamut (5′-AGGGAAGGTA-3′) is present once in 11/12 type II TCRs and characterized by a single base alteration (G to T). The archetype and type II TCRs contain an SP-1 site (5′-AGGGAGGAGC-3′) at nt 38 to 47 relative to Mad-1. The NF-1 motif (5′-TGGCTGCCAGCCA-3′) is present three times in Mad-1 (nt 39 to 51, nt 137 to 149 and nt 257 to 269) and contains two inserted GF_1 sites (5′-GCCAGCCA-3′) at nt 44 to 51 and nt 142 to 149. At position 262 to 269 there is an AP-1 like site (5′-TGTCACCA-3′). At positions nt 56 to 62 and positions nt 154 to 161 there are two AP-1 (5′-TGAGTCA-3′) sites. A tst-1/oct-6 site B is present at nt 89 to 104 and 187 to 202 (5′-GCCAGTAAACAAAGCA-3′). A pseudo-NF-1 (5′-TGGAAAGCAGCCA-3′) site is found at nt 213 to 225.

In 12 of 17 CSF samples (70.5%) rearranged TCRs that could be classified as type II were detected (Figure 3). The remaining five sequences were of the Mad-1 type (Figure 2). These Mad-1 (like) sequences were consistently amplified from five unrelated individuals from five different cities over a period of 9 months. These were amplified from the CSF of three AIDS patients, a breast cancer case and from a patient with astrocytoma. This patient had MRI findings suggestive of PML. Of the three AIDS patients, only one had a MRI study suggestive of PML. Of these five type I TCRs, three were identical to Mad-1. One contained an insertion of thymidine at position 187 (127D1). The other (138D1) had a substitution of cytidine for guanosine at nt 90, of adenine for cytydine at nt 202, of adenosine for guanosine at nt 266 and cytydine for guanosine at nt 268, causing a loss of the two oct-6/tst-1 sites B in region C/E and the NF-1 and AP-1 sites in region F (Figure 2).

The size of the type II TCRs (n=12) ranged from 211 to 372 bp with a median and a mean length of 298 bp (Figure 3).

Of these 12 sequences, 11 showed a complex rearrangement, one showed a simple rearrangement. Three general patterns of rearrangement were seen (Figures 2 and 3). The so called 'long duplication pattern' including a duplication of region C, deletion of region D and duplication of region E was seen in 5/17 (29%) CSF-derived sequences, i.e. in all Mad-1 and Mad-1 like sequences (Figure 2). A pattern identified by the retention of region D with an insertion of sequences either before or after it, the so-called 'D-retaining' pattern could be identified in 10/12 (83%) of the TCR type II sequences. In 6/10 (60%) of these sequences a pattern characterized by insertions in region D and the creation of juxtapositioned NF-1, GF_1 and AP-1 binding sites (112D1 with an additional GF_1 site) in conjunction with at least one NF-1 and AP-1 like binding site in region F was seen (Figure 3). Two sequences (134D1 and 137D1) showed only an AP-1 site, the remaining two sequences showed either one single GF_1 site (110D1) or no binding site (108D1) at all in the D region.

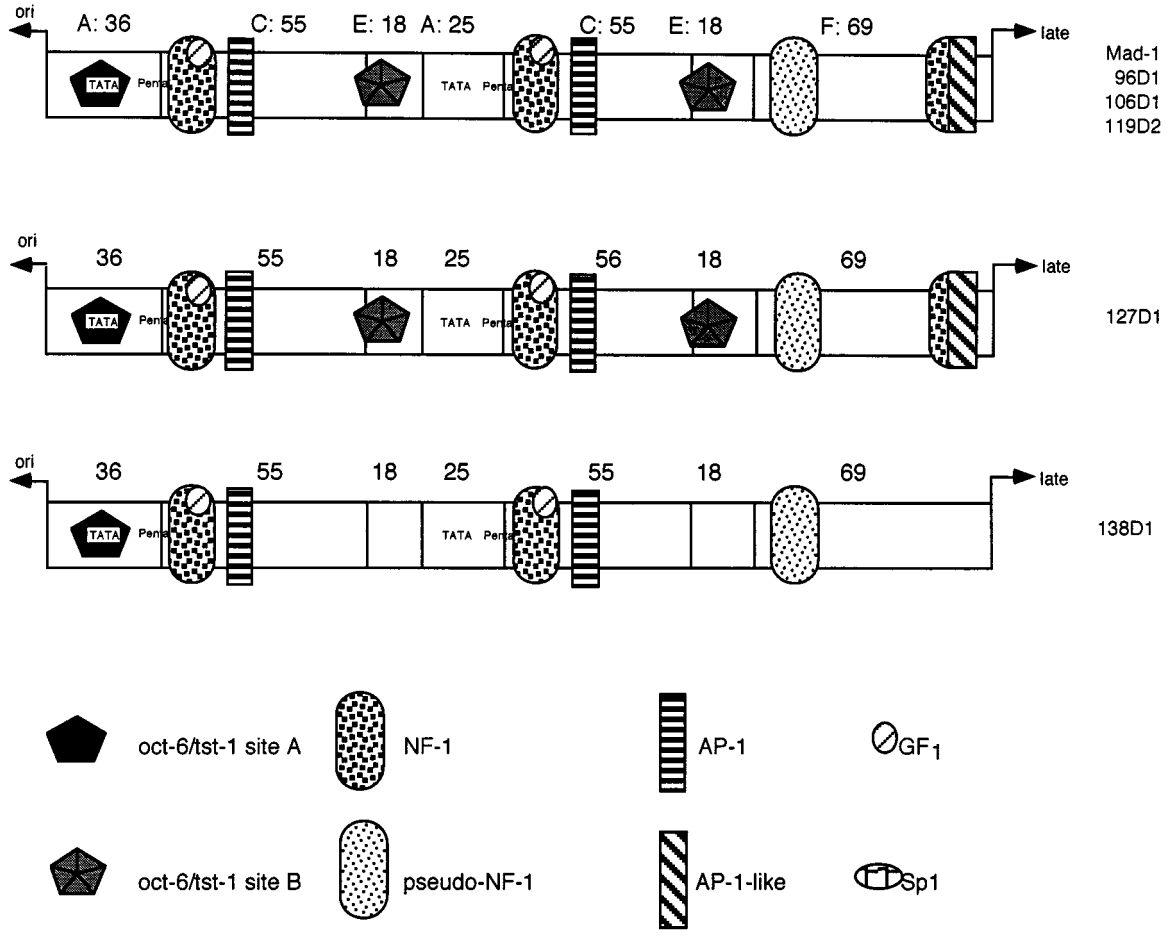

Figure 2 Schematic diagram of the Mad-1 and Mad-1 like CSF sequences (type I TCR). Mad-1 is shown at the top, the sequences identical to Mad-1 are given as well. The sequence is numbered according to Frisque *et al* (1984). Regions A to F and their sizes are indicated above each drawing.

One of the sequences (120D1, Figure 3) had a deletion in region A of two nucleotides (nt 34 – 35) thus altering the pentanucleotide sequence. From a total of 17 CSF-derived sequences, two had short deletions of four to five nucleotides in region C (Figure 3). Deletions were seen in region D (120D1 and 123D1) and E (93D1 and 108D1) in 2/17 CSF-derived sequences each, while 10/17 CSF-derived

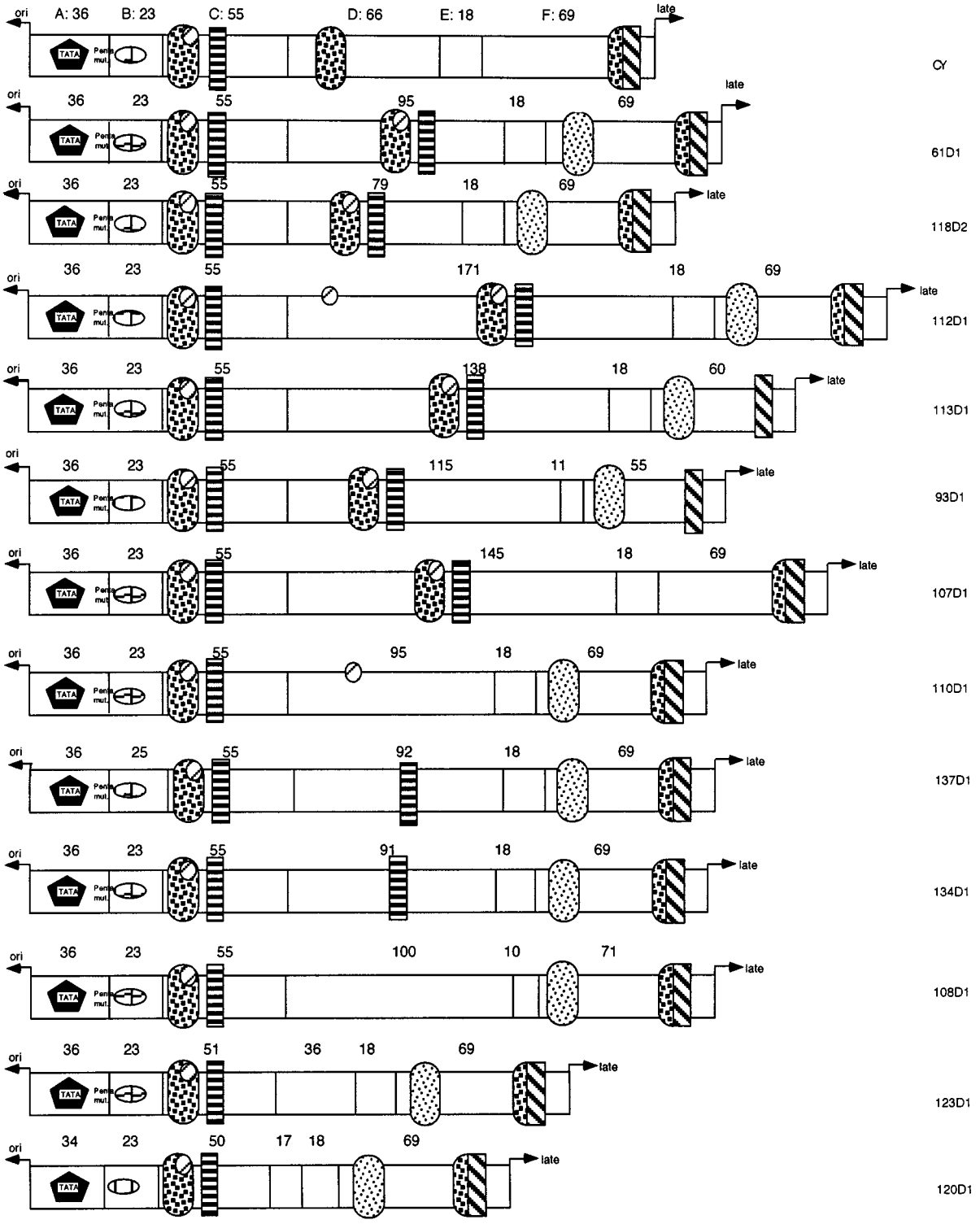

Figure 3 Schematic diagram of the rearranged CSF JCV-TCR type II sequences. The CY archetype is shown at the top. The sequence is numbered according to Frisque *et al* (1984). The rearranged CSF sequences are shown below the archetype.

Table 1 Distribution of mutations in regions A to F of type II TCR CSF amplicons (n=12)

	Region					
	A	*B*	*C*	*D*	*E*	*F*
Duplication	0	4	9	9	0	0
Deletion	1	0	2	2	2	2
Insertion	0	1	0	10	0	1

Table 2 Frequency of DNA binding motifs in 17 CSF amplicons

DNA binding motif	*Frequency of occurrence*		
	1	*2*	*3*
oct-6/tst-1 A	17	0	0
oct-6/tst-1 B	0	4	0
NF-1	0	9	8
pseudo-NF-1	16	0	0
GF_1	5	11	1
AP-1/c-jun	4	13	0
AP-1 like/c-jun	16	0	0
Pentanucleotide	0	5	0
Pentamut	11	0	0
TATA-Box	12	5	0
Sp1	12	0	0

Sequences of each DNA-binding site/motif are given in Figure 1 and the legend to Figure 1.

sequences (59%) had insertions in region D, all of which occurred in type II TCR (Figure 3, Table 1). A long duplication was observed in sequence 113D1, 61D1 and 93D1 encompassing a segment of the last portion of region B and first portion of Region D (Figure 3, Table 1). A triplication was found in one sequence (112D1) encompassing the junction of regions C and D (within 24 bp).

The frequency of occurrence of several DNA binding motifs in 17 CSF-derived sequences is given in Table 2. The most frequent transcription factor binding sites found were NF-1, followed by GF_1 and AP-1 sites. Oct-6/tst-1 site A, AP-1 like and pseudo-NF-1 sites occurred only once. Region A contained a binding site for oct-6/tst-1 site A in all CSF-derived sequences. An Sp1 site was observed in 12/12 (Figure 3) rearranged CSF sequences, but in none of the Mad-1 like sequences (Figure 2). In contrast, the pentanucleotide repeat sequence appeared twice only in the five Mad-1 like sequences and once in 11/12 type II sequences with a G to T substitution at position 37 relative to Mad-1 designated as pentamut (Figure 3). Region C was invariable in all sequences. The oct-6/tst-1 site B was only present in 4/5 TCR type I sequences (Figure 2).

Of 12 type II TCR CSF-derived sequences, six had juxtapositioned NF-1, GF_1 and AP-1 sites in region D, while a single AP-1 site was present in two instances in region D (134D1, 137D1). One sequence (112D1) showed a third, isolated GF_1 in region D. Another sequence (110D1) lacked the juxtapositioned NF-1, GF_1 and AP-1 site in region D but displayed an isolated GF_1-site in region D (Figure 3). One type II sequence (108D1) completely lacked binding sites in region D.

Region F contained a pseudo-NF-1 in 16/17 CSF-derived sequences.

Discussion

Direct cycle sequencing of PCR product was chosen as it is fast and reliable for DNA sequence analysis (Agostini *et al*, 1996a,b). In addition, cloning has shown a preponderance of a single TCR sequence in the majority of samples analysed so far (Ault and Stoner, 1993; Ciappi *et al*, 1999; Newman and Frisque, 1999; Sugimoto *et al*, 1998; Yogo *et al*, 1991). The ratio of nucleotide substitution errors produced by Taq polymerase is approximately 5×10^{-6} thus making direct sequencing of PCR products preferable to the sequencing of PCR clones (Arnheim and Erlich, 1992). Compared to other studies (Garcia de Viedma *et al*, 1999; Koralnik *et al*, 1999a; Vago *et al*, 1996; Weber *et al*, 1996) a much lower percentage (56.7% *versus* ~80%) of positive PCR results was obtained when targeting the TCR. The reason for the lower sensitivity observed in this study is best explained by the different regions analysed. The regulatory region is highly variable and has a high average GC content of 53% for the archetype sequence which could explain the much lower limit of detection and the ensuing reduced sensitivity reported here (Agostini *et al*, 1997; Ault and Stoner, 1993; Ciappi *et al*, 1999; Newman and Frisque, 1999; Yogo *et al*, 1994).

Sequence analysis of the JCV-TCR revealed two TCR types. The type I TCR is characterized by the fusion of the TATA box and following sequences leading to a repetition of elements that include the TATA box (Agostini *et al*, 1997; Ault and Stoner, 1992; Elsner and Dörries, 1998; Martin *et al*, 1985). For CSF, type I was found in five sequences and in none of the urine samples analysed. Type II TCR has one TATA box proximal to the early mRNA initiation site that is not included in the repeated elements. Of the 17 cases positive for JCV-DNA in CSF, 13 patients were HIV infected. The other four patients suffered from leukaemia, breast cancer, astrocytoma and multiple sclerosis (MS). Interestingly, in five cases, type I TCRs were amplified (Figure 2). Of these, three were identical to the prototype Mad-1 isolate, while two (127D1 and 138D1) differed slightly from Mad-1. The finding of three type I TCRs identical with Mad-1 and two closely related type I TCRs was unexpected. As these sequences were unambiguously identified in both strands sequenced and type II TCRs were identified concomitantly, contamination appears highly unlikely. One explanation could be that the type I TCR of the Mad-1 strain is biologically

advantageous and reflects a virus adaptation to the replication in human glial tissue. Based on 11/14 (79%) probable cases it appears that detection of a TCR type II sequence in CSF is more likely to support the diagnosis of PML while the detection of a TCR type I (Mad-1) sequence, seen in 3/3 possible PML cases may indicate temporarily replication only or reflects visceral replication, as reported recently for AIDS patients (Caldarelli-Stefano *et al*, 1999). As no biopsy or autopsy specimen for either group was available for sequencing, this hypothesis can not be validated. The detection of a rearranged TCR in an MS case was unexpected. On reexamination of a CSF sample from the same patient obtained 6 weeks after the first spinal tap JCV-DNA was not detected. These findings add a note of caution and suggest that a single positive CSF-PCR may not be sufficient for the diagnosis of PML (Ciappi *et al*, 1999). The rare detection of JCV-DNA in the CSF of patients with MS may be taken as evidence of a temporary viral replication in a state of altered cellular immunity. These findings do not imply a role of JCV in the aetiology for MS as previously suggested and recently reported (Ferrante *et al*, 1998; Johnson, 1975; Sarchielli *et al*, 1993; Stoner, 1993).

The high incidence of PML in AIDS patients as compared to patients with other immunosuppressive diseases suggests a molecular interaction between HIV and JCV contributing to the pathogenesis of PML in AIDS patients. Two regions of the JCV promoter at position nt 79–94 and nt 181–196 corresponding to regions C and E, respectively, have a high homology with the HIV-1 Tat-responsive element (TAR) and this JCV TAR homologue is responsive to HIV-1 Tat (Chowdhury *et al*, 1993). In the present study, duplications were found in region C in 82% (14/17) of CSF-derived sequences. Our findings support a functional role for the region from nt 74–84 as it is conserved in all sequences but not for nt 159–171 which is highly variable. Further studies of a significant number of PML patients suffering from various immunosuppressive conditions with detailed information about their immunological status and the presence of viral coinfections are important for the understanding of the mechanisms contributing to the replication of JCV and ensuing rearrangement of the JCV-TCR.

The previously described 'long duplicate' pattern could be identified in 5/5 (100%) of TCR type I or Mad-1 and Mad-1 like sequences, respectively (Ault and Stoner, 1993). In contrast to some reports, however, the 'D-retaining' pattern was detected most frequently (Ault and Stoner, 1993; Ciappi *et al*, 1999), as also reported by others (Sugimoto *et al*, 1998). This was found for 12 CSF derived sequences and in all 14 urine derived sequences. Twelve of 14 urine sequences were of the archetype and two urine-derived sequences showed rearrangements. This was not surprising as the rearranged form can also be present in the kidney and urine of immunocompromised individuals (Ault *et al*, 1994; Elsner and Dörries, 1998; White *et al*, 1992). Of these two cases, rearranged sequences were also detected in the CSF. The corresponding CSF sequences also possessed a type II TCR.

The rearranged JCV-TCR sequences were aligned with the archetype CY and divided in six regions as proposed, in order to identify rearrangement patterns of the TCR (Ault and Stoner, 1993; Sugimoto *et al*, 1998; Yogo *et al*, 1990). These were unique for each of the 12 rearranged sequences in CSF. This is in agreement with other reports showing highly divergent JCV regulatory regions in PML patients (Ault and Stoner, 1993; Ciappi *et al*, 1999; Elsner and Dörries, 1998; Sugimoto *et al*, 1998). In only one instance a deletion of two base pairs in region A was observed (Figure 3, Table 1). Region A contains an oct-6/tst-1 A binding site overlapping with the TATA box. Oct-6/tst-1 increases viral DNA replication by the synergistic interaction with the large T antigen and has been shown to bind to two sites in the transcriptional control region (Wegner *et al*, 1993). The oct-6/tst-1 motif site A was present in 17/17 amplicons, while the oct-6/tst-1 motif site B was found twice in 4/5 type I TCRs, i.e. in the Mad-1 like sequences. The TATA box appears to be important for the early transcription initiation and seems to confer glial specificity to the viral early promoter (Ahmed *et al*, 1990; Krebs *et al*, 1995; Tada *et al*, 1989). The TATA box is followed by a pentameric sequence AGGGA. The pentanucleotide sequence stimulates the viral DNA replication mediated by the JCV T antigen (Chang *et al*, 1994; Lynch and Frisque, 1990). The proximal TATA box and the pentanucleotide sequence were retained in all Mad-1 like CSF sequences and the distal TATA box was missing in all archetype and rearranged sequences (Flaegstad *et al*, 1991; Newman and Frisque, 1997; Yogo *et al*, 1991). This indicates that the second TATA box is not required for the transcription of the late genes (Frisque *et al*, 1984). Region B was retained in all TCRs sequenced analysed so far (Ault and Stoner, 1993; Ciappi *et al*, 1999; Newman and Frisque, 1997, 1999). Experimental findings have allocated a role for the pentanucleotide sequence in the regulation of JCV early gene expression, while Sp1 appears to regulate glial-specific gene expression concomitantly with the NF-1 sites in the tandem repeats (Chang *et al*, 1994; Henson *et al*, 1992; Henson, 1994).

The beginning of region C contains consensus NF-1, GF_1 and AP-1/c-jun binding sites (de Vries *et al*, 1985; Kerr and Khalili, 1991; Leegwater *et al*, 1985). Region C seems to be important for JCV expression in human brain cells owing to its NF-1, GF_1 and AP-1/c-*jun* sites. The NF-1 sites play a critical role in the brain-specific transcription from the early JCV promoter-enhancer (Kumar *et al*,

1996; Sumner *et al*, 1996). NF-1 was the most frequent motif observed in the 17 CSF amplicons. This motif appeared three times in 8/17 CSF sequences and it was present twice in 9/17 sequences. The frequency of NF-1 binding sites provides evidence for the importance of this motif in brain-specific regulation of the transcription of JCV early and late gene expression (Kumar *et al*, 1993; Zoltick *et al*, 1995). The most important NF-1 binding sites are the two sites inside the 98 bp tandem repeats (Amemiya *et al*, 1992; Kumar *et al*, 1993). In 6/12 type II TCRs an NF-1 site was retained in region D and always associated with a GF_1 and an AP-1 site (Figure 3). *In vitro* data have frequently assigned a prominent role of NF-1 for the regulation of JCV expression (Amemiya *et al*, 1992; Atwood *et al*, 1995; Kumar *et al*, 1994, 1996). Although the NF-1 site outside the tandem repeats (positions 213–225 according to Frisque (Frisque *et al*, 1984)) has been found to be less important, the findings of an additional NF-1 site towards the late genes in 14/17 (82%) CSF-derived sequences (4/5 Mad-1; 10/12 type II TCRs) argues for a functional role of these sites in the replication of JCV, most likely of the late genes (Amemiya *et al*, 1992; Atwood *et al*, 1995; Kumar *et al*, 1996; Shirohara *et al*, 1997).

Furthermore, region C seems to determine tissue specificity of JCV gene expression (Ault and Stoner, 1993; Ciappi *et al*, 1999; Sumner *et al*, 1996). Insertions were observed mostly in region D while deletions in this region were present in only two of 12 type II TCR sequences (Table 1). Deletions reported in this region do not imply that region D is not functional (Ault and Stoner, 1993; Ciappi *et al*, 1999). It appears that JCV is able to balance deleted elements by duplications and these rearrangement patterns are responsible for the growth of the virus in brain (Agostini *et al*, 1997; Ault and Stoner, 1993; Elsner and Dörries, 1998; Raj and Khalili, 1995). Region E was retained in all isolates sequenced so far.

Region F was present in most sequences with the exception of two sequences where short deletions were detected as reported by others (Ault and Stoner, 1993; Ciappi *et al*, 1999; Newman and Frisque, 1999). Region F contains sites for the NF-1 motif and the AP-1 like/c-*jun* binding site (Amemiya *et al*, 1992). The rearranged CSF sequences contained both binding sites with the exception of two sequences (93D1, 113D1). In these two sequences, an AP-1 like sequence was positioned towards the late genes. Based on the results of the sequence analysis of multiple clones from various tissues from two patients it is proposed that in regions E and F, rearrangements can occur at random (Newman and Frisque, 1999). The findings reported here and by others, however, suggest this to be a rare event (Ault and Stoner, 1993; Ciappi *et al*, 1999).

Based on the analysis of the distribution of mutations in the regions A to F regions A and C appear to be indispensable for JCV replication in brain tissue (Ault and Stoner, 1993; Ciappi *et al*, 1999; Newman and Frisque, 1999; Raj and Khalili, 1995). Further studies of the functional role of DNA binding motifs and factors in the regulation of JCV replication in PML should also use the most frequent 'D-retaining' pattern seen in the TCR type II identified here.

Materials and methods

Patients and samples

CSF and/or urine samples of 70 patients clinically suspected of having PML were analysed prospectively for JCV-DNA. All in all, 28 urine and 69 CSF specimens were analysed. The samples were stored at −80°C. The diagnosis of PML was based on clinical features and neuroimaging findings. Definite PML cases are defined as patients submitted to brain biopsy or autopsy with histological confirmation of the diagnosis of PML. Probable cases were defined as patients with MRI findings suggestive of PML, clinical signs and symptoms compatible with the diagnosis of PML and with at least two positive CSF-PCR results for JCV-DNA.

The findings of an intrathecal humoral immune response to VP1 as evidenced by an antibody specificity index (ASI) exceeding 1.5 was included for the diagnosis of probable PML, even in those cases, in whom the PCR was negative (Weber *et al*, 1997). Possible cases were defined as patients with MRI findings not compatible with PML or MRI not available, clinical signs and symptoms compatible with PML and with at least two positive CSF-PCR results. Patients with MRI findings not supportive of PML, clinical signs and symptoms compatible with PML with at least two negative CSF-PCR results for JCV-DNA were grouped as other cases. Of all patients, 59 were HIV-positive and 11 had other underlying immunosuppressive conditions. JCV-DNA was amplified in specimens obtained from 22 patients. A total of 14 urine specimens, 17 CSF specimens and six paired CSF/urine samples were available for direct cycle sequencing of JCV-TCR.

JCV-TCR PCR

Urine specimens (10–20 ml) were centrifuged at 500 r.c.f. for 10 min. The urine pellet was suspended in phosphate-buffered saline (PBS) pH 7.2 and stored at −80°C. The pellet was boiled for 5 min at 95°C to destroy possible heat-sensitive inhibitors before being used in the PCR reaction. CSF-DNA was purified using the QIAamp® Blood kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and stored at −20°C until DNA amplification.

Nested PCR (nPCR)

The JCV regulatory region was amplified from CSF-DNA by nPCR, using the external primers JC4850

(5′-ATTCTCTTCATCTTGTCTTCGTC-3′) and JC559 (5′-GCAGCCTCAGAAACAGTAGCAAC-3′) and the internal primers JC5041 (5′-GCAAAAAAGGGAAA-AACAAGGG-3′) and JC288 (5′-CAGAAGCCTTACG-TGACAGCTGG-3′). The limit of detection for each PCR reaction was monitored by limiting dilution using plasmid DNA (JCV-Mad-1) serially diluted in log 10 steps as standard. The first round of PCR was carried out in a total volume of 50 μl with a final concentration of 70 pM of each outer primer, 5 μl of 10x Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 1.5 mM $MgCl_2$), 2.5 mM of each deoxynucleoside triphosphate (dNTP). Each reaction mixture contained 10 μl of sample DNA and was overlaid with 50 μl of mineral oil. Reactions were heated to 85°C for 5 min, and rapidly chilled on ice. Following this pre-heating step, 2 units of Taq DNA Polymerase (Pharmacia Biotech, Freiburg, Germany) were added and samples were subjected to thermal cycling, using an UNO-Thermocycler™ (Biometra, Göttingen, Germany). The PCR for the outer primer pair consisted of 25 cycles. Denaturation was carried out at 95°C for 45 s. Primer annealing was performed at 55°C for 1 min and extension at 70°C for 2 min. Each successive extension was prolonged by 1 s and the last cycle was extended for 5 min. The samples were stopped at 4°C.

The second nested reaction contained 50 pM of the internal primers and the other component as described above. A 2 μl sample of the first amplification product was transferred to the second PCR mixtures. This PCR was run for 35 cycles and cycling parameters were 95°C for 45 s, 55°C for 1 min and 72°C for 1 min. In the first cycle, denaturation was extended for 5 min and each successive extension was prolonged by 1 s, with an additional 5 min added to the last cycle. PCR was stopped at 4°C.

All experiments were done in parallel with positive, negative controls and 10^6, 10^3 molecules of JCV plasmid DNA.

'Manual hot start PCR'

A 'manual hot start' PCR was developed to amplify the JCV-TCR in urine samples, using 60 pM of the primers JC4981 (5′-CATGGATTCCTCCCTATT CA-GCA-3′) and JC290L (5′-CACAGAAGCCTTACGT-GACAGCT-3′) under the same conditions as the first round of the nested PCR described above. An initial experiment using 3, 5, 7 and 10 μl of urine pellet were used in the PCR in order to find the optimal amount of template. PCR was performed with an UNO-Thermocycler™ (Biometra, Göttingen, Germany) for 35 cycles. Each cycle consisted of a 45 s 95°C denaturation, 1 min 55°C annealing, and 1 min 70°C extension, which was successively prolonged by 1 s, followed by a single 5 min extension at 70°C. Reactions were stopped by chilling on ice at 4°C.

Purification of PCR products

The PCR products were analysed by electrophoresis on 2% agarose gel stained with 0.5 μg/ml ethidium bromide and the desired DNA fragment was excised from the agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), as described by the manufacturer. The purified double-strand DNA (dsDNA) was resuspended in H_2O_{bidest} and then used as template for sequencing.

Cycle sequencing

Both negative and positive strand of the purified PCR products were sequenced using the primers JC5041, JC4981, JC290 and JC288, as previously described (Weber *et al*, 1994). The cycle sequencing reaction was performed according to the manufacturer's instruction using the ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Kit (Applied Biosystems, Weiterstadt, Germany), with 50–100 pM dsDNA and 10–15 pM sequencing primer. Fluorescence-based DNA sequence analyses were obtained on an ABI 377 DNA Sequencer (Applied Biosystems, Weiterstadt, Germany).

Computer analysis

Multiple sequences were first analysed with MacVector™ Sequence Analysis Software version 6.5 (Oxford Molecular Group PLC, USA). The sequences were aligned either with the CY archetype sequence or prototype Mad-1 strain when a duplicated TATA box was present (Frisque *et al*, 1984; Yogo *et al*, 1990). In order to identify repeat sequences, a second analysis was done with Lasergene Software for Macintosh and Power PC, Megalign 3.12 (DNASTAR Inc., Madison, USA). A dot plot was created for each sequence by comparing a sequence to itself with a percentage of homology set at 90% or higher and a minimal length of the region set at 10 nucleotides.

The sequences reported here have been deposited in the GenBank using BankIt. The submission numbers are 331403 for 61D1, 331431 for 118D2, 331426 for 112D1, 331427 for 113D1, 331417 for 93D1, 331420 for 107D1, 331424 for 110D1, 331439 for 137D1, 331438 for 134D1, 331423 for 108D1, 331436 for 123D1, 331429 for 120D1, 331442 for 127D1 and 331443 for 138D1.

Acknowledgements

The authors thank Dr T Rosenkranz, Department of Neurology, Allgemeines Krankenhaus St. Georg, Hamburg, Germany for providing patient samples and clinical data. We gratefully acknowledge the support for DNA sequencing by the members of the Institut für Zellbiologie und Klinische Neurobiologie Universitäts Krankenhaus Eppendorf, Hamburg, Germany. We thank

Dr F Buck for many helpful discussions and suggestions. This study was supported by a grant from the Deutsche Forschungsgemeinschaft to T Weber (We 1297/3-1) and in part by a grant from the Werner Otto Stiftung to T Weber. B Vaz was supported by the Deutsche Akademische Austauschdienst (grant 415/br-4-schr). M Pickhardt was supported by the Werner Otto Stiftung.

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