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RECOMBINANT JC VIRAL DNA: VERIFICATION AND PHYSICAL MAP OF PROTOTYPE

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SUMMARY: Genomic DNA of the human polyomavirus JC was molecularly cloned from DNA extracted from primary human fetal glial cells infected with prototype (MAD-1) virus and from diseased brain tissue from which JC virus originally was isolated. This report documents that these recombinant MAD-1 DNAs are identical, are prototypical, and may serve as unambiguous references to distinguish the variant DNAs produced by independent isolates of JC virus. Possible ambiguity in other recombinants of MAD-1 DNA is discussed. A new restriction endonuclease cleavage map of MAD-1 DNA was derived for 52 sites cleaved by eight enzymes.

The human polyomavirus JC circulates widely in the human population, presumably by inapparent infections during early childhood (1). The virus very likely is the causative agent of PML*, a slowly demyelinative disease of immunocompromised adults (2), and it produces neurological tumors in hamsters (1,2), owl monkeys (3), and squirrel monkeys (4). JCV has been isolated from diseased brain tissues of patients with PML and has been propagated in PHFG cell cultures (1,5). The viral genome is covalently-closed circular, double stranded DNA containing approximately 5100 bp (6,7). By restriction endonuclease cleavage analyses of viral DNAs molecularly cloned from diseased brains, it is clear that multiple variants of the genome exist in the human population (8,9).

Because of the great difficulty in the isolation and propagation of JCV, studies of its genome are being facilitated by molecular cloning of the DNA

*Abbreviations used: PML, progressive multifocal leukoencephalopathy; JCV, JC virus; PHFG, primary human fetal glial; bp, base pairs; MOI, multiplicity (ies) of infection.

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(8,10; Grinnell et al., submitted for publication). In view of the following considerations (9,11; Martin et al., submitted for publication) it is imperative that recombinant JCV DNA be thoroughly characterized and identified as prototypical, variant, or defective: (1) There are at least three classes of JCV isolates, based on restriction endonuclease cleavage patterns of the respective DNAs. (2) The DNAs of some -- but not all -- isolates propagated through even two or three passages in vitro at low MOI are heterogeneous in size and include presumably defective molecules. (3) Passage of the virus in vitro can induce cryptic alterations in the full-length genome.

This manuscript describes and authenticates, well-characterized prototypical (MAD-1) JCV DNA to serve as unambiguous reference DNA to which JCV variants and defectives and other polyomaviral genomes may be compared. To facilitate the clear identification and comparison of DNAs of other JCV isolates, a new restriction endonuclease cleavage map of MAD-1 DNA was derived for the enzymes Bam Hl (1 site), Hpa II (1 site), Xba I (2 sites), Ava II (3 sites), Pvu II (3 sites), Sst I (4 sites), Hae III (16 sites) and Alu I (22 sites).

MATERIALS AND METHODS

Sources of Viral DNAs. JCV DNAs were obtained from infected PHFG cell cultures and from diseased brain tissues. PHFG cell cultures (12) were infected with isolates MAD-1 (13) and MAD-2 (5) partially purified as described elsewhere (6). The isolates had been passed routinely in typical cultures (12) 8 times and 5 times, respectively, at MOI of 0.1 infectious unit per cell. Thereafter, both isolates were passed 3 times in exceptional, spongioblast-rich cultures (7) at MOI of 0.01. The resultant JCV DNAs were slightly heterogeneous in size but were considerably more homogeneous than the typical DNAs obtained during routine passage (7,14). The original brain tissues from which prototype (MAD-1) JCV had been isolated were stored at -70°C.

Preparation of Viral DNA. Supercoiled (form I) viral DNA was purified from lysates of brain homogenates and from lysates of infected PHFG cells by two consecutive equilibrium centrifugations in CsCl-ethidium bromide solutions, as described previously (7).

Molecular Cloning of Viral DNA. MAD-1 and MAD-2 viral DNAs derived from tissue cultures were cloned at their unique Pst I cleavage sties in the bacterial plasmid pBR322 by established methods. The recombinant DNAs are herein referred to as REC MAD-1_{TC}(Pst) and REC MAD-2_{TC}(Pst). MAD-1 DNA obtained from brain tissue was cloned in pBR322 at its unique Eco R1 site [REC MAD-1_{B}(R1)]. Some of the studies were facilitated by subcloning the JCV DNA insert of REC MAD-1_{TC}(Pst) into pBR322 at the Eco R1 site [REC MAD-1_{TC}(R1)]. Recombinant DNAs were propagated in E. coli HB101.

Preparation of Recombinant DNAs. Recombinant JCV-pBR322 plasmids were extracted from chloramphenicol-amplified bacterial cultures by the method of

Holmes and Quigley (15). The DNA preparations were banded twice in CsCl-ethidium bromide solutions.

Analyses with Restriction Endonucleases. Recombinant JCV DNAs were cleaved with restriction endonucleases purchased from Bethesda Research Laboratories, Inc., according to the producer's specifications. Digestion products were analyzed by electrophoresis in 1% or 2% agarose gels (7) and in 5% acrylamide gels (16). Sizes of restriction fragments were determined by the method of Southern (17).

Ordering Restriction Fragments. The order in which restriction fragments are located around the circular genome of JCV was deduced from appropriate double-digestions with restriction enzymes, as described before (7), or by the strategy of Smith and Birnstiel (18), as described here. Linear MAD-1_{TC} and MAD-1_B inserts were excised, respectively, from REC MAD-1_{TC}(R1) and REC MAD-1_B(R1). The recombinant DNAs were cleaved with Eco R1, and the JCV-specific inserts were isolated in low melting-point agarose (FMC Corp.) gels for further purification (19). Labeling of 5' termini of the Eco R1-linearized MAD-1 inserts was obtained by the Maxam-Gilbert procedure (20) with commercial enzymes (Bethesda Research Laboratories, Inc.) and [γ^{32}P] ATP (3000 Ci/mmole; New England Nuclear). The end-labeled DNAs were digested to completion with either Hpa II (0.81 map unit) or with Pst I (0.32 map unit). The resultant fragments were purified and were subjected to partial digestions with Xba I, Sst I, Hae III, or Alu I. Partial digestions were achieved in 30-min reactions (25 µl, 37°C) containing a fragment labeled at one end (~20000 CPM), pBR322 DNA (1 µg) and from 0.1 to 2 times the amount of enzyme required to completely digest the pBR322 DNA. The digestion products were separated in 2% agarose gels and were visualized by autoradiography of the dried gels (7,18).

RESULTS AND DISCUSSION

The observations presented here document three recombinant JCV MAD-1 DNAs as prototypical and representative of a major class of JCV isolates. Viral DNA was extracted from PHFG cultures infected with prototype (MAD-1) virus (MAD-1_{TC}; ref. 13). The MAD-1_{TC}DNA was cloned at its single Pst I site (0.315 map unit; ref. 7) into pBR322. Two clones -- REC MAD-1_{TC}(Pst) clones 1-47 and 3-34 -- were selected for characterization as prototype DNA. MAD-1_{TC} DNA is typically heterogeneous in size (7,14) and therefore includes molecules with deletions and probably other alterations. Furthermore, brief passage of JCV in vitro at low MOI can induce modifications of the genomes (Martin et al., submitted for publication). Therefore, clones 1-47 and 3-34 were compared with DNA cloned directly from diseased brain tissue from which MAD-1 virus originally was isolated [REC MAD-1_B(R1)] and with cloned DNA of another isolate (MAD-2) of the prototype class [REC MAD-2_{TC}(Pst)].

Figure 1 depicts typical electrophoretic analyses of REC MAD-1_{TC}(Pst)/1-47 and 3-34 and of REC MAD-2_{TC}(Pst)/1-4 and 3-17. Clone 1-47 contains a full-length MAD-1 insert oriented in one direction with respect to pBR322;

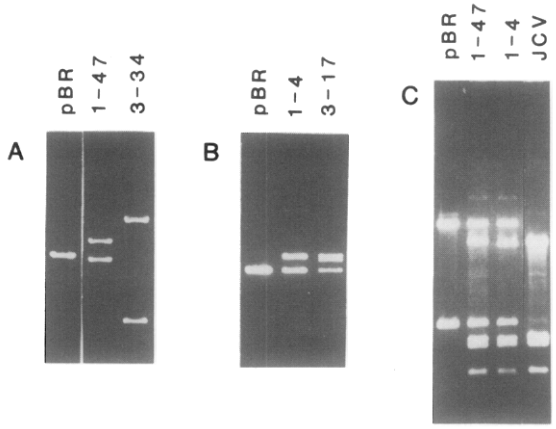

FIGURE 1. Identity of REC MAD-1_{TC}(Pst) and REC MAD-2_{TC}(Pst) with each other and with MAD-1 viral DNA. DNAs were prepared as described in Materials and Methods. Portions (1 µg) were digested with restriction endonucleases and were displayed in 1% agarose gels (12). (A) REC MAD-1_{TC}(Pst) clone 1-47 and clone 3-34 were cleaved with Eco RI. The position of Eco RI-linearized pBR322 is shown. (B) pBR322, REC MAD-2_{TC}(Pst) clone 1-4, and clone 3-17 were cleaved with Pst I. (C) The Pst I-Hinc II double-digest profiles of pBR322, REC MAD-1_{TC}(Pst) clone 1-47, REC MAD-2_{TC}(Pst) clone 1-4, and MAD-1 viral DNA illustrate the identity of the JCV inserts with viral DNA.

clone 3-34 is oriented in the opposite direction (Fig. 1A). The full-length MAD-2 inserts of clones 1-4 and 3-17 (Fig. 1B) are in the same orientation as that of 1-47 (not shown). (Clone 3-17 contains an additional insert of MAD-2 DNA bearing a 200-bp deletion.) Figure 1C illustrates that REC MAD-1_{TC}(Pst) and REC MAD-2_{TC}(Pst) were found to be identical with each other and with MAD-1 viral DNA. Furthermore MAD-1 viral DNA hybridized equally well with both MAD-1 and MAD-2 recombinants (results not shown). Independent measurements of full-lengths and of fragment sizes revealed no significant differences among MAD-1 and MAD-2 inserts and MAD-1 viral DNA (data not shown). The fragment sizes agree very well with sizes obtained in this study (not shown) and with those reported previously (7) for MAD-1 viral DNA and may be computed from the mapping data presented below (Table 1). Full-length, Pst I-linearized recombinant MAD-1 and MAD-2 DNAs measured 5128 ± 88 bp in size (15 determinations with 8 DNA preparations). These results, obtained with λ DNA markers, agree very well with a genome size of 5125 ± 105 bp for MAD-1 viral DNA obtained with SV40 DNA markers and reported previously (7). Thus the length of the prototype JCV genome in these studies is greater by several hundred base pairs than the length of MAD-1 DNA which has been reported by Howley et al. (8,21).

TABLE 1. Locations of restriction endonuclease cleavage sites[a] in prototype JCV DNA.

Bam HI	Eco RI[b]	Hpa I[c]	Hpa II
0.505 ± 0.015	0.000	0.850	0.814 ± 0.012
Pst I[c]	**Xba I**	**Ava II**	**Hind III[c]**
0.315	0.887 ± 0.014	0.149 ± 0.006	0.550
	0.968 ± 0.004	0.763 ± 0.008	0.630
		0.952 ± 0.007	0.670
Pvu II	**Hinc II[c]**	**Sst I**	
0.100 ± 0.015	0.145	0.662 ± 0.018	
0.236 ± 0.017	0.355	0.672 ± 0.004	
0.716 ± 0.014	0.855	0.724 ± 0.005	
	0.975	0.805 ± 0.021	

Hae III		Alu I	
0.091 ± 0.004	0.657 ± 0.012	0.033 ± 0.002	0.635 ± 0.011
0.236 ± 0.008	0.682 ± 0.010	0.100 ± 0.003	0.679 ± 0.012
0.253 ± 0.010	0.734 ± 0.006	0.113 ± 0.005	0.696 ± 0.010
0.266 ± 0.010	0.815 ± 0.006	0.142 ± 0.006	0.721 ± 0.007
0.320 ± 0.008	0.863 ± 0.004	0.162 ± 0.008	0.737 ± 0.006
0.337 ± 0.010	0.904 ± 0.003	0.213 ± 0.008	0.761 ± 0.006
0.425 ± 0.007	0.914 ± 0.003	0.226 ± 0.009	0.782 ± 0.006
0.595 ± 0.009	0.955 ± 0.002	0.242 ± 0.012	0.796 ± 0.010
		0.315 ± 0.017	0.808 ± 0.007
		0.476 ± 0.016	0.835 ± 0.007
		0.548 ± 0.017	0.888 ± 0.006

[a] Cleavage sites are given as the fractional distance from the Eco RI site for the unit length. The values for all sites except those of Hae III and Alu I represent 5-7 independent determinations involving both MAD-1_{TC} and MAD-1_{B} DNA. The values were determined from measurements on photographs of ethidium bromide-stained gels, as described in Materials and Methods. Hae III and Alu I sites were determined on MAD-1_{B} DNA in three independent end-labeling-partial digest experiments, three agarose gels, and two acrylamide gels for each enzyme (see Materials and Methods).

[b] chosen arbitrarily as 0.000/1.000.

[c] reported previously (7).

To verify further that REC MAD-1_{TC}(Pst) clones 1-47 and 3-34 have not been altered from full-length, prototype JCV DNA, they were compared with REC MAD-1_{B}(RI) derived directly from viral DNA present in diseased brain of the prototype case (13). When the MAD-1 insert of REC MAD-1_{TC}(Pst) was subcloned at the Eco RI site [REC MAD-1_{TC}(RI)], it was shown visually to be identical with REC MAD-1_{B}(RI) (Fig. 2). No significant differences were noted for restriction sites mapped previously (7) or for the additional sites reported below (Table 1).

Table 1 lists restriction enzyme cleavage sites for MAD-1 DNA; Fig. 3 is a pictorial map. [Results with a few of these enzymes were reported previously (7) but were confirmed in this study and are included for conven-

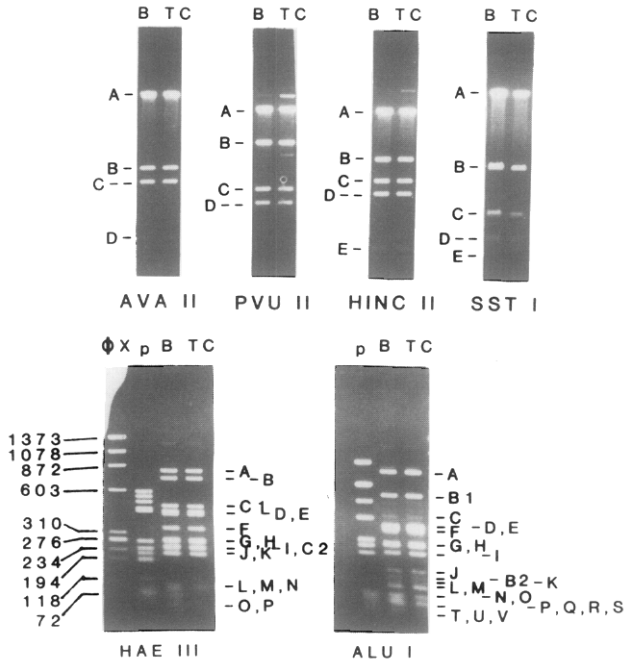

FIGURE 2. Identity of the JCV DNA inserts in REC MAD-1_{TC}(R1) and REC MAD-1_{B} (R1). The viral DNA in each recombinant was excised with Eco R1 and was isolated in an agarose gel. Pieces of the gel containing the inserts were melted and were extracted with phenol-chloroform to recover the DNA. Portions (1 µg) of the MAD-1_{TC} and MAD-1_{B} preparations were digested with the indicated restriction enzymes and the products were separated by agarose gel electrophoresis. Numbers to the left in the lower panel are bp. ØX, ØX 174 replicative form; p, pBR322; B, MAD-1_{B}; TC, MAD-1_{TC}.

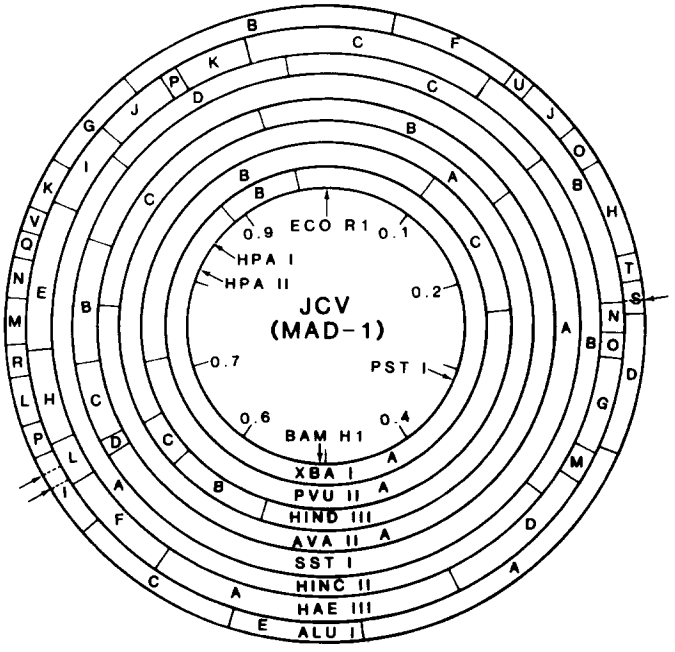

FIGURE 3. A physical map of the genome of prototype (MAD-1) JCV. The dashed lines (arrows) in the Alu I ring indicate presumptive sites which were not detected experimentally due to their proximity to adjacent sites. Their existence is presumed from the fact that the Alu I recognition sequence is contained within the recognition sequences for Hind III, Pvu II, and Sst I.

ience.] The cleavage sites were localized around the circular genome by appropriate double-digestions or by partial digestions of fragments labeled at one end with ^{32}P. Specifically, the sites cleaved by Hind III (ref. 7) and by Xba I, Sst I, Hae III, and Alu I (this paper) were mapped by the partial digestion strategy. It is noteworthy that the Pvu II sites on the recombinant prototype JCV DNAs described here are significantly different from the MAD-1 DNA cloned by others (cf. ref. 8). [Pvu II discriminates prototype and variant JCV DNAs (8,9).]

The sizes in bp of individual fragments may be computed from a unit length of 5125 ± 105 bp (7) or from 5128 ± 88 bp (this study). The standard deviations of map sites listed in Table 1 are consequences of pooling data from two systems of analyses: agarose and acrylamide gels. Measurements obtained within either system alone have standard deviations of less than 0.005 map unit. The discrepancies between each system are produced by differences in migration of certain fragments in agarose and in acrylamide. For example, Hae III fragment A (884 ± 99 bp) migrates slightly faster than an 872 bp marker in 2% agarose gels but slightly slower than that marker in 5% or 8% acrylamide gels.

There is one fragment of MAD-1 DNA which is peculiar. Alu I fragment C (451 ± 43 bp) always appears as a submolar fragment in both agarose (Fig. 2) and acrylamide gels (not shown). The fragment does not seem to be simply a partial digestion product since it is highly reproducible. Sites at map positions 0.548 and 0.635 (Table 1) are evident from end-labeling/partial digestion experiments, and no intervening sites are apparent. One possible explanation is that there are in fact several Alu I sites within fragment C. These may be preferentially cleaved in sub-genomic DNA and therefore would not be detected in the end-labeling/partial digestion experiments. However they may be more resistant to cleavage in the context of the complete genome.

MAD-1 DNA excised from REC MAD-1_B(R1) described here induced T- and V-antigen synthesis and produced infectious virus in a permissive cell culture system (PHFG; results not shown). The MAD-1_{TC}DNA in a recombinant constructed

by Howley et al. transformed a semi-permissive cell culture system (human amnion; ref. 10). The differences in genome lengths and in Pvu II sites reported by our group and by Howley's group may reflect a difference in infectivity. This discrepancy illustrates the importance of clear identification of a recombinant JCV DNA as either a prototype, a variant, or a defective.

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