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Genotype Profile of Human Polyomavirus JC Excreted in Urine of Immunocompetent Individuals

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The human polyomavirus JC (JCV) causes the central demyelinating disease progressive multifocal leukoencephalopathy in about 5% of AIDS patients. To characterize the type profile of JCV in a control population in the United States, 54 females (10 to 79 years of age; average age, 43.4 years) and 51 males (18 to 94 years of age; average age, 47.9 years) were examined for the excretion of different genotypes of JCV in their urine by PCR followed by direct cycle sequencing. The group consisted of 89 patients of a general medical clinic in addition to 16 healthy volunteers. The overall incidence of JC viruria was 43 of 105 (40.9%) subjects, with a marked increase for those subjects above the age of 30 years. Two men were found to excrete two different types of JCV at the same time, indicating double infections. Of the three different genotypes of JCV identified to date, type 1 strains (European) were the most common in this cohort (64% of total strains) followed by type 2 (East Asian) (18%). No type 3 (East African) strains were detected. Indirect evidence for the existence of JCV type 3 was found in seven individuals (16%) in the form of a type 1/3 recombinant (also called type 4). In addition, a single example of JCV which differs from types 1, 2, and 3 and may represent a phylogenetically older type (type 5) was found in a 59-year-old African-American. Delineation of sequence variations between JCV types is essential for the design of primers for sensitive PCR with clinical samples.

The polyomavirus JC (JCV) is one of the most prevalent human DNA viruses in the population worldwide. It is closely related to the other human polyomavirus, BK, and to simian virus 40. Seroepidemiological studies have shown that more than 70% of the population generates a humoral immune response against JCV, mostly acquired in late childhood (24, 31). Recently three genotypes of JCV have been characterized. Single strains isolated in Europe and the United States fall within the type 1 [prototype JCV(Mad-1) (11)] or the type 2 [prototype JCV(GS/B) (17)] group (5, 13). Asian JCV genomes from Japan and China are without exception consistent with the type 2 sequence. JCV of East African origin is characteristic for type 3 (1). Types 2 and 3 are more closely related to each other than either one is to type 1.

After the infection of an individual by an unknown route, the virus persists in the kidneys and possibly other internal organs. In adults with immunodeficiency, JCV can be reactivated in oligodendrocytes and astrocytes, causing the central demyelinating disease progressive multifocal leukoencephalopathy (PML) (18, 30). The incidence of PML among AIDS patients in the United States and Europe is about 5% on the basis of neuropathological findings (7). Reactivation in the majority of cases is associated with the rearrangement of the regulatory region from which the genome is transcribed bidirectionally. In contrast, JCV excreted in the urine shows predominantly a nonrearranged, archetypal regulatory region (34). In addition to these sequence variations in the regulatory region arising within the PML patient by a process of deletion and duplication, stable genotypes arose by viral evolution in geographically isolated populations. For the examination of larger numbers of sequences, the amplification of short PCR fragments followed by direct cycle sequencing has been found useful to determine the viral genotype. The available sequence data for typing are concentrated in the coding region, especially the capsid protein VP1 and the 5′ end of the early regulatory protein, large T antigen (V-T-intergenic region). To the left of the origin of DNA replication in the noncoding regulatory region, three additional type-determining sites correlate well with the coding region scheme. Crossovers of typing sites in single strains have been reported (5, 13). The origins of these are unknown, but they may reflect occasional double infections.

Clinically, 25% of all AIDS patients who develop PML present with this central nervous system disease (7). Initial symptoms depend on the localization of the demyelinating foci but usually progress to severe impairment of visual, motor, or intellectual functions within months. Prolonged clinical courses of PML beyond 1 year are uncommon (8). The diagnosis of PML is based mainly on the clinical presentation, the detection by magnetic resonance imaging of demyelinating lesions, and the neuropathology of the brain biopsy (6). Amplification of viral DNA by PCR can be used to confirm the existence of JCV in the brain or the cerebrospinal fluid.

JCV sequences analyzed for type definitions were obtained from brains of PML patients (5, 33). The incidence of excretion of JCV types in an ethnically diverse population such as in the United States, which potentially harbors all three types, is unknown. This knowledge and the determination of type-specific sequence variations will be important to design correct primer sequences for sensitive PCR methods to diagnose PML or detect JCV in urine. In addition, type profiles in clinical samples will facilitate the identification of possible pathogenetic differences among JCV types. We therefore examined 105 individuals for their excretion of JCV in urine. Beside the distribution of JCV types 1, 2, and 3, evidence for a recombinant type circulating in the U.S. population and a possible ancestral sequence antedating types 2 and 3 are described in this work.

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TABLE 1. JCV primers for PCR and cycle sequencing

Primer	Sequence (5′ to 3′)	Position (nt)[a]	L[b] (nt)
JRR-25	CATGGATTCCTCCCTATTCAGCA	4981–5003	28
JRR-1	CTTCTGAGTAAGCTTGGAGGCGG	5103–5125	23
JRR-8	GGCGAAGAACCATGGCCAGCTGG	289–267	23
ECO-11	AGATACATTTGAAAGTGACTCCC	1663–1685	23
ECO-7	AGAATTCCACTACCCAATCTAAATGAGGAT	1721–1751	31
ECO-12	TGGAATTCTGGCCACACTGTAACAAG	1729–1704	26
JLP-1	CTCATGTGGGAGGCTGT(G/T)ACCT	1769–1790	22
JLP-4	ATGAAAGCTGGTGCCCTGCACT	1897–1876	22
VPV-9	TTTTGGGACACTAACAGGAG	2107–2126	20
VPV-9/2	CACTGTTGTGGCAGTGTTTGTT	2176–2155	22
VPV-10	TCCCTCAAAAACTCTAACCTC	2455–2435	21
BAM-1	GGGATCCTGTGTTTTCATCATCACTGGC	4306–4333	28
BAM-2	AGGATCCCAACACTCTACCCCACC	4313–4290	24

[a] nt, nucleotide. Numbering is based on the sequence of JCV(Mad-1).
[b] L, length of oligonucleotide.

MATERIALS AND METHODS

Participants. Single urine samples were obtained from 105 individuals from Pennsylvania and Maryland (suburban Washington, D.C.), of which 89 were collected at a general medical clinic. Sixteen healthy volunteers were included in the study. The age of the 54 female donors ranged from 10 to 79 years, with an average of 43.4 years and a median of 41 years. The 51 male donors, with an age range of 18 to 94 years, had an average age of 47.8 years with a median of 47 years. Five individuals were African-American.

Samples. Fresh urine specimens (40 to 100 ml) were stored at 4°C until they were centrifuged at 10,000 × *g*. The pellet from each specimen was washed in sterile phosphate-buffered saline before overnight digestion with proteinase K in a total volume of 100 to 250 μl as described previously (1). Upon centrifugation, 2 to 5 μl of the supernatant was used for PCR.

PCR. A 129-bp fragment from the VP1 coding region was amplified with the primer pair JLP-1 and JLP-4 (JLP-1&4). For oligonucleotide sequences see Table 1. A low level of background amplification was achieved by a 50-cycle, two-step program of 1 min at 63°C for annealing and elongation and 30 s at 94°C for denaturation, and this was followed by 10 min at 72°C and termination of the reaction at 4°C by using the UlTma DNA polymerase with 3′-to-5′ exonuclease (proofreading) activity (Perkin Elmer Cetus, Norwalk, Conn.). This PCR product includes four known typing sites which distinguish JCV types 1, 2, and 3 (1, 13). The regulatory region was amplified with the primer pair JRR-1&8 in a 50-cycle, three-step program with 1 min at 60°C for annealing, 1 min at 72°C for elongation, and 1 min at 94°C for denaturation. Reactions were started at 80°C (hot start) by adding the DNA polymerase.

PCR of the complete JCV genome. JCV-positive samples were further processed for complete PCR amplification of the JCV genome as described previously (2). Briefly, the DNA extracts were digested with the restriction enzymes *Bam*HI and *Eco*RI before PCR amplification with overlapping primer pairs BAM-1&2 and ECO-3&4, which flank the respective restriction enzyme sites (Table 1). The combination of thermostable DNA polymerases with and without 3′-to-5′ exonuclease activity and buffer additives facilitating strand separation (GeneAmp XL PCR; Perkin Elmer Cetus) was used for a total of 39 cycles with increasing annealing-extension time (6 to 12 min) at 64°C and denaturation (30 s) at 94°C followed by final extension (10 min) at 72°C. The reaction was stopped at 4°C.

Cycle sequencing. Upon purification by preparative agarose gel electrophoresis or the Qiagen (Chatsworth, Calif.) procedure, PCR-amplified fragments including the complete genome PCR product were used as template for direct cycle sequencing with primers ECO-11, JLP-1, JLP-4, VPV-9, VPV-9/2, and VPV-10 for the VP1 coding region and primers JRR-1, JRR-25, and JRR-8 for the regulatory region (Table 1). Primers were end labeled with [^{33}P]ATP (Amersham, Arlington Heights, Ill.) mixed with 2 to 5% of the cleaned template, chain-terminating nucleotides, and a thermostable DNA polymerase (SequiTherm cycle sequencing; Epicentre Technologies, Madison, Wis.). The annealing temperature in the 30-cycle reaction was 60°C, except that for VPV-9/2 (65°C). The products were analyzed on a 6% polyacrylamide-urea gel as described in detail previously (1, 25).

Reference sequences. The following is a list of GenBank accession numbers for regions referred to in this work: JCV archetypal regulatory region JCV(CY), M35834 (34); JCV coding region JCV(Mad-1), J02227 (11), JCV(GS/B), M20322 (17); consensus sequences for the V-T-intergenic regions of types 1, 2, and 3, U21839, U21840, and U21841, respectively (see reference 5 for types 1 and 2 and reference 1 for type 3); for the JLP-1&4 fragments of types 1, 2, and 3, U21842, U21843, and U21844, respectively (see reference 1). Numbering of the coding region JCV sequence is based on the sequence of JCV(Mad-1). Regulatory region numbering is based on the archetypal sequence of JCV(CY).

Statistical and sequence analyses. Sequence analysis utilized version 7-UNIX programs of the Genetics Computer Group, Inc., on a Silicon Graphics IndigoR4000 computer. Primer design and analysis were assisted by OLIGO version 5.0 (NBI, Plymouth, Minn.). Statistical parameters were analyzed by the chi-square test and the Fisher exact test provided by the SigmaStat program.

RESULTS

Urinary excretion of JCV. Of 105 individuals tested by PCR amplification of a 129-bp fragment from the capsid protein VP1 coding region, 41% (43 of 105) were shown to excrete JCV DNA in urine. On average, males (49% [25 of 51]) had a higher excretion rate than did females (33% [18 of 54]). Two men were doubly infected (see below). The incidence of JCV excretion is related to age (Table 2). Excretion appears to be minimal during childhood and adolescence, although this series focused on adults. The probability of finding JCV in a single urine sample increases to 42% (females) and 69% (males) in the fifth decade of an individual's life. Determining whether fewer men and women between the ages of 51 and 60 years excrete JCV than do individuals in their 40s and 60s will require a larger series of samples. The differences noted here are not statistically significant.

JCV type distribution. The type of JCV excreted by the 43 individuals was determined by cycle sequencing of the JLP-1&4-amplified fragments from two directions. Within this part of the VP1 coding region, the known JCV types 1, 2, and 3 are clearly distinguishable at the four positions 1804, 1818, 1869, and 1870. In addition, it was possible to identify two subtypes of JCV type 1, types 1A and 1B, on the basis of nucleotide positions 1843 and 1850, as shown in Fig. 1. The partial type 1A sequence is consistent with the prototype JCV(Mad-1). The significance of this 129-bp PCR product for the JCV type

TABLE 2. Age dependence of JCV excretion in females and males

Age group (yr)	No. positive/total no. of individuals (%)[a]	
	Female	Male
≤20	0/3 (0)	0/3 (0)
21–30	1/8 (13)	2/6 (33)
31–40	5/14 (36)	3/7 (43)
41–50	5/12 (42)	9/13 (69)
51–60	2/9 (22)	5/12 (42)
61–70	2/3 (67)	4/6 (67)
≥71	3/5 (60)	2/4 (50)

[a] Number of JCV-positive individuals/total number of individuals examined.

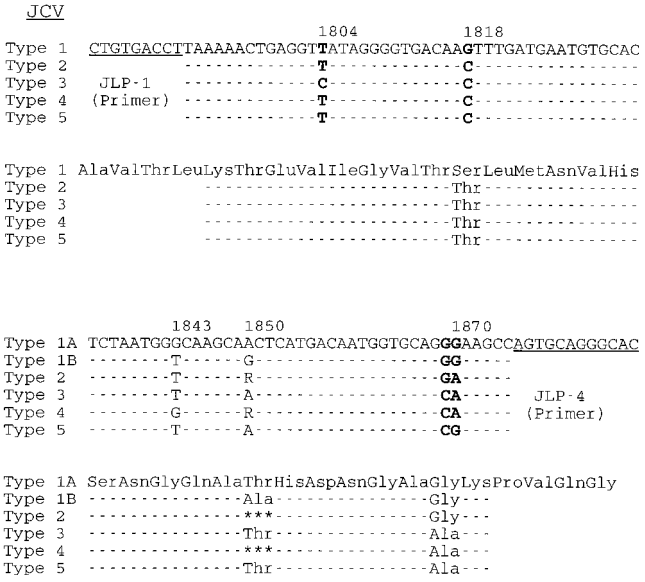

FIG. 1. Sequence of the 129-bp PCR product as amplified by primer pair JLP-1&4. Typing sites allow us to distinguish JCV types 1 through 5 and two subtypes of type 1. Type 4 represents seven strains with identical recombinations of types 1 and 3. The strain tentatively called type 5 possibly diverged from a phylogenetically older strain of JCV. Major typing sites are shown in boldface type. R, A or G. Numbering is based on the sequence of JCV(Mad-1).

determination was confirmed by analysis of an additional 600 bp from the V-T-intergenic region with the complete genome PCR products of 10 samples representing the different major JCV types. The typing results from the short and long fragments agreed with previously published typing schemes (5, 13). Table 3 illustrates the overall type distribution among the 45 examples of JCV excreted by 43 individuals. No significant correlation of JCV type of excretion and age of the individual was detectable (Table 4). The dominant JCV type, type 1, was found in 64% (29 of 45) of the strains, and of these 34% (10 of 29) were type 1A and 66% (19 of 29) were type 1B. Of the detected strains, 18% (8 of 45) showed a type 2 sequence pattern. No JCV type 3 strains as previously described in urines from human immunodeficiency virus type 1-positive patients from Tanzania were identified in this group of 105 individuals (1).

Double infections. A 23-year-old man appeared to be infected by two different strains of JCV which could be characterized as JCV types 1A and 2. In the urine of another 38-year-old man, sequences consistent with JCV types 1A and 1B could be identified. This increases the total number of strains identified in 43 JCV-positive patients to 45. Characterization of these and other double infections by direct cycle sequencing will be outlined elsewhere (unpublished data).

TABLE 3. Distribution of five JCV types in 105 individuals

JCV type	JCV-positive individuals	
	No.	% of total[a]
1A	10	22
1B	19	42
2	8	18
3		
4	7	16
5[b]	1	2
Total	45	100

[a] All together, types 1A and 1B accounted for 64% of the total JCV types detected.
[b] Tentative designation.

TABLE 4. Summary by age group of JCV types excreted

Age group (yr)	No. of samples with detected JCV type					
	1A[a]	1B[a]	2	3	4	5
21–30	2	1	1			
31–40	2	3	1		3	
41–50	4	7	2		1	
51–60		3	1		2	1
61–70		4	1		1	
≥71	2	1	2			
Total	10	19	8	0	7	1

[a] All together, types 1A and 1B accounted for 29 of the total JCV types detected.

Apparent recombinant JCV type 1/3. In addition to excreting JCV of the described types 1 and 2, seven individuals excreted JCV with a previously unknown sequence within the JLP-1&4-amplified fragment, as illustrated in Fig. 1 (referred to as type 1/3 or type 4). At position 1804, all seven strains shared deoxythymidine (T) with types 1 or 2 but showed a type 3-like sequence at the other three typing sites (positions 1818, 1869, and 1870). The complete viral DNA from three samples was amplified by PCR and partially cycle sequenced within the VP1 coding region. It became clear that for at least 115 bp upstream of the 1804 position, all type 4 sequences were identical to those of type 1 strains. Downstream of the JLP-1&4-amplified fragment, type 4 strains shared an additional type 3 site at position 1959. After these consecutive four type 3-determining sites (1818 to 1959), the type 4 sequence crossed back to the type 1 consensus sequence before position 1993, as shown in Fig. 2. Within the next 738 bp, type 4 differs only at three single typing sites from the type 1 consensus sequence. At sites 2227 and 2661 in all other JCV types (see Fig. 4), T can be found. Crossovers at position 2227 have been previously described (5). At position 2356, the T in type 4 strains is shared by some type 2 strains (5, 13). The sequence to the left of origin of DNA replication was also analyzed in the complete genome PCR products of the type 4 strains, with primer JRR-25 for cycle sequencing, and was found to be identical to type 1 (A, C, and C at positions 5017, 5026, and 5039, respectively).

Regulatory region. Cycle sequencing of the noncoding regulatory region included 13 products of complete genome PCR amplification and seven 308-bp fragments amplified by the primer pair JRR-1&8, and of these strains 11 have been classified as type 1 (1 type 1A and 10 type 1B) and four, four, and one have been classified as types 2, 1/3, and 5, respectively. Eighteen strains analyzed showed an archetypal configuration without duplications or deletions. The regulatory region of a JCV type 2 strain (208) amplified from the urine of a 50-year-old man by short-range PCR showed a deletion of two nucleotides at positions 221 and 222 (archetypal sequence numbering [Fig. 3]). The possible type 5 sequence will be described in detail below.

Sixteen of the regulatory region sequences from strains of JCV types 1, 2, and 4 were identical (Fig. 3). These provide a consensus sequence for the archetypal regulatory region which differs from the prototype type 2 Japanese strain JCV(CY) (34), and from four JCV regulatory region sequences from

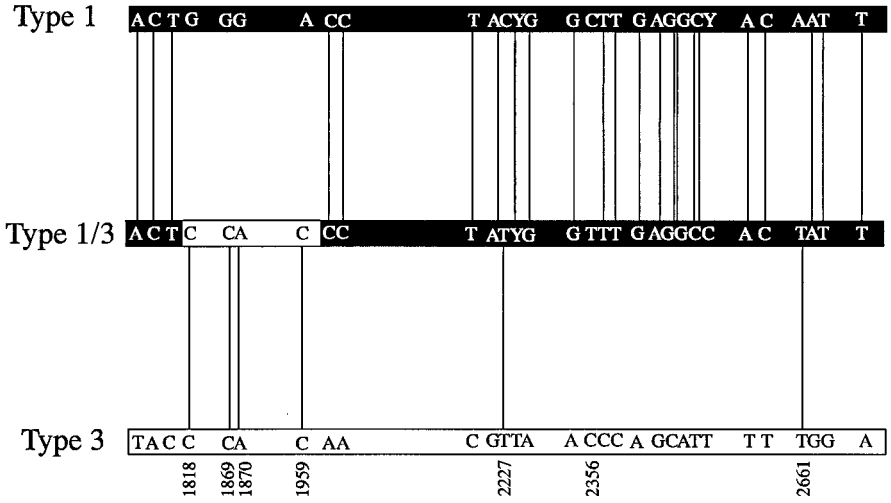

FIG. 2. The type 1/3 (type 4) sequence of the VP1 coding region contains parts of JCV types 1 and 3, most likely as a result of recombination. Vertical lines indicate typing sites by which JCV types 1 and 3 are distinguishable, and these sites are shared by three type 4 strains as analyzed by complete genome amplification and partial cycle sequencing. T at positions 2227 and 2661 can be found in all types except type 1. At position 2356, type 4 differs from types 1 and 3. Y, T or C. Numbering is based on the sequence of JCV(Mad-1).

pregnant women in the United States (19), as well as from East African type 3 strains (1). All these replace the deoxyadenosine (A) at position 217 in the new consensus sequence with deoxyguanosine (G). However, the type 2 strain (208) with the short deletion and an additional point mutation at position 219 shared with these strains the G at position 217. Other single base pair substitutions at sites of polymorphism described previously (19, 27, 34) were found at positions 108 and 159 in a type 1 (108) and a type 2 (207) strain, respectively (Fig. 3).

```
JCV
                1                                                  50
Archetype       GCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGG
Type 1 (11)     --------------------------------------------------
Type 2 (4)      --------------------------------------------------
Type 4 (4)      --------------------------------------------------
Type 5 (1)      --------------------------------------------------

                                                               100
                CTAAAACTGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCA
Type 1 (11)     --------------------------------------------------
Type 2 (4)      --------------------------------------------------
Type 4 (4)      --------------------------------------------------
Type 5 (1)      --------------------------------------------------

                     AAG^T                      C                150
                ACCAGCTGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATA
Type 1 (10)     --------------------------------------------------
  # 108         -------A--------------------------------------------
Type 2 (4)      --------------------------------------------------
Type 4 (4)      --------------------------------------------------
Type 5 (1)      -------T--------------------------------------------

                 C      A^G                                      200
                GTGAAACCCCTCCCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGG
Type 1 (11)     --------------------------------------------------
Type 2 (3)      --------------------------------------------------
  # 207         --------A-------------------------------------------
Type 4 (4)      --------------------------------------------------
Type 5 (1)      --------------------------------------------------

                              G            C              A     250
                AAGTGGAAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGG
Type 1 (11)     --------------------------------------------------
Type 2 (3)      --------------------------------------------------
  # 208         ----------------G-A-..-----------------------------
Type 4 (4)      --------------------------------------------------
Type 5 (1)      ----------------G---------------------------------

                           270
                CTTGTCACCAGCTGGCCATG
Type 1 (11)     --------------------
Type 2 (4)      --------------------
Type 4 (4)      --------------------
Type 5 (1)      --------------------
```

FIG. 3. Consensus regulatory region sequence (archetype) compared with sequences of different JCV types, including 11 type 1 strains, 4 type 2 strains, 4 type 4 strains, and 1 strain with archetypal configuration, tentatively called JCV type 5. The type 2 strain 208 showed a 2-bp deletion at position 221 to 222. Superscripted nucleotides indicate polymorphisms as reported previously (1, 9, 19, 34). Numbering is based on the sequence of JCV(CY).

Possible JCV type 5. The partial sequence of the VP1 and T antigen coding region and the associated intergenic region of a JCV strain detected in the urine of a 59-year-old African-American man is shown in comparison with the consensus sequences of types 1, 2, and 3 in Fig. 4. In this segment of 948 bp, JCV types 1, 2, and 3 are distinguishable at 34 major typing sites. In this segment, the possible type 5 sequence (500) differed at 26 sites from the JCV type 1 consensus sequence, at 12 sites from the type 2 sequence, and at 9 sites from the type 3 sequence. Two sites (positions 2407 and 2633) within the V-T-intergenic region analyzed were unique to this strain. In contrast to the mostly silent or conservative predicted amino acid changes between types 1 and 2, substitution of A by deoxycytidine (C) at position 1959 in type 3 and 5 strains changed the predicted amino acid sequence from lysine (K) to threonine (T). An additional change of a charged and a neutral amino acid, as indicated in Fig. 4, could be predicted on the basis of nucleotide changes at position 2462 in the carboxy-terminal VP1. In JCV types 3 and 5, this triplet coded for glutamine (Q) instead of glutamic acid (E) as in types 1 and 2.

The regulatory region of JCV type 5 was of the expected archetypal structure. The sequence left of the origin of DNA replication, including the three typing sites, was found to be identical to that of type 3. However, the regulatory region differed from that of type 3 strains at position 133 but not at 217 and shows a previously undescribed T at position 108 in the polymorphic triplet (nucleotides 107 to 109, Fig. 3) (1, 9, 19, 34).

DISCUSSION

This study delineates for the first time the JCV type profile in the urine of control individuals in the United States and allows precise primer design for maximum sensitivity of PCR amplification from clinical samples. The dominant genotype found in nearly two-thirds of the strains excreted by this cohort was JCV type 1, followed by JCV type 2 and a recombinant,

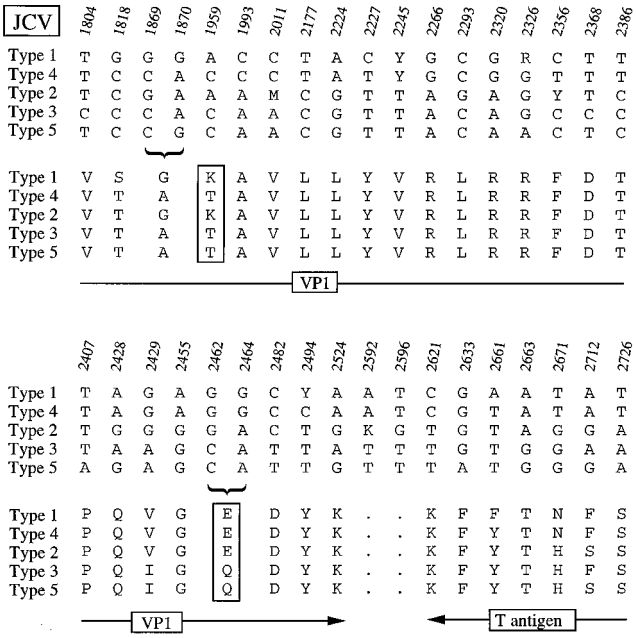

FIG. 4. Typing sites within the VP1 and T antigen coding region and the associated intergenic region of the strain tentatively called JCV type 5 compared with the consensus sequence at the typing sites of JCV types 1 through 3, including the recombinant type 4 (type 1/3). Note that the nucleotide changes at positions 1959 and 2462 result in complementary amino acid changes (framed) with regard to the VP1 net charge in JCV types 1, 2, 3, and 5 but not in type 4. Numbering (shown above the DNA sequences) is based on the sequence of JCV(Mad-1). M, A or C; Y, T or C; R, A or G. Amino acids are shown in one-letter code.

type 1/3 (or type 4). Although the JCV type 3 recently characterized in East Africa was not identified in this study, we have subsequently identified a type 3 sequence in the urine of an African-American multiple sclerosis patient from Los Angeles, California (unpublished data). The low proportion of type 2 strains was unexpected on the basis of the original type-defining work on brains of PML patients, in which the occurrence of JCV type 1 was nearly equal to that of type 2 (5). Additional data on genotypes of JCV amplified from brain tissue and cerebrospinal fluid of AIDS patients with PML indicate that there is, in fact, a significant difference between the genotype profiles of the viruses obtained from control urine samples and those in PML patient brain and CSF samples (unpublished data).

In our experience, JLP-1&4 is a sensitive primer pair for diagnostic PCR, yielding a fragment of 129 bp (Table 1). The JLP-1 primer is synthesized with a redundancy at position 1786 to accommodate the mutation of G to T, which is found at this site in some type 2 strains. A variety of primers from both early and late coding regions have been proposed for diagnostic use on the basis of the Mad-1 sequence of JCV (type 1) (4, 29, 32). Although these primers amplify both type 1 and type 2 sequences, some of them might be improved by introducing sequence redundancy to accommodate sequence differences between the genotypes. For example, the primers designated V (position 2481 to 2500) (29) and JC-26 (position 2450 to 2469) (32) in the VP1 gene each span two or three type-determining sites (Fig. 4). In addition, the primer designated T (position 4481 to 4500) (29) and the primer JEP-1 (position 4283 to 4322) (4) as well as the probe JC-4849 (position 4849 to 4878) (32) each show a difference in sequence between Mad-1 and GS/B strains at a single site. The demonstration that these sites represent type-specific changes rather than random mutations awaits the determination of a consensus sequence for each genotype. It should be noted that the primers in the most widely used primer pair for JCV detection in clinical samples, PEP-1 and PEP-2 (3, 4, 10, 22, 23, 28), have identical sequences in both Mad-1 (type 1) and GS/B (type 2) strains.

The JCV strain amplified from the urine of a 59-year-old African-American man represents a different type or subtype of the virus, tentatively called type 5. On the basis of the portion of the sequence analyzed in this study, JCV type 5 is more closely related to type 3 as identified in Tanzania, East Africa, and to type 2 than it is to type 1. Since it shares unique typing sites within the coding region with strains of all these types, it could be derived from an ancestral sequence of JCV that antedates the divergence of types 2 and 3. In addition, type 5 has a unique T at position 108 in the regulatory region which has not been previously described in strains from the United States, Europe, or Asia. It is possible that this rare type may have been brought to North America by early migration from Asia or later migration from Africa. Another explanation for this unusual genome would be a late origin from multiple recombinations between different genotypes with additional point mutations. Supplementary sequence data from the type 5 genome should decide its early or late origin and its designation as a major type or subtype of JCV type 3.

In this cohort, JCV DNA was excreted in the urine with an incidence of 30 to 50%, confirming that viral DNA is highly prevalent in the urine of nonimmunocompromised individuals without neurological symptoms (14, 15, 20, 26). This excretion increases with age to nearly 50% for patients above the age of 30 and may be somewhat higher for men than for women in the 41-to-60-year-old age group (although this difference is not statistically significant). As shown by complete genome PCR amplification from urine (2), the viral DNA is not fragmented. It is likely that complete viral particles are excreted, as originally shown by electron microscope studies (12).

In contrast to the JCV coding region, which shows sequence variations, the JCV regulatory region is highly conserved among different genotypes. This reflects the complex regulatory functions in the bidirectionally transcribed genomes of human polyomaviruses. A few exceptions, which may be based on phylogenetic differences, exist. For example, only in JCV type 3 strains from Tanzania was C found at position 133. At position 217, no G has been identified in any type 1 strains. Both of these sites are located in or near NF-1 binding motifs (TGG. . .CCA) (16, 19), and functional type-specific differences within the regulatory region are possible.

During the sequencing of the VP1 coding region of JCV types 1 and 2, strains with occasional nucleotide exchanges at typing sites, so-called crossovers (5), were found. These crossovers in single JCV strains led to the hypothesis of double infections with different JCV types. In the present study, two men were found to excrete different types or subtypes of JCV at the same time. In addition, the identification of recombinant JCV type 4 (type 1/3) in the urine of 7 of the 105 individuals supports the idea of double infection with two JCV types. In contrast to the occasional single nucleotide exchange, this recombination of a JCV type 1 with about 150 bp from a type 3 DNA resulted in a stable JCV genome which spread widely in the population. Sequencing the entire type 4 genome may provide clues to the basis for its biological success.

One interesting aspect of coding region recombination becomes clear when the net charge of the predicted amino acid sequence is examined. At two positions, the type-specific change in the nucleotide sequence leads to the loss of charged amino

acids in strains of types 3 and 5 (Fig. 4, framed amino acids). The exchange of basic lysine for the neutral threonine is associated with the loss of glutamic acid in favor of the neutral glutamine residue, leaving the net charge unchanged in types 3 and 5. In recombinant type 4 strains, the change of lysine to threonine but not glutamic acid to glutamine can be predicted to leave a +1 net charge compared with all other JCV types.

The JLP-1&4-amplified fragment of the VP1 coding region where this apparent recombination phenomenon occurred was also shown to be a site of deletions, as detected in two AIDS patients with rapid, progressive courses of PML (25). This coincidence confirms that this region can be a site of DNA breakage and rejoining in the viral genome. In the mouse polyomavirus, viral replication is enhanced by deletions in the structural protein coding region (21). It will be of interest to identify any other locations within the JCV genome, besides the regulatory region and the VP1 gene, where deletions and recombinations occur.

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