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Characterization of JC Papovavirus Adapted to Growth in Human Embryonic Kidney Cells

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Human papovavirus JC virus was adapted to growth in human embryonic kidney (HEK) cells. After eight passages, the HEK-adapted JC virus produced high virus yields and was capable of forming plaques in HEK monolayer cultures. Eleven plaque-purified stocks were prepared and characterized. Biologically, the plaque-purified virus induced tumor and viral antigens in HEK cells earlier and in a higher percentage of cells than uncloned virus. Cytopathic changes were also evident sooner and were more extensive. The DNA from uncloned as well as plaque-purified isolates was analyzed by restriction endonuclease cleavage followed by gel electrophoresis. The DNA from uncloned HEK-adapted virus was heterogeneous. Plaque-purified virus isolates yielded DNA which, although much less heterogeneous than the uncloned stock, still consisted of two or more species of viral DNA.

It is well established that the human papovavirus JC virus (JCV) has a highly restricted host range. Efficient replication with high virus yields has been observed only in primary human fetal glial (PHFG) cells, which have a high proportion of spongioblasts (12). Since the original isolation of JCV in 1971 (13), other strains have been recovered from brain tissue of patients with progressive multifocal leukoencephalopathy (1, 2, 14, 17). Without exception, all isolations were made in PHFG cells, and attempts to culture the virus in a variety of other types of cells were unsuccessful (12).

We recently reported on the replication of JCV in human amnion cells, but the yields were not comparable to those achieved in PHFG cells (16). In a continuation of these studies exploring other, more readily available cells for their ability to support JCV replication, we have successfully adapted JCV to growth in human embryonic kidney (HEK) cells. Biological and biochemical differences were noted in the adapted strain (JCV-HEK) compared with the wild-type MAD-1 strain propagated in PHFG cells. The most notable biological difference was the ability of the HEK-grown virus to form plaques. These results are reported in this communication.

MATERIALS AND METHODS

Cells. HEK cells were purchased from Microbiological Associates, Rockville, Md., and were used at passages 2 through 5. Cells were maintained in Eagle minimum essential medium supplemented with 5% fetal bovine serum.

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Virus. Prototype JCV (MAD-1) was obtained from B. Padgett and propagated in PHFG cells. The stock virus had a hemagglutination (HA) titer of 320 U/0.2 ml.

Plaque formation. Monolayer cultures of HEK cells in 60-mm plastic dishes were inoculated with 0.5 ml of serial 10-fold dilutions of JCV-HEK. After 1 h of adsorption, the cultures were overlaid with 5 ml of medium containing 0.9% agarose and Eagle medium with 5% fetal bovine serum. Additional overlay medium was added at 5 and 12 days; the cultures were stained with medium containing 0.005% neutral red after 3 weeks.

Serological tests. For fluorescent-antibody tests, the indirect procedure was used, employing fluorescein-conjugated goat anti-hamster and anti-rabbit globulin. HA and HA inhibition tests were performed with human type O erythrocytes at 4°C. JCV antiserum was prepared by inoculating rabbits intravenously with 5,000 HA units of purified virus. The rabbits were bled and serum was collected 2 weeks after inoculation. Anti-JC T-antibody was obtained from hamsters bearing JCV tumors.

Virus purification. Infected cells which detached and floated in the medium were collected by centrifugation at 3,000 rpm. When cultures showed extensive cytopathic effects, the cells were removed by scraping and added to the previously collected, floating infected cells. The cells were suspended in 0.25% trypsin and 0.02% EDTA. Sodium deoxycholate (1% final concentration) was added to lyse the cells. After incubation at 37°C for 30 min, the suspension was clarified by centrifugation at 10,000 rpm for 10 min. The supernatant fluid was layered onto a 3-ml cushion of cesium chloride (1.34 g/ml) and centrifuged for 2 h at 35,000 rpm in an SW40 rotor. The virus band which formed below the interface was removed, and the virus was purified by isopycnic banding in cesium chloride in an SW50.1 rotor at 35,000 rpm for 18 h. The virus band was removed and dialyzed against phosphate-buffered

saline (pH 7.2) for 12 h.

Preparation of JCV DNA. JCV-HEK DNA was prepared by the method of Hirt (5) from infected cells which had been collected as described for virus purification. Supercoiled form I DNA was isolated by the dye (ethidium bromide)-buoyant density method (15). JCV (MAD-1) DNA, used as a reference, was extracted from purified virions grown in PHFG cells. The MAD-1 strain used in this experiment was from a virus stock which yielded homogeneous viral DNA and was a generous gift from B. Padgett.

Cleavage of JCV DNA with restriction endonucleases. Besides enzymes and DNA, the reaction mixtures contained 100 mM Tris-hydrochloride (pH 7.5), 50 mM NaCl, and 5 mM $MgCl_2$ for *Eco*RI (New England BioLabs, Beverly, Mass.), 20 mM Tris-hydrochloride (pH 8.0), 100 mM NaCl, and 7 mM $MgCl_2$ for *Bam*HI (Bethesda Research Laboratories, Inc., Rockville, Md.), and 7 mM Tris-hydrochloride (pH 7.5), 60 mM NaCl, 7 mM $MgCl_2$, and 100 μg of bovine serum albumin per ml for *Hin*dIII (New England BioLabs). The mixtures were incubated at 37°C for 30 min for *Eco*RI or 60 min for *Bam*HI and *Hin*dIII.

The uncleaved and cleaved DNA samples (25 μl) were adjusted to 8% sucrose and 0.025% bromophenol blue and subjected to electrophoresis in 1.2% agarose slab gels at 21°C. The running Tris-borate buffer (pH 8.2) contained 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA. Gels were stained by soaking in Tris-borate buffer with 1 μg of ethidium bromide per ml for about 20 min. DNA bands were visualized and photographed on a short-wave length UV transilluminator. The six *Hin*dIII fragments of simian virus 40 (SV40) DNA were used as molecular weight standards.

RESULTS

JCV replication in HEK cells. HEK cells grown on cover slips were infected with 80 HA units of prototype JCV (MAD-1). After 30 min of adsorption at 37°C, Eagle medium with 5% serum was added. At various times after infection, cover slips were removed, air dried, and fixed in acetone-methanol (1:1). The cells were then stained for tumor (T)- and viral (V)-antigens. The results of this test are shown in Table 1. A few cells were positive for T-antigen 10 days after infection, but were negative for V-antigen. Two weeks after infection, there was an increase in the number of T-antigen-positive cells, and a small percentage (0.5%) of the cells were also positive for V-antigen. The percentage of cells which stained for both types of antigens continued to increase with time, and, by 25 days, 30% of the cells were positive for T-antigens and 10% were positive for V-antigen. Cultures in 75-cm^2 flasks which had been infected at the same time were also observed carefully for cytopathic effect, but no distinctive changes were noted and the cultures appeared healthy. However, numerous floating cells were seen above the monolayer of healthy cells 2 to 3 weeks after infection. These were collected by centrifugation whenever the medium was changed. After 2 months, the floating cells were pooled, sonicated briefly, and treated with 50 U of receptor-destroying enzyme for 18 h at 37°C. After centrifugation for 5 min at 3,000 rpm, the supernatant was tested for HA activity and found to be positive at a titer of 160 U/0.2 ml. The hemagglutinin was inhibited by JCV but not by BK virus antiserum.

TABLE 1. *JCV T- and V-antigen synthesis in HEK cells*

Day	Passage 1[a]		Passage 2		Passage 3	
	T	V	T	V	T	V
5	0	0	20	0.5	10	2
10	0.3	0	20	1	50	25
15	10	0.5	40	10	75	50
20	25	5	80	25	80	50
25	30	10	90	30	ND	ND
40	30	5	ND	ND	ND	ND

[a] Inoculum: first passage, 80 HA units; second passage, 160 HA units; third passage, 160 HA units. Data indicate percent positive cells. ND, Not done.

Further serial passages in HEK cells were made, following the same procedure described above. T- and V-antigens were detected as early as 5 days on the second passage, and by 25 days, 90% of the cells were positive for T-antigens and 30% were stained by V-antigens (Table 1). By the third passage, 50% of the cells were positive for V-antigen by 15 days, whereas most of the cells now stained for T-antigens (Fig. 1). Cytopathic effect was detected by 5 to 7 days. The cellular changes consisted of scattered, round, highly refractile cells. Bizarre-shaped as well as giant cells were also observed. These cells became detached and floated in the medium.

In PHFG cells infected with JCV, the hemagglutinin is strongly cell associated and is rarely found in the supernatant fluid (12). By contrast, after seven passages in HEK, JCV hemagglutinin was detected in the supernatant fluid with titers of 1:80 to 1:320. Most of the virus still remained cell associated, and purified banded virus was obtained from infected cells according to the procedure described in Materials and Methods. The yields of JCV varied, depending on the quality of the HEK cells available. With exceptional cultures, it was possible to obtain virus yields of 150 μg per 150-cm^2 flask. Examination of purified virions by electron microscopy revealed typical papovavirus particles.

Plaque formation of JCV-HEK. After eight passages, attempts were made to develop a plaque assay for JCV in HEK cells. Discrete plaques measuring 1 to 3 mm developed after 3 weeks (Fig. 2). Several stocks of JCV-HEK were assayed, and the titers ranged from 2×10^3 to 4.5×10^4/ml. By contrast, the prototype MAD-

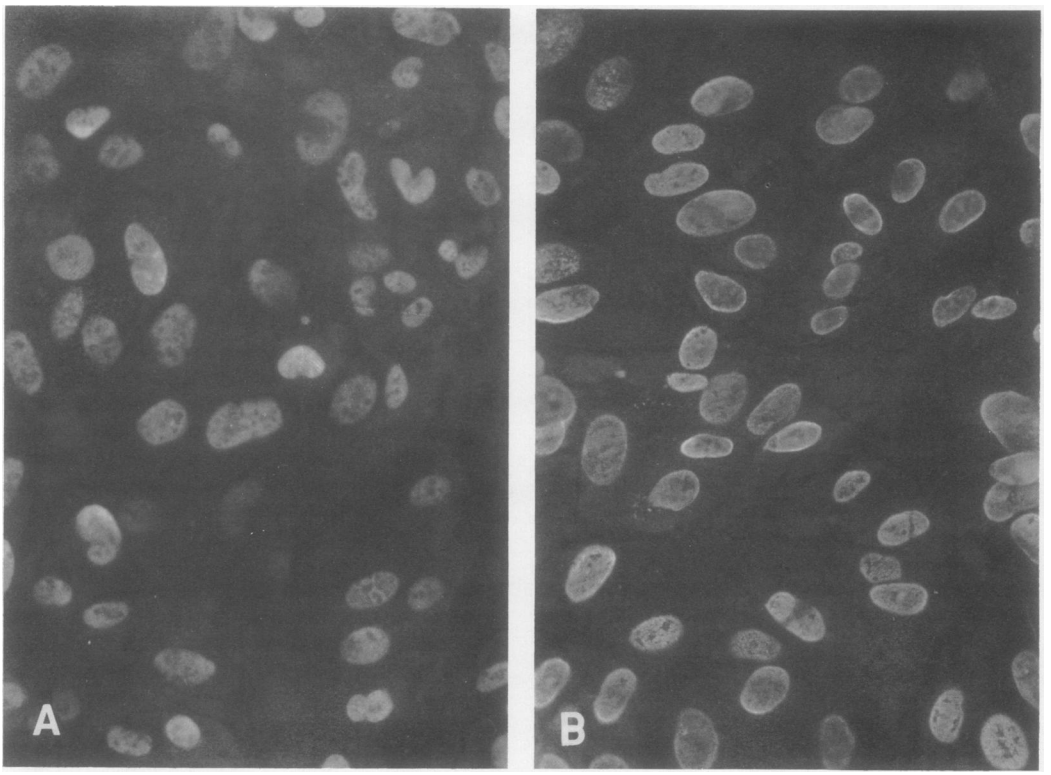

FIG. 1. *Immunofluorescent staining of T-antigens (A) and V-antigens (B) in JCV-infected HEK cells. Cover slip cultures of HEK cells were infected with JCV-HEK, passage 3. Cells were stained 15 days after infection. Magnification, ×640.*

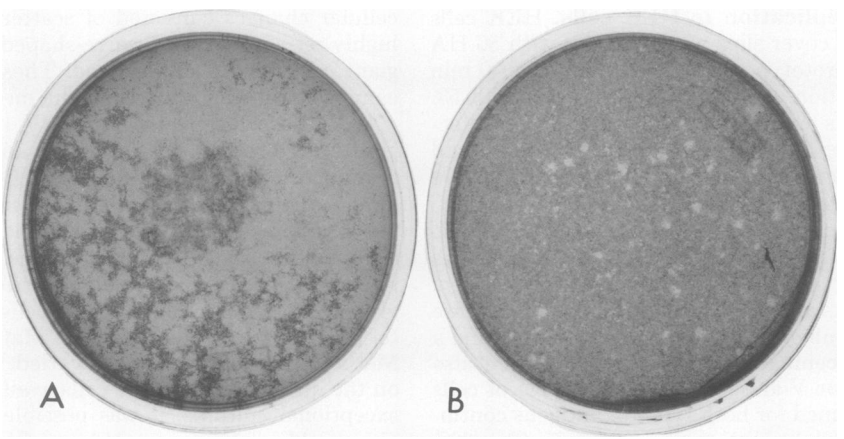

FIG. 2. *Plaque formation of JCV in HEK cells. Plates were inoculated with 10^{-2} dilution (A) and 10^{-3} dilution (B) of JCV-HEK.*

1 strain, which had been grown only in PHFG cells, failed to produce plaques in HEK cells even at high virus concentrations. The progeny from 11 different plaques were picked, and stocks were prepared. These plaque-purified stocks were identified as JCV by HA inhibition and fluorescent-antibody tests using monospecific antiserum. Fluorescent-antibody tests of plaque-purified stocks for their ability to induce T- and V-antigens revealed that these antigens were detected much earlier and the intensity of staining was greater (Table 2). Furthermore,

TABLE 2. *Induction of T- and V-antigens by plaque isolates in HEK cells*

JCV plaque isolate	HA titer	% Positive cells[a]	
		T	V
Pl-3	1,280	70	50
Pl-4	160	50	25
Pl-5	640	50	30
Pl-6	1,280	60	40
Pl-7	160	70	40
Pl-9	640	70	30
Pl-10	160	70	40
Pl-12	160	40	5

[a] Percent positive cells 4 days after infection.

cytopathic effects appeared sooner and were more extensive. Cytoplasmic vacuolization was frequently observed.

Restriction enzyme analysis of JCV-HEK DNA. (i) Uncloned JCV-HEK. HEK cells infected with passage-9 virus were extracted for viral DNA (JCV-HEK P_{10}) by employing Hirt's procedure (5). Uncleaved viral DNAs as well as DNAs cleaved with *Hin*dIII were analyzed by agarose gel electrophoresis together with DNA from JCV (MAD-1) grown in PHFG cells. SV40 and BK virus DNAs were included for comparison. Uncleaved form I JCV-HEK P_{10} DNA formed a broad band which migrated faster than SV40 or JCV (MAD-1) DNA (Fig. 3A). JCV (MAD-1) DNA yielded three fragments after *Hin*dIII digestion, A (4,480 base pairs [bp]), B (440 bp), and C (230 bp) as reported previously by Martin et al. (9). By contrast, the *Hin*dIII fragments of JCV-HEK P_{10} were very different (Fig. 3B). Fragments B and C could be found, but full-sized A fragments were not. There were multiple bands between the positions for fragments A and B. The C fragment, presumably containing the origin of DNA replication (8), appeared to be enriched. These results indicate that the passage 10 virus consisted of a heterogeneous population of virions as reported previously for JCV grown in PHFG cells (9) or human amnion cells (16). Similar results were obtained with another DNA sample from passage 9.

(ii) Plaque-purified JCV-HEK. DNA was prepared from HEK cells infected with 11 different stocks of plaque-purified JCV. Figure 4 shows the electrophoretic patterns of uncleaved DNAs. Some of them formed a single sharp band only (Pl-3, 6), or with minor bands (Pl-4, 7). Others formed multiple or broader bands (Pl-5, 8, 9, 10, 12, 17, 20). The DNA bands from all plaque isolates moved faster than the form I DNA of SV40 or JCV (MAD-1). Figure 5 shows electrophoretic patterns of representative DNAs cleaved with *Eco*RI and *Bam*HI. Digestion with *Eco*RI revealed that most of the DNAs from plaque-purified JCV were susceptible to this enzyme. Only Pl-12 and 20 contained *Eco*RI-resistant fractions in detectable amounts (data not shown for Pl-20). All of the *Eco*RI-cleaved DNAs moved faster than the SV40 DNA (Fig. 5) or JCV (MAD-1) linear DNA (data not shown), indicating that they were of shorter length. Digestion with *Bam*HI showed that the DNAs tested (Pl-3, 4, 5, 6, 7, and 8) were composed of susceptible and resistant fractions. These results indicate that the DNAs from plaque-purified JCV contain at least two species of DNA.

The *Hin*dIII cleavage patterns of the plaque isolates were simpler than that of the uncloned JCV-HEK DNA (Fig. 3), but differed from each other as well as from JCV (MAD-1). Figure 6 shows the representative cleavage patterns. As noted for the uncloned DNA, none of the DNAs from plaque-purified virus contained full-length A fragments. All the DNAs except Pl-3 generated two fragments of 2,800 to 3,000 bp and two fragments at positions for B and C fragments. In addition they produced fragments differing in size (600 to 1,200 bp) and quantity with each isolate. Since the full length of JCV DNA is

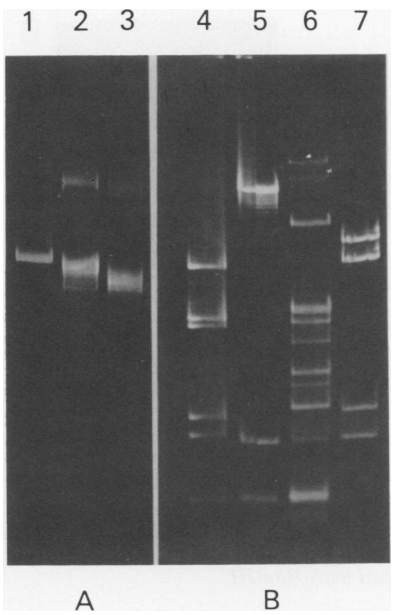

FIG. 3. *DNA from JCV-HEK. (A) Uncleaved DNAs; SV40 (lane 1), JCV (MAD-1) (lane 2), and JCV-HEK P_{10} (lane 3). Electrophoresis was done at 130 V for 190 min. (B) HindIII-cleaved DNAs; SV40 (lane 4), JCV (MAD-1) (lane 5), JCV-HEK (lane 6), and BK virus (lane 7). Electrophoresis was done at 100 V for 190 min. SV40 and BK virus DNAs were prepared from infected CV-1 (TC7) and HEK cells, respectively.*

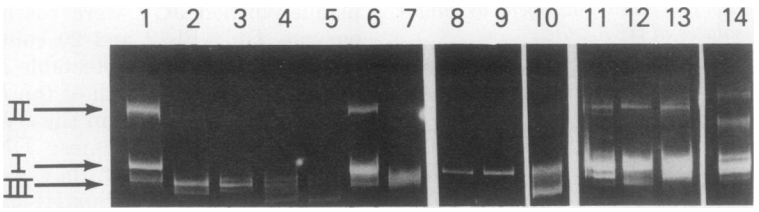

FIG. 4. *DNA from plaque-purified JCV. DNAs of SV40 (lane 1); JCV-HEK (Pl-4 (lane 2); Pl-7 (lane 3); Pl-10 (lane 4); Pl-12 (lane 5); JCV (MAD-1) (lane 6); JCV-HEK P_{10}, uncloned (lane 7); Pl-3 (lane 8); Pl-6 (lane 9); Pl-20 (lane 10); Pl-5 (lane 11); Pl-8 (lane 12); Pl-9 (lane 13); and Pl-17 (lane 14) were subjected for electrophoresis at 130 V for 190 min. Arrows indicate the positions for forms I, II, and III of SV40 DNA. JCV (MAD-1) apparently contained some defective DNA.*

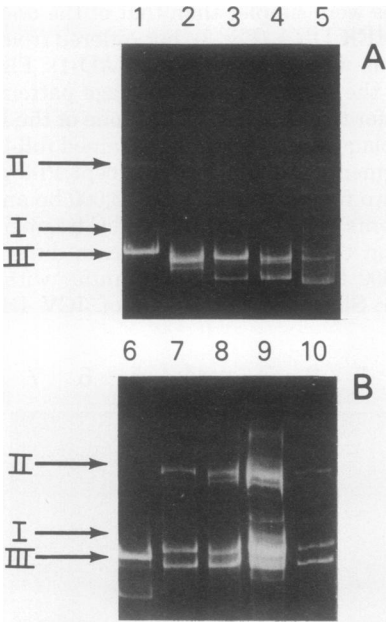

FIG. 5. *Digestion of plaque-purified JCV DNA with endonucleases EcoRI and BamHI. Arrows indicate the positions for forms I, II, and III of SV40 DNA. (A) Digestion with EcoRI. Digested DNAs of SV40 (lane 1), JCV-HEK Pl-4 (lane 2), Pl-7 (lane 3), Pl-10 (lane 4), and Pl-12 (lane 5) were subjected to electrophoresis at 130 V for 240 min. (B) Digestion with BamHI. Digested DNAs of SV40 (lane 6), JCV-HEK Pl-3 (lane 7), Pl-4 (lane 8), Pl-5 (lane 9), and Pl-6 (lane 10) were subjected to electrophoresis at 130 V for 240 min. The JCV DNA, which migrated more slowly than form III SV40 DNA, is form I DNA uncleaved with BamHI.*

about 5,100 bp (9), the two 2,800- to 3,000-bp fragments must have originated from different molecules.

Figure 6 also shows the *Hin*dIII + *Eco*RI cleavage patterns of DNAs from JCV (MAD-1) and the representative plaque isolates. JCV (MAD-1) DNA has an *Eco*RI-susceptible site within the *Hin*dIII A fragment (9); therefore, combined digestion with the two enzymes generated four fragments, A_1 (2,820 bp), A_2 (1,620 bp), B (440 bp), and C (230 bp), as shown in Fig. 6, lane 6. The plaque isolates were found to contain the fragments corresponding to A_1 and A_2 in size, although in Pl-17 (data not shown) and Pl-20 (Fig. 6, lane 15) the amount of A_1 fragments was reduced considerably. From the sizes of A_1 and A_2 fragments, they must have originated from different 2,800- to 3,000-bp fragments of *Hin*dIII. The combined digestion also showed that the 2,800-bp fragments of Pl-3 consisted of two DNAs, *Eco*RI-cleaved and uncleaved.

In an attempt to produce homogeneous stocks, JCV Pl-3 and 6 were passaged at very low multiplicities of infection (1 to 10 HA units per culture). After a long period of 2 months for complete cell lysis, the viral DNAs were analyzed. The *Hin*dIII and *Hin*dIII + *Eco*RI cleavage patterns were identical with those of DNAs obtained at higher multiplicities of infection.

Some of the plaque-purified stocks (Pl-4, 7, 10, and 12) were replaqued on HEK cells, and seven new plaques were isolated. All the DNAs from the newly isolated stocks had the same common characteristics described above. Digestion with *Hin*dIII produced two large fragments of 2,800 to 3,000 bp, the B and C fragments, and other fragments which differed from sample to sample. Although the viral DNAs lacked *Hin*dIII A fragments, they were revealed to have A_1 and A_2 fragments after *Eco*RI digestion. The cleavage patterns of the two new isolates originating from Pl-4 and 7 resembled those of Pl-6.

DISCUSSION

In a previous report (12), it was shown that JCV has an extremely restricted host range and that the original prototype virus, MAD-1 strain, replicated well only in PHFG cells; HEK cells did not support the growth of this virus. Limited growth of JCV was observed in human amnion cells (16). Attempts to circumvent the block in viral replication through the use of viral DNA

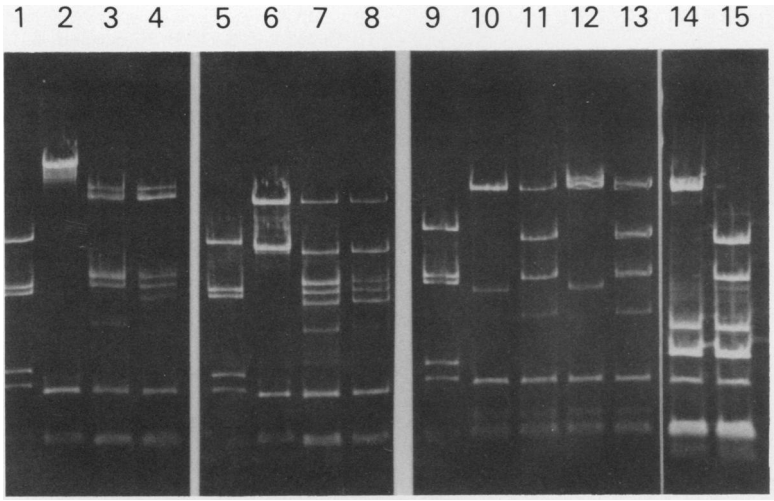

FIG. 6. *Cleavage of plaque-purified JCV DNA with HindIII and with EcoRI + HindIII. Electrophoresis was done at 100 V for 190 min. Lane 1, SV40 DNA (digested with HindIII); lane 2, JCV (MAD-1) DNA (HindIII); lane 3, JCV-HEK Pl-4 DNA (HindIII); lane 4, Pl-7 DNA (HindIII); lane 5, SV40 DNA (HindIII); lane 6, JCV (MAD-1) DNA (EcoRI + HindIII); lane 7, Pl-4 DNA (HindIII + EcoRI); lane 8, Pl-7 DNA (EcoRI + HindIII); lane 9, SV40 DNA (HindIII); lane 10, Pl-3 DNA (HindIII); lane 11, Pl-3 DNA (EcoRI + HindIII); lane 12, Pl-6 DNA (HindIII); lane 13, Pl-6 DNA (EcoRI + HindIII); lane 14, Pl-20 DNA (HindIII); lane 15 Pl-20 DNA (EcoRI + HindIII).*

in transfection studies using HEK cells also failed (4). We have found that although HEK cells are quite resistant to JCV, the virus can be adapted to growth in these cells, and after eight passages, relatively high yields can be obtained. Furthermore, unlike the prototype virus, the adapted strain is capable of forming plaques in HEK cells. By several criteria, the virus was identified as JCV. These included standard HA inhibition and fluorescent-antibody tests using specific antisera, and restriction enzyme analysis of viral DNA.

JCV DNA obtained from virus grown in PHFG cells has been reported to be heterogeneous by several investigators (4, 6, 10, 11). Similarly, the JCV grown in HEK cells also yielded heterogeneous viral DNA, and surprisingly, the virus obtained after plaque purification again yielded DNA which, although considerably less heterogeneous than the parental virus, was not completely homogeneous. It thus appears that JCV is inherently unstable and continuously produces particles which contain DNA of various sizes. Although SV40, polyoma virus, and BK virus produce particles containing heterogeneous DNA which are defective (3, 7, 18), it is not clear whether such particles of JCV are also defective. In studying the infectivity of JCV DNA, Frisque et al. (4) did not find large differences in the infectivity of heterogeneous DNA as compared to homogeneous DNA preparations as far as their ability to induce viral antigens was concerned.

DNAs from plaque-purified JCV were slightly shorter than JCV (MAD-1) DNA and consisted of two or more species of DNA. Despite the lack of a full-length *Hin*dIII A fragment, most of the DNA from plaque-purified virus yielded A_1 and A_2 fragments probably originating from different DNA molecules upon combined digestion with *Hin*dIII and *Eco*RI. These characteristics were unchanged after low-multiplicity passage or after recloning by the plaque method. The results of these experiments suggest that the plaques may have been formed by complementation between defective particles. Alternatively, we postulate that there may be various viable deletion mutants in stocks adapted to growth in HEK cells. To determine which of these hypotheses is applicable, the various species of DNA must be isolated and characterized.

Although plaque-purified virus did not yield completely homogeneous DNA, its replication in HEK cells was more efficient and the growth cycle was considerably shortened. This was evident by the appearance of earlier, more extensive cytopathic effects and complete cell lysis, resulting in greater virus yields. Thus, plaque-purified JCV stocks will facilitate biological and biochemical studies of this interesting and important virus.

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