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Molecular Cloning and Physical Mapping of Murine Cytomegalovirus DNA

ANGELIKA EBELING,[1] GÜNTHER M. KEIL,[1] ELISABETH KNUST,[2]† AND ULRICH H. KOSZINOWSKI[1]*

Federal Research Institute for Animal Virus Diseases, D-7400 Tübingen,[1] and Institut für Klinische Virologie, University of Erlangen-Nürnberg, D-8500 Erlangen,[2] Federal Republic of Germany

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Murine cytomegalovirus (MCMV) Smith strain DNA is cleaved by restriction endonuclease *Hin*dIII into 16 fragments, ranging in size from 0.64 to 22.25 megadaltons. Of the 16 *Hin*dIII fragments, 15 were cloned in plasmid pACYC177 in *Escherichia coli* HB101 (*recA*). The recombinant plasmid clones were characterized by cleavage with the enzymes *Xba*I and *Eco*RI. In addition, fragments generated by double digestion of cloned fragments with *Hin*dIII and *Xba*I were inserted into the plasmid vector pACYC184. The results obtained after hybridization of ^{32}P-labeled cloned fragments to Southern blots of MCMV DNA cleaved with *Hin*dIII, *Xba*I, *Eco*RI, *Bam*HI, *Apa*I, *Cla*I, *Eco*RV, or *Kpn*I allowed us to construct complete physical maps of the viral DNA for the restriction endonucleases *Hin*dIII, *Xba*I, and *Eco*RI. On the basis of the cloning and mapping experiments, it was calculated that the MCMV genome spans about 235 kilobase pairs, corresponding to a molecular weight of 155,000,000. All fragments were found to be present in equimolar concentrations, and no cross-hybridization between any of the fragments was seen. We conclude that the MCMV DNA molecule consists of a long unique sequence without large terminal or internal repeat regions. Thus, the structural organization of the MCMV genome is fundamentally different from that of the human cytomegalovirus or herpes simplex virus genome.

Murine cytomegalovirus (MCMV) has been classified as a member of the *Betaherpesvirinae* (23). In mice, MCMV infection resembles human cytomegalovirus (HCMV) infection in humans in the main biological effects, and MCMV has therefore been used as a model for studying cytomegalovirus infection and persistent herpesvirus infections in general (12). Although a considerable body of information on virus-cell and virus-host interactions has been collected, latent infections and persistent infections with very low levels of virus multiplication have so far resisted investigation. With the advent of techniques for locating the viral genome and its products more precisely, new attempts to gain insight into the regulation of the latent stage are possible. The HCMV genome was recently cloned (10, 11, 20, 29, 30), and the temporal pattern of transcriptional activity is under study (6, 31, 32).

To serve as a model for the study of persistency and latency, MCMV must be mapped and cloned to provide the tools for further investigation. To date very little is known about the molecular properties of the MCMV genome. A duplex DNA structure with a mean guanine-plus-cytosine content of 59% has been reported (19). Velocity sedimentation, experiments revealed a molecular weight of 132×10^6 (19) or 136×10^6 (17). In this paper, we describe the cloning of the *Hin*dIII fragments of MCMV in the plasmid vector pACYC177 and construction of the cleavage maps for *Hin*dIII, *Xba*I, and *Eco*RI.

MATERIALS AND METHODS

Virus and cell culture. MCMV (mouse salivary gland virus, strain Smith, ATCC VR-194) was obtained from the American Type Culture Collection, Rockville, Md., and propagated on BALB/c mouse embryo fibroblasts. Fibroblasts were prepared from 15- to 17-day-old BALB/c mouse embryos by trypsinization (0.25% trypsin, 0.125% EDTA, sterilized by filtration through a 100-nm filter [Millipore Corp., Bedford, Mass.] and subsequent selection during two 4- to 6-day culture passages in minimum essential medium with Earles salts complemented with 5% fetal calf serum (Seromed, Munich, Germany), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.35 mg of L-glutamine per ml in a humidified 5% CO_2 atmosphere at 37°C. The fibroblasts were infected for large-scale virus propagation by adding infected cells to noninfected cells. They were seeded in 60-mm plastic dishes

† Present address: Institut für Entwicklungsphysiologie, University of Cologne, D-5000 Cologne, Federal Republic of Germany.

(Greiner, Nürtingen, Germany) and infected 20 h later with 0.5 PFU/cell while being centrifuged at 800 × *g* for 30 min. This method of infection results in a 30- to 80-fold increase in infectivity (14). At 1 h postinfection, the cells were trypsinized and added to trypsinized noninfected cells in a ratio of 1:100. This cell suspension was seeded in Roux flasks. Two days later cytopathic effects were seen in the monolayers, and after 3 days all cells were detached from the surface.

Preparation of viral DNA. Cell culture fluid from infected cells was collected and centrifuged for 20 min at 6,000 rpm in a Beckman JA10 rotor to eliminate cell debris. The supernatant was removed, and, after centrifugation at 13,000 rpm in a Beckman JA14 rotor for 3 h, the pellet was suspended in a small volume of phosphate-buffered saline and homogenized by 30 to 50 strokes in a Dounce homogenizer. Cellular DNA was degraded by incubation with DNase I (100 μg/ml) (Boehringer Mannheim, Germany) for 1 h at 37°C. The reaction mixture was layered onto a 15% sucrose cushion in phosphate-buffered saline and centrifuged for 1 h at 25,000 rpm in a Beckman SW41 rotor. The resulting pellet was lysed in 2 ml of 20 mM Tris-hydrochloride (pH 8.5) containing 2% sodium lauroyl sarcosinate and proteinase K (1 mg/ml) (Serva, Heidelberg, Germany) and incubated at 56°C for 1 h. The lysate was layered onto 8.7 ml of a CsCl solution at a density of 1.832 g/ml and centrifuged to equilibrium at 33,000 rpm for 60 h in a Beckman 50 Ti rotor. The DNA banded at a density of about 1.718 g/ml. Pooled fractions were dialyzed against 20 mM Tris-hydrochloride (pH 8.5), and the final concentration of DNA was determined. The purity was tested by restriction enzyme digestion.

Viral DNA labeled in vivo with ^{32}P was prepared by adding 0.5 mCi of ^{32}Pi per ml to infected cells at 16 h postinfection. The cells were harvested at 24 h postinfection by trypsinization, washed twice in phosphate-buffered saline, and lysed with 2% sodium lauroyl sarcosinate in 10 mM Tris-hydrochloride–10 mM EDTA (pH 7.5). The lysate was made up to 100 μg/ml with RNase A (Sigma Chemical Co., Munich Germany). After 1 h at 37°C, proteinase K was added to a final concentration of 1 mg/ml, and the reaction mixture was incubated at 56°C for 1 h. The DNA was extracted twice with phenol, twice with chloroform-isoamyl alcohol (96:4), and finally with ether. After precipitation with 2.5 volumes of ethanol at −20°C, the DNA was resuspended in 20 mM Tris-hydrochloride (pH 8.5), and 2 ml (250 μg of DNA per ml) was centrifuged to equilibrium in CsCl as described above.

Restriction endonuclease digestion and electrophoresis. Restriction endonucleases *Hin*dIII, *Eco*RI, *Eco*RV, *Cla*I, *Apa*I, *Bgl*II, *Bam*HI, *Nci*I, *Sph*I, and *Taq*I were obtained from Boehringer Mannheim; *Kpn*I, *Sal*I, and *Xba*I were from Bethesda Research Laboratories, Bethesda, Md. *Eca*I and *Sma*I were purified by the method of Roberts (22). Digestions were performed as specified by the suppliers. The fragments obtained were sized on 0.4, 0.6, or 0.8% horizontal agarose gels. The bacteriophage lambda DNA molecular weight standards I, II, III, and IV (Boehringer Mannheim) and the *Hae*III fragments of phage φX174 DNA (Enzo Biochem, New York, N.Y.) served as molecular weight markers. The agarose gels and 5 to 20% polyacrylamide gradient gels (15) were stained with ethidium bromide, destained in water, and photographed with Polaroid type 665 or 667 film.

Exonuclease III digestion. A 40-μg amount of DNA was incubated with 2 U of exonuclease III per μg of DNA in the recommended buffer (Bethesda Research Laboratories), and the digestion was allowed to proceed for up to 60 min. The enzyme reaction was terminated by extraction with an equal volume of chloroform-isoamyl alcohol (96:4). After precipitation with 2.5 volumes of ethanol, the pellet was resuspended in the appropriate enzyme buffer and analyzed by electrophoresis after cleavage with restriction enzymes.

Labeling of terminal fragments with the Klenow fragment of DNA polymerase I. Virion DNA was digested with exonuclease III for up to 30 min. After ethanol precipitation the DNA was suspended in nick-translation buffer containing 0.035 M Tris-hydrochloride (pH 7.5), 0.01 mM dATP, 0.01 mM dGTP, 2.5 mM dithiotreitol (DTT), and 5 mM $MgCl_2$, and the protruding ends were filled with 1 U of DNA polymerase I (Klenow fragment; Boehringer Mannheim) per μg in a 25-μl volume containing 25 μCi of [α-^{32}P]dCTP and 25 μCi of [α-^{32}P]dTTP (Amersham Buchler, Braunschweig, Germany) for 30 min at room temperature. The radiolabeled DNA was separated by chromatography over Sephadex G-50 columns. Samples containing 10^6 cpm of incorporated activity were precipitated with ethanol and, after resuspension in the appropriate buffer, cleaved with *Hin*dIII, *Xba*I, or *Eco*RI and subjected to electrophoresis on 0.4 or 0.8% agarose gels. Gels were dried under vacuum and subjected to autoradiography. Virion DNA not subjected to exonuclease III treatment and DNA cleaved with *Hin*dIII, *Xba*I, or *Eco*RI followed by Klenow fragment labeling served as controls for monitoring the integrity of the DNA and the efficiency of the polymerase reaction.

Hybridization procedures. DNA fragments separated in agarose gels were denatured, neutralized in situ, and transferred to nitrocellulose filters (0.2-μm pore size; Schleicher & Schüll, Dassel, Germany) by the method described by Southern (24). The filters were baked at 80°C in a vacuum oven, preannealed for 4 to 6 h at 68°C in a solution containing 0.02% each of bovine serum albumin (BSA), Ficoll, and polyvinylpyrrolidone; 5× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate); and 100 μg of yeast RNA per ml. The DNA bound to filters was then hybridized, using the same buffer with 0.1% sodium dodecyl sulfate (SDS) and 20 mM sodium phosphate buffer (pH 7.0) added, to the ^{32}P-labeled virion or cloned MCMV DNA at 68°C for 24 h. After hybridization, the unannealed DNA was removed by washing the filters three times for 15 min each in 2× SSC–0.1% SDS at 60°C, three times for 15 min each in 1× SSC–0.1% SDS at room temperature, and three times for 15 min each in 0.1× SSC–0.1% SDS at room temperature. The dried filters were subjected to autoradiography with Curix MR 600 intensifying screens on Kodak X-Omat S film.

Construction of recombinant plasmids. MCMV DNA was cleaved with the restriction enzyme *Hin*dIII in buffer containing 33 mM Tris-acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 100 μg of BSA per ml, and 0.5 mM DTT (TA buffer) (P. O'Farrell, Bethesda Research Laboratories Focus, vol. 3, no. 3, p. 1–3, 1981). After digestion at 37°C for 1 h, ATP, DTT, and BSA were adjusted to final concentrations of 1 mM, 10 mM, and 50 μg/ml, respectively; 1

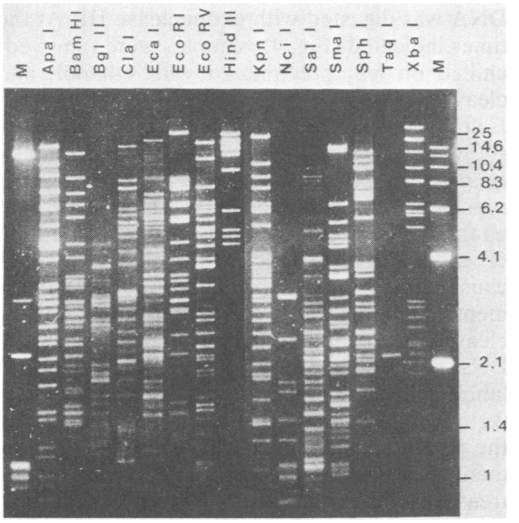

FIG. 1. Restriction enzyme analysis of MCMV DNA. MCMV DNA (1 μg) was cleaved with the restriction enzymes indicated. The resulting fragments were subjected to electrophoresis on 0.6% agarose gels and visualized with ethidium bromide. The molecular weight marker (lanes M) was a mixture of linear oligomeres of phage lambda *dv*21 DNA. Fragment sizes are given in Md.

U of polynucleotide kinase (Bethesda Research Laboratories) was added, and the mixture was incubated for 30 min at 37°C. Plasmid pACYC177 (3) was digested with *Hin*dIII, and the 5'-terminal phosphates were removed by incubation with 1 U of bacterial alkaline phosphatase (Bethesda Research Laboratories) for 30 min at 65°C to prevent recircularization. The two parts (1 μg of MCMV DNA to 0.5 μg of pACYC177 DNA) were mixed and extracted twice with phenol and ether, and after precipitation with 2.5 volumes of ethanol the resulting pellet was dissolved in TA buffer containing 1 mM ATP, 10 mM DTT, and 50 μg of BSA per ml and ligated with 2 U of T4 DNA ligase (Bethesda Research Laboratories) at 4°C for up to 3 days. The ligated plasmid was transformed into *E. coli* HB101 (*recA*) by the Ca^{2+} shock method (18). Transformants were selected on agar plates containing 100 μg of ampicillin per ml and tested for sensitivity to 50 μg of kanamycin per ml (3). Positive colonies were picked and grown overnight in LB medium (10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract, and 8 g of NaCl per liter) with 100 μg of ampicillin per ml under continuous agitation at 37°C. The method of Birnboim and Doly (1) was used for rapid screening for inserted viral fragments. For the purification of larger amounts of plasmid DNA, the method of Holmes and Quigley (13) was used. After lysis of bacteria with lysozyme and Triton X-100, chromosomal DNA and debris were pelleted by low-speed centrifugation. The plasmid DNA was recovered from the supernatant by isopropanol precipitation and suspended in 8.7 ml of 20 M Tris-hydrochloride–1% sodium lauroyl sarcosinate (pH 8.5)–9.4 g of CsCl–900 μl of ethidium bromide (20 mg/ml) and centrifuged to equilibrium for 48 h at 40,000 rpm in a Beckman 50 Ti rotor. The supercoiled plasmid DNA was collected under UV light at 360 nm, the ethidium bromide was removed by successive extractions with isoamyl alcohol, and the DNA solutions were dialyzed against 20 mM Tris-hydrochloride (pH 8.5). The isolated plasmids were cleaved with *Hin*dIII and fractionated on 0.6% agarose gels in Tris-acetate buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.8). DNA (recombinant plasmid DNA or MCMV DNA) was labeled to high specific activity (5×10^7 to 1×10^8 cpm/μg) for hybridization procedures with [α-^{32}P]dCTP and [α-^{32}P]dTTP by established methods. (21).

The *Hin*dIII fragments of MCMV DNA with internal cleavage sites for *Xba*I were digested with both *Hin*dIII and *Xba*I. The resulting *Hin*dIII-*Xba*I fragments were subcloned in the plasmid vector pACYC184 (3), which has a single *Hin*dIII site within the tetracycline resistance gene and a single *Xba*I site outside this region. Transformants of *E. coli* HB101 (*recA*) were selected on agar plates containing 20 μg chloramphenicol per ml, and sensitivity to tetracycline (100 μg/ml) was tested. Positive colonies were grown overnight in LB medium with chloramphenicol (20 μg/ml).

RESULTS

Restriction enzyme analysis. MCMV DNA was incubated with various restriction endonucleases, and the digestion products were analyzed by agarose slab gel electrophoresis (Fig. 1). Most of the enzymes cleaved MCMV DNA into more than 30 fragments. Fragments obtained after *Hin*dIII cleavage appeared to be the most suitable for cloning in plasmids, because only 16 fragments were generated, 8 of them ranging between 10 and 22 megadaltons (Md), and none of the residual fragments was smaller than about 0.6 Md. Fragments generated by *Xba*I and

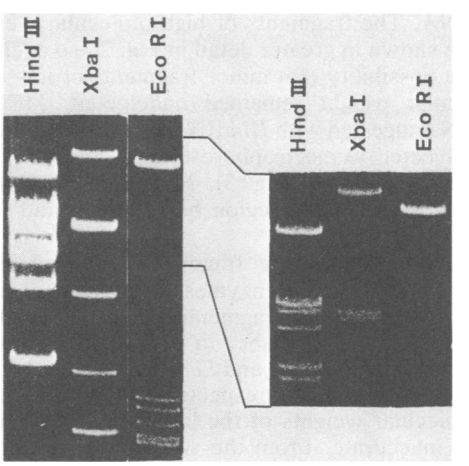

FIG. 2. Restriction enzyme analysis of the high-molecular-weight fragments of MCMV DNA. MCMV DNA (0.25 μg) was cleaved with the enzymes indicated. The resulting fragments were electrophoresed on 0.4% agarose gels for 24 (left) or 32 (right) h.

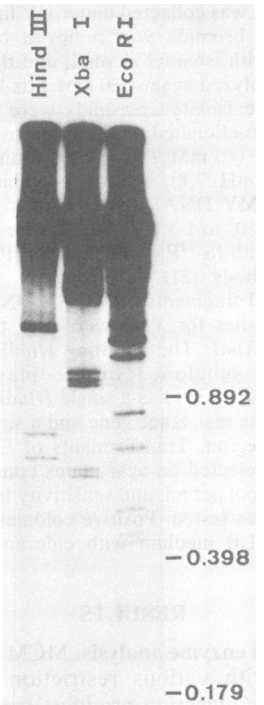

FIG. 3. Restriction enzyme analysis of the small restriction enzyme fragments of ^{32}P-labeled MCMV DNA. The DNA was cleaved with the restriction enzymes indicated and electrophoresed on a 5 to 20% polyacrylamide gradient gel. Bands were visualized by autoradiography of the dried gel. Sizes are shown in Md.

*Eco*RI digestion seemed to be appropriate for the construction of physical maps of MCMV DNA. The fragments of high molecular weight are shown in greater detail in Fig. 2. To exclude the possibility that minor fragments of low molecular weight remained undetected, MCMV DNA digested with *Hin*dIII, *Xba*I, or *Eco*RI was subjected to electrophoresis on 5 to 20% polyacrylamide gels (Fig. 3). No *Hin*dIII fragment was found in the region between 0.6 and 0.04 Md.

Table 1 lists the fragments obtained after cleavage with the enzymes *Hin*dIII, *Xba*I, and *Eco*RI. Individual fragments were designated by letters of the alphabet in order of decreasing size. *Hin*dIII, *Xba*I, and *Eco*RI generated 16, 25, and 33 fragments, respectively. The indicated molecular weights of the larger fragments may be inaccurate. From the sum of the molecular weights of the fragments, a genome size of about 155 Md or 235 kilobase pairs (kbp) could be calculated. Data obtained from experiments described below corroborated these calculations.

Identification of terminal fragments. MCMV DNA was digested with exonuclease III. At the times indicated (Fig. 4), samples were removed, chilled on ice, precipitated with ethanol, and cleaved with *Hin*dIII, *Xba*I, or *Eco*RI. *Hin*dIII-E and -N, *Xba*I-L and -S, and *Eco*RI-F and -G showed increased mobility, diminished intensity, or both in the gels and were thus assumed to represent terminal fragments. The intensity of all fragments decreased somewhat with the time of exposure to exonuclease III, probably because of nicks within the DNA. In other experiments, MCMV DNA was labeled after exonuclease III digestion with the Klenow fragment of DNA polymerase I. Although all fragments were labeled to some extent, the terminal fragments were much more intensively labeled, confirming the results of the exonuclease digestion experiments (data not shown). MCMV DNA not pretreated with exonuclease III was resistant to repair synthesis with the Klenow fragment. The fact that only two terminal fragments could be

TABLE 1. *Hin*dIII, *Xba*I, and *Eco*RI fragment sizes

Fragment	Size					
	*Hin*dIII [Md (kbp)]		*Xba*I [Md (kbp)]		*Eco*RI [Md (kbp)]	
A	22.25	(33.7)	28.7	(43.5)	25.0	(37.9)
B	18.1	(27.4)	17.9	(27.1)	9.5	(14.4)
C	17.8	(26.97)	17.0	(25.75)	9.0	(13.6)
D	16.75	(25.4)	13.5	(20.45)	8.6	(13.0)
E	15.3	(23.2)	10.3	(15.6)	8.35	(12.65)
F	14.8	(22.4)	8.55	(12.95)	8.2	(12.4)
G	13.3	(20.2)	8.55	(12.95)	7.0	(10.6)
H	10.8	(16.4)	6.35	(9.6)	6.95	(10.5)
I	6.3	(9.55)	6.15	(9.3)	6.85	(10.38)
J	5.4	(8.2)	6.05	(9.2)	6.2	(9.4)
K	5.2	(7.9)	5.3	(8.0)	6.1	(9.2)
L	4.8	(7.3)	3.0	(4.5)	6.05	(9.16)
M	1.8	(2.7)	2.9	(4.4)	5.0	(7.6)
N	1.5	(2.3)	2.7	(4.1)	4.5	(6.8)
O	0.7	(1.1)	2.6	(3.9)	3.9	(5.9)
P	0.64	(0.97)	2.4	(3.6)	3.7	(5.6)
Q			2.35	(3.55)	3.5	(5.3)
R			2.3	(3.5)	3.3	(5.0)
S			2.15	(3.25)	3.3	(5.0)
T			2.0	(3.0)	3.05	(4.6)
U			1.4	(2.1)	2.95	(4.5)
V			1.15	(1.7)	2.6	(3.9)
W			1.1	(1.6)	2.3	(3.5)
X			0.58	(0.88)	1.8	(2.7)
Y			0.57	(0.86)	1.7	(2.6)
Z					1.65	(2.5)
a					1.45	(2.2)
b					0.78	(1.2)
c					0.63	(0.96)
d					0.47	(0.71)
e					0.42	(0.63)
f					0.4	(0.6)
g					0.26	(0.39)
Total	155.4	(235.5)	155.5	(235.6)	155.5	(235.6)

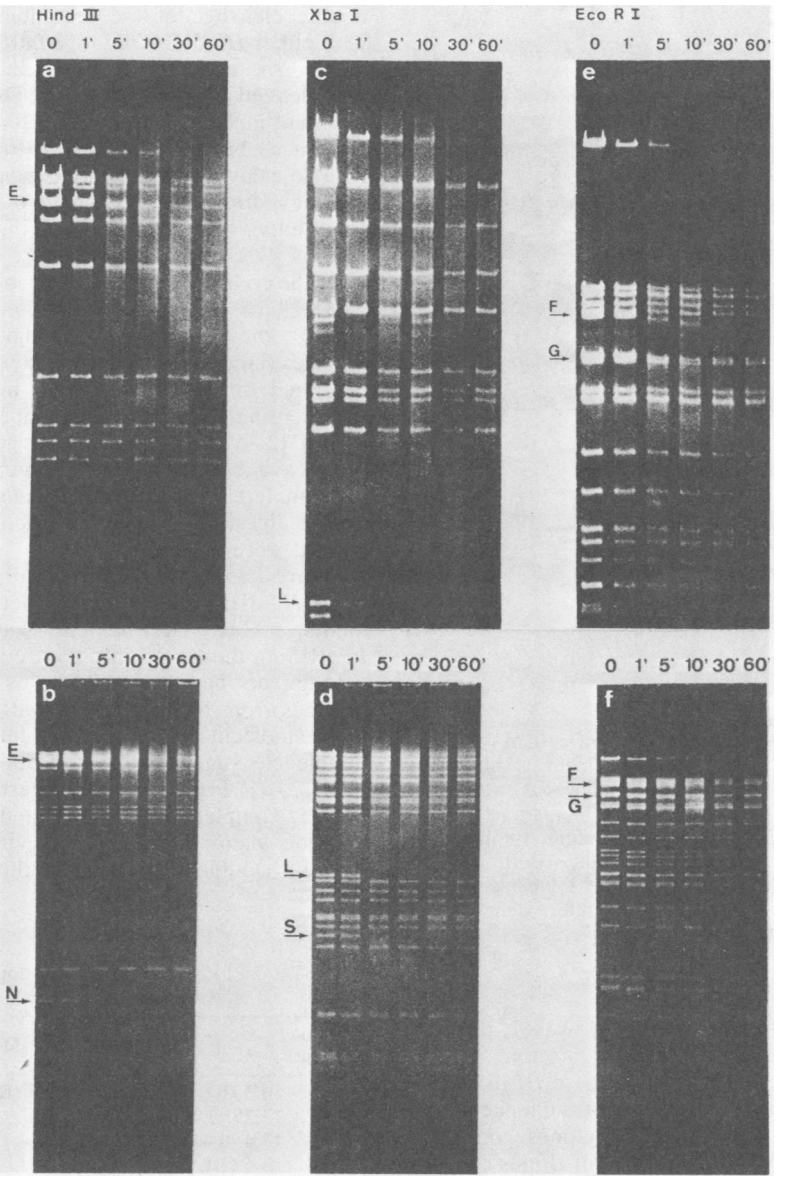

FIG. 4. Identification of the terminal fragments of MCMV DNA for the restriction enzymes *Hin*dIII, *Xba*I, and *Eco*RI. MCMV DNA was incubated with exonuclease III as described in the text. At the times indicated (minutes), samples were digested with (a and b) *Hin*dIII, (c and d) *Xba*I, or (e and f) *Eco*RI. The fragments were electrophoresed on 0.4 (top) or 0.8% (bottom) agarose gels. Bands were visualized with ethidium bromide. Lettered arrows indicate the terminal fragments.

identified after treatment with each of the three endonucleases argues against there being isomeric forms of MCMV DNA.

Molecular cloning of *Hin*dIII fragments. *Hin*dIII fragments were cloned in the plasmid vector pACYC177. This vector was selected to facilitate further experiments for the construction of cleavage maps because it lacks recognition sites for *Xba*I and *Eco*RI. Within the 450 recombinant plasmids characterized, all fragments except the terminal *Hin*dIII-E fragment were represented (Fig. 5). Most of these cloning experiments were performed with unfractionated DNA. Attempts to clone *Hin*dIII-E after isolation from the gel failed, although control experiments with the gel-purified F fragment

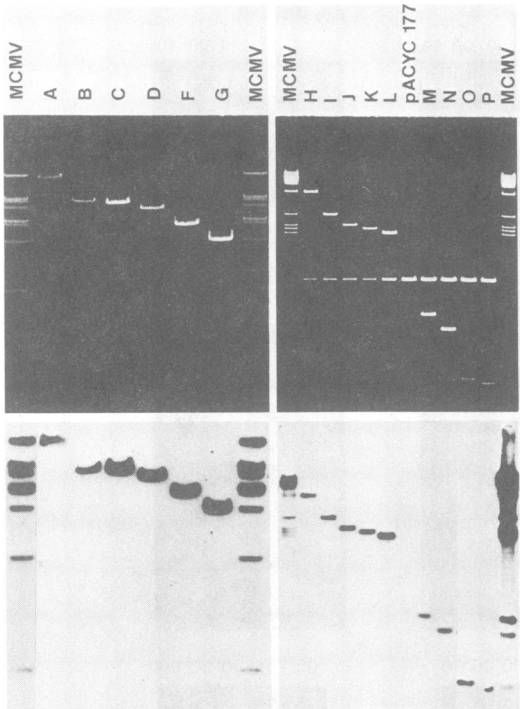

FIG. 5. Representative set of MCMV-*Hin*dIII clones. MCMV DNA and recombinant plasmids were cleaved with *Hin*dIII and electrophoresed on 0.4 (large fragments, left) or 0.6% (right) agarose gels. (Top) Bands were visualized with ethidium bromide and transferred to nitrocellulose filters. (Bottom) After hybridization to nick-translated ^{32}P-labeled MCMV DNA (about 5×10^7 cpm/μg), the filters were exposed for autoradiography.

were repeatedly successful. However, the *Hin*dIII-N fragment, which was determined to be a terminal fragment by exonuclease III digestion, could be cloned. This was not a chance occurrence, since the number of clones containing *Hin*dIII-N was similar to that of clones carrying other individual fragments. The cloned inserts were first identified by their electrophoretic mobility in comparison with digested virion DNA, followed by Southern blot hybridization with ^{32}P-labeled MCMV DNA to verify the viral origin of the integrated DNA. To demonstrate the purity of the cloned fragments, the plasmids were labeled with ^{32}P and hybridized to filters containing *Hin*dIII-digested virion DNA. Each clone hybridized only to a single *Hin*dIII fragment identical in size, allowing unambiguous assignment. This again indicated that there is no inversion within the MCMV genome.

Characterization of the cloned *Hin*dIII fragments. The *Xba*I and *Eco*RI fragments mapping within the *Hin*dIII clones were determined by double cleavage of the recombinant plasmids with either *Hin*dIII-*Xba*I or *Hin*dIII-*Eco*RI (Table 2). As an example, pAM121 (D fragment) was cleaved by *Xba*I into *Xba*I-D and a second fragment including both the vector and the viral sequences bracketing *Xba*I-D (Fig. 6, lane c). Double cleavage of this clone gave *Xba*I-D and two new fragments (2.75 and 0.5 Md) absent from either *Hin*dIII or *Xba*I digests of total MCMV DNA, which must have been derived from the ends of *Hin*dIII-D (lane b). Cleavage of pAM123 (B fragment) with *Xba*I resulted in *Xba*I-P, the vector and the adjacent viral sequences (lane f). After double cleavage, *Xba*I-P remained uncleaved, and two new fragments representing the viral sequences bracketing *Xba*I-P (2.45 and 13.25 Md) were generated (lane e). Cleavage of pAM127 (C fragment) revealed no complete *Xba*I fragment (lane i), and after double digestion two new fragments of 17.4 and 0.4 Md were obtained (lane g).

Figure 7 and Table 3 show examples of analogous *Hin*dIII-*Eco*RI double cleavage experiments. pAM13 (A fragment) was cleaved by *Eco*RI into the EcoRI into the EcoRI M, U, and c fragments and the vector plus the viral sequences from both ends of *Hin*dIII-A (lane g), whose molecular sizes could be determined after double cleavage as 8.25 and 5.4 Md (lane f). pAM123 (B fragment) was digested by *Eco*RI into the complete *Eco*RI D, L, and W fragments and the vector plus adjacent viral sequences (lane k). Double digestion gave the *Eco*RI D, L,

TABLE 2. Fragment sizes after double cleavage with *Hin*dIII and *Xba*I

*Hin*dIII fragment	*Xba*I fragments (Md)[a]
A	10.3 (E), 6.05 (J), 2.85, 2.3 (R), 0.75
B	13.25, 2.45, 2.4 (P)
C	17.4, 0.4
D	13.5 (D), 2.75, 0.5
E	ND[b]
F	6.9, 3.5, 2.0 (T), 1.4 (U), 1.0 (W)
G	—[c]
H	—
I	5.2, 1.1
J	3.53, 1.87
K	2.35 (Q), 1.5, 1.15 (V), 0.2[d]
L	—
M	—
N	—
O	—
P	0.45, 0.19[d]

[a] Included *Xba*I fragments are shown in parentheses.
[b] ND, Not done.
[c] —, No *Xba*I site present.
[d] Calculated size.

and W fragments (lane i) and the incomplete *Xba*I fragments (0.75 and 0.35 Md) from the ends of *Hin*dIII-B. pAM121 (D fragment) contained the *Eco*RI K, Q, a, and d fragments and the vector plus adjacent viral sequences (lane n) with molecular sizes of 3.05 and 2.2 Md (lane m).

Mapping the *Hin*dIII, *Xba*I, and *Eco*RI restriction sites. The alignment of fragments generated by *Hin*dIII, *Xba*I, and *Eco*RI was analyzed by hybridizing ^{32}P-labeled fragments to Southern blots with total MCMV DNA cleaved with either *Hin*dIII, *Xba*I, *Eco*RI, or *Bam*HI. Identical digests of DNA were separated on the same agarose gel for either 24 h, to prevent the loss of smaller fragments from the gel, or 40 h, to provide a better separation of comigrating fragments of high molecular weight. Gels were stained and photographed, and DNA was transferred to nitrocellulose filters. Individual gels included three identical sets of DNA for each of the four enzymes. One set was hybridized against ^{32}P-labeled total MCMV DNA, and the other sets were hybridized against ^{32}P-labeled

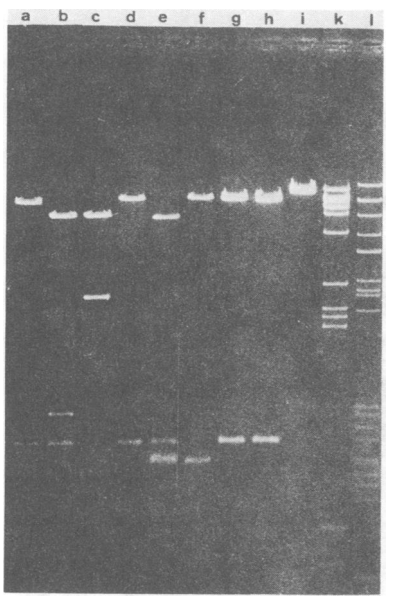

FIG. 6. Restriction enzyme analysis of cloned *Hin*dIII fragments after digestion with *Hin*dIII and *Xba*I. Fragments were separated on 0.6% agarose gels and stained with ethidium bromide. Fragments smaller than 1 Md could be seen, but their fluorescent intensity was insufficient for them to be recorded on this photograph. Lanes: pAM121 (D fragment) cleaved with (a) *Hin*dIII, (b) *Hin*dIII and *Xba*I, and (c) *Xba*I; pAM123 (B fragment) cleaved with (d) *Hin*dIII, (e) *Hin*dIII and *Xba*I, and (f) *Xba*I; pAM127 (C fragment) cleaved with (g) *Hin*dIII, (h) *Hin*dIII and *Xba*I, and (i) *Xba*I; MCMV DNA cleaved with (k) HindIII and (l) *Xba*I.

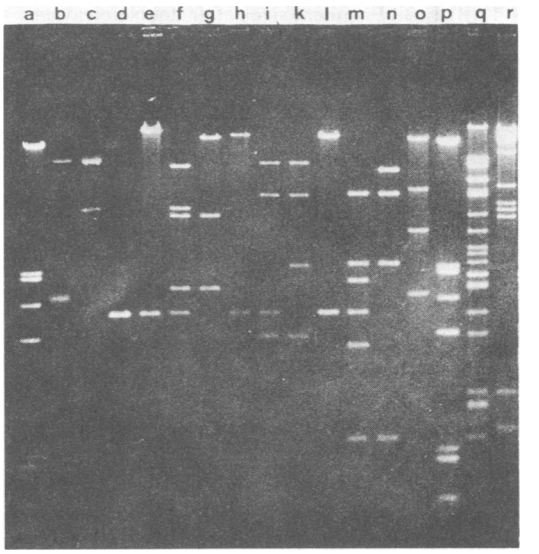

FIG. 7. Restriction enzyme analysis of cloned *Hin*dIII fragments after digestion with *Hin*dIII and *Eco*RI. Fragments were separated on 0.6% agarose gels and stained with ethidium bromide. Fragments smaller than 1.0 Md could be seen, but their fluorescent intensity was insufficient for them to be recorded on this photograph. Lanes: a, lambda DNA cleaved with *Hin*dIII and *Eco*RI; pACYC177 (b) untreated, (c) treated with *Eco*RI, and (d) cleaved with *Hin*dIII; pAM13 (A fragment) cleaved with (e) *Hin*dIII, (f) *Hin*dIII and *Eco*RI, and (g) *Eco*RI; pAM123 (B fragment) cleaved with (h) *Hin*dIII, (i) *Hin*dIII and *Eco*RI, and (k) *Eco*RI; pAM121 (D fragment) cleaved with (l) *Hin*dIII, (m) *Hin*dIII and *Eco*RI, and (n) EcoRI; lambda DNA cleaved with (o) *Hin*dIII and (p) *Hin*dIII and *Eco*RI; MCMV DNA cleaved with (g) *Eco*RI and (r) *Hin*dIII.

cloned fragments (Fig. 8 and Table 4). Individual BamHI fragments are not designated with letters in this paper since hybridization to these fragments served only to confirm nearest-neighbor relationships. As an example, pAM123 (B fragment) hybridized with *Hin*dIII-B (Fig. 8, lanes r and v), with *Xba*I fragments A, K, and P (lanes s and w), and with *Eco*RI fragments C, D, L, W, and y (lanes u and y).

The data obtained from the hybridizations and the double cleavages allowed linear arrangement of the majority of the *Hin*dIII fragments (Fig. 9). However, the correct sequence of some fragments still remained obscure. Therefore ^{32}P-labeled *Hin*dIII clones were hybridized to virion DNA cleaved with other enzymes that also generate a number of large fragments that can be easily distinguished. Digests with *Apa*I, *Cla*I, *Eco*RV, and *Kpn*I were separated on agarose gels (Fig. 10), transferred to nitrocellulose fil-

TABLE 3. Fragment sizes after double cleavage with *Hin*dIII and *Eco*RI

*Hin*dIII fragment	*Eco*RI fragments (Md)[a]
A	8.25, 5.4, 5.0 (M), 2.95 (U), 0.65 (c)
B	8.6 (D), 6.05 (L), 2.3 (W), 0.75, 0.35
C	16.3, 1.5
D	6.1 (K), 3.5 (Q), 3.05, 2.2, 1.45 (a), 0.47 (d)
E	ND[b]
F	6.85 (I), 3.4 (R), 2.35, 2.2
G	8.7, 4.6
H	6.45, 4.25
I	4.65, 1.65
J	2.6 (V), 1.6, 1.2
K	2.35, 1.8 (X), 0.42 (e), 0.65
L	—[c]
M	1.46, 0.3
N	—
O	—
P	—

[a] Included *Eco*RI fragments are shown in parentheses.
[b] ND, Not done.
[c] —, No *Eco*RI site present.

ters, and hybridized to the ^{32}P-labeled clones. To obtain internal markers for the identification of individual fragments, a short (5 min) prehybridization with ^{32}P-labeled virion DNA was carried out before hybridization with ^{32}P-labeled fragments. This procedure was done for all *Hin*dIII clones to confirm the alignment described below. As an example, the correct sequence of *Hin*dIII fragments I, O, and P was determined as follows. pAM28 (O fragment) and pAM58 (P fragment) hybridized to one *Cla*I fragment (Fig. 10, lanes f and k) to which pAM117 (I fragment) did not hybridize (lane o); therefore *Hin*dIII-O and -P are adjacent. pAM28 (O fragment) and pAM117 (I fragment), but not pAM58 (P fragment), hybridized to a common *Eco*RV fragment (lanes g, l, and p); therefore the sequence must be I-O-P or P-O-I.

*Hin*dIII-N and -A, but no other *Hin*dIII fragment, hybridized to identical *Apa*I, *Cla*I, *Eco*RV, and *Kpn*I fragments that were all smaller in size than the *Hin*dIII-A fragment (data not shown). Thus, *Hin*dIII-N could be identified as a

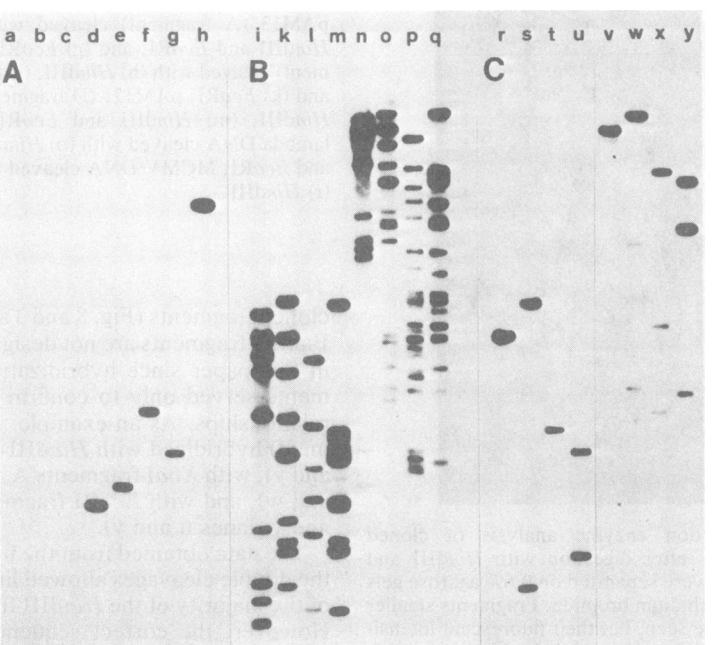

FIG. 8. Representative autoradiograms of Southern blot hybridizations used to establish linkage groups. MCMV DNA (0.25 μg) was cleaved with *Hin*dIII (lanes a, e, i, n, r, v), *Xba*I (lanes b, f, k, o, s, w), *Bam*HI (lanes c, g, l, p, t, x), or *Eco*RI (lanes d, h, m, q, u, y) and electrophoresed on 0.6% agarose gels for 40 (a–d, i–m, and r–u) or 16 h (e–h, n–q, and v–y). Transferred fragments were hybridized to ^{32}P-labeled DNA from pAM35 (N fragment) (A), MCMV (B), or pAM123 (B fragment) (C). No hybridization of pAM35 to *Hin*dIII, *Xba*I, or *Bam*HI fragments is shown in lanes a through c because it only hybridized to the smaller fragments obtained with these enzymes shown in lanes d through h.

TABLE 4. Hybridization of *Hin*dIII clones to *Xba*I and *Eco*RI fragments

*Hin*dIII fragment	Hybridization to *Xba*I fragments	Included *Xba*I fragments	Hybridization to *Eco*RI fragments	Included *Eco*RI fragments
A	E, J, K, R, S	E, J, R	C, G, M, U, c	M, U, c
B	A, K, P	P	C, D, L, W, Y	D, L, W
C	B, C	—	A, P	—
D	A, B, D	D	B, K, P, Q, a, d	K, Q, a, d
E	ND[a]	J, L, M, X, Y	ND	F, O, Z, b, f, g
F	C, F/G, T, U, W	T, U, W	I, H, R, T	I, R
G	C	—	A, H	—
H	A	—	B, N	—
I	H, N	—	J, S	—
J	F/G, N	—	E, S, V	V
K	F/G, F/G, Q, V	Q, V	E, T, X, e	X, e
L	F/G	—	E	—
M	A	—	N, Y	—
N	S	—	G	—
O	H	—	J	—
P	H, O	—	J	—

[a] ND, Not done.

direct neighbor of *Hin*dIII-A. Linkage between *Hin*dIII-A and *Hin*dIII-B was revealed by hybridization of both fragments to *Xba*I-K and *Eco*RI-C. The HindIII-M and *Hin*dIII-H fragments are included in the *Xba*I-A fragments. The hybridization of *Hin*dIII-H to *Eco*RI-B and of *Hin*dIII-D to *Eco*RI-B, and additional hybridization experiments with MCMV DNA cleaved with *Apa*I, *Cla*I, *Eco*RV, or *Kpn*I, disclosed that the correct sequence is *Hin*dIII-B, -M, -H, -D. *Hin*dIII-D was connected to *Hin*dIII-C by hybridization to *Eco*RI-P. The transition of *Hin*dIII-C to *Hin*dIII-G was confirmed by the hybridization to *Eco*RI-A, and the junction of *Hin*dIII-G to *Hin*dIII-F was proven by hybridization to *Eco*RI-H. *Hin*dIII-F was joined to *Hin*dIII-K by hybridization to *Eco*RI-T and confirmed by nearest-neighbor hybridization to *Eco*RV and *Kpn*I fragments.

The *Hin*dIII-K, -L, -J alignment was revealed by hybridization to *Eco*RI-E. Association between *Hin*dIII-J and *Hin*dIII-I was shown by hybridization to the *Xba*I-N and *Eco*RI-S fragments. The sequence *Hin*dIII-I, -O, -P was discussed above. The terminal *Hin*dIII-E remained the only *Hin*dIII fragment whose position could not be directly demonstrated by blotting experiments. Its identification as a terminal fragment meant it could only be positioned at the free end, and the following informations supported this conclusion. The *Xba*I-O fragment, 2.6 Md, hybridized only with the *Hin*dIII-P fragment containing only one *Xba*I site. The shared sequence between *Xba*I-O and *Hin*dIII-P spanned only 0.19 Md. Since no other cloned *Hin*dIII fragment hybridized to *Xba*I-O, the residual sequence of this fragment must be colinear with *Hin*dIII-E. In addition, this alignment of the *Hin*dIII-E fragment was confirmed by hybridization of the *Hin*dIII fragments I, O, and

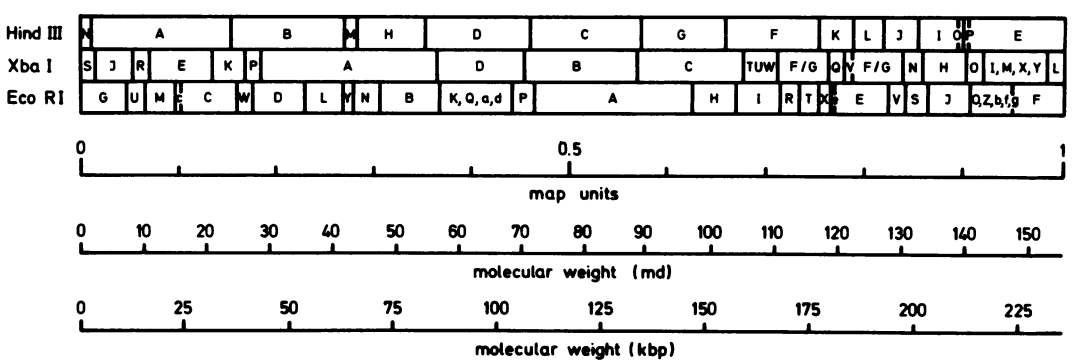

FIG. 9. Map of the MCMV Smith strain genome for the enzymes *Hin*dIII, *Xba*I, and *Eco*RI.

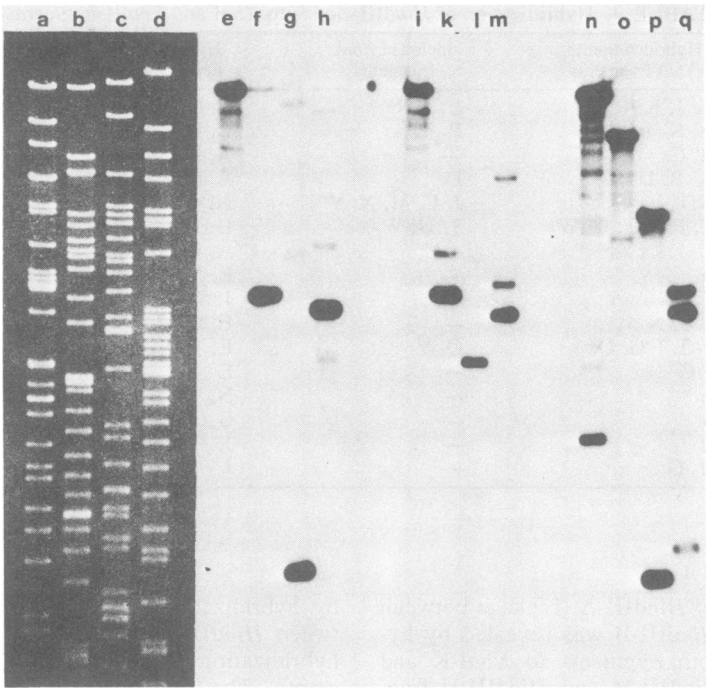

FIG. 10. Representative gels for the analysis of neighboring fragments. MCMV DNA (0.5 μg) was cleaved with *Apa*I (a, e, i, n), *Cla*I (b, f, k, o), *Eco*RV (c, g, 1, p), or *Kpn*I (d, h, m, q), and the restriction fragments were electrophoresed on 0.6% agarose gels. DNA on gel strips (lanes a through d) was transferred to nitrocellulose filters and hybridized to ^{32}P-labeled MCMV DNA for 5 min. After this prehybridization, the DNA was removed and the filters were hybridized overnight against ^{32}P-labeled cloned fragments. Lanes e through h, Autoradiogram of the hybridization of pAM28 (O fragment); i through m, autoradiogram of pAM58 (P fragment); n through q, autoradiogram of pAM117 (I fragment).

P to the largest *Bam*HI fragment, to which no other *Hin*dIII fragments hybridized. Therefore the remaining part (about 7.5 Md) of this *Bam*HI fragment should map within *Hin*dIII-E.

The data allowed the construction of physical maps for *Xba*I and *Eco*RI also, with the exception of accurate orientation of the *Xba*I and *Eco*RI fragments included within the *Hin*dIII fragments A, B, D, E, and F. Double cleavage of pAM13 (A fragment) with *Xba*I and *Eco*RI resulted in eight fragments (data not shown). With the exception of *Eco*RI-c, all genuine *Xba*I and *Eco*RI fragments were cleaved into smaller fragments. The only possible orientation for the *Xba*I and *Eco*RI fragments included in *Hin*dIII-A is shown (Fig. 9). The double digestion of pAM123 (B fragment) with *Xba*I and *Eco*RI cleaved *Xba*I-P, *Eco*RI-D, and EcoRI-W into smaller fragments, but left *Eco*RI-L intact. The orientation of the EcoRI fragments within *Hin*dIII-B is also shown (Fig. 9). Double digests of pAM84 (F fragment) with *Xba*I and *Eco*RI gave no information on the linear arrangement of *Xba*I fragments T, U or W, but allowed the alignment of the *Eco*RI fragments mapping within *Hin*dIII-F. The orientation of the *Xba*I fragments included in *Hin*dIII-E and the sequence of the *Eco*RI fragments within *Hin*dIII-D and -E could not be determined.

The correct alignment was ascertained by hybridization experiments with *Hin*dIII-*Xba*I subclones (Fig. 11). Hybridization of one subclone of pAM84 (F fragment) to *Hin*dIII-F, *Xba*I-C, and *Eco*RI-H and -I is shown. Another subclone hybridized to *Hin*dIII-F, *Xba*I-F/G, and *Eco*RI fragments I, R, T. The transitions for the *Hin*dIII fragments A, B, C, D, F, K, I, J, and P were confirmed with subclones. All hybridizations with the subclones verified the arrangement given in the map.

DISCUSSION

In this communication we describe the construction of recombinant plasmids containing the restriction fragments of MCMV DNA that include about 90% of the total coding capacity of the genome. The *Hin*dIII fragments were inserted into the plasmid vector pACYC177, and

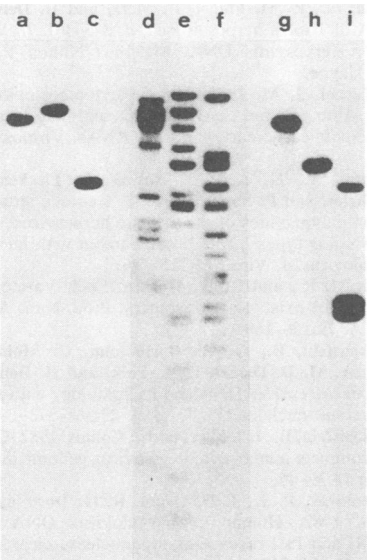

FIG. 11. Hybridization of the subclones derived from *Hin*dIII-F after *Hin*dIII and *Xba*I double cleavage. MCMV DNA (0.25 μg) was cleaved with *Hin*dIII (a, d, g), *Xba*I (b, e, h), or *Eco*RI (c, f, i). After electrophoresis on 0.6% agarose gels, the fragments were transferred to nitrocellulose filters and hybridized with ^{32}P-labeled DNA from (a through c) subclone pAM84-3 (left-end *Hin*dIII-*Xba*I fragment), (d through f) MCMV DNA, or (g through i) subclone pAM84-5 (right-end *Hin*dIII-*Xba*I fragment).

*Hin*dIII-*Xba*I double cleavage fragments were inserted into the plasmid vector pACYC184. Hybridization of the radioactively labeled cloned fragments to blots of MCMV DNA cleaved with various endonucleases allowed the identification of the cloned fragments and the construction of the physical map of the genome for the restriction enzymes *Hin*dIII, *Xba*I, and *Eco*RI.

Of the 16 fragments obtained after digestion with *Hin*dIII, only 1, the terminal *Hin*dIII-E of 15.3 Md, could not be cloned. This was expected, since terminal fragments of a linear genome generally resist cloning. Surprisingly, the *Hin*dIII-N fragment (1.5 Md), which appeared to be the other terminus, could be cloned with ease. This phenomenon is not without precedent and was reported previously for the cloning of varicella-zoster virus DNA (8, 28). The explanation for this finding is that *Hin*dIII-N is flanked by a very small terminal fragment. We could not find this fragment in gels run to identify small fragments and concluded that it was smaller than 40 kd. In some experiments, asymmetrical susceptibility of the terminal fragments to exonuclease III treatment was observed. The *Hin*dIII-N fragment was degraded more slowly during digestion than the *Hin*dIII-E fragment was. This resistance cannot be fully explained even by the presence of small terminal fragment beyond *Hin*dIII-N and is similar to observations made for exonuclease digestion of terminal fragments obtained with channel catfish virus DNA and herpes simplex virus DNA (4, 27, 34).

The three restriction endonucleases *Hin*dIII, *Xba*I, and *Eco*RI cleave the MCMV genome into 16, 25, and 33 fragments, respectively. The cleavage pattern was identical in different DNA preparations, and no change could be observed after transfection and plaque purification. From the mobility of the restriction fragments in agarose gels, nearly identical estimates for the size of the MCMV genome, about 155 Md (corresponding to 235 kbp), could be determined after digestion with any of the three enzymes. According to our estimate, the genome of MCMV Smith strain is about 20 Md larger than previously described (19). The difference between the two calculations may be due in part to the different techniques used. However, herpes simplex virus type 1 DNA was also determined to be only 87 Md (19). Since the accurate value is approximately 100 Md (25), the earlier estimate of the size of the MCMV genome was probably also too low. Substantial differences between the molecular weight calculations made by sucrose gradient centrifugation and by gel electrophoresis were described for another herpesvirus as well (33).

By our estimate, the overall size of the MCMV genome is almost identical to that of the HCMV genome. Surprisingly, the genomic structure differs from that of HCMV, as MCMV lacks the inverted repeat sequences described for HCMV and other herpesviruses (6, 7, 25, 26). This conclusion is based on the findings that (i) after exonuclease treatment and labeling of the termini, only two terminal fragments could be identified, (ii) no submolar fragments were observed, (iii) cloned fragments hybridized only to single *Hin*dIII fragments of identical electrophoretic mobility, and (iv) no backfolding structures were seen in electron microscopic studies (P. Sheldrick and N. Berthelot, personal communication). Neither terminal nor internal redundant sequences were indicated by our data. We can certainly exclude the type of highly repetitive terminal sequences found in the genomes of *Herpesvirus saimiri* (2) and *Herpesvirus ateles* (9), since the subterminal *Hin*dIII-N fragment of 1.5 Md did not hybridize with any other *Hin*dIII fragment. With the exception of *Xba*I-F/G, all fragments obtained after digestion with the three enzymes could be resolved to equimolar bands. Iterative sequences were not revealed by these enzymes, since in the search

for the terminal fragment flanking *Hin*dIII-N no molar fragment of 40 kd was seen, and smaller fragments of higher molarity would have been detected (Fig. 3). Regions larger than 1.5 Md within *Hin*dIII fragments that could contain repeated sequences were excluded for cloned fragments by digestion with other enzymes which generate small fragments (*Bgl*II, *Hin*fI, *Hpa*II; data not shown). The largest fragment that could still contain repetitions is the *Xba*I-I fragment of about 6 Md which maps within *Hin*dIII-E. This excludes large regions containing iterated sequences of the type found in Epstein-Barr virus DNA (16).

Thus, MCMV DNA molecules seem to consist of a single long unique sequence. Among the characterized herpesviruses, this type of DNA structure was suggested only for the Tupaia herpesviruses (5) and bears some resemblance to that of channel catfish virus, which in addition is characterized by terminal repeat regions of identical orientation (4).

The organization of the MCMV genome reveals major differences between MCMV and HCMV. However, since the biological aspects of MCMV studied to date are very similar to those of HCMV, whether the observed structural differences have any biological importance must be determined. Because the genomic structure by itself is a weak base for taxonomic associations, the question arises of whether MCMV should be classified by biological or structural criteria.

With the help of the recombinant clones and the map of the genome, localization of the MCMV genome and definition of its activity during persistent and latent infections is now open to investigation.

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