Comparison of Infectious JC Virus DNAs Cloned from Human Brain

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We cloned JC virus DNA obtained directly from brain tissue of 10 cases of progressive multifocal leukoencephalopathy and compared DNAs by restriction endonuclease mapping. Before cloning, each DNA preparation was homogeneous with respect to restriction patterns, but with the cloned DNAs we found variability in three regions of the genome among DNAs from different cases. There was a region of hypervariability between 0.67 and 0.725 map units; no two DNAs were exactly alike in this region. We determined that the origin of DNA replication also was in this region at 0.69 ± 0.02 map units. In 4 of the 10 DNAs examined there was a deletion of approximately 75 base pairs between 0.14 and 0.235 map units, the region presumed to contain the codons for the C-terminal ends of the structural protein Vp1 and for T antigen. JC virus DNA from these same four cases had an additional *HincII-HpaI* site at 0.895 map units in the presumptive Vp3 and Vp2 coding regions. Overall, no two JC virus genomes were identical although all were from fatal central nervous system infections and were infectious in vitro. Our restriction patterns suggest that there are two subtypes of JC virus circulating in the population.

JC virus (JCV) is the most commonly isolated virus from progressive multifocal leukoencephalopathy (PML), an uncommon but destructive demyelinating disease of the central nervous system. Although PML is rare, JCV is ubiquitous in the human population; approximately 75% of adults possess antibodies to the virus (28). Primary infection by JCV is usually experienced before adulthood and apparently results in long-term persistence of the virus (29). Serological studies of renal transplant patients (14, 18) and JCV viruria in pregnant women (7, 10) suggest that the virus persists in renal tissue and can be reactivated under certain conditions. However, even in populations in which reactivation is known to occur with some frequency (e.g., renal transplant recipients) PML is a rare event, and it is not known whether the disease results from the reactivation of persistent virus or from primary infection while immune functions are depressed. Overall, the pathogenesis of nervous system infection is not clear. Certainly one factor in the development of PML is the status of the individual's immune system, as PML generally occurs in persons with concurrent conditions that impair immunity or in persons undergoing immunosuppressive therapy. However, an equally important factor could relate to strain differences in JCV.

With a number of viruses, it has been possible to correlate specific genetic types with specific

disease manifestations and to apply this information to pathogenesis. For example, among the DNA viruses, comparative restriction endonuclease mapping studies of the genomes of herpesviruses have demonstrated strain differences, not detectable serologically, that have been useful in understanding the spread of the virus and in studying reactivation of latent virus (2, 4, 22, 36). Similar molecular epidemiological studies have correlated specific genetic subtypes of adenovirus type 19 with epidemic keratoconjunctivitis (41) and specific papillomavirus types with specific kinds and locations of papillomas (3). A similar approach might be useful in understanding certain aspects of PML not approachable by conventional methods. However, there have been relatively few isolations of JCV from either brain or urine and even fewer studies of the DNA of such isolates. Because of this, there is little information on the natural genetic variation of JCV DNA. In fact, comparative studies of culture-grown JCV DNA would be difficult to interpret because passage in cell culture introduces heterogeneity (13, 25) not present in the viral DNA in brain (15). Therefore, DNA extracted directly or cloned directly from organ material should be used. Such DNA from brain tissue of two PML cases has been examined (35), but the DNAs were not shown to be either infectious or representative of the JCV DNA in the brains. The two DNAs were found

to be identical to prototype MAD-1 DNA except for minor variation in the region between 0.67 and 0.74 map units. From analogy with simian virus 40 (SV40) it is thought that the origin of JCV DNA replication is located in the general region showing the variation mentioned above (24). Naturally occurring variation around the origin is seen with regularity in SV40 and in the human polyomavirus BK virus (BKV) (19, 37, 40, 43), and Howley et al. (19) have suggested that this region in the papovaviruses is susceptible to drift. Therefore, it would not be surprising that JCV DNA should show variation between 0.67 and 0.74 map units if the origin is located nearby. Thus, this type of variability might be of limited use in molecular epidemiological studies.

We recently isolated JCV DNA from 13 cases of PML and showed that each preparation was homogeneous (15). To provide more information on the natural variability in JCV and to determine the feasibility of applying molecular epidemiology to this virus, we cloned 10 of the DNAs as representatives of the virulent JCV DNA in PML and analyzed each by restriction mapping. We found that JCV DNA can be divided into two subtypes based on their *HpaI*, *HincII*, and *PvuII* restriction patterns. Additionally, JCV DNAs of both subtypes varied in the region between 0.67 to 0.72 map units; no two DNAs were identical. We determined that the origin of DNA replication was in fact in this region. In spite of this variability, all cloned DNAs were infectious in primary human fetal glial (PHFG) cell cultures.

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MATERIALS AND METHODS

Cell culture and virus. PHFG cell cultures were prepared from brain tissue of abortions and maintained as previously described (26). Culture-passaged virus was the prototype MAD-1, originally isolated by inoculating an extract of PML brain into PHFG cells (30). Cultures were infected with 100 to 200 hemagglutinating units of MAD-1 and incubated 3 to 4 weeks with weekly changes of medium (minimal essential medium with 3% newborn calf serum).

PML brain tissues. Tissues used for JCV DNA isolation were from autopsy of confirmed PML patients. Clinical diagnosis was confirmed by indirect fluorescent antibody tests with antisera specific for JCV (31, 42) and by characteristic histopathology. PML cases were designated by number with case 1 corresponding to the brain tissue from which the prototype JCV MAD-1 was isolated. The other cases used in this study were described elsewhere (15).

Preparation of JCV DNA. JCV DNA was extracted

from infected PHFG cells and PML brain tissue by the method of Hirt (17). Supercoiled DNA was purified by ethidium bromide buoyant density centrifugation (32) and stored in TE buffer (10 mM Tris-hydrochloride, pH 7.8, 1 mM EDTA). The JCV DNAs from each PML brain were designated as Br followed by the case number.

Restriction enzyme cleavage. Restriction endonucleases were obtained from Bethesda Research Laboratories. Unless otherwise stated, the amount of enzyme used was 2 times the commercial unit necessary to digest 0.5 μ g of DNA completely. Reactions were carried out under conditions specified by the manufacturer for each specific enzyme. However, when a DNA sample was digested by more than one enzyme, both were added simultaneously to the DNA in buffer containing 10 mM Tris-hydrochloride (pH 7.6)–7 mM $MgCl_2$ –50 mM NaCl–1 mM dithiothreitol and 1 μ g of autoclaved gelatin (Difco Laboratories) per ml. Digestions were carried out for 2 to 4 h at 37°C and terminated by incubation for 10 min at 60°C. Samples were separated on 1.4% agarose or 8% polyacrylamide gels (Sigma Chemical Co.). Electrophoresis (E) buffer consisted of 10 mM Tris-acetate (pH 7.8), 5 mM sodium acetate, and 1 mM EDTA. A constant current of 25 mA per gel was maintained for 6 to 8 h at room temperature. Gels were stained with ethidium bromide (0.5 μ g/ml) and photographed under short-wave UV light. Full-length linear JCV DNA was separated from *EcoRI*-digested plasmid by electrophoresis on 0.7% low-melting-temperature agarose gels (Seaplaque, Marine Colloids) in E buffer. The DNA, stained with ethidium bromide, was visualized under UV light. The DNA-containing slice was excised from the gel and melted at 60°C. The mixture was extracted three times with phenol, twice with chloroform-isoamyl alcohol (24:1 vol/vol), and the DNA was precipitated with ethanol. The DNA was collected by centrifugation and the resulting pellet was suspended in TE buffer.

Molecular cloning. The procedure for cloning was essentially that described by Israel et al. (23). Br-DNAs 1, 8, 9, 10, and 11 were cloned in collaboration with R. Frisque (Pennsylvania State University). The plasmid pBR322 was prepared by the method of Guerry et al. (16) and digested with *EcoRI*. The digested DNA was extracted twice with phenol and once with chloroform-isoamyl alcohol (24:1 vol/vol). It was precipitated for 2 h at –70°C with 2 volumes of 100% ethanol, and the linear DNA was collected by centrifugation. The resulting pellet was suspended in 10 mM Tris-hydrochloride (pH 9.0). To reduce self ligation of the plasmid, the 5' phosphates were removed by incubating the *EcoRI*-linearized DNA with 50 units of calf alkaline phosphatase (Worthington Diagnostics) for 45 min at 37°C.

Purified calf alkaline phosphatase-treated pBR322 was ligated to the *EcoRI*-digested Br-DNA by incubation at 14°C for 12 h with T4 ligase (New England Biolabs) in buffer containing 50 mM Tris-hydrochloride (pH 7.8), 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, 0.2 mM EDTA, and 50 μ g of bovine serum albumin per ml. Ligated DNA preparations were used to transform ampicillin-sensitive *Escherichia coli* K-12 strain HB101 under essentially the conditions described by Cohen et al. (6). Transformation buffer contained 100 mM RbCl, 45 mM $MnCl_2$, 35 mM potassium acetate, 10 mM $CaCl_2$, 5 mM $MgCl_2$, and

0.5 mM LiCl in 15% ultrapure sucrose (pH 5.80). Ampicillin-resistant colonies were screened for JCV DNA by agarose gel electrophoresis of undigested and *EcoRI*-digested plasmid DNA that was extracted by a rapid isolation method (12). Colonies containing recombinant plasmids were grown in LB broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) and plasmid copy number amplified with chloramphenicol (5). Supercoiled recombinant DNA was isolated as described by Guerry et al. (16) and purified by CsCl density gradient centrifugation. Cloned JCV DNAs isolated from brain tissue were designated as BrC followed by the PML case number.

Infectivity of Br-DNAs. Linear BrC-DNA was purified away from the plasmid and the infectivity was determined in PHFG cells by the calcium precipitation procedure described by Frisque et al. (13). Virion antigen production was assayed by the indirect immunofluorescent antibody method. Antisera were obtained from M. Proctor (University of Wisconsin) and were either JCV specific or against the polyomavirus common antigen (39).

Radiolabeling of JCV DNA. PHFG cells were inoculated with JCV MAD-1 at a multiplicity of infection of approximately 0.1. At 8, 9, or 10 days after inoculation, separate cultures were pulse-labeled for 8 min by the addition of 100 μ Ci of [*methyl*- 3 H]thymidine per ml followed by 3 μ g of unlabeled thymidine per ml in excess cold medium. Cultures had been grown in medium containing dialyzed fetal calf serum for 1 day before labeling and in Hanks balanced salt solution (GIBCO Laboratories) 1 h before labeling. Uniformly labeled JCV DNA was produced by growing virus in the presence of 10 μ Ci of [3 H]thymidine per ml. JCV DNA was extracted from the cells as described above.

RESULTS

Molecular cloning of Br-DNAs. Supercoiled JCV DNA was separated from brain tissue of 10 PML cases and each was shown previously to be homogeneous in length (15). To compare these DNAs adequately, we cloned each into pBR322 at the unique *EcoRI* restriction site. *E. coli* was transformed with ligated Br-pBR322 DNA preparations, and colonies were screened for recombinants by gel electrophoresis of bacterial lysates. Depending on the sample, 10 to 40% of the ampicillin-resistant colonies contained single inserts of JCV DNA; full-length JCV DNA could be recovered from each clone by *EcoRI* digestion. The orientation of the JCV DNA insert was determined by cleavage of each recombinant DNA with *PstI*. JCV and pBR322 DNAs each have a single recognition site for *PstI* such that cleavage of a recombinant gives two fragments of 7.1 and 2.4 kilobase pairs (orientation 1) or 5.2 and 4.3 kilobase pairs (orientation 2). Both of these orientations were found among our clones.

Infectivity of BrC-DNAs. The infectivity of the BrC-DNAs was evaluated in multiplying PHFG cell cultures. No infectivity could be detected in the presence of pBR322. Therefore, recombinant molecules were digested with *EcoRI* and

the linear JCV DNA was separated from the plasmid as described above. At 9 days after transfection, BrC-DNA from each PML case had induced JCV virion antigen production. Infectivities ranged from 0.72×10^3 to 2.5×10^3 fluorescent cell units per μ g of DNA (Table 1). These values were approximately 15- to 25-fold less than infectivities of supercoiled Br-DNA. To ensure that infectious progeny virions were being produced, we transfected cells with DNA and after 9 days processed the cells for passage of virions as previously described (27). Fresh PHFG cell cultures were inoculated with these preparations and assayed for viral antigen production as above. Each of our 10 BrC-DNA preparations produced progeny virions that could be passaged; therefore, we were confident that our clones were representative of the viral DNA in the brain of each case.

Physical map of Br-1 DNA. The restriction cleavage sites on JCV DNA for *EcoRI*, *PstI*, *HpaI*, *HincII*, *HindIII* (25), and *PvuII* (21) have been determined. These enzymes were used alone and in combination to compare our recombinant DNAs. However, all published sites have been established with culture-passaged prototype MAD-1 DNA or a clone thereof. It is now known that passage in culture can result in alterations in cleavage sites (J. Martin, personal communication). Therefore, we first compared Br-1 DNA with MAD-1 DNA (MAD-1 virus was originally derived from Br-1 virus) to determine whether their cleavage maps were identical. Br-1 and MAD-1 DNAs exhibited the same restriction patterns with *EcoRI*, *PstI*, *HpaI*, *HincII*, and *HindIII*, and our map sites were the same as

TABLE 1. Infectivity of cloned brain-extracted JCV DNA in PHFG cells

BrC-DNA ^a	Fluorescent cell units per:	
	Cover slip per 5 μ g of DNA	μ g of DNA ^b
1	1,957 $\pm$ 191	7.0×10^2
6	3,846 $\pm$ 352	1.4×10^3
8	2,217 $\pm$ 146	7.9×10^2
9	3,716 $\pm$ 284	1.3×10^3
10	3,303 $\pm$ 270	1.2×10^3
11	2,320 $\pm$ 134	8.3×10^2
16	7,168 $\pm$ 970	2.5×10^3
18	2,994 $\pm$ 282	1.1×10^3
19	5,617 $\pm$ 342	2.0×10^3
21	4,106 $\pm$ 199	1.5×10^3

^a BrC-DNAs were numbered according to the case of PML from which they were derived. BrC-1 is from the same case from which MAD-1 was isolated.

^b Determined by multiplying the number of fluorescent cell units per coverslip by 1.78 (1.78 = area of well divided by the area of coverslip).

TABLE 2. Sizes of Br-1 DNA fragments generated by *PvuII* alone and with *EcoRI*, *HpaI*, or *BamHI*

Enzyme(s) used	Fragment	Length	
		Kilobase pairs ^a	% JCV genomes ^b
<i>PvuII</i>	A	2.45	48.0
	B	1.98	38.5
	C	0.72	13.5
<i>PvuII</i> + <i>EcoRI</i>	A	2.49	48.5
	B1	1.48	29.0
	B2	0.49	9.5
	C	0.71	14.0
<i>PvuII</i> + <i>HpaI</i>	A	2.43	47.5
	B1	1.28	25.0
	B2	0.67	13.0
	C	0.71	14.0
<i>PvuII</i> + <i>BamHI</i>	A1	1.37	27.0
	A2	1.10	21.5
	B	1.98	38.5
	C	0.70	13.5

^a Sizes were estimated from mobilities on 1.2% agarose gels compared with the mobilities of SV40 *HindIII* fragments (10).

^b Percentage of JCV genome to the nearest 0.5%, calculated using a value of 5.13 kilobase pairs for JCV (25).

those reported by Martin et al. (25). The single *BamHI* site was located at 0.51 map units. However, the *PvuII* map sites for both Br-1 and our MAD-1 DNAs were different from those previously reported for MAD-1 (21). DNA from two additional preparations of MAD-1, of different passage histories, had the same *PvuII* sites as Br-1. The fragment sizes of Br-1 DNA cleaved with *PvuII* and combinations of *PvuII* with *EcoRI*, *HpaI*, or *BamHI* are shown in Table 2. From these data, the *PvuII* sites in Br-1 and our MAD-1 DNAs are at 0.095, 0.235, and 0.725 map units. A restriction map of prototype Br-1 DNA, opened at the *EcoRI* site, is shown in Fig. 1.

Representative nature of clones. Although previously we found no evidence of a heterogeneous population of JCV DNA in brain (15), we examined each BrC-DNA to determine whether it was representative of the Br-DNA from which it was derived. DNA from the brain of case 16 contained two full-length populations of JCV DNA, one with a larger *HincII* A fragment. We have been able to clone only the DNA with the shorter *HincII* A fragment for this study. In seven cases we had sufficient supercoiled Br-DNA to compare directly each Br-DNA restriction pattern with the BrC-DNA recombinants derived from it. Within an individual case, the restriction patterns of the supercoiled Br-DNA

FIG. 1. Restriction endonuclease cleavage map of prototype Br-1 DNA with the *EcoRI* site at 0.0 map units.

and BrC-DNA recombinants were identical. In cases where we had insufficient supercoiled DNA, we examined several clones derived from the Br-DNA of that case. For example, four separately isolated recombinants of Br-16, digested with a combination of *PvuII* and *HindIII*, showed identical restriction enzyme patterns (Fig. 2). The reason that this enzyme combination was used is discussed below. A similar analysis of BrC-1 (four clones), BrC-9 (two clones), BrC-11 (three clones), and BrC-19 (two

FIG. 2. Representative nature of JCV DNA clones from an individual PML case. BrC-16 DNAs (a through d) were isolated from separate ampicillin-resistant colonies after transformation with Br-pBR322 DNA. DNA samples from each colony were digested with *PvuII* plus *HindIII* and separated on an 8% polyacrylamide gel. The position of representative fragments of *HaeIII*-digested pBR322 are indicated and served as markers. Refer to the legend to Fig. 3 for fragment identification.

FIG. 3. Digestion of BrC-1 recombinant DNA of both orientations with *PvuII* plus *HindIII*. (a) Restriction fragments separated on an 8% polyacrylamide gel and (b) the location of each fragment in the recombinant molecules. Size of each fragment in kilobase pairs was determined relative to the *HindIII* plus *HincII* SV40 fragments (10) on 1% agarose gels and pBR322 *HaeIII* fragments on 8% polyacrylamide gels.

clones) also demonstrated that DNA clones derived from the same PML case were identical with respect to the restriction enzymes used in this study. These data, in conjunction with our infectivity studies, indicate that our clones from a particular PML case are representative of the JCV DNA in the brain of that individual.

Comparison of BrC-DNAs. Although we examined our JCV-pBR322 recombinant molecules with all of the enzymes shown in Fig. 1, a useful enzyme combination for comparative purposes was *PvuII* plus *HindIII*. Cleavage of Br-1 DNA with this combination, in addition to other fragments, produces several small fragments around the presumptive origin of replication, where we might expect to see some variability. Our standard for comparison was the prototype BrC-1. Figure 3 shows BrC-1 recombinants of both orientations cleaved with the *PvuII*-*HindIII* combination. The cleavage products (Fig. 3a) with approximate map locations and fragment sizes (Fig. 3b) are shown. Note that the two orientations are easily distinguishable by the sizes of the two junction fragments. If our cloned JCV DNA from different cases of PML were the same, we would expect these same

restriction patterns for either orientation. However, an examination of all of the BrC DNAs with the *PvuII*-*HindIII* combination revealed that JCV DNA from only one other case, BrC-11, had a restriction pattern similar to BrC-1, although the D fragment was slightly smaller (Fig. 4). All other BrC-DNAs showed changes in the D fragment as is shown for BrC-9, -10, -18, and -19 in Fig. 4 and for BrC-16 in Fig. 2. This fragment is the region between 0.67 and 0.72 map units on the JCV genome. The additional small fragments, exemplified by BrC-9, -10, and -18 in Fig. 4, ranged in size from 60 to 125 base pairs (bp) and were derived from fragment D. We determined that the additional fragments resulted from additional *PvuII* sites by digesting the DNAs with *HindIII* and *PvuII* alone and by partial digest patterns with *PvuII* (not shown). The number and approximate size of fragments between 0.67 and 0.725 map units for all of our BrC clones are summarized in Table 3. Although it appears that only BrC-1 and -11 are similar in this region, one of the additional *PvuII* sites in BrC-8, -9, -10, -16, and -18 is located at the same position, 0.695 map units, and the 125-bp fragment in these DNAs is located between this site

FIG. 4. Digestion of BrC DNAs with *PvuII* plus *HindIII*. *HaeIII*-digested pBR322 served as size markers and pBR322 digested with *PvuII* plus *HindIII* served as a control. BrC-DNAs 9, 10, 11, and 19 were orientation 1 whereas BrC-DNAs 1 and 18 were orientation 2. Fragments were identified as in the legend to Fig. 3.

and the *HindIII* site at 0.67 map units. Besides the changes in the number of *PvuII* sites in six of the BrC DNAs (Table 3), BrC-19 (Fig. 4) and BrC-22 (not shown) both have a major deletion in fragment D of 85 and 30 bp, respectively. Also, as shown in Table 3, the sum of the fragments indicated that several isolates had additions or deletions within this region. Because we would not have resolved fragments smaller than 30 bp on our gels, additional *PvuII* sites could be present and account for the apparent deletions. In view of the above data, it is apparent that the region in JCV DNA between 0.67 and 0.725 is hypervariable.

In addition to this somewhat random variation in a small segment of the genome, we have found changes in two other regions of the genome in four of our BrC-DNAs. In Fig. 4, the B fragment of BrC-10, 18, and 19, corresponding to 0.095 to 0.235 map units on the Br-1 JCV genome, is approximately 75 bp smaller than the B fragment of the other BrC-DNAs. This also was observed for BrC-8 (not shown). We did not observe any additional fragments that would suggest the smaller size was due to an additional *PvuII* site near the end of the B fragment. Partial digest results confirmed this. The B fragment containing the deletion has a *HincII* site at 0.15 map units. By cleavage with this enzyme, we were able to localize the deletion to a region between 0.15 and 0.235 map units (not shown). A significant finding was that these same four DNAs,

TABLE 3. *PvuII*-*HindIII* fragments between 0.67 and 0.725 map units

BrC	No. of fragments ^a (sizes in bp)
1	1 (280)
6	2 (125, 60)
8	3 (125, 90, 80)
9 ^b	4 (125, 100, 70)
10 ^b	4 (125, 85, 75)
11	1 (270)
16	2 (125, 90)
18	4 (125, 90, 65, 60)
19	1 (195)
22	1 (250)

^a Sizes were estimated from mobilities on 8% acrylamide gels compared with *HaeIII*-digested pBR322 markers. Values are means of two determinations to the nearest 5 bp. Although the measured difference in size between certain fragments was small, each was migrating visibly different on the gel.

^b Partial digest results indicate that there is an additional *PvuII* site approximately 15 bp before the site at 0.725 map units. Our gels would not have resolved a fragment this small.

BrC-8, -10, -18, and -19, all had an additional *HincII* site (recognition sequence GTPyPuAC) in a different region of the genome, and in all four this *HincII* site was a *HpaI* site (recognition sequence GTTAAC). Figure 5 shows a *HpaI* digest of *EcoRI* linearized BrC-1, -10, -18, and -19 purified away from the plasmid. As expected, cleavage of BrC-1 (lane 1) resulted in two fragments, A and B, of 4,380 bp and 775 bp, respectively. The B fragment maps between 0.85

FIG. 5. Cleavage of *EcoRI* linear BrC-1, -10, -18, and -19 with *HpaI*. The BrC-DNAs were excised and purified away from the plasmid, digested with *HpaI*, and the fragments separated on a 7% acrylamide gel.

TABLE 4. Comparison of restriction fragments of Br-1 and Br-10 with *HincII* and *HpaI* plus *EcoRI*

DNA	Enzyme(s) used	Fragments	Size ^a (bp)
Br-1	<i>HincII</i>	A	2,540
		B	1,070
		C	870
		D	640
Br-10	<i>HincII</i>	A	2,550
		B	1,020
		C	860
		D1	400
		D2	230
Br-1	<i>HpaI</i> + <i>EcoRI</i>	A	4,370
		B	780
Br-10	<i>HpaI</i> + <i>EcoRI</i>	A	4,350
		B1	540
		B2	230

^a Fragment sizes were estimated from the relative mobilities of SV40 *HindIII* fragments (10).

(*HpaI* site) and 0.0 (*EcoRI* site) map units (Fig. 1). However, cleavage of BrC-10, -18, and -19 resulted in three fragments, with the B fragment containing the additional *HpaI* site. This site was located by comparing the fragment sizes after digestion of the DNA with *HincII* and with *EcoRI* plus *HpaI*, and is shown for Br-10 in Table 4, with Br-1 included for comparative purposes. Fragment *HincII*-D2 and *HpaI*-*EcoRI*-B2 were of the same size, indicating a shared site location. Analysis of the fragment sizes placed the *HincII*-*HpaI* site at 0.895 map units without ambiguity in all four DNAs.

The results of our restriction mapping for all 10 BrC-DNAs, showing the location of the differences from BrC-1 with relation to the early and late protein coding regions, are summarized in Fig. 6. JCV can be divided into two subtypes based on the consistent and identical differences in 4 of 10 isolates, BrC-8, -10, -18, and -19.

Location of the origin of DNA replication. We were interested in the location of the variability among isolates relative to the origin of DNA replication. As mentioned above, homology studies suggest that the genomic organization of JCV would be the same as SV40 and BKV (24). The origin in both SV40 and BKV is reported to be at 0.67 map units (9, 20). To determine whether the origin in the JCV genome was near 0.67, MAD-1 DNA was pulse-labeled and cleaved into six fragments (Fig. 7b). After separation of these fragments by gel electrophoresis, the relative incorporation of label in each fragment was determined by comparison with the same fragment from uniformly labeled DNA. As shown in Fig. 7a, the lowest relative incorpo-

ration was in fragment F and the highest in fragment A. By extrapolation of a plot of fragment versus incorporation, the biologically active origin of replication was located at 0.69 ± 0.02 map units. Because of the small number of fragments examined, this value is not exact but does place the origin in the region suggested by the hybridization studies. As would be expected, replication proceeds bidirectionally and, from the approximately equivalent slopes of the plot in Fig. 7a, proceeds at approximately the same rate in both directions. Therefore, replication would terminate near 0.19 map units.

DISCUSSION

We examined DNA from 10 fatal cases of PML in the form of representative, infectious cloned DNA molecules and found natural variation in limited regions of the genome. Based on the restriction patterns of the enzymes used, JCV can be divided into two subtypes. In addition, we clearly demonstrated hypervariability in a region near the origin of DNA replication.

The subdivision of JCV into two types was based on the presence of an additional *HincII*-*HpaI* site at 0.895 map units and a 75-bp deletion

FIG. 6. Summary of the differences of each BrC-DNA relative to Br-1. Early and late coding regions, assumed from homology studies (24), are indicated along with the origin of DNA replication (0 end). The number of additional *PvuII* sites is shown in parentheses.

FIG. 7. Relative incorporation of thymidine in fragments derived from pulse-labeled JCV DNA. DNA was pulse-labeled as described in the text and sequentially digested with *EcoRI*, *PstI*, *HpaI*, and *HindIII*. Fragments (a) were separated on a 0.7% agarose gel (Seaplaque), and each fragment was excised from the gel and melted at 90°C in the presence of 0.1 N HCl. Relative incorporation (b) was determined by comparison to uniformly labeled DNA digested as above. The results of a duplicate experiment are shown, with (○) having 2,364 total CPM and (●) having 2,162 total CPM.

between 0.145 and 0.235 map units in the JCV genome of 4 of the 10 PML cases examined. The probability of these specific changes occurring independently is extremely unlikely and it would appear that there are at least two genomic populations of JCV circulating in humans.

The differences in the genomes of the two subtypes may manifest themselves in detectable biological changes. From our location of the replication origin and the colinear alignment of the genomes of JCV, BKV, and SV40 (24), we can assume that the late and early mRNAs of JCV map very closely to those of SV40 and BKV (19). This would place the additional *HincII-HpaI* site within the codons for the JCV structural proteins Vp2 and Vp3 and the deletion between 0.15 and 0.235 map units in a region including the codons for the C-terminal ends of Vp1 and the large T (tumor) antigen. Therefore, it is reasonable that these 2 types of JCV may have different biological properties if the genome differences alter the proteins. The non-coding region between the C-terminal ends of Vp1 and T antigen in SV40 is very small (around 30 bp) (19), so with a 75-bp deletion we might expect a size alteration in either Vp1 or T antigen. In SV40, the viable deletion mutant dl1294 (40) at 0.15 map units shortens Vp1 by

4,000 daltons and there are viable deletions in SV40 strains at 0.17 to 0.18 and at 0.21 map units (8), the latter of which results in a shorter T antigen.

Because of a high degree of variability around the origin in our cases (nine distinctly different restriction patterns), changes in this small region apparently are of little biological importance. All of these DNAs were from fatal cases of PML and we emphasize that from any one case, the DNA population was homogeneous. It was not surprising to find variation around the origin as this is the region that shows a high degree of variation in the primate polyomaviruses (21, 34, 37, 43). In SV40 and BKV, this is a region of repetitive DNA (38). However, it was surprising that no two cases showed exactly the same changes in this region (although BrC-1 and BrC-11 were very similar). The minor variability in the two JCV DNAs previously reported by Rentier-Delrue et al. (35) also was in this general region, and from their data, neither DNA appears to be the same as any of ours. However, even though there is little evolutionary constraint in this region, even within the same host species (19), the changes between 0.67 and 0.725 in our JCV DNA were not totally random as six of the DNAs shared a *PvuII* site at 0.695 map

units. We did not find direct evidence of repeated sequences; photometric scanning of our gels indicated that all of the fragments produced by *PvuII-HindIII* digestion were equimolar. However, in SV40 and BKV DNAs there are bases separating the repeats (38) so we might not expect to see fragments of equal size by cleavage in a repetitive region. Further studies, preferably sequencing, will be needed to determine whether the region between 0.67 and 0.725 map units of JCV DNA contains repetitive sequences.

The extreme heterogeneity around the origin of JCV DNA is of interest. One might expect that in a virus with a hypervariable region, a DNA population, such as in the brain, would contain a mixture of variants, but the supercoiled DNA from an individual PML brain is homogeneous with respect to this region. While not totally inconceivable, it is difficult to imagine that every JCV DNA from brain will be different. However, because JCV can persist for long periods in an individual, variation, especially in a hypervariable region, might occur in vivo with amplification and selection of variants over time. We recently identified small amounts of JCV DNA by Southern blot hybridization in extraneural tissues of several of the PML cases examined in this study (B. W. Grinnell, B. L. Padgett, and D. L. Walker, manuscript in preparation). If it is possible to clone such extraneural viral DNA, a comparison of it with the DNA from brain of the same case will tell us whether extensive variation occurs within a given individual.

On a population basis, the identification of two types of JCV must be kept in mind for future comparative studies of JCV isolates from brain and from other sources, such as urine (1, 33). The subtyping of JCV will aid in our understanding of its natural history and open the way for limited molecular epidemiology studies.

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LITERATURE CITED

- Beckman, A. M., K. V. Shah, and B. L. Padgett. 1982. Propagation and primary isolation of papovavirus JC in epithelial cells derived from human urine. *Infect. Immun.* 38:774-777.
- Buchman, T. G., B. Roizman, G. Adams, and H. Stover. 1978. Restriction endonuclease fingerprinting of herpes simplex virus DNA: a novel epidemiological tool applied to a nosocomial outbreak. *J. Infect. Dis.* 138:488-498.
- Campo, M. S., M. H. Moar, H. M. Laird, and W. F. H. Jarrett. 1981. Molecular heterogeneity and lesion site specificity of cutaneous bovine papillomaviruses. *Virology* 113:323-335.
- Centifanto-Fitzgerald, Y. M., T. Yamaguchi, H. E. Kaufman, M. Tognon, and B. Roizman. 1982. Ocular disease pattern induced by herpes simplex virus is genetically determined by a specific region of viral DNA. *J. Exp. Med.* 155:475-489.
- Clewell, D. B. 1972. Nature of Col E₁ plasmid replication in *Escherichia coli* in the presence of chloramphenicol. *J. Bacteriol.* 110:667-676.
- Cohen, S. N., A. C. Y. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistant bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. U.S.A.* 69:2110-2114.
- Coleman, D. V., M. R. Wolfendale, R. A. Daniel, N. K. Dhanjal, S. D. Gardner, P. E. Gibson, and A. M. Field. 1980. A prospective study of human polyomavirus infection in pregnancy. *J. Infect. Dis.* 142:1-8.
- Crawford, L. V., C. N. Cole, A. E. Smith, E. Paucha, P. Tegtmeier, K. Rundell, and P. Berg. 1978. The organization and expression of simian virus 40's early genes. *Proc. Natl. Acad. Sci. U.S.A.* 75:117-121.
- Danna, K. J., and D. Nathans. 1972. Bidirectional replication of simian virus 40 DNA. *Proc. Natl. Acad. Sci. U.S.A.* 69:3097-3100.
- Daniel, R., K. Shah, D. Madden, and S. Stagno. 1981. Serological investigation of the possibility of congenital transmission of papovavirus JC. *Infect. Immun.* 33:319-321.
- Danna, K. J., G. H. Sack, and D. Nathans. 1973. Studies of simian virus 40 DNA VII. A cleavage map of the SV40 genome. *J. Mol. Biol.* 78:363-376.
- Davis, R. W., M. Thomas, J. Cameron, T. P. St. John, S. Scherer, and R. A. Padgett. 1980. Rapid DNA isolations for enzymatic and hybridization analysis. *Methods Enzymol.* 65:404-411.
- Frisque, R. J., J. D. Martin, B. L. Padgett, and D. L. Walker. 1980. Infectivity of the DNA from four isolates of JC virus. *J. Virol.* 32:476-482.
- Gardner, S. D. 1977. Implication of papovaviruses in human disease, p. 41-84. *In* E. Kurstak and C. Kurstak (ed.), *Comparative diagnosis of viral disease*, vol. 1. Academic Press, Inc., New York.
- Grinnell, B. W., J. D. Martin, B. L. Padgett, and D. L. Walker. 1982. Is progressive multifocal leukoencephalopathy a chronic disease because of defective interfering particles or temperature sensitive mutants of JC virus? *J. Virol.* 43:1143-1150.
- Guerry, P., D. J. LeBlanc, and S. Falkow. 1973. General method for the isolation of plasmid deoxyribonucleic acid. *J. Bacteriol.* 116:1064-1066.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26:365-369.
- Hogan, T. F., E. C. Borden, J. A. McBain, B. L. Padgett, and D. L. Walker. 1980. Human polyomavirus infections with JC virus and BK virus in renal transplant patients. *Ann. Intern. Med.* 92:373-378.
- Howley, P. M. 1980. Molecular biology of SV40 and the human polyomaviruses BK and JC, p. 489-538. *In* G. Klein (ed.), *Viral oncology*. Raven Press, New York.
- Howley, P. M., M. A. Israel, M.-F. Law, and M. A. Martin. 1979. A rapid method for detecting and mapping homology between heterologous DNAs. *J. Biol. Chem.* 254:4876-4883.
- Howley, P. M., F. Rentier-Delrue, C. A. Heilman, M.-F. Law, K. Chowdhury, M. A. Israel, and K. K. Takemoto. 1980. Cloned human polyomavirus JC DNA can transform human amnion cells. *J. Virol.* 36:878-882.
- Huang, E. S., C. A. Alford, D. W. Reynolds, S. Stagno, and R. F. Pass. 1980. Molecular epidemiology of cytomegalovirus infections in women and their infants. *N. Engl. J. Med.* 303:958-962.
- Israel, M. A., H. W. Chan, W. P. Rowe, and M. A. Martin. 1979. Molecular cloning of polyoma virus DNA in *Escherichia coli*: plasmid vector system. *Science* 203:883-887.
- Law, M.-F., J. D. Martin, K. K. Takemoto, and P. M. Howley. 1979. The colinear alignment of the genomes of papovaviruses JC, BK and SV40. *Virology* 96:576-587.
- Martin, J. D., R. J. Frisque, B. L. Padgett, and D. L.

- Walker. 1979. Restriction endonuclease cleavage map of the DNA of JC virus. *J. Virol.* 29:846-855.
26. Osborn, J. E., S. M. Robertson, B. L. Padgett, D. L. Walker, and B. Weisblum. 1976. Comparison of JC and BK human papovaviruses with simian virus 40: DNA homology studies. *J. Virol.* 19:675-684.
 27. Padgett, B. L., C. M. Rogers, and D. L. Walker. 1977. JC virus, a human polyoma virus associated with progressive multifocal leukoencephalopathy: additional biological characteristics and antigenic relationships. *Infect. Immun.* 15:656-668.
 28. Padgett, B. L., and D. L. Walker. 1973. Prevalence of antibodies in human sera against JC virus, an isolate from a case of progressive multifocal leukoencephalopathy. *J. Infect. Dis.* 127:467-470.
 29. Padgett, B. L., and D. L. Walker. 1980. Human papovavirus JCV: natural history, tumorigenicity, and interaction with human cells in culture, p. 319-327. *In* Virus in naturally occurring cancers, vol. 7. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
 30. Padgett, B. L., D. L. Walker, G. M. ZuRhein, R. J. Eckroade, and B. H. Dessel. 1971. Cultivation of papovaviral virus from human brain with progressive multifocal leukoencephalopathy. *Lancet* i:1257-1260.
 31. Padgett, B. L., D. L. Walker, G. M. ZuRhein, A. E. Hodach, and S. M. Chou. 1976. JC papovavirus in progressive multifocal leukoencephalopathy. *J. Infect. Dis.* 133:686-690.
 32. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in Hela cells. *Proc. Natl. Acad. Sci. U.S.A.* 57:1514-1521.
 33. Rand, K. H., K. P. Johnson, L. J. Rubinstein, J. S. Wolinsky, J. B. Penny, D. L. Walker, B. L. Padgett, and T. C. Merigan. 1977. Adenine arabinoside in the treatment of progressive multifocal leukoencephalopathy: use of virus-containing cells in the urine to assess response to therapy. *Ann. Neurol.* 1:458-462.
 34. Reddy, V. B., B. Thimmappaya, R. Dhar, B. Subramanian, S. Zain, J. Pan, P. K. Ghosh, M. L. Celma, and S. M. Weissman. 1978. The genome of simian virus 40. *Science* 200:494-502.
 35. Rentier-Delrue, F., A. Lubiniecki, and P. M. Howley. 1981. Analysis of JC virus DNA purified directly from human progressive multifocal leukoencephalopathy brains. *J. Virol.* 38:761-769.
 36. Roizman, B., and T. Buchman. 1979. The molecular epidemiology of herpes simplex virus. *Hosp. Pract.* 14:95-104.
 37. Seif, I., G. Khoury, and R. Dhar. 1979. Genome of the human papovavirus BKV. *Cell* 18:963-977.
 38. Seif, I., G. Khoury, and R. Dhar. 1980. Sequence and analysis of the genome of human papovavirus BK, p. 897. *In* J. Tooze (ed.), Molecular biology of tumor viruses, part 2. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
 39. Shah, K. V., H. L. Ozer, H. N. Ghazey, and T. J. Kelly, Jr. 1977. Common structural antigen of papovaviruses of the simian virus 40-polyoma subgroup. *J. Virol.* 21:179-186.
 40. Tegtmeier, P. 1980. Genetics of SV40 and polyoma virus, p. 297. *In* J. Tooze (ed.), Molecular biology of tumor viruses, part 2. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
 41. Wadell, G., and J. C. de Jong. 1980. Restriction endonucleases in identification of a genome type of adenovirus 19 associated with keratoconjunctivitis. *Infect. Immun.* 27:292-296.
 42. Walker, D. L. 1978. Progressive multifocal leukoencephalopathy: an opportunistic viral infection of the central nervous system, p. 207-329. *In* P. J. Vinken and G. W. Bruyn (ed.), Handbook of clinical neurology, vol. 34, part II. North-Holland Publishing Co., New York.
 43. Yang, R. C. A., and R. Wu. 1979. BK virus DNA: complete nucleotide sequence of a human papovavirus. *Science* 206:456-462.