Genes in the *Hind*III J Fragment of the Murine Cytomegalovirus Genome Are Dispensable for Growth in Cultured Cells: Insertion Mutagenesis with a *lacZ/gpt* Cassette

JEFFREY VIEIRA,^{1†} HELEN E. FARRELL,² WILLIAM D. RAWLINSON,² AND EDWARD S. MOCARSKI^{1*}

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305-5402,¹
and Laboratory of Molecular Biology, Medical Research Council, and Department of Pathology,
University of Cambridge, Cambridge, CB2 2QH England²

Received 1 February 1994/Accepted 6 May 1994

The organization and function of the genes encoded within the *Hind*III J region of the murine cytomegalovirus genome were analyzed by transcript mapping and cDNA isolation, nucleotide sequence analysis and identification of open reading frames (ORFs), and construction of recombinant viruses carrying insertions disrupting five of the seven ORFs. This region was found to encode five β transcripts and one γ transcript in addition to two β transcripts previously mapped to the *sgg1* locus. Seven open reading frames were identified, and one was recognized as a homolog of a human cytomegalovirus US22 gene family. The five largest ORFs contained within the *Hind*III J fragment (*sgg1*, HJ4, HJ5, HJ6, and HJ7) were each disrupted by the insertion of a *lacZ/gpt* genetic marker cassette. The growth kinetics of all recombinant viruses were investigated and found to be the same as wild-type parental virus, indicating that these five ORFs were dispensable for growth in cell culture.

Human cytomegalovirus (CMV) is a ubiquitous pathogen causing disease in immunocompromised individuals and infants (1). The replication and biology of CMV have been the subject of considerable study, as reviewed in reference 35. Biologically related betaherpesviruses infect a wide range of different mammals (42, 56). Strict species specificity has prevented the study of human CMV in any animal model, and related viruses such as murine CMV have been used to provide insights into the tissue tropism, pathogenesis, and latency of this group of viruses (20, 23, 34, 43, 51). Human CMV shares the following characteristics with murine CMV: a large (approximately 230-kb) double-stranded DNA genome (10, 33, 36), amino acid sequence similarity in the predicted protein products of many genes (11, 22, 28, 46, 63), biological aspects of tissue tropism and latency (17, 24, 39), and a virion structure and temporal regulation of gene expression typical of herpesviruses (16, 35). Recently, the nucleotide sequences of the genomes of both human (5) and murine (47) CMV have been determined, and both viruses appear to encode a largely colinear set of approximately 200 genes.

Many genes in human or murine CMV have been found to be dispensable for growth in cultured cells (19, 29, 30). Their presence on the viral genome indicates that they may be needed to perform functions only involved in modulating the interaction with the respective human or animal host. Murine CMV offers the opportunity to study the function of such genes in the natural host (the mouse). Thus, viruses carrying mutations in genes found to be dispensable for growth in cell culture may be evaluated for an impact in the natural host, as has been done for the salivary gland growth gene 1 (*sgg1*) (30).

The *lacZ* gene has been used to introduce insertion muta-

tions into recombinant herpes simplex virus (15) and CMV (29, 55). The *Escherichia coli gpt* gene, first used as a selection marker in mammalian cells (37), has been employed as a genetic marker in poxviruses (12) and Marek's disease virus (31). In this paper, a dual marker cassette consisting of the *gpt* and *lacZ* genes has been used to construct recombinant murine CMV with insertions in the *Hind*III J region, a portion of the murine CMV genome that carries a cluster of genes with only marginal similarity to any portion of the human CMV genome. The selection system that we employed allows for rapid and convenient selection and identification of recombinant viruses. The genes found to be dispensable for growth in cultured cells in this region may be predicted to impact on the interaction of murine CMV with the host animal.

MATERIALS AND METHODS

Cells and viruses. Murine CMV was grown on NIH 3T3 cells (ATCC CRL1658) in Dulbecco's modified Eagle medium (Gibco/BRL, Bethesda, Md.) supplemented with 10% NuSerum (Collaborative Research, Waltham, Mass.), penicillin (100 U/ml), and streptomycin (100 μ g/ml). The wild-type parental murine CMV used in these studies was K181⁺ (58), a plaque-purified derivative of K181 (originally obtained from M. Colin Jordan).

Plasmid constructs. The murine CMV DNA *Hind*III J fragment was isolated from an agarose gel and cloned into the plasmid vector pMT11 (54). This plasmid, designated pON449, was digested with *Sau*3A in the presence of ethidium bromide (0.9 μ g/ μ g of DNA, 1 h, 37°C) to generate random, predominantly singly cut molecules (40), which were purified by electrophoresis in an agarose gel and, after purification, ligated to a similarly purified 4.8-kbp *Bam*HI fragment from pON855 containing a *lacZ/gpt* insert. The ligation mixture was used to transform *E. coli* DH5 α , and clones containing the *lacZ/gpt* insert were identified as blue colonies (*lacZ*⁺) on Luria broth agar plates containing 5-bromo-3-chloro-indolyl- β -D-galactopyranoside (X-Gal). Seven plasmids, pON868, pON869, pON870,

* Corresponding author. Phone: (415) 723-6435. Fax: (415) 723-1606. Electronic mail address: MK.ESM@Forsythe.Stanford.EDU.

† Present address: Virology Division, Departments of Microbiology and Medicine, University of Washington, Pacific Medical Center, Seattle, WA 98144.

pON871, pON872, pON873, and pON874, with *lacZ/gpt* inserts in the J fragment generated from this procedure were used to construct recombinant viruses. A 700-bp *Bam*HI/*Hind*III fragment from *Hind*III L, representing the region immediately adjacent to *Hind*III J, was cloned into pBluescriptKS⁺ (Stratagene) and denoted pON4448.

The construction of the *lacZ/gpt* insert of pON855 was done as follows: pSV2gpt (37) was digested with *Dra*I and *Hpa*I and recircularized to create pON818. A 0.8-kb *Bgl*II/*Bam*HI fragment, containing the *gpt* gene from pON818, was inserted into *Mlu*I-digested pRB103 (44) after all ends were made blunt with a Klenow fragment and deoxynucleoside triphosphates to generate pON848. A 1-kb *Pst*I/*Sma*I fragment from pON848 was inserted into *Pst*I/*Stu*I-digested pUC6S (62) to make pON852, a plasmid which contains the *gpt* gene under control of the herpes simplex virus type 1 *tk* promoter and simian virus 40 early polyadenylation signal. The *lacZ* segment was constructed by inserting a 3.5-kb *Xho*I/*Hind*III fragment from pON840 (18) into *Xho*I/*Hind*III-digested pH β APr-1 (14) to generate pON845. A *Nhe*I/*Dra*I fragment containing *lacZ* was taken and inserted into *Bam*HI/*Xba*I-digested pON829 (18) (with the *Bam*HI site filled in) to create pON847, which has the *lacZ* gene expressed from a chimeric human/rat β -actin promoter and the simian virus 40 late poly(A) signal. A *Bam*HI site present between the β -actin promoter and the *lacZ* gene was destroyed by cutting pON847 with *Bam*HI, filling in the ends with a Klenow fragment and deoxynucleoside triphosphates, and ligating the plasmid to create pON847b. A 3.8-kb *Stu*I/*Dra*I fragment from pON847b containing the *lacZ* construct was inserted into *Pvu*II-digested pON852 to create pON853. pON853 was digested with *Bam*HI, and the *lacZ/gpt* segment was inserted into *Bam*HI-digested pOK7 (61) to make pON855, which contains the *lacZ/gpt* cassette flanked by *Bam*HI sites. Restriction enzymes, ligase, and the Klenow fragment of DNA polymerase I were purchased from New England Biolabs.

cDNA cloning. A plasmid cDNA library generated from RNA isolated from NIH 3T3 cells 8 h postinfection (p.i.) with murine CMV (26) was plated at 800 to 1,000 colonies per dish, and 8×10^3 to 10×10^3 colonies were screened by colony blot hybridization (49) with an [α -³²P]dCTP (Amersham Corp., Arlington Heights, Ill.)-labeled *Clal*/*Hind*III probe representing sequences to the right of *sgg1* in pON449 generated by random priming (13). The library was made with the vector pME18S, derived from pcD-SR α (59). Two plasmids were isolated and characterized. pON880 containing the 1.9-kb cDNA and pON882 containing the 1.3-kb cDNA. To confirm their map position, restriction maps were constructed and the nucleotide sequence of the ends of the CMV cDNA fragments in each of these clones was determined following the Sequenase protocol (U.S. Biochemical Corp., Cleveland, Ohio), with primers complementary to vector sequences flanking the cDNA insertion site (18).

RNA isolation and analysis. Total cellular RNA was isolated from either uninfected or murine CMV-infected (multiplicity of infection [MOI] of 10 PFU per cell) NIH 3T3 cells, and following guanidine isothiocyanate solubilization and sedimentation through a CsCl cushion (7), RNA (10 μ g per lane) was separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose (49), and immobilized by UV cross-linking (Stratalinker; Stratagene, La Jolla, Calif.). [α -³²P]dCTP-labeled DNA fragments were generated by random priming (13) and hybridized to the RNA as described previously (27). Strand-specific RNA probes were generated by using T7 or SP6 polymerase and hybridized as described previously (45). RNA was also made from cells

infected (MOI of 10 PFU per cell) in the presence of cyclohexamide (50 μ g/ml) from 1 h before infection to 6 h p.i., at which time cells and RNA were harvested.

Viral DNA isolation and analysis. NIH 3T3 cells (70 to 80% confluent) were infected at an MOI of 0.1 PFU per cell. At 2 days post-100% cytopathic effect, the medium was collected from the culture, and the cells were removed by centrifugation (1,000 $\times$ g, 5 min). Virions were isolated from the medium by centrifugation (27,000 $\times$ g, 45 min). The pellet was resuspended in 400 μ l of 5 mM NaCl–5 mM Tris-HCl (pH 7.5)–1 mM EDTA (STE), 10 μ l of 10-mg/ml proteinase K solution and 45 μ l of 10% sodium dodecyl sulfate were added, and the mixture was incubated for 1 h at 60°C. It was then extracted twice with an equal volume of phenol-chloroform (50:50) and once with chloroform. The DNA was ethanol precipitated at 25°C for 1 h, and the pellet was recovered by centrifugation, washed with 70% ethanol, dried, and resuspended in 200 μ l of STE. After restriction enzyme digestion, viral DNAs were subjected to electrophoresis in a 0.6% agarose gel followed by hybridization as described previously (60), with pON449 DNA [α -³²P]dCTP labeled by random priming.

Generation of recombinant virus. In order to release the murine CMV DNA from the vector, plasmid DNAs were digested with *Hind*III, extracted with phenol-chloroform (50:50) and chloroform, ethanol precipitated, and then resuspended in STE before transfection. Recombinant viruses were generated by either of two methods, calcium phosphate precipitation (6) or electroporation. For those viruses made following calcium phosphate transfection, NIH 3T3 cells 24 h postplating (60 to 70% confluent) were infected at an MOI of 2 to 5 PFU per cell with murine CMV, and at 3 h p.i. plasmid DNA was transfected into the cells as previously described (6), except that the cells were incubated with the calcium phosphate DNA mixture in a 5% CO₂ incubator. Transfections were carried out in either 100-mm-diameter dishes with 10 ml of medium or six-well dishes with 1.5 ml per well, with a total of approximately 2 μ g of DNA per ml.

Electroporation. NIH 3T3 cells (60 to 70% confluent) were harvested by trypsinization, mixed with 2 volumes of medium, pelleted at 500 $\times$ g, and resuspended in OptiMEM (Gibco) at a concentration of 5×10^6 to 8×10^6 cells per ml. Cells (0.7 ml) were transferred to a 0.4-cm-diameter cuvette, 30 μ g of DNA was added in a 30- μ g volume, and the cuvette was shaken to mix. Electroporation was immediately carried out at 220 V and 960 mF on a Bio-Rad gene pulser. The cells were then plated with medium in a 100-mm-diameter dish. After 6 to 12 h, the cells were infected at an MOI of 2 to 5 PFU per cell with murine CMV. At 2 h p.i., the cells were trypsinized and distributed to a six-well dish so that six independent pools of recombinant virus were generated.

Selection for expression of the *gpt* gene. At 3 days p.i., medium from the infected cultures was used to infect fresh cells (~80% confluent). At 3 h p.i., the growth medium was replaced with medium containing 12.5 μ g of mycophenolic acid (Sigma stock: 5 mg/ml in 0.1 N NaOH) per ml and 100 μ g of xanthine (Sigma cell culture reagent stock: 5 mg/ml in 0.1 N NaOH) per ml. At 3 days p.i., medium and cells were harvested, sonicated, and used to infect fresh cells (50 to 100% of the harvested virus from the selection step was used for the next infection). For the isolation of RM868, RM869, RM871, RM872, and RM873, the selection in medium with mycophenolic acid and xanthine was repeated a total of three times, and recombinant viruses were identified as blue-stained plaques following overlay with 0.5% agarose-containing medium supplemented with X-Gal at 150 μ g/ml (29, 55). For the isolation of RM874 and RM870, the second round of selection in

FIG. 1. Transcripts encoded in the *HindIII* J region. Whole-cell RNA was isolated at 8 and 24 h p.i. from infected NIH 3T3 cells and compared with RNA from uninfected cells (lanes labeled 3T3) as indicated above the lanes. The RNA was electrophoretically separated by formaldehyde-containing agarose gel electrophoresis, transferred to nitrocellulose, and hybridized. Autoradiograms are shown. The panels A, B, C, and D correspond to fragment probes diagrammed in Fig. 2. The position of the 18S and 28S rRNAs and the apparent sizes of the viral RNA bands (in kilobases) are indicated adjacent to each set of lanes.

medium containing mycophenolic acid and xanthine was followed by filtration through a 0.22- μ m-pore-size filter and plaque purification in the absence of X-Gal. Virus identified in this manner was found to be *lacZ* as well as *gpt* positive.

Nucleotide sequence of the *HindIII* J region. A sonicated DNA fragment library of purified murine CMV was constructed in M13mp18, and a single-stranded DNA template was prepared as described previously (2, 53). The sequence

was determined by the dideoxy chain termination method (50) with either an (α - 35 S)-dATP protocol (2) or an automated DNA sequencer (Applied Biosystems 373A) using linear amplification sequencing (48) and fluorescently tagged primers (52). Deaza-dGTP was used in place of dGTP as a means of resolving compression that resulted from C:G-rich stretches. The DNA sequence was read from the autoradiographs with an automated gel reader (Amersham). Regions of the *HindIII* J region that were not represented on both strands following this initial analysis were determined by reverse-primer sequencing with fluorescently tagged primers (53) or by cycle sequencing with custom primers and fluorescently labeled dideoxy terminators according to the manufacturer's instructions (Applied Biosystems). Data were assembled by using the program xbp (9) and analyzed for the presence of open reading frames (ORFs) and transcription control signals with the program DIANA (8).

RESULTS

Transcripts of the *HindIII* J region. We first identified β (delayed early) or γ (late) transcripts encoded within the *HindIII* J fragment by hybridizing infected cell RNA from cells at 8 and 24 h p.i. with probes prepared from this region. There were no apparent α (immediate early) transcripts encoded in *HindIII* J (21) and the only gene previously mapped within this region was *sgg1*, a β gene encoding two 3' coterminal transcripts with sizes of 1.5 and 1.8 kb (26, 30). The approximate map positions and sizes of the transcripts were determined by RNA blot analysis (Fig. 1). [α - 32 P]dCTP-labeled double-stranded DNA probes were generated from gel-purified fragments of pON449 (*HindIII* J) or pON880 (a cDNA clone of the 1.9-kb β transcript) after digestion with various restriction

FIG. 2. Murine CMV *HindIII* J region. The top line represents a *HindIII* restriction map of the murine CMV (K181) genome (33). The expanded region depicts the J fragment. (A) Probes. The positions of DNA fragments used as DNA or RNA probes in RNA blot analysis are shown and labeled A to G. (B) Transcripts. The positions, directions, sizes, and temporal classes of expression (β [early] and γ [late]) are summarized from the data presented in this study. The *sgg1* transcripts are described elsewhere (26, 30). The start sites of the β 4.1-, 6.0-, and 7.4-kb transcripts lie to the right of the *HindIII* J/I junction (indicated by ---). (C) Restriction map for *Bam*HI (B), *Bgl*II (Bg), *Eco*RI, *Hind*III (H), *Stu*I (S), *Sac*I (S1), and *Xba*I (X), with the position of one *Sac*II (S2) site shown. (D) Insertions. The sites of the *lacZ/gpt* segment insertions within the *HindIII* J fragment are summarized. The clones pON868 to pON874 were used to create the corresponding recombinant viruses RM868 to RM874. (E) Arrangement of predicted ORFs with the positions of putative TATA boxes for leftward-oriented promoters (indicated by leftward-pointing arrows) and potential polyadenylation signals (indicated by vertical arrows). Only leftward-oriented signals have been shown.

FIG. 3. Comparison of genomic and cDNA clones to map the β 1.3- and 1.9-kb transcripts. (A) Schematic diagram of a 4-kb region of pON449 encompassing the β 1.3- and 1.9-kb transcripts. The cDNA clones of the β 1.9-kb transcript, pON880, and the β 1.3-kb transcript, pON882, are depicted above and below the genomic region, respectively. Restriction sites shown above the line are present on viral DNA and cDNA, and sites shown below the line for pON880 and pON882 represent those in the vector sequences flanking the insert. Probes 1 and 2, different *Bam*HI/*Sac*I fragments of pON449 used as [α - 32 P] dCTP-labeled probes on the DNA blots depicted in panels C and E, respectively. (B and D) ethidium bromide-stained gels; (C and E) autoradiographs following hybridization analysis. (B and C) Lanes: 1, pON449 digested with *Bam*HI and *Sac*I; 2, pON882 digested with *Bam*HI and *Sac*I; 3, pON449 digested with *Bam*HI and *Stu*I; 4, pON882 digested with *Bam*HI and *Stu*I. The positions of the expected *Bam*HI/*Sac*I and *Bam*HI/*Stu*I fragments are indicated by arrowheads. (D and E) Lanes: 1, pON449 digested with *Eco*RI and *Bgl*II; 2, pON880 digested with *Eco*RI and *Bgl*II; 3, pON449 digested with *Bam*HI, *Eco*RI, and *Sac*I; 4, pON880 digested with *Eco*RI and *Pst*I. The relevant *Eco*RI/*Bgl*II, *Eco*RI, and *Eco*RI/*Pst*I fragments of pON880 and the relevant *Eco*RI/*Sac*I and *Eco*RI/*Bam*HI fragments of pON449 are indicated by arrowheads. Additional hybridizing species were detected in all of these analyses because of the sequence homology between the probes and other fragments in the restriction digests. Restriction enzyme abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Stu*I; S1, *Sac*I.

enzymes. Probe A, a *Hind*III/*Eco*RI fragment (Fig. 2), detected the 1.5- and 1.8-kb β transcripts previously shown to be encoded by the *sgg1* gene (26, 30). A 0.85-kb γ transcript was detected with this probe only at late times (24 h p.i.) (Fig. 1A). This transcript was mapped to a region downstream of *sgg1*, crossing the *Hind*III J/L junction. The map position and orientation of the 0.85-kb γ transcript was determined with a strand-specific riboprobe G, representing sequences from within the *Hind*III L fragment adjacent to *Hind*III J, as depicted in Fig. 2. Hybridization to this probe indicated that the direction of transcription was leftward on the genome (data not shown), the same direction as *sgg1* (Fig. 2). The additional bands in the region of the 28S rRNA detected with double-stranded DNA probe A at 24 h p.i. (Fig. 1A) were not reproducibly detected with single-stranded riboprobes (data not shown) and were not studied further.

RNA blot analysis with double-stranded DNA probes and single-stranded RNA probes was employed to identify transcripts to the right of the *sgg1* gene within *Hind*III J. As shown in Fig. 1B and C and depicted in Fig. 2, double-stranded DNA probe B, a *Sac*II/*Stu*I fragment, or probe C, a *Stu*I/*Bam*HI fragment, detected 1.3- and 4.2-kb β transcripts at either 8 or 24 h p.i. To further map these transcripts, strand-specific riboprobes equivalent to probe C were generated, and only the 1.3-kb β transcript was reproducibly detected at 8 and 24 h p.i.

(data not shown), and this transcript was oriented on the viral genome as depicted in Fig. 2. To further investigate the 3' end of the 1.3-kb β transcript and to determine whether the 4.2-kb β transcript was located downstream of the 1.3-kb transcript, we used a probe representing of the left half of probe B, a *Sac*II/*Bgl*II fragment, and this probe failed to detect the 4.2-kb β transcript or any other transcripts at either 8 or 24 h p.i. (data not shown). Thus, the region between the *Sac*II and *Bgl*II sites does not appear to be represented in mature transcripts at early or late times p.i. Double-stranded probe D, an *Eco*RI/*Pst*I fragment from pON880 (Fig. 3A), was prepared and shown to detect four β transcripts with apparent sizes of 1.9, 4.1, 6.0, and 7.4 kb (Fig. 1D), and they were all detected with a strand-specific probe representing the same sequences as probe D, consistent with the orientation shown in Fig. 2. The 4.2-kb β transcript that had been detected with double-stranded DNA probes B and C consistently migrated at a different position from that of the 4.1-kb β transcript detected with single- and double-stranded probe D, and thus we do not believe that these transcripts are related.

The complexity of transcripts in the *Hind*III region suggested that RNA splicing might be occurring in this region. The plasmid-based cDNA library was made by using polyadenylated RNA at 8 h p.i. (26), and clones were isolated by screening with [α - 32 P]dCTP-labeled *Clal*/*Hind*III probe F (Fig. 2). Approximately 0.4% of the clones in the library hybridized to this probe were specific for the 1.9-kb β transcript, and approximately 0.025% were specific for the 1.3-kb β transcript. Several of these clones were analyzed by restriction enzyme digestion and hybridization, and two nearly full-length copies were chosen for further work (Fig. 3A). Restriction digests of the genomic clone, pON449, and the cDNA clone, pON882, were compared and found to be colinear within the region represented by the β 1.3-kb transcript (Fig. 3B and C), indicating that the transcript was not likely to be spliced. Restriction patterns of pON880 and pON449 were also compared, and the region represented by the β 1.9-kb transcript was also found to be colinear with the genome (Fig. 3D and E), suggesting that it too was unspliced. Finally, the nucleotide sequence of the ends of the cDNA inserts (data not shown) was used to confirm the positions and orientations of these two transcripts and also identified the amino termini of the HJ5 and HJ6 ORFs within pON882 and pON880, respectively. Thus, full-length cDNA clones for the 1.3- and 1.9-kb β transcripts were not apparently spliced.

Identification of ORFs. The DNA sequence of the *Hind*III J region was derived from the data base of a current project to determine the complete DNA sequence of the murine CMV (Smith) genome. The size of the *Hind*III J fragment was identical in the Smith and K181 viral genomes (10, 33), and restriction sites mapped in this region of strain K181 (by enzyme digestion) were all found in the same location in the nucleotide sequence from strain Smith (Fig. 2). The principle criterion used to distinguish coding sequences in this fragment was the presence of an ORF of at least 300 nucleotides that did not overlap any other ORF by more than 60% of its length. The ORFs selected by these criteria were examined for the presence of initiator methionine codons whose context sequence resembled RNNATG or NNNATGG (25) and for constraints likely to be imposed by coding by the positional base preference method (57). Seven ORFs were identified, and all were oriented in the same (leftward) direction on the viral genome. In the absence of a full sequence analysis of the entire murine CMV genome, they have been temporarily designated HJ1 through HJ7. The sequence analysis, including the arrangement of predicated ORFs, predicated TATA boxes, and

poly(A) signals are diagrammed in Fig. 2. Each of these ORFs is diagrammed from a predicted or known initiator methionine codon. On the basis of the comparisons of the transcripts that have been mapped in this region, predicted ORFs and other signals, several conclusions may be drawn. (i) The 367-amino-acid (aa) protein predicted from the HJ2 ORF is related to the *sgg1* gene that has been characterized in the K181 strain of murine CMV (26). The 1.5- and 1.8-kb *sgg1* β transcripts are both spliced and encode a 37-kDa protein by using a 312-aa ORF, with 305 aa derived from the 367-aa ORF (26). The product expected from the Smith strain is highly related and is predicted to differ by 1 aa (Arg-263 $\rightarrow$ His). (ii) HJ3 is predicted to encode a 107-aa protein, which has been previously found (for the K181 strain) in the β 1.8-kb transcript upstream of the *sgg1* ORF (26). HJ3 also has only 1-aa substitution between virus strains (Leu-59 $\rightarrow$ Val). (iii) The HJ5 ORF is contained within the β 1.3-kb transcript and is predicted to encode a 318-aa protein. (iv) The HJ6 ORF is contained within the β 1.9-kb transcript and is predicted to encode a 569-aa protein. (v) The HJ7 ORF may be encoded by the β 4.1-kb transcript or one of the other transcripts (β 6.0- or 7.4-kb transcript) predicted to initiate in the adjacent *HindIII* I fragment (Fig. 2). The precise mapping of the transcript(s) encoding this ORF requires additional work. (vi) Proteins of 109 aa and 253 aa are predicted from HJ1 ORF and HJ4 ORF, respectively; however, neither HJ1 nor HJ4 has been assigned to a transcript at this point. The HJ4 ORF lies in a region covered by the *SacII*-*Bgl*II half of probe B. This region did not encode any detectable RNA transcripts in infected cells at either 8 or 24 h p.i. Furthermore, insertion mutant RM874 (discussed below), which placed an insertion within this ORF, did not alter any of the transcripts detected by surrounding probes C or A. Finally, without more precise mapping of the 5' end of the γ 0.85-kb transcript, we can speculate only that it may encode HJ1 or another ORF entirely contained within the *HindIII* L fragment (Fig. 2).

Possible TATA boxes were predicted for the ORFs by scanning the sequence upstream from the predicted initiator methionine codons, and candidates were found adjacent to the β 1.3-, 1.5-, 1.8-, and 1.9-kb transcripts and the γ 0.85-kb transcript. Only ORF HJ4, which has not been assigned to a transcript, lacked a readily identified consensus TATA box, suggesting that it might be expressed via alternative transcription initiation signals or might be part of a low-abundance spliced transcript. Several potential poly(A) sites in *HindIII* J fit the consensus (AAUAAA). In agreement with the size and number of transcripts that have been mapped, HJ3 and HJ7 appeared to share 3' termination signals with ORFs HJ2 and HJ6, respectively, a feature that has been found in the organization of other herpesvirus genomes (5, 32).

The amino acid sequences encoded by the seven ORFs were screened against the SWISSPROT library by using the FASTA program (41). The product of ORF HJ7 possesses homology with the US22 gene family of human CMV (optimized FASTA score of 402 against the human CMV US22 ORF by using an alignment k-tuple value of 2). The products of the remaining ORFs were not found to exhibit significant similarity to ORFs in human CMV or other herpesviruses or to other predicted or known proteins in the sequence data bases.

Construction of recombinant viruses. Insertion mutations were introduced into the murine CMV genome by utilizing a cassette containing both the *E. coli gpt* and *lacZ* genes. Strong selection methods for *gpt* gene expression made it possible to generate recombinant viruses by infection combined with transfection of plasmid DNA, rather than by the cotransfection method used previously with the *lacZ* gene (29, 55). First, a

FIG. 4. Insertions within *HindIII* J. (A) Ethidium bromide-stained *Pst*I digestion of pON449, pON871, pON872, and pON874 and *Pst*I/*HindIII* double digestion of pON868, pON869, pON870, pON873, and pON874. Size markers (in kilobase pairs) are indicated on the left, and the vector band released by *HindIII* digestion is indicated on the right. In the *Pst*I lanes, the lower band represents the fragment length from the insertion site to the right end of *HindIII* J. In the *Pst*I + *HindIII* lanes, the lower bands (disregarding the vector band) represent the fragment length from the insertion site to the left side of *HindIII* J. (B) Diagram of the *HindIII* J fragment (pON449) showing the position and orientation of each *lacZ/gpt* insert. The positions of the *Pst*I and *HindIII* sites are shown. (C) Schematic diagram of *lacZ/gpt* construct present in pON855 used for insertions into the murine CMV genome. The positions of the *Bam*HI and *Pst*I sites are shown.

series of plasmid constructs with single insertions in the murine CMV *HindIII* J fragment were created by partial digestion of pON449 with *Sau*3A followed by the insertion of a DNA cassette containing the *E. coli lacZ* gene, expressed from the β -actin promoter and *gpt* gene, expressed from the herpes simplex virus type 1 *tk* promoter (Fig. 4C). Seven plasmids, pON868, pON869, pON870, pON871, pON872, pON873, and pON874, with *lacZ/gpt* insertions in *HindIII* J were made and used to generate recombinant viruses. The site of insertion and orientation of the inserts were identified by digestion of pON871, pON872, and pON874 with *Pst*I and pON868, pON869, pON870, and pON873 with *Pst*I and *HindIII* (Fig. 4A). In the parent plasmid pON449, there was a single *Pst*I site in the vector adjacent to the right-hand side of the *HindIII* J insert, and there was a unique *Pst*I site at the *lacZ* end of the *lacZ/gpt* insert (Fig. 4B). All constructs were found to contain the expected insertions, except pON869, in which the insertion replaced a 1.5-kb deletion probably resulting from digestion of pON449 at two sites during *Sau*3A digestion.

The inserts within these constructs disrupted the following transcripts and ORFs: pON868 disrupted the *sgg1* ORF (HJ2) near its amino terminus; pON869 deleted the β 1.3-kb transcript, completely removing the HJ5 ORF and disrupting the carboxyl terminus of the HJ6 ORF; pON870 disrupted the β 1.3-kb transcript near the 5' end; pON871 and pON872 were insertions at two different sites disrupting the β 1.9-kb transcript and the HJ6 ORF; and pON873 disrupted the β 4.1-, 6.0-, and 7.4-kb transcripts within the carboxyl-terminal region of the HJ7 ORF. pON874 disrupted the HJ4 ORF. The orientation of the insert in each construct is summarized in Fig. 4B, and the positions of insertions relative to transcripts and ORFs are summarized in Fig. 2.

These constructs were digested with *HindIII* to release the

FIG. 5. Viral DNA restriction digests. (A) Parental murine CMV (MCMV), RM868, RM870, RM871, RM872, RM873, and RM874 DNA and pON869 and pON874 were digested with *Hind*III and separated by electrophoresis on a 0.6% agarose gel, stained with ethidium bromide, and visualized with UV illumination. The positions of murine CMV *Hind*III fragments A to M are indicated on the left of the lanes, and *Hind*III-digested λ DNA is in the first lane. (B) Autoradiograph of hybridization analysis of DNA described for panel A with an [α - 32 P]dCTP-labeled *Hind*III J probe. The position of the parental *Hind*III J fragment is indicated between the sets of lanes.

murine CMV DNA from vector sequences and then introduced into the viral genome by one of two methods. RM868, RM869, RM871, RM872, and RM873 were constructed by introducing the plasmids by calcium phosphate precipitation (6) into NIH 3T3 cells that had been first infected with murine CMV. RM870 and RM874 were constructed by introducing pON870 and pON874 by electroporation into NIH 3T3 cells, which were infected 6 to 12 h later. The progeny virus from these transfection/infection cultures was passaged twice (for RM870 and RM874) or three times (for RM868, RM869, RM871, RM872, and RM873) in the presence of mycophenolic acid and xanthine, thereby selecting for a pool of viruses carrying the *gpt* gene. The resulting pools were between 20 and 40% recombinants after two rounds of selection and greater than 50% after three rounds as determined by blue plaque phenotype after X-Gal overlay staining. Recombinant viruses were then plaque purified prior to further analysis (29).

Recombinant viral DNA analysis. In order to examine the genome structure of the recombinant viruses, viral DNA was

isolated and then subjected to restriction enzyme digestion. DNA from each of the recombinant viruses was compared with parental murine CMV DNA, pON869, and pON874 after digestion with *Hind*III and separation of fragments by agarose gel electrophoresis (Fig. 5A). An autoradiograph from the hybridization of the *Hind*III-digested DNAs with a *Hind*III J fragment probe showed that, as expected, all the insertions were within the J fragment, that the recombinant J fragment in the viruses matched the size of the fragment in the plasmids used to construct them, and that no wild-type J fragment was detectable in the DNA prepared from the recombinant viruses (Fig. 5B). The comparison of *Hind*III digests, as well as *Eco*RI and *Xba*I digests (data not shown) of recombinant and parental viral DNA, revealed that for RM868, RM870, RM871, RM872, RM873, and RM874, no additional deletions or insertions had occurred outside of the expected insertions in *Hind*III J; however, RM869 carried a spontaneous 8.3-kb deletion within the *Hind*III E fragment in addition to the *lacZ/gpt* insertion.

Transcript analysis of recombinant virus. To examine the impact of insertions on expression of transcripts in the recombinant viruses, RNA blot analysis was carried out (Fig. 6). The probes used here were described above (Fig. 2). RNA isolated at 8 and 24 h p.i. from RM868-, RM870-, RM871-, RM873-, and RM874-infected cells was compared with RNA from cells infected with parental virus.

When RM868-infected cell RNA was hybridized with probe A, the *sgg1* transcripts were disrupted, and the γ 0.85-kb transcript remained intact (Fig. 6A). Although the insertion in RM874 was in a region to which no transcripts had been mapped, RNA from RM874-infected cells was hybridized with probes A, B, and C in order to investigate any effect the insertion may have had on transcripts in the region. Probe A showed no differences in transcription in the region of the *sgg1* transcripts (Fig. 6A). Probes B and C showed normal expression of the β 1.3- and 4.2-kb transcripts but also detected a 1.85-kb transcript not made by parental virus (Fig. 6B and C). By using single-stranded RNA probes, this transcript was shown to arise from the same DNA strand as the β 1.3-kb transcript, did not hybridize with *lacZ* sequences, and apparently terminated at the poly(A) signal present at the 3' end of the *lacZ* gene (data not shown).

FIG. 6. RNA blot analysis of recombinant viruses. Autoradiographs compare expression from parental wild-type (MCMV) and recombinant viruses. Infected NIH 3T3 cell RNA was isolated at 8 and 24 h p.i. as indicated above the lanes. Hybridization was done with one of the [α - 32 P]dCTP-labeled probes, A, B, C, D or E, diagrammed in Fig. 2 and corresponding to those to the panels shown here. The positions of the 18S and 28S rRNAs and the sizes of the viral RNAs (in kilobases) are indicated adjacent to each set of lanes. (A) Probe A hybridization with parental virus, RM868, and RM874 RNA. (B) Probe B hybridization with parental virus and RM874. (C) Probe C hybridization with parental virus, RM870, and RM874. (D) Probe D hybridization with parental virus, RM871, and RM873. (E) Probe E hybridization with parental virus, RM871, and RM873 on RNA at 8 h p.i.

FIG. 7. Temporal expression of *lacZ* from within recombinant RM873. Autoradiograph of RNA blot analysis of whole-cell RNA isolated from cells infected with RM873 at 2.5, 8, and 24 h p.i. and at 6 h p.i. in the presence of cycloheximide. Hybridization was done with an [α - 32 P]dCTP-labeled *lacZ* probe, a 3.2-kb *HindIII*/*EcoRI* fragment of pON1 (54). (A) RM873-infected cell RNA isolated at 2.5 h p.i., parental wild-type virus (K181)-infected cell RNA isolated at 8 h p.i., and RM873-infected cell RNA from cells treated with cycloheximide (CH) from 1 h before infection until 6 h p.i. (B) RM873-infected cell RNA isolated at 8 and 24 h p.i. The positions of the 18S and 28S rRNAs are indicated between the sets of lanes.

When RM870-infected cell RNA was hybridized with probe C, the β 1.3- and 4.2-kb transcripts were absent (Fig. 6C), whereas transcripts in regions flanking the insert remained intact (data not shown). RM871- and RM873-infected cell RNA was hybridized with probe D to determine whether these insertions disrupted expression of the β 1.9-kb transcript. The transcript was disrupted in RM871 but was present in normal levels at both early and late times in RM873 (Fig. 6D). In RM870, a faint 0.9-kb transcript, with the size expected for a transcript terminated by the poly(A) signals flanking the marker genes the insert site, was present. When hybridized with probe E, shortened versions of the β 4.1-, 6.0-, and 7.4-kb transcripts were detected consistent with the use of the simian virus 40 poly(A) signal (Fig. 6E). These results demonstrated that the insertions within the *HindIII* J fragment truncated or disrupted each of the major transcripts and large ORFs detected in this region.

To assess the temporal expression the β -actin promoter from within the context of the viral genome, a *lacZ* probe was hybridized to immobilized RNA from RM873 infected cells held in the presence of cycloheximide for 6 h or with RNA made at 2.5, 8, and 24 h p.i. in the absence of drug (Fig. 7). RNA from the β -actin promoter was detected at 6 h p.i. in the presence of cycloheximide, as well as at 8 and 24 h p.i. The detection of a transcript in the presence of cycloheximide indicated that this gene was being expressed as an α gene from within the viral genome; however, the lack of detection of this transcript in the absence of drug at 2.5 h p.i. suggests that expression was not vigorous. The increase in expression between 8 and 24 h suggests that viral transactivators or replication plays a role in the activity of the β -actin promoter resident on the viral genome. These data are consistent with expression of the β -actin promoter being constitutive throughout the viral replication cycle.

Growth kinetics of recombinant viruses. Growth curves were carried out in order to determine whether the insertion mutations affected the ability of recombinant viruses to grow in cell culture. Stocks of all recombinant viruses were prepared, and their growth rates were compared with that of parental wild-type murine CMV. NIH 3T3 cells were infected at an MOI of approximately 0.1 PFU per cell, and virus titers were determined from these cultures at various times after infection (Fig. 8). In this analysis, there was no detectable effect of any

FIG. 8. Growth kinetics of recombinant viruses. NIH 3T3 cells were infected at an MOI of 0.1 PFU per cell, and virus titers were determined at the indicated times. The datum points were generated from the average of titers from three separate cultures of each virus for each time. (A) Growth kinetics of RM868, RM871, RM872, and RM873 compared with wild-type murine CMV (MCMV), with titers determined following sonication of cells and medium to release virus. (B) Growth kinetics of RM868, RM870, and RM874 compared to wild-type murine CMV with (MCMV), titers of virus released into the culture medium of infected cultures.

of these mutations on viral growth, indicating that the genes which we disrupted in these viruses do not encode functions that are important for growth in cell culture.

DISCUSSION

We have truncated or disrupted six known or putative murine CMV genes (HJ1, *sgg1*, HJ3, HJ4, HJ5, HJ6, and HJ7) within or overlapping the *HindIII* J fragment. Our results suggest that intact versions of these genes are not required for viral replication in NIH 3T3 cells. The approach reported here allows the rapid and efficient isolation of recombinant CMV by using the strong selection afforded by the *gpt* gene and the blue plaque phenotype of *lacZ*⁺ viruses.

The design of the *lacZ/gpt* cassette takes into consideration its use in the context of a herpesvirus genome. We chose to use the herpes simplex virus type 1 *tk* promoter to drive expression of *gpt* and the cellular β -actin promoter to drive *lacZ* because these promoters ensure adequate levels of each marker without introducing a strong enhancer that could alter expression from viral genes flanking the insert. Neither the β -actin gene (38) nor the herpes simplex virus type 1 *tk* gene has been reported to carry strong enhancers, but, as expected, the β -actin gene promoter was constitutively active when expressed from within the viral genome. In addition, the β -actin promoter was chosen to direct expression of *lacZ* because it was known to be broadly active in different types of cells and has been shown to allow ready identification of infected cells in infected mouse tissues (26).

The *lacZ/gpt* cassette is flanked by simian virus 40 sequences with convergent early and late poly(A) signals that function in either orientation (4). This feature was included to facilitate

the isolation of inserts in a targeted gene without altering expression of nearby genes that may share a common poly(A) signal with the disrupted gene. Many herpesvirus genes share common poly(A) signals, such as is the case with *sgg1* and HJ3 and apparently with HJ6 and HJ7. Introduction of poly(A) signals along with the *lacZ/gpt* cassette prevented the unintentional disruption of 3' coterminal genes. Thus, in RM868, the 1.5-kb transcript and HJ2 ORF are disrupted, whereas a truncated 1.8-kb transcript normally 3' coterminal with the 1.5-kb transcript would still be expected to encode the HJ3 ORF. This arrangement also terminates transcription that might run into the insert and helps to keep expression of the marker genes independent of the site of insertion.

Methods were developed by using the *lacZ/gpt* cassette for the efficient and rapid disruption of genes in the viral genome. The strong selection potential of the *gpt* gene makes it possible to generate recombinant viruses by virus infection rather than by the previous method of cotransfection of viral DNA along with recombinant plasmid that had relied on the *lacZ* gene alone (29, 55), thereby eliminating the need to purify viral DNA. Because viral DNA is not included, the delivery of the recombinant plasmid is simplified. Here, plasmid DNA was successfully delivered by calcium phosphate precipitation or electroporation before or after viral infection. This method is very efficient even though the levels of recombinant viruses in the initial pool is typically lower than that after cotransfection (0.01 to 0.0001% versus 1.0 to 0.01%). Recombinant viruses were rapidly enriched to 20 to 40% of the population after two cycles of growth under *gpt* selection, which can be accomplished within the time it takes for transfected DNA to generate virus. All methods of constructing murine CMV recombinants that have been tried thus far, including use of the *gpt* gene in the absence of the *lacZ* gene, have yielded a proportion of recombinants, such as recombinant RM869 described here or recombinant RM427 described previously (26, 30), that carry adventitious deletions. There is currently no method available that yields recombinant viruses without risk of such secondary mutations, and therefore, genome structure must be carefully analyzed after isolation.

We made seven viruses with *lacZ/gpt* insertions and were guided by genes present in the region that had been identified by transcript mapping and nucleotide sequence analysis. The alignment of transcripts and ORFs indicated the following assignments. ORFs HJ2 and HJ3 are present in the *sgg1* locus and are encoded by the β 1.5- and 1.8-kb transcripts (26, 30). ORFs HJ5, HJ6, and HJ7 are positioned such that they would be encoded by the β 1.3-, 1.9-, and 4.1-kb transcripts, respectively. No transcript that would encode the HJ4 ORF has been unambiguously detected, and there is no apparent TATA box upstream of this ORF. The identification of a transcript capable of expressing the HJ1 ORF cannot be positively made until the map position of the γ 0.85-kb transcript is better defined. It is possible that the γ 0.85-kb transcript may encode a portion of HJ1, an ORF located in the adjacent *HindIII* L fragment (Fig. 2), or a spliced product combining both. It is known from analysis of RM461 (58), a virus that carries an insertion at the *HindIII* site between the L and J fragments, that the γ 0.85-kb transcript may be disrupted at this site without any reduction of growth of virus in cell culture or in infected animals.

The seven ORFs that have been assigned to the *HindIII* J region were identified from sequence analysis of the Smith strain of murine CMV. The two strains of virus in widespread use, Smith and K181, exhibit occasional restriction site polymorphism (10, 33), and that which is known about the nucleotide sequence conservation between the two strains shows

variation between complete and 96.3% identity (11, 46, 63). Here, we found a high level of retention of restriction sites throughout the *HindIII* J region and a similarly high level of identity in the *sgg1* gene (26). Thus, it appears as though the nucleotide sequence generated for the Smith strain of murine CMV is very useful for predicting gene structure in the K181 strain.

Except for RM869, all of the recombinant viruses that we describe here had an intact genome structure and lacked adventitious deletions or insertions. RM869 carried a spontaneous, unintentional 8.3-kb deletion in the *HindIII* E fragment. This deletion occurred in the same region as a deletion previously identified in the Vancouver variant of the Smith strain (3). It is not known whether the increase in genome size that results from insertions or the recombination process itself increases the risk of generating such mutations. Secondary mutations in recombinant murine CMV constructed by cotransfection have been observed previously (30), and the current method of infection/transfection did not significantly decrease their frequency. We had initially sought to develop a method of constructing recombinant virus in which infection rather than viral DNA transfection is used to reduce the likelihood of adventitious mutations. When six or more independent pools of recombinant viruses are selected for *gpt* expression, the predicted, correct genome structure is generally observed in 50% of the pools. Thus, the current infection/transfection method is no better than cotransfection with *lacZ* with regard to the generation of adventitious mutations.

RNA analysis showed that the expected transcripts and ORFs within the *HindIII* J fragment had been either truncated or disrupted. The insertion in RM868 interrupts the β 1.5- and 1.8-kb transcripts and is within the HJ2 ORF. Although it terminates the β 1.8-kb transcript, it does not disrupt the HJ3 ORF. RM874 would be expected to truncate ORF HJ4 near its carboxyl terminus, although transcripts capable of encoding this ORF have not been detected. The insertion in RM870 interrupts the β 1.3-kb transcript near the translation initiation site and would therefore prevent expression of the HJ5 ORF. The HJ5 ORF is also disrupted in RM869 via the deletion. RM871 disrupts the β 1.9-kb transcript and interrupts the HJ6 ORF in the amino-terminal half of the protein. While this insertion also terminates the β 4.1-, 6.0-, and 7.4-kb transcripts, it would not be expected to completely destroy the expression of the HJ7 ORF. The insertion of RM873 is in the carboxyl terminus of the HJ7 ORF and would be expected to encode a truncated HJ7 protein.

One unexpected result from the RNA analysis was the detection of a novel RNA species in RM874. The insertion site in this virus occurred in a region in which no clear transcript had been detected in wild-type murine CMV, and although it did not affect previously identified transcripts flanking the insertion, a new transcript that terminated at the insert was found. The poly(A) sites carried on the *lacZ/gpt* may have contributed to the detection of this transcript. Its size was consistent with its being 5' coterminal with the β 1.3-kb transcript and extending through the poly(A) signal at the end of the HJ5 ORF, although an explanation for why this should occur is not obvious from the sequences involved. It is possible that the *lacZ/gpt* insert has activated some previously undetected promoter in the region or that adding a poly(A) signal stabilizes a truncated version of the 4.2-kb transcript that was tentatively mapped in the region.

The growth kinetics of these recombinant viruses were similar to that of parental wild-type murine CMV. Thus, the β 1.3 (HJ5)- and 1.9 (HJ6)-kb genes are in a category with the previously identified *ie2* (29), *sgg1* (30), and γ 0.85-kb (58)

genes and are dispensable for growth in cell culture. Given these results, an analysis of the function of these genes in mice needs to be carried out to determine whether they play roles during interaction with the natural host.

ACKNOWLEDGMENTS

We appreciate the technical support of Maria Kirichenko. J.V. was a postdoctoral trainee in the Host Parasite Interactions Training Program (T32 AIO7328). This work was supported by Public Health Service grants (AI30363 and AI28341).

REFERENCES

- Alford, C. A., and W. J. Britt. 1993. Cytomegalovirus, p. 227–255. In B. Roizman, R. J. Whitley, and C. Lopez (ed.), *The human herpesviruses*. Raven Press, New York.
- Bankier, A. T., and B. G. Barrell. 1989. Sequencing single stranded DNA using the chain termination method, p. 37–78. In C. J. Howe and E. S. Ward (ed.), *Nucleic acids sequencing: a practical approach*. IRL Press, Oxford.
- Boname, J. M., and J. K. Chantler. 1992. Characterization of a strain of murine cytomegalovirus which fails to grow in the salivary glands of mice. *J. Gen. Virol.* **73**:2021–2029.
- Carswell, S., and J. C. Alwine. 1989. Efficiency of utilization of the simian virus 40 late polyadenylation site: effects of upstream sequences. *Mol. Cell. Biol.* **9**:4248–4258.
- Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. I. Hutchison, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell. 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154**:125–170.
- Chen, C. A., and H. Okayama. 1988. Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *BioTechniques* **6**:632–638.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299.
- Crook, J., T. Horsnell, and B. G. Barrell. 1994. Personal communication.
- Dear, S., and R. Staden. 1991. A sequence assembly and editing program for efficient management of large projects. *Nucleic Acids Res.* **19**:3907–3911.
- Ebeling, A., G. M. Keil, E. Knust, and U. H. Koszinowski. 1983. Molecular cloning and physical mapping of murine cytomegalovirus DNA. *J. Virol.* **47**:421–433.
- Elliott, R., C. Clark, D. Jaquish, and D. H. Spector. 1991. Transcription analysis and sequence of the putative murine cytomegalovirus DNA polymerase gene. *Virology* **185**:169–186.
- Falkner, F. G., and B. Moss. 1990. Transient dominant selection of recombinant vaccinia viruses. *J. Virol.* **64**:3108–3111.
- Feinberg, A. P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **137**:266–267.
- Gunning, P., J. Leavitt, G. Muscat, S. Y. Ng, and L. Kedes. 1987. A human beta-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc. Natl. Acad. Sci. USA* **84**:4831–4835.
- Ho, D. Y., and E. S. Mocarski. 1988. β -Galactosidase as a marker in the peripheral and neural tissues of the herpes simplex virus-infected mouse. *Virology* **167**:279–283.
- Ho, M. 1991. *Cytomegalovirus: biology and infection*, 2nd ed. Plenum Publishing Corp., New York.
- Hudson, J. B. 1979. The murine cytomegalovirus as a model for the study of viral pathogenesis and persistent infections. *Arch. Virol.* **62**:1–29.
- Jenkins, D. E., C. L. Martens, and E. S. Mocarski. The human cytomegalovirus *ie2* gene product produced late in infection: a transactivator as well as a repressor of gene expression. *J. Gen. Virol.*, in press.
- Jones, T. R., and V. P. Muzithras. 1992. A cluster of dispensable genes within the human cytomegalovirus genome short component: IRS1, US1 through US5, and the US6 family. *J. Virol.* **66**:2541–2546.
- Jordan, M. C. 1983. Latent infection and the elusive cytomegalovirus. *Rev. Infect. Dis.* **5**:205–215.
- Keil, G. M., K. A. Ebeling, and U. H. Koszinowski. 1984. Temporal regulation of murine cytomegalovirus transcription and mapping of viral RNA synthesized at immediate-early times after infection. *J. Virol.* **50**:784–795.
- Keil, G. M., K. A. Ebeling, and U. H. Koszinowski. 1987. Immediate-early genes of murine cytomegalovirus: location, transcripts, and translation products. *J. Virol.* **61**:526–533.
- Klotman, M. E., S. C. Henry, R. C. Greene, P. C. Brazy, P. E. Klotman, and J. D. Hamilton. 1990. Detection of mouse cytomegalovirus nucleic acid in latently infected mice by in vitro enzymatic amplification. *J. Infect. Dis.* **161**:220–225.
- Koszinowski, U. H., M. Del Val, and M. J. Reddehase. 1990. Cellular and molecular basis of the protective immune response to cytomegalovirus infection. *Curr. Top. Microbiol. Immunol.* **154**:189–220.
- Kozak, M. 1989. The scanning model for translation: an update. *J. Cell Biol.* **108**:229–241.
- Lagenaur, L. A., J. Vieira, C. L. Martens, and E. S. Mocarski. The structure and function of the murine cytomegalovirus *sgg1* gene: a determinant of viral growth in salivary gland acinar cells. Submitted for publication.
- Leach, F. S., and E. S. Mocarski. 1989. Regulation of cytomegalovirus late-gene expression: differential use of three start sites in the transcriptional activation of ICP36 gene expression. *J. Virol.* **63**:1783–1791.
- Loh, L. C., N. Balachandran, and W. J. Britt. 1991. Characterization of a membrane-associated phosphoprotein of murine cytomegalovirus (pp50) and its immunological cross-reactivity with a human cytomegalovirus protein. *Virology* **183**:181–194.
- Manning, W. C., and E. S. Mocarski. 1988. Insertional mutagenesis of the murine cytomegalovirus genome: one prominent alpha gene (*ie2*) is dispensable for growth. *Virology* **167**:477–484.
- Manning, W. C., C. A. Stoddart, L. A. Lagenaur, G. B. Abenes, and E. S. Mocarski. 1992. Cytomegalovirus determinant of replication in salivary glands. *J. Virol.* **66**:3794–3802.
- Marshall, D. R., J. D. Reilly, X. Liu, and R. F. Silva. 1993. Selection of Marek's disease virus recombinants expressing the *Escherichia coli* gpt gene. *Virology* **195**:638–648.
- McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69**:1531–1574.
- Mercer, J. A., J. R. Marks, and D. H. Spector. 1983. Molecular cloning and restriction endonuclease mapping of the murine cytomegalovirus genome (Smith strain). *Virology* **129**:94–106.
- Mercer, J. A., C. A. Wiley, and D. H. Spector. 1988. Pathogenesis of murine cytomegalovirus infection: identification of infected cells in the spleen during acute and latent infections. *J. Virol.* **62**:987–997.
- Mocarski, E. S. 1993. Cytomegalovirus biology and replication, p. 173–226. In B. Roizman, R. Whitley, and C. Lopez (ed.), *The human herpesviruses*. Raven Press, New York.
- Mosmann, T. R., and J. B. Hudson. 1973. Some properties of the genome of murine cytomegalovirus (MCMV). *Virology* **54**:135–149.
- Mulligan, R. C., and P. Berg. 1981. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci. USA* **78**:2072–2076.
- Ng, S.-Y., P. Gunning, S.-H. Liu, J. Leavitt, and L. Kedes. 1989. Regulation of the human β -actin promoter by upstream and intron domains. *Nucleic Acids Res.* **17**:601–615.
- Osborn, J. E. 1982. Cytomegaloviruses and other herpesviruses, p. 267–293. In H. L. Foster, J. D. Small, and J. G. Fox (ed.), *The mouse in biomedical research*. Academic Press, New York.
- Parker, R. C., R. M. Watson, and J. Vinograd. 1977. Mapping of closed circular DNA by cleavage with restriction endonucleases and calibration by agarose gel electrophoresis. *Proc. Natl. Acad. Sci. USA* **74**:851–855.

41. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**:2444–2448.
42. **Plummer, G.** 1973. Cytomegaloviruses of man and animals. *Prog. Med. Virol.* **15**:92–125.
43. **Pomeroy, C., P. J. Hilleren, and M. C. Jordan.** 1991. Latent murine cytomegalovirus DNA in splenic stromal cells of mice. *J. Virol.* **65**:3330–3334.
44. **Post, L. E., A. J. Conley, E. S. Mocarski, and B. Roizman.** 1980. Cloning of reiterated and nonreiterated herpes simplex virus 1 sequences as BamHI fragments. *Proc. Natl. Acad. Sci. USA* **77**:4201–4205.
45. **Products, B. R.** 1989. IX. Single stranded DNA template preparation from M13 vectors or pGEM-Zf phagemids, p. 75–80. *In* Promega protocols and application guide. Promega Corp., Madison, Wis.
46. **Rapp, M., M. Messerle, B. Buhler, M. Tannheimer, G. M. Keil, and U. H. Koszinowski.** 1992. Identification of the murine cytomegalovirus glycoprotein B gene and its expression by recombinant vaccinia virus. *J. Virol.* **66**:4399–4406.
47. **Rawlinson, W. D., H. E. Farrell, and B. G. Barrell.** 1993. Global comparison of the DNA sequences of HCMV (AD169) and MCMV (Smith)—preliminary analysis, p. 55–62. *In* S. A. Plotkin and S. Michelson (ed.), *Multidisciplinary approaches to understanding cytomegalovirus disease*. Elsevier Science Publishers, Amsterdam.
48. **Rosenthal, A., O. Coutelle, and M. Craxton.** 1993. Large-scale production of DNA sequencing templates by microtitre format PCR. *Nucleic Acids Res.* **21**:173–174.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
51. **Shanley, J. D., M. C. Jordan, M. L. Cook, and J. G. Stevens.** 1979. Pathogenesis of reactivated latent murine cytomegalovirus infection. *Am. J. Pathol.* **95**:67–80.
52. **Smith, L. M., J. Z. Sanders, R. J. Kaiser, P. Hughes, C. Dodd, C. R. Connell, C. Heiner, S. B. Kent, and L. E. Hood.** 1986. Fluorescence detection in automated DNA sequence analysis. *Nature (London)* **321**:674–679.
53. **Smith, V., M. Craxton, A. T. Bankier, C. M. Brown, W. D. Rawlinson, M. S. Chee, and B. G. Barrell.** 1993. Preparation and fluorescent sequencing of M13 clones: microtiter methods. *Methods Enzymol.* **218**:173–187.
54. **Spaete, R. R., and E. S. Mocarski.** 1985. Regulation of cytomegalovirus gene expression: α and β promoters are *trans* activated by viral functions in permissive human fibroblasts. *J. Virol.* **56**:135–143.
55. **Spaete, R. R., and E. S. Mocarski.** 1987. Insertion and deletion mutagenesis of the human cytomegalovirus genome. *Proc. Natl. Acad. Sci. USA* **84**:7213–7217.
56. **Staczek, J.** 1990. Animal cytomegaloviruses. *Microbiol. Rev.* **54**:247–265.
57. **Staden, R.** 1984. Measurements of the effects that coding for a protein has on a DNA sequence and their use for finding genes. *Nucleic Acids Res.* **12**:551–567.
58. **Stoddart, C. A., R. D. Cardin, J. M. Boname, W. C. Manning, G. B. Abenes, and E. S. Mocarski.** Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. Submitted for publication.
59. **Takebe, Y., M. Seiki, J. Fujisawa, P. Hoy, K. Yokota, K. Arai, M. Yoshida, and N. Arai.** 1988. SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **8**:466–472.
60. **Tsao, S. G., C. F. Brunk, and R. E. Pearlman.** 1983. Hybridization of nucleic acids directly in agarose gels. *Anal. Biochem.* **131**:365–372.
61. **Vieira, J.** 1988. Ph.D. thesis. The University of Minnesota, Minneapolis.
62. **Vieira, J., and J. Messing.** 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. *Gene* **100**:189–194.
63. **Xu, J., P. B. Dallas, P. A. Lyons, G. R. Shellam, and A. A. Scalzo.** 1992. Identification of the glycoprotein H gene of murine cytomegalovirus. *J. Gen. Virol.* **73**:1849–1854.