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Cloning and molecular characterization of the murine herpesvirus 68 genome

S. Efstathiou,* Y. M. Ho and A. C. Minson

Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, U.K.

Murine herpesvirus 68 (MHV-68) is a naturally occurring herpesvirus of small free-living rodents. In order to facilitate the molecular characterization of the virus genome, a library of cloned restriction fragments has been produced and restriction enzyme cleavage maps deduced for the enzymes *Bam*HI, *Eco*RI and *Hin*dIII. The MHV-68 genome comprises a region of unique DNA of approximately 118 kbp which is flanked by variable numbers of a 1·23 kb repeat unit. The organization of the MHV-68 genome is, therefore, most similar to that of the lymphotropic γ_2 group of herpesviruses which include herpesvirus saimiri and herpesvirus ateles.

Introduction

Animal models have been of great importance in studies of herpesvirus pathogenesis both in providing systems by which to study basic immunological and virological aspects of acute and latent infection and for the evaluation of chemotherapeutic and vaccination strategies. The laboratory mouse has been of particular value in such studies largely because of our detailed knowledge of its immunology, the existence of many uniform genetically defined strains and its modest cost. Perhaps the best examples are those models which have been used to study acute and latent herpes simplex virus (HSV) infections. HSV is a neurotropic alphaherpesvirus which when inoculated into mice produces an infection which closely mimics that observed in man (Wildy *et al.*, 1982). In the case of human cytomegalovirus (HCMV), a betaherpesvirus, its strict species specificity has precluded its use in experimental animals. For this reason, murine cytomegalovirus (MCMV), which resembles its human counterpart both genetically and in many aspects of its pathogenesis, has been used as a model by which to study betaherpesvirus infection (Hudson, 1979; Mercer *et al.*, 1988). At present, no murine animal model exists for the study of gammaherpesvirus pathogenesis. We are interested in developing novel murine animal systems by which to study herpesvirus pathogenesis and in this paper describe some molecular characteristics of a naturally occurring herpesvirus of rodents.

During field studies in the Bratislava region of Czechoslovakia, five virus isolates were recovered from two species of small free-living rodents, the bank vole (*Clethrionomys glareolus*) and the yellow-necked mouse (*Apodemus flavicollis*) (Blaskovic *et al.*, 1980). These isolates were successfully propagated in newborn laboratory mice and electron microscopic studies of infected rabbit embryo fibroblasts provided morphological evidence that these viruses belonged to the family herpesviridae (Blaskovic *et al.*, 1980; Ciampor *et al.*, 1981). All five isolates were shown to be antigenically related (Svobodova *et al.*, 1982*b*) and to induce cytopathic effects characteristic of herpesvirus infection in various epithelial and fibroblastoid cell lines. It was noted that the morphogenesis of these isolates most resembled that of herpes simplex virus (Ciampor *et al.*, 1981) and the kinetics of growth and range of permissive host cells suggested that the five isolates be classified as members of the alphaherpesvirus subgroup (Svobodova *et al.*, 1982*a*). On the basis of this initial classification it was considered that these virus isolates could provide a useful murine model for the study of natural alphaherpesvirus pathogenesis. However, studies on the pathogenesis of one isolate designated murine herpesvirus 68 (MHV-68), which was isolated from the bank vole, demonstrated that both newborn and 5- to 10-day-old outbred laboratory mice infected orally or intranasally developed a severe exudative pneumonia with haematogenous dissemination of virus (Rajcani *et al.*, 1985; Blaskovic *et al.*, 1984). Virus antigen was detected in many different cell types of infected animals including alveolar epithelium, heart muscle, tubular epithelium and lymphocytes (Rajcani *et al.*, 1985). Infected mice failed to demonstrate neurological disease and the consistent finding was that infection by the respiratory route was efficient, resulting in an exudative pneumonia at high virus doses followed by transient viraemia during

which infectious virus could be detected in many tissues (Rajcani *et al.*, 1985; N. P. S. Chandra *et al.*, unpublished observations). Taken together these studies suggest that MHV-68 infection of laboratory mice could provide a useful model for the study of viraemia but that it is unlikely to provide a suitable system to investigate neurotropic alphaherpesvirus pathogenesis. Furthermore, these observations raised doubts as to the initial classification of MHV-68 as an alphaherpesvirus.

In order to understand the relationship of MHV-68 with other well characterized members of the alpha-, beta- and gammaherpesvirus subgroups we have undertaken a detailed analysis of the genome of this virus. A genomic library of cloned restriction fragments has been produced and used both to construct *Bam*HI, *Eco*RI and *Hin*dIII restriction maps and to elucidate the structure of the virus genome.

Methods

Viruses and cells. Stocks of MHV-68 (Rajcani *et al.*, 1985) were kindly provided by J. Lesso (Comenius University, Czechoslovakia). Prior to use, this original virus stock was plaque-purified twice by limiting dilution assay and clone g2.4 used in subsequent studies. Virus stocks were prepared by infection of BHK-21 C13 cells at a low multiplicity of infection (0·1 p.f.u./cell). Cell-associated virus was disrupted by sonication and dispensed in 0·2 ml volumes and stored at −70 °C until required. Virus infectivity was measured by plaque titration using BHK-21 C13 cells grown in Eagle's medium containing 10% (v/v) tryptose phosphate broth and 10% (v/v) calf serum (ETC) at 37 °C in equilibrium with humidified 5% CO_2 for 3 days.

Virus DNA preparation. High M_r infected cell DNA was prepared as follows. Monolayers of BHK-21 C13 cells were infected at a multiplicity of 0·1 p.f.u./cell. Three to 4 days after infection cells were harvested, washed in phosphate-buffered saline and lysed by resuspension in 0·5% (v/v) SDS in 10 mM-Tris–HCl, 50 mM-EDTA pH 8. The lysate was then treated with Pronase (1 mg/ml) at 37 °C for 5 h followed by two phenol/chloroform extractions. The DNA was ethanol-precipitated, spooled and redissolved in 10 mM-Tris–HCl, 1 mM-EDTA pH 7·5.

To obtain purified virion DNA, virus particles were recovered from the medium of infected BHK-21 C13 cells by centrifugation at 25000 r.p.m. in a Beckman SW28 rotor. The pelleted virus was resuspended in 0·5% (v/v) SDS in 10 mM-Tris–HCl, 50 mM-EDTA pH 8. The suspension was then treated with Pronase (1 mg/ml) at 37 °C for 5 h followed by two phenol/chloroform extractions. The virion DNA was then ethanol-precipitated and redissolved in 10 mM-Tris–HCl, 1 mM-EDTA pH 7·5. Virion DNA was banded by isopycnic centrifugation in CsCl density gradients with an initial density of 1·72 g/ml formed in 10 mM-Tris–HCl, 1 mM-EDTA pH 7·5 containing 3 μg/ml ethidium bromide. Banded virion DNA was visualized by u.v. light and harvested. Following butanol extraction and overnight dialysis against 100 mM-Tris–HCl, 1 mM-EDTA pH 7·5 the DNA was ethanol-precipitated and redissolved in 10 mM-Tris–HCl, 1 mM-EDTA pH 8·0.

Agarose gel electrophoresis. Restriction enzyme digestion was carried out as suggested by the manufacturers (Bethesda Research Laboratories and Boehringer-Mannheim). Electrophoresis was carried out in horizontal 0·8, 1·0 or 1·2% agarose gels at 5 V/cm in 40 mM-Tris–acetate pH 7·8, 5 mM-sodium acetate, 1 mM-EDTA. DNA bands were visualized by u.v. light after staining with ethidium bromide (1 μg/ml).

Gel purification of specific DNA fragments was performed using NA45 paper following the manufacturer's protocols (Schleicher & Schuell), or using the phenol/freeze method (Tautz & Renz, 1983).

Cloning of virus restriction fragments. The vectors used in this study were pUC13 and pBR322. Vector DNA was prepared by the alkaline lysis method (Birnboim & Doly, 1979) and digested with the appropriate restriction enzyme. Digested vectors were treated with alkaline phosphatase using the manufacturer's protocols (Boehringer-Mannheim). Virus restriction fragments were ligated with the appropriate linearized, phosphatase-treated vector and transformed into *Escherichia coli* strains TG1 or TG2 using the Hanahan transformation method (Hanahan, 1983). DNA was prepared from the transformants digested with the appropriate restriction enzyme and screened by size on agarose gels. As the majority of virus restriction fragments used for the cloning were prepared from infected BHK-21 cell DNA, transformants were prescreened by colony filter hybridization as described by Maniatis *et al.* (1982) in order to identify legitimate MHV-68-derived clones.

Hybridization procedures. Restriction-digested DNA was transferred to nitrocellulose (BA85, Schleicher & Schuell) or GeneScreen (NEN) by the technique of Southern (1975) or using an LKB Vacublot system. DNA fragments to be used as hybridization probes were labelled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) by extension of a random primer (Feinberg & Vogelstein, 1983). Prehybridization and hybridization of filters were performed as described previously (Efstathiou *et al.*, 1986). Autoradiography was carried out at −70 °C using Kodak X-Omat S film.

Results

Restriction enzyme analysis and cloning of the MHV-68 genome

Fig. 1 shows *Bam*HI, *Eco*RI and *Hin*dIII restriction enzyme patterns of CsCl-purified MHV-68 virion DNA. The length of restriction fragments was determined by agarose gel electrophoresis (Tables 1 to 3). In the case of fragments larger than 10 kb, their size was determined by restriction analysis of individual cloned fragments and for fragments for which clones were not available, their size was ultimately deduced from the final restriction map. For the cloning of virus restriction fragments either high M_r infected BHK cell DNA or CsCl-purified virion DNA was digested with the relevant restriction enzyme and electrophoresed in 0·8% agarose gels. Individual restriction fragments were purified and cloned into the vector pUC13. The production of large amounts of virus DNA in infected cell DNA preparations made the cloning of the majority of restriction fragments possible without the necessity for prior purification of virion DNA. Using this approach, legitimate MHV-68 clones were formally identified by differential colony blot hybridization using either radiolabelled MHV-68-infected cell DNA or uninfected BHK cell DNA as probes. Approximately 90% of all clones derived using this strategy contained MHV-68 DNA. For restriction

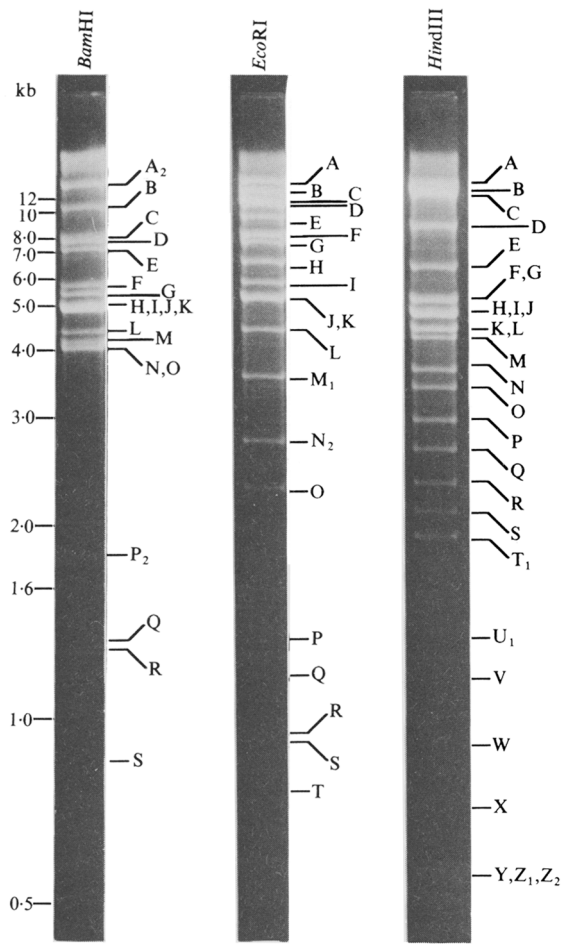

Fig. 1. *Bam*HI, *Eco*RI and *Hin*dIII restriction profiles of MHV-68 CsCl-purified virion DNA. The letter designation given to each restriction fragment is shown.

fragments which appeared to be multimolar upon u.v. visualization of ethidium bromide-stained gels, several representative clones were obtained and the identity of different cloned fragments distinguished following digestion with *Alu*I. Cloning of the 13·9 kb *Hin*dIII-A fragment proved unsuccessful presumably due to the inability of pUC13 to carry such a large insert stably. However, a 12·5 kb *Eco*RI/*Hin*dIII subclone of the *Hin*dIII-A fragment was successfully cloned into this vector. *Bam*HI, *Eco*RI and *Hin*dIII libraries comprising 67·5 kb, 64·68 kb and 114·18 kb respectively were generated. Internal *Bam*HI, *Eco*RI and *Hin*dIII restriction sites were determined for each clone (data not shown) and in order to determine the relationship of restriction fragments each clone was hybridized to Southern blots of restriction enzyme-digested MHV-68 DNA and the size of each cross-hybridizing band determined. These results are summarized in Tables 1 to 3. In some cases, the letter designation given to fragments detected by Southern blot hybridization was deduced both by size estimation of the hybridizing fragments and on the basis of internal restriction sites within cloned fragments.

*Bam*HI, *Eco*RI and *Hin*dIII restriction maps deduced from these data are shown in Fig. 2(*a*). Confirmation of the identity of the left-hand terminal fragments required the isolation of a 1·8 kb *Pst*I fragment which spanned the junction between unique and repetitive DNA. Alignment of the right-hand terminal fragments was made possible by the isolation of a 2·6 kb *Sph*I fragment which overlapped these submolar terminal fragments and the most proximal unimolar restriction fragments. A number of small *Bam*HI fragments <200 bp lie between *Bam*HI-T and -E covering a region of approx. 1·2 kb overlapping the *Eco*RI-K and *Hin*dIII-C fragments. So far we have been unable to place *Eco*RI-U on the MHV-68 *Eco*RI restriction map. It should be noted that at present we have no clone that spans the *Eco*RI-A and -L fragments. Although the 12·5 kb *Eco*RI/*Hin*dIII subclone of the *Hin*dIII-A fragment is thought to terminate at the *Eco*RI site defining the left end of *Eco*RI-L, we have no direct evidence to support this and it is possible that *Eco*RI-U lies between *Eco*RI-A and -L.

Arrangement of terminal repetitive sequences

It became apparent from the hybridization of cloned fragments to restriction enzyme-cleaved MHV-68 DNA that a number of clones detected a complex series of submolar fragments forming ladder-like patterns. For example, hybridization with the 6·1 kb *Hin*dIII-E cloned fragment (Fig. 3*a*) resulted in the detection of a high M_r (>18 kb) smear of *Bam*HI fragments, the lowest member of which is designated $A_1^\dagger$. In addition, this clone detected a submolar *Eco*RI ladder series, the lowest member, designated $N_1^\dagger$, having an estimated size of 2·8 kb. Each member of this ladder series increased in size sequentially by approximately 1·2 kb. A molar fragment corresponding to the 12·5 kb *Eco*RI-B fragment was also detected by this clone. Hybridization of *Bam*HI- and *Eco*RI-digested MHV-68 DNA with the 0·5 kb *Hin*dIII-Z_2 cloned fragment resulted in the detection of a subset of submolar ladders different to those detected by the 6·1 kb *Hin*dIII-E fragment. The lowest fragments forming the *Bam*HI and *Eco*RI ladders had sizes of 2·76 kb (designated *Bam*HI-$P_1^\dagger$) and 3·24 kb (designated $M_2^\ddagger$) respectively. The ladders detected by the 0·5 kb *Hin*dIII-Z_2 fragment also increased in size sequentially by approx. 1·2 kb (Fig. 3*b*). From the genomic map summarized in Fig. 2, it is apparent that the 6·1 kb *Hin*dIII-E fragment and the 0·5 kb *Hin*dIII-Z_2 fragments lie at opposite ends of the MHV-68 genome and the detection of subsets of ladders by each fragment is consistent with a model of the genome having a unique

Table 1. *Restriction fragments hybridizing with each probe*

Probe		*Bam*HI		*Eco*RI	
*Hin*dIII fragment	Size (kb)	Size (kb)	Letter designation	Size (kb)	Letter designation
A*	13·90	13·4, 5·0, 4·1, 1·7, 1·3	A_2, K, N, P_2, Q	14·4	A
B	13·10	5·2, 5·0	G, H, I	7·8, 5·25, >3·24†	F, J, $M_2^‡$
C	12·40	7·4, 7·0, <0·3	D, E	7·5, 5·17, 2·3	G, K, O
D	8·35	5·6, 5·2, 4·15	F, G, M	10·7, 5·25, 0·9	D, J, R
E	6·10	>18·3	$A_1^†$	12·5, >2·82	B, $N_1^†$
F	5·20	5·0	J	9·3, 3·6, 0·85	E, M_1, S
G	5·20	>18·3	$A_1^†$	12·5, 11·6	B, C
H	4·90	13·4	A_2	6·4	H
I	4·83	4·4, 0·8, 0·4	L, S, T	11·6, 5·17	C, K
J	4·80	>18·3	$A_1^†$	12·5	B
K	4·50	9·84	B	9·3, 1·35, 1·16	E, P, Q
L	4·50	7·5	C	5·65	I
M	4·27	7·5, 7·38	C, D	2·72, 2·3, 0·75	N_2, O, T
N	3·68	>18·3, 4·05	$A_1^†$, O	11·6	C
O	3·40	9·84	B	9·3	E
P	2·97	13·4	A_2	4·4	L
Q	2·65	4·1	N	14·4, 3·6	A, M_1
R	2·34	9·84	B	9·3	E
S	2·10	4·4, 4·05	L, O	11·6	C
T_1	1·92	5·6, 1·25	F, R	10·7	D
U_1	1·34	7·5	C	5·65, 0·75	I, T
V	1·15	5·6	F	10·7	D
W	0·80	13·4, 1·25	A_2, R	6·4	H
X	0·68	5·6	F	10·7	D
$Y^‡$	0·50	–§	–	–	–
$Z_1^‡$	0·50	–	–	–	–
Z_2	0·50	>2·76	$P_1^†$	>3·24	$M_2^‡$

* Hybridization was performed using a cloned 12·5 kb *Eco*RI/*Hin*dIII subfragment of *Hin*dIII-A.
† Lowest sized number of a submolar terminal fragment ladder series.
§ Not determined.
‡ Fragment not cloned.

stretch of DNA which is flanked by variable numbers of a 1·2 kb repeat unit. Equal hybridization was observed to each fragment within a *Bam*HI-$P_1^†$, *Eco*RI-$M_2^‡$ and *Eco*RI-$N_1^†$ ladder series (Fig. 3*a* and *b*) indicating the equal representation of molecules with each number of repeat units. A schematic representation of the structure of the MHV-68 genome based on these data is shown in Fig. 2(*b*).

Digestion of MHV-68 DNA with a variety of restriction enzymes resulted in the appearance of a multimolar fragment of size 1·2 kb following digestion with *Pst*I (data not shown). This fragment was considered to represent a complete repeat unit. Hybridization of *Bam*HI-, *Eco*RI- and *Hin*dIII-digested CsCl-purified MHV-68 virion DNA with this cloned repeat unit resulted in the detection of double ladders (Fig. 3*c*); consistent with the proposed structure of the virus genome (Fig. 2*b*). This repeat unit therefore detected both variable ends of the MHV-68 genome. It is difficult to estimate the exact number of repeats present at each end of the genome, but at least 10 distinctly resolved bands are detected for each ladder series. Table 4 summarizes the data obtained from a number of clones that hybridize to ladders from either the left or right end of the genome. The mean repeat unit size estimated by hybridization to restriction digests with each of the probes shown in Table 4 is 1237 bp. Hybridization of *Pst*I-digested MHV-68 DNA with the *Pst*I 1·2 kb repeat unit resulted in the detection of three bands, the multimolar 1·2 kb repeat unit and unimolar 1·8 and 0·6 kb fragments (Fig. 4*a*). This result is consistent with the 1·8 and 0·6 kb *Pst*I fragments representing junctions between unique and repeat DNA sequences. The 1·8 kb *Pst*I junction fragment was cloned into pUC13 and hybridized to restriction digests of MHV-68 DNA. This fragment detected the submolar *Bam*HI-$A_1^†$, *Eco*RI-$N_1^†$ and *Hin*dIII-$U_2^‡$ ladders and the unimolar 6·1 kb *Hin*dIII-E fragment and therefore spans the junction between unique and repeat sequences at the left end of the genome (Fig. 5*a*). Faint bands corresponding to the ladders representing the variable size right-hand termini were also detected by this cloned fragment since by

Table 2. *Restriction fragments hybridizing with each probe*

Probe		*Bam*HI		*Hin*dIII	
*Eco*RI fragment	Size (kb)	Size (kb)	Letter designation	Size (kb)	Letter designation
A*	14·40	–†	–	–	–
B	12·50	>18·3‡	A†	6·1, 5·2, 4·8	E, G, J
C*	11·60	–	–	–	–
D	10·70	5·6, 4·15, 1·25	F, M, R	8·35, 1·92, 1·15, 0·68, 0·50	D, T_1, V, X, Z_1
E*	9·30	–	–	–	–
F	7·80	5·0	H, I	13·1	B
G*	7·50	–	–	–	–
H	6·40	13·4, 1·25	A_2, R	4·9, 0·8, 0·5	H, W, Y
I	5·65	7·5	C	4·5, 1·34	L, U_1
J	5·25	5·2, 5·0	G, H	13·1, 8·35	B, D
K*	5·17	–	–	–	–
L	4·40	13·4	A_2	13·9, 2·97	A, P
M_1	3·60	5·0, 4·1	J, N	5·2, 2·65	F, Q
N_2	2·72	7·5, 7·38	C, D	4·27	M
O	2·30	7·38	D	12·4, 4·27	C, M
P	1·35	9·84, 7·5	B, C	4·5	K
Q	1·16	9·84	B	4·5	K
R*	0·90	–	–	–	–
S	0·85	5·0	J	5·2	F
T*	0·75	–	–	–	–
U*	0·65	–	–	–	–

* Fragment not cloned.
† Not determined.
‡ Lowest sized member of a submolar terminal fragment ladder series.

Table 3. *Restriction fragments hybridizing with each probe*

Probe		*Eco*RI		*Hin*dIII	
*Bam*HI fragment	Size (kb)	Size (kb)	Letter designation	Size (kb)	Letter designation
A_2*	13·40	–†	–	–	–
B	9·84	9·3, 1·35, 1·16	E, P, Q	4·5, 3·4, 2·34	K, O, R
C	7·50	5·65, 2·72, 1·35, 0·75	I, N_2, P, T	4·5, 4·27, 1·34	K, L, M, U_1
D	7·38	7·5, 2·72, 2·3	G, N_2, O	12·4, 4·27	C, M
E	7·00	7·5, 5·17	G, K	12·4	C
F	5·60	10·7	D	8·35, 1·92, 1·15, 0·68, 0·5	D, T_1, V, X, Z_1
G*	5·33	–	–	–	–
H	5·00	7·8, 5·25	F, J	13·1	B
I	5·00	7·8, >3·24‡	F, M_2‡	13·1	B
J	5·00	9·3, 3·6, 0·85	E, M_1, S	5·2	F
K*	5·00	–	–	–	–
L	4·40	11·6	C	4·83, 2·1	I, S
M	4·15	10·7	D	8·35	D
N	4·10	14·4, 3·6	A, M_1	13·0, 2·65	A, Q
O*	4·05	–	–	–	–
P_2	1·77	14·4	A	13·9	A
Q*	1·30	–	–	–	–
R*	1·25	–	–	–	–
S	0·80	11·6, 5·17	C, K	4·83	I
T*	0·50	–	–	–	–

* Fragment not cloned.
† Not determined.
‡ Lowest sized member of a submolar terminal fragment ladder series.

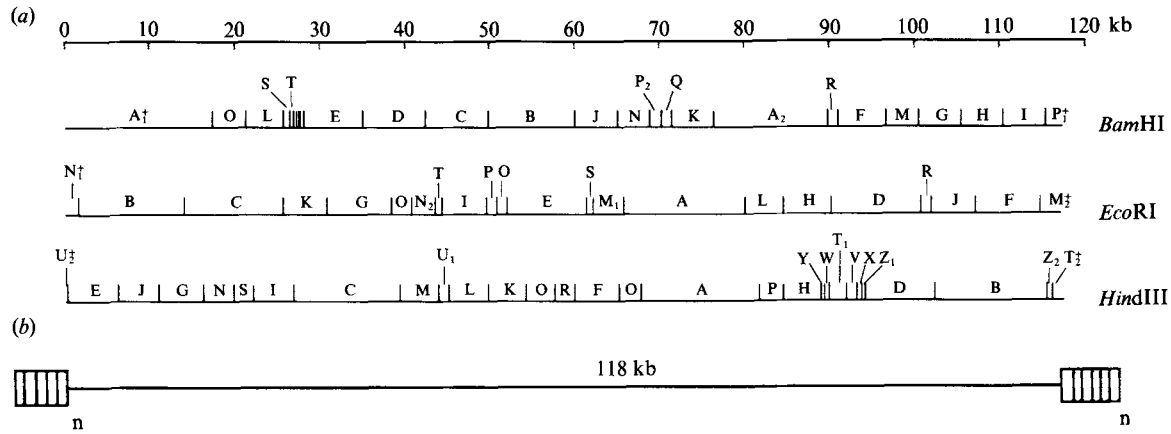

Fig. 2. (*a*) *Bam*HI, *Eco*RI and *Hin*dIII restriction maps of the MHV-68 genome. (*b*) Schematic representation of the structure of the MHV-68 genome which consists of approx. 118 kbp of unique sequence DNA which is flanked at each end by variable numbers of a 1·2 kb repeat unit.

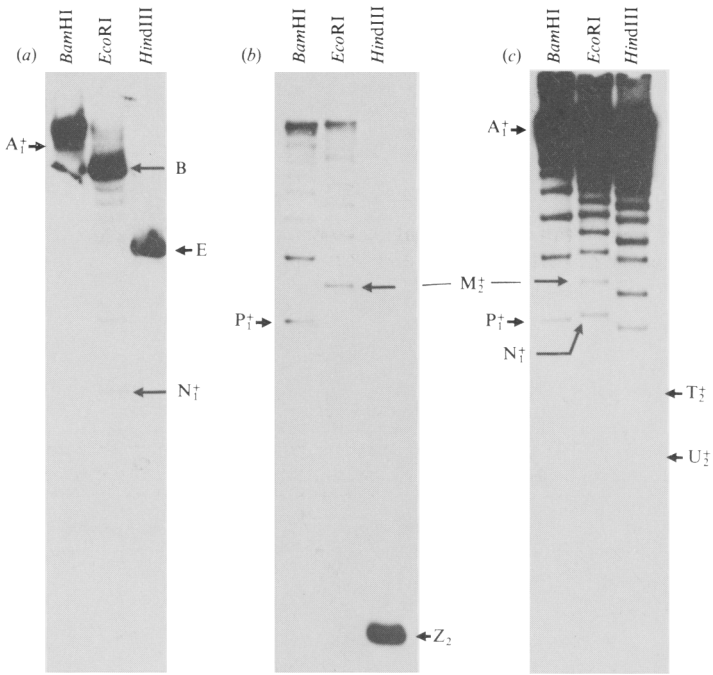

Fig. 3. Southern blot hybridization analysis of *Bam*HI-; *Eco*RI- and *Hin*dIII-digested MHV-68 CsCl-purified virion DNA with (*a*) the 6·1 kb *Hin*dIII-E fragment (see Table 1), (*b*) the 0·5 kb *Hin*dIII-Z_2 fragment (see Table 1) and (*c*) the cloned 1·2 kb *Pst*I repeat unit (see Table 4).

definition it contains repetitive DNA sequences which are present at each end of the genome. The 0·6 kb *Pst*I fragment detected by the cloned repeat unit is considered to represent the right-hand junction between repetitive and unique DNA, although we cannot formally discount the possibility that this fragment represents the physical ends of the genome. Cloning and further characterization of this fragment was not undertaken since it is of insufficient size to span past the right-hand 1·8 kb *Hin*dIII-$T_2^‡$ terminal fragment and would therefore be of little value in confirming the positions of either the right-hand submolar terminal fragments or the 0·5 kb *Hin*dIII-Z_2 fragment. Confirmation of the position of these fragments proved difficult due to the absence of conveniently sized restriction fragments which span this right-hand junction. Fig. 4(*b*) shows a Southern blot of MHV-68 DNA cleaved with a variety of restriction enzymes and probed with either the 0·5 kb *Hin*dIII-Z_2 fragment or the 1·2 kb *Pst*I repeat unit. Of the seven enzymes used in this experiment only *Sma*I and *Hpa*II were found to cleave within the virus repeats. The remainder of the enzymes cleaved outside the repeat unit, resulting in the detection of double ladders when using the cloned *Pst*I repeat unit as a probe. The recognition sites of *Sma*I and *Hpa*II are CCCGGG and CCGG respectively, therefore the presence of multiple *Sma*I and *Hpa*II cleavage sites within the 1·23 kb repeat unit suggests that it is likely to have a relatively high G+C content.

Although *Sph*I did not cleave within the repeat unit, an *Sph*I fragment of 2·6 kb was detected using the 0·5 kb *Hin*dIII-Z_2 fragment as probe. This 2·6 kb *Sph*I fragment was cloned into pBR322 and the position of internal *Bam*HI, *Eco*RI and *Hin*dIII restriction sites determined in order to confirm both the location of the 0·5 kb *Hin*dIII-Z_2 fragment and the position of the right-hand terminal *Bam*HI-$P_1^†$, *Eco*RI-$M_2^‡$ and *Hin*dIII-$T_2^‡$ fragments.

Fig. 5(*b*) shows the relationship of this clone to both the submolar terminal and most proximal unimolar restriction fragments, thus confirming the location of the *Hin*dIII-Z_2 fragment. As summarized in Table 4, the cloned *Sph*I 2·6 kb fragment hybridized to the *Bam*HI-$P_1^†$ and *Eco*RI-$M_2^‡$ right-hand submolar terminal ladder series in addition to the molar 0·5 kb *Hin*dIII-Z_2, 13·1 kb

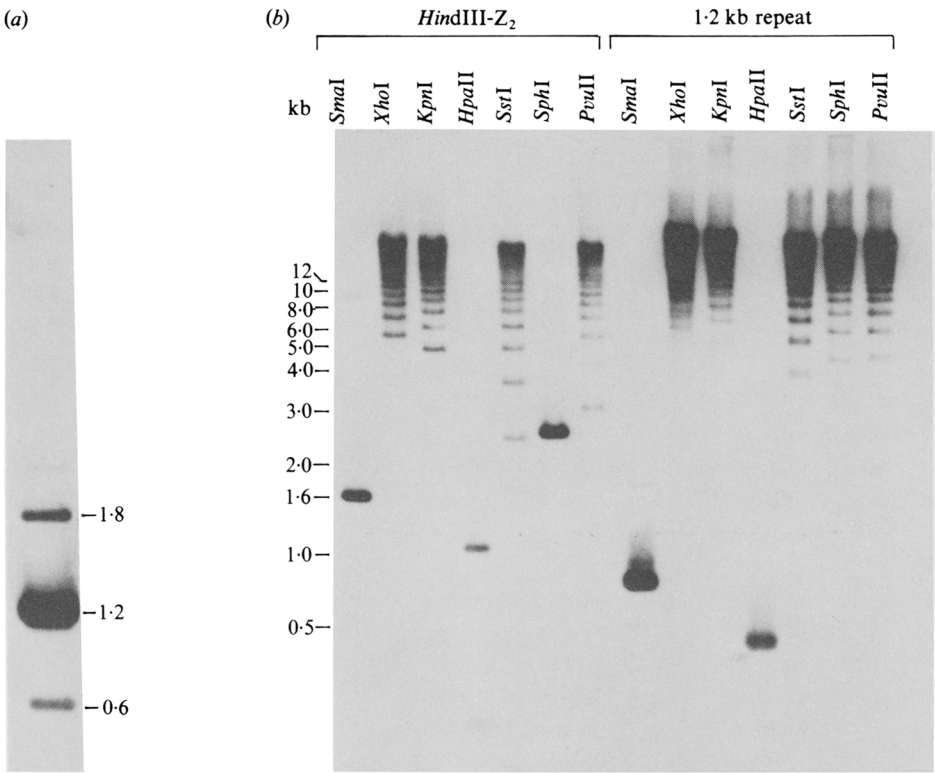

Fig. 4. Southern blot hybridization of (*a*) *Pst*I-digested MHV-68 DNA probed with the cloned *Pst*I 1·2 kb repeat unit. (*b*) MHV-68 DNA cleaved with a variety of restriction enzymes and probed with either *Hin*dIII-Z_2 or the 1·2 kb *Pst*I repeat unit.

Table 4. *Detection of submolar terminal ladders*

	Left end			Right end		
Probe	*Bam*HI-A$_1^\dagger$	*Eco*RI-N$_1^\dagger$	*Hin*dIII-U$_2^\ddagger$	*Bam*HI-P$_1^\dagger$	*Eco*RI-M$_2^\ddagger$	*Hin*dIII-T$_2^\ddagger$
*Hin*dIII-B*	−†	−	−	−	+	−
*Hin*dIII-E	+	+	−	−	−	−
*Hin*dIII-G	+	−	−	−	−	−
*Hin*dIII-J	+	−	−	−	−	−
*Hin*dIII-Z_2	−	−	−	+	+	−
*Bam*HI-I	−	−	−	−	+	−
*Eco*RI-B	+	−	−	−	−	−
*Pst*I 1·8 kb	+	+	+	+	+	+
*Pst*I 1·2 kb	+	+	+	+	+	+
*Sph*I 2·6 kb	−	−	−	+	+	−

* Detection of the *Eco*RI-M$_2^\ddagger$ terminal ladder was accomplished using a 0·9 kb *Eco*RI/*Hin*dIII subfragment of the *Hin*dIII-B clone.
† Negative hybridization.

*Hin*dIII-B, 7·8 kb *Eco*RI-F and 5·0 kb *Bam*HI-I fragments. The mapping of internal *Hin*dIII sites within the *Sph*I 2·6 kb fragment revealed that the overlap of this clone with the terminal 1·8 kb *Hin*dIII-T$_2^\ddagger$ fragment is <100 bp. Such a small overlap is the most likely explanation for our inability to detect the *Hin*dIII-T$_2^\ddagger$ right-hand ladder series when using the *Sph*I 2·6 kb fragment as a probe.

From size estimates of both the unimolar *Bam*HI, *Eco*RI and *Hin*dIII restriction fragments and the smallest submolar terminal fragments (Tables 1 to 3), the size of MHV-68 unique DNA was estimated as 118·4 kb, 118·54 kb and 117·7 kb respectively for the enzymes *Bam*HI, *Eco*RI and *Hin*dIII (an average of 118·2 ± 0·37 kbp). The difference in size estimates calculated for each enzyme is most likely due to the accumulation of small

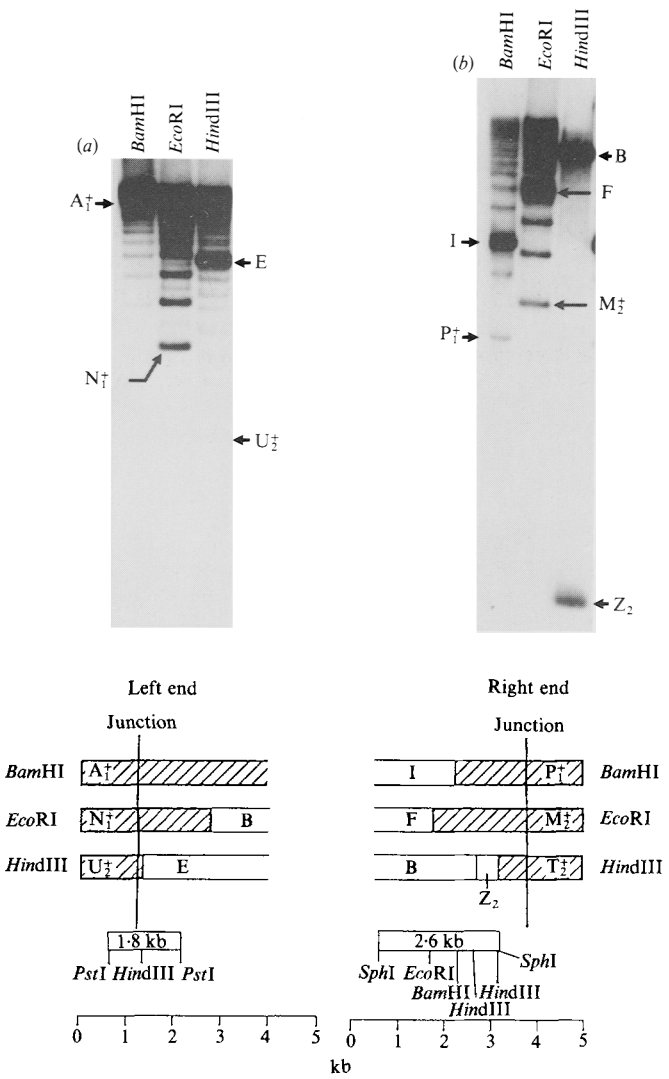

Fig. 5. Determination of the relationship of submolar terminal fragments (shown as hatched boxes) to the most proximal unimolar restriction fragments. (*a*) Hybridization of *Bam*HI-, *Eco*RI- and *Hin*dIII-digested MHV-68 DNA with the 1·8 kb *Pst*I cloned fragment resulting in the detection of the *Bam*HI-A†, *Eco*RI-N† and *Hin*dIII-U‡ terminal ladder series and the molar 6·1 kb *Hin*dIII-E fragment. The presence of a single *Hin*dIII site within the cloned *Pst*I-1·8 kb fragment which spans the repeat/unique junction links the *Hin*dIII-E fragment to the *Hin*dIII-U‡ submolar terminal ladder series. (*b*) Restriction digests of MHV-68 DNA hybridized with the 2·6 kb *Sph*I cloned fragment resulting in the detection of the terminal *Bam*HI-P† and *Eco*RI-M‡ ladder series and the unimolar *Bam*HI-I, *Eco*RI-F and *Hin*dIII-B and -Z_2 fragments.

sizing errors of individual clones in addition to small fragments less than 0·5 kb which may have been missed. As the genome of MHV-68 is flanked by variable copies of the 1·23 kb repeat unit, the actual size of packaged virion DNA will be significantly greater than 118·2 kb.

Discussion

Genomic maps of MHV-68 have been determined for the restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII and restriction fragments representing most of the virus genome have been cloned. Of particular interest was the finding that the MHV-68 genome consists of a unique stretch of DNA of 118·2 kb which is flanked by variable numbers of a 1·23 kb repeat unit. Thus the structure of the MHV-68 genome is strikingly similar to the genomes of members of the lymphotropic γ_2 herpesvirus subgroup which include herpesvirus saimiri (HVS) (Stamminger *et al.*, 1987), herpesvirus ateles (HVA) (Fleckenstein *et al.*, 1978), herpesvirus aotus type 2 (Fuchs *et al.*, 1985), herpesvirus sylvilagus (Medveczky *et al.*, 1989) and alcelaphine herpesvirus-1 (Bridgen *et al.*, 1989). For example, the genome of the best studied member of the γ_2 herpesvirus subgroup, HVS, consists of a 111 kb central segment of A + T-rich DNA (L-DNA) flanked by variable terminal 1·44 kb repeats of G + C-rich DNA (H-DNA) (Bankier *et al.*, 1985; Stamminger *et al.*, 1987). In every HVS H-DNA repeat unit there is a packaging/cleavage site and it is considered that mature genomes are formed from a concatemeric intermediate by initial cleavage at any one such site followed by further cleavage at a specific repeat unit dictated by a headful packaging mechanism. Thus, the length and number of repeats per genome is constant although the actual number of repeats present at each end of the genome is variable (Bornkamm *et al.*, 1976; Stamminger *et al.*, 1987). Whether the number of repeats in the MHV-68 genome remains constant is not known. However, the constant periodicity of the MHV-68 terminal ladder series indicates that the repeats are arranged as tandem blocks at each end of the genome. Whether these repeat units have a function in the cleavage and packaging of genomic length DNA is under investigation.

MHV-68 unique DNA has a G+C content of approximately 45% as determined by nucleotide sequence analysis (Efstathiou *et al.*, 1990) and although the % G+C content of the virus repeat unit has yet to be determined, the presence of multiple *Sma*I and *Hpa*II cleavage sites, whose recognition sites are CCCGGG and CCGG respectively, suggests that this unit has high G+C content. Further analysis of MHV-68 DNA by CsCl centrifugation and sequencing of the repeat unit should determine whether, as for HVS and other γ_2 herpesviruses, the MHV-68 genome consists of low G+C-containing unique DNA flanked by G+C-rich repeats.

Of a series of herpesviruses isolated from murid species, MHV-68 recovered from *C. glareolus* (subfamily microtinae) has been studied in most detail with regards to its biological properties (Blaskovic *et al.*, 1984;

Rajcani *et al.*, 1985) and is the subject of study in this paper. A number of antigenically related herpesviruses recovered from *A. flavicollis* (subfamily murinae) have also been described (Blaskovic *et al.*, 1980; Svobodova *et al.*, 1982*a*, *b*; Ciampor *et al.*, 1981) and studies on one of these isolates, strain Sumava Af, have shown that this virus genome has a mean G+C content of approximately 60% and a size of 135 kb (Blaskovic *et al.*, 1988). A comparison of the MHV-68 and MHV Sumava Af genomes is at present speculative since the exact relationship between these viruses isolated from rodents with a different phylogenetic history is not known. However, the estimates of both the mean % G+C content and size of the MHV Sumava Af strain DNA would be consistent with the MHV genome containing multiple repeats of a high % G+C content.

In addition to structural similarities between the genome of MHV-68 and the genomes of members of the γ_2 herpesvirus subgroup, analysis of short DNA sequences has identified a number of open reading frames which show the greatest similarity to the homologous proteins of the gammaherpesviruses HVS and Epstein–Barr virus (EBV) than to the alphaherpesvirus herpes simplex virus or the betaherpesvirus HCMV (Efstathiou *et al.*, 1990). Furthermore, the relative arrangements of the genes identified indicate that MHV-68 has a genome organization similar to that described for EBV and HVS (Gompels *et al.*, 1988).

Classification of the alpha-, beta- or gammaherpesvirus subgroups is currently based upon differences in the growth of the viruses *in vitro* and in the nature of those tissues involved during both acute and latent infection *in vivo* (Roizman, 1982; Honess & Watson, 1977; Honess, 1984). Members of the gammaherpesvirus subgroup are associated with lymphoproliferative diseases in their natural or experimental hosts and lymphoid cell lines containing multiple copies of episomal DNA can be frequently established from peripheral blood lymphocytes of the infected host. Thus, these viruses are termed the lymphotropic gammaherpesviruses. These viruses can be further divided into the γ_1 or B cell-tropic (e.g. EBV) or γ_2 or T cell-tropic (e.g. HVS) subdivisions (Honess, 1984). An additional feature of the γ_2 herpesviruses is their ability to infect fibroblasts productively from a number of species. MHV-68 is also known to replicate in a variety of cell types in tissue culture (Svobodova *et al.*, 1982*a*) but its lymphotropic properties have yet to be clearly defined. The virus is known to cause viraemia in experimentally infected animals and virus antigen has been detected in many cell types including lymphocytes (Rajcani *et al.*, 1985; Blaskovic *et al.*, 1984). The scarcity of data regarding the nature of those cells involved during either acute or latent infection *in vivo* prevents the classification of MHV-68 to the gammaherpesvirus subgroup. However, on the basis of both its genome structure and organization (Efstathiou *et al.*, 1990), MHV-68 is clearly most closely related to members of the gammaherpesvirus subgroup and we would therefore predict that MHV-68 will have biological properties characteristic of the lymphotropic herpesviruses. Further studies on the pathogenesis of MHV-68 are required to determine whether it is indeed a lymphotropic herpesvirus and to assess its potential as a model with which to study gammaherpesvirus infection.

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