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Murine Cytomegalovirus with a Deletion of Genes Spanning *Hin*dIII-J and -I Displays Altered Cell and Tissue Tropism

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Murine cytomegalovirus (MCMV) gene products dispensable for growth in cell culture are likely to have important functions within the infected host, influencing tissue tropism, dissemination, or immunological responses against the virus. To identify such genes, our strategy was to delete large regions of the MCMV genome likely to contain genes nonessential for virus replication in NIH 3T3 cells. Mutant virus RV7 contained a deletion of 7.7 kb spanning portions of MCMV *Hin*dIII-J and -I. This virus grew comparably to wild-type (WT) virus in NIH 3T3 fibroblasts, primary embryo fibroblasts, and bone marrow macrophages. However, RV7 failed to replicate in target organs of immunocompetent BALB/c mice and severe combined immunodeficient mice, which are exquisitely sensitive to MCMV infection. This defect in in vivo growth may be related to the observation that RV7 grew poorly in the peritoneal macrophage cell line IC-21, which is highly permissive for growth of WT MCMV. Two other mutant viruses with an insertion or smaller deletion in the region common to the RV7 deletion grew comparably to WT virus in the macrophage cell line and replicated in salivary gland tissue. The poor growth of RV7 in IC-21 cells was due to a block in immediate-early gene expression, as levels of RNA from immediate-early gene IE1 were reduced eightfold compared with levels for WT virus in macrophages infected with RV7. Consequently, levels of RNA from early and late genes were also reduced. The lower expression of IE1 in RV7-infected IC-21 macrophages was not due to defective entry of virus into the cells, as equal amounts of viral DNA were present in cells 3 h after infection with RV7 or WT MCMV. These studies demonstrate that deletion of sequences in *Hin*dIII-J and -I confer altered cell and tissue tropism.

Cytomegalovirus (CMV) is a significant human pathogen in immunocompromised individuals, causing severe and often life-threatening illness (1). Individuals at high risk for CMV disease include the immunologically immature fetus and newborn, patients undergoing immunosuppressive therapy for cancer or organ transplantation (24), and those with progressive immunodeficiency diseases, such as AIDS (20). Strict species specificity has hindered the study of human CMV (HCMV) in animals, and infection of mice with murine CMV (MCMV) has been used extensively as a model for studying the pathogenesis of CMV infection (8, 34, 46, 57). The human and murine viruses are biologically similar in replication and pathogenesis (25, 45) and have homologous genomes (52), which display similar genetic organizations and encode analogous gene products with similar functions (19, 31, 43, 44, 50, 51, 68). The genomes of HCMV and MCMV are linear, double-stranded DNA molecules approximately 230 kb in length and encode more than 200 genes (10, 52), many of which are arranged collinearly on the HCMV and MCMV genomes.

To better understand the pathogenic mechanisms of CMV infection, viral gene products involved in modulating interactions between the virus and its host must be identified and characterized. Many genes in HCMV and MCMV are nonessential for virus replication in cultured cells in vitro (9, 28, 29, 35, 39, 65). These genes likely have specific functions in vivo and contribute to viral pathogenesis. Nonessential virus genes may influence virus-host interactions in vivo by encoding pathogenic factors, such as those governing virulence (11, 42, 64), persistence, latency (55), immune evasion (21, 22, 27, 63, 69), tissue tropism (35), dissemination, and transmission. It is unlikely that nonessential genes would be conserved in the virus if they were not purposeful in the intact host. Infection of mice with MCMV carrying mutations in nonessential genes offers the ideal opportunity to examine the functions of these genes during pathogenesis in the natural host.

The genes encoded within one nonessential region of the MCMV genome, the *Hin*dIII J fragment, have been identified by transcript mapping and nucleotide sequence analysis (65). The *Hin*dIII-J region contains seven open reading frames (ORFs), each shown by insertion mutagenesis to be nonessential for MCMV replication in NIH 3T3 cells (9, 65). This region of the viral genome encodes eight transcripts; however, the products of only three of these transcripts have been characterized to date (35, 62). Two transcripts, expressed from the same MCMV gene (*sgg1*), are overlapping and encode a 37-kDa protein involved in virus replication in the salivary gland (35). Another transcript, expressed from the MCMV *fcr1* gene, encodes an Fc receptor for murine immunoglobulin G and produces glycoproteins of 86 to 88 and 105 kDa (62). The five remaining transcripts encoded within the *Hin*dIII J fragment are uncharacterized in terms of the protein products that they encode and their functions during MCMV infection. The three

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largest transcripts encoded by the *Hin*dIII J fragment are overlapping, have the same polyadenylation signal, and initiate in the adjacent *Hin*dIII-I region of the MCMV genome (62, 65). Presently, little else is known of the transcripts or viral gene products expressed from the *Hin*dIII-I region.

The purpose of this study was to identify nonessential genes of MCMV and characterize their roles in virus-host interactions. The *Hin*dIII J and I fragments of the MCMV genome were targeted for mutagenesis in this study because they correspond in map units to a dispensable cluster of 14 genes in the unique short component of HCMV strain AD169 (28, 29). Our strategy was to generate large deletions of MCMV in order to disrupt the expression of multiple MCMV gene products simultaneously, as more than one gene product may act cooperatively to produce a detectable phenotype (27). Our data indicate that the nonessential region from *Hin*dIII-J extends into *Hin*dIII-I up to 0.866 map units. One mutant, recombinant virus 7 (RV7), with a deletion of 7.7 kb of viral sequences spanning *Hin*dIII-J and -I, failed to replicate in immunocompetent and severe combined immunodeficient (SCID) mice in spite of its ability to replicate in primary fibroblasts and bone marrow macrophages in vitro. However, RV7 grew poorly in a peritoneal macrophage cell line, which is fully permissive for wild-type (WT) virus, as a result of a block in immediate-early IE gene (IE gene) expression. These studies, therefore, have identified a nonessential region of MCMV *Hin*dIII-J and -I whose gene products regulate cell and tissue tropism.

MATERIALS AND METHODS

Cells. Murine NIH 3T3 fibroblasts (American Type Culture Collection [ATCC] CRL1658; ATCC, Rockville, Md.) were propagated in Dulbecco's modified essential medium (DMEM; Mediatech, Herndon, Va.) supplemented with 10% heat-inactivated bovine calf serum (HyClone Laboratories, Logan, Utah) and 1% L-glutamine (Gibco/BRL, Grand Island, N.Y.). IC-21 cells, a simian virus 40-transformed peritoneal macrophage cell line derived from C57BL/6 mice (41), were obtained from ATCC and were propagated in RPMI medium (Mediatech) supplemented with 10% heat-inactivated fetal calf serum (Gibco/BRL) and 1% L-glutamine. Primary fibroblasts, derived from the embryos of timed-pregnant BALB/c.ByJ mice (Jackson Laboratory, Bar Harbor, Maine) on day 19 of gestation, were cultured in DMEM containing 20% heat-inactivated fetal calf serum, 1% L-glutamine, and 50 μg of gentamicin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml.

Virus. The parental WT MCMV used in this study was of the Smith strain (ATCC VR-194). Stocks of WT and mutant viruses were prepared in and titers were determined on NIH 3T3 cells as previously described (8). Mock preparations of virus consisted of culture supernatants from noninfected NIH 3T3 cells.

Mice. BALB/c.ByJ mice, either 20-day-old weanlings (males) or timed-pregnant animals, were purchased from Jackson Laboratory and were housed in microisolator cages with sterile food, water, and bedding. Adult female BALB/c mice were used to harvest bone marrow cells. CB17 SCID mice were bred and housed as previously described (23).

Plasmid constructs. Plasmid cloning was conducted by standard methods (38). All restriction endonucleases were purchased from Promega (Madison, Wis.), and digestions were done according to the manufacturer's suggestions, using supplied buffers.

A genomic library of *Hin*dIII-digested MCMV DNA was cloned into the plasmid vector pGEM-4Z (Promega). The *Hin*dIII-F, -I, -J, and -L genomic clones were used in this study.

The plasmid designated pe1/βglu (Fig. 1A) was constructed in the pGEM-7Z vector (Promega) and contains the β-glucuronidase (β-glu) reporter gene under the control of the MCMV early region 1 (e1) promoter. This e1/β-glu cassette contains 2.2 kb of the β-glu gene (derived by digestion of plasmid pgHBg10pA [kindly provided by Thomas Jones, American Cyanamid Co., Pearl River, N.Y.] with *Nco*I and *Sst*I), coupled to 0.2 kb of the e1 promoter. The e1 promoter was derived from the 5.5-kb *Pst*I fragment of the MCMV *Hin*dIII-F genomic clone, which contains the entire e1 transcription unit (7). The promoter consists of an *Sst*I-to-*Nco*I fragment containing the first 200 nucleotides upstream of the start site of transcription (which is located at the *Nco*I site). The entire 2.4-kb e1/β-glu cassette can be removed from pe1/βglu by digestion with *Sst*I.

A recombination plasmid, designed to generate an insertion mutation within the MCMV *Hin*dIII-J genomic clone and designated pJβglu-5 (Fig. 1C), was constructed by inserting the e1/β-glu cassette into the second *Sst*I site within *Hin*dIII-J. Recombination plasmids, developed to generate deletion mutations within the MCMV *Hin*dIII-J and -I genomic clones and designated pJβglu-6, pJIβglu-7, and pJIβglu-9 (Fig. 1C), were constructed by deleting selected genomic sequences and replacing them with the e1/β-glu cassette. To facilitate the generation of these recombination vectors, a plasmid (designated p3.4Jβglu; Fig. 1B) that contains only the first 3.4 kb of MCMV sequences from *Hin*dIII-J coupled to the e1/β-glu cassette was constructed. This plasmid was generated by completely digesting MCMV *Hin*dIII-J with *Sst*I, isolating the large 6.3-kb band (consisting of vector sequences and the first 3.4 kb of *Hin*dIII-J sequences), and cloning the e1/β-glu cassette into the *Sst*I site. Plasmid p3.4Jβglu was then partially digested with *Sst*I, and linearized molecules were ligated to other various *Sst*I-digested fragments from *Hin*dIII-J or -I. pJβglu-6, pJβglu-7, and pJβglu-9 were constructed as indicated in Fig. 1.

Isolation of MCMV DNA. Intact, infectious MCMV DNA was prepared from virions pelleted from a WT virus stock by sorbitol cushion density gradient centrifugation as previously described (29).

Construction of recombinant MCMV. Recombinant MCMV was generated by cotransfecting infectious MCMV DNA and linearized recombination plasmids of interest into NIH 3T3 cells. A total of 7 μg of DNA (3 μg of MCMV DNA and 4 μg of linearized plasmid DNA) was transfected into 5×10^5 cells in suspension, using a standard calcium phosphate precipitation technique (2). Blue plaques, identified by the addition of substrate for the β-glu enzyme (5-bromo-4-chloro-3-indolyl-β-D-glucuronide; Biosynth Inc., Zurich, Switzerland) underwent at least five rounds of plaque purification before a high-titer stock of each recombinant virus was prepared.

Southern blot analysis. DNA was prepared from mock-, WT-, or RV-infected NIH 3T3 cells as previously described (59). Southern blotting was conducted as described by Maniatis et al. (38). Probes of MCMV *Hin*dIII-J and -I were prepared from gel-purified fragments (8.1 and 9.7 kb, respectively) following *Hin*dIII digestion of these genomic clones. A probe of the β-glu gene was prepared from the gel-purified 2.2-kb *Nco*I-to-*Sst*I fragment of plasmid pe1/βglu. The Boehringer Mannheim Genius system was used for hybridization, with probes labeled with digoxigenin by random priming and chemiluminescence detection using Lumi-Phos 530.

Sequencing and identification of potential ORFs. The complete DNA sequence of MCMV Smith strain was determined by using a whole-virion shotgun cloning approach as has been described elsewhere (15, 65). Sucrose gradient-purified virion DNA was cloned into M13 by using standard techniques, and the sequencing reactions were performed by standard dideoxy-chain termination methods (5). The DNA sequence was generated by using both fluorescent and radioactive labels (56), with single-stranded templates (from M13) and double-stranded templates generated by PCR. Sequences were assembled by using the Staden package on a Sun computer (Sun Microsystems, Mountain View, Calif.) (16, 17). Potential ORFs within *Hin*dIII-I were identified by criteria used to study the entire genome. Initial analysis was performed with the programs NIP (58), acedb (18), and DIANA (13). This analysis identified ORFs with lengths of at least 300 nucleotides and overlap of less than 60%, and it was assumed that the longest ORF was coding and that mRNA splicing did not occur.

In vitro growth of MCMV mutants. Cells were infected at a multiplicity of 0.1 PFU (for multistep growth curves) or 5 PFU (for single-step growth curves) per cell with either WT or mutant MCMV. At the indicated day postinfection, virus was harvested from both cells and supernatant, and infectious virus was quantitated by standard plaque assay on NIH 3T3 cells. The limit of detection was 10 PFU/ml.

Growth of MCMV in bone marrow macrophages. Bone marrow macrophages were generated by culturing bone marrow cells (10^7 cells per 10-cm-diameter non-tissue culture-treated dish; Sarstedt, Newton, N.C.) in low-endotoxin DMEM (Biofluids, Rockville, Md.) containing 2 mM L-glutamine, 10% fetal calf serum (HyClone), 5% low-endotoxin horse serum (Sigma), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 20% L-cell conditioned medium (LCM) for the first 7 days or 10% LCM thereafter (4). LCM was made by culturing NCTC clone 929 fibroblasts (ATCC CCL 1) in DMEM containing 10% fetal calf serum for 7 to 10 days.

Bone marrow macrophages were harvested by incubation in low-endotoxin phosphate-buffered saline-EDTA (Sigma) on ice for 15 min followed by scraping. Cells in suspension and on ice were infected with MCMV or RV7 for 1 h at a multiplicity of 0.05 PFU per cell. Cells were then washed and cultured in dishes (6 by 6 cm; 10^6 cells per dish) in bone marrow macrophage medium at 37°C in 5% CO_2. At 0, 2, and 5 days after infection, viral titers in duplicate dishes were determined by plaque assay as described previously (23).

In vivo growth of MCMV mutants. BALB/c.ByJ mice, at 21 days of age, were injected intraperitoneally with 1.5×10^5 PFU of tissue culture-passaged virus. At the indicated times postinoculation, submaxillary salivary glands, spleens, livers, and lungs were harvested as a 20% (wt/vol) tissue homogenate, and titers were determined on NIH 3T3 cells by standard plaque assay. The limit of detection was 10 PFU/ml of tissue homogenate.

Adult CB17 SCID mice (three mice per group) were infected intraperitoneally with 10^4 PFU of tissue culture-passaged MCMV or RV7. Ten days later, spleens were harvested and virus titers were determined as previously described (23). To compare levels of lethality of WT MCMV and RV7 in SCID mice, animals (eight per group) were inoculated intraperitoneally with 7.8×10^5 PFU of either WT MCMV or RV7 and observed daily for survival.

Northern (RNA) blot analysis. Cells were mock infected or infected with WT or mutant MCMV at a multiplicity of 4 PFU per cell. To assess predominantly immediate-early transcription, total RNA was harvested from cultures at 3 h

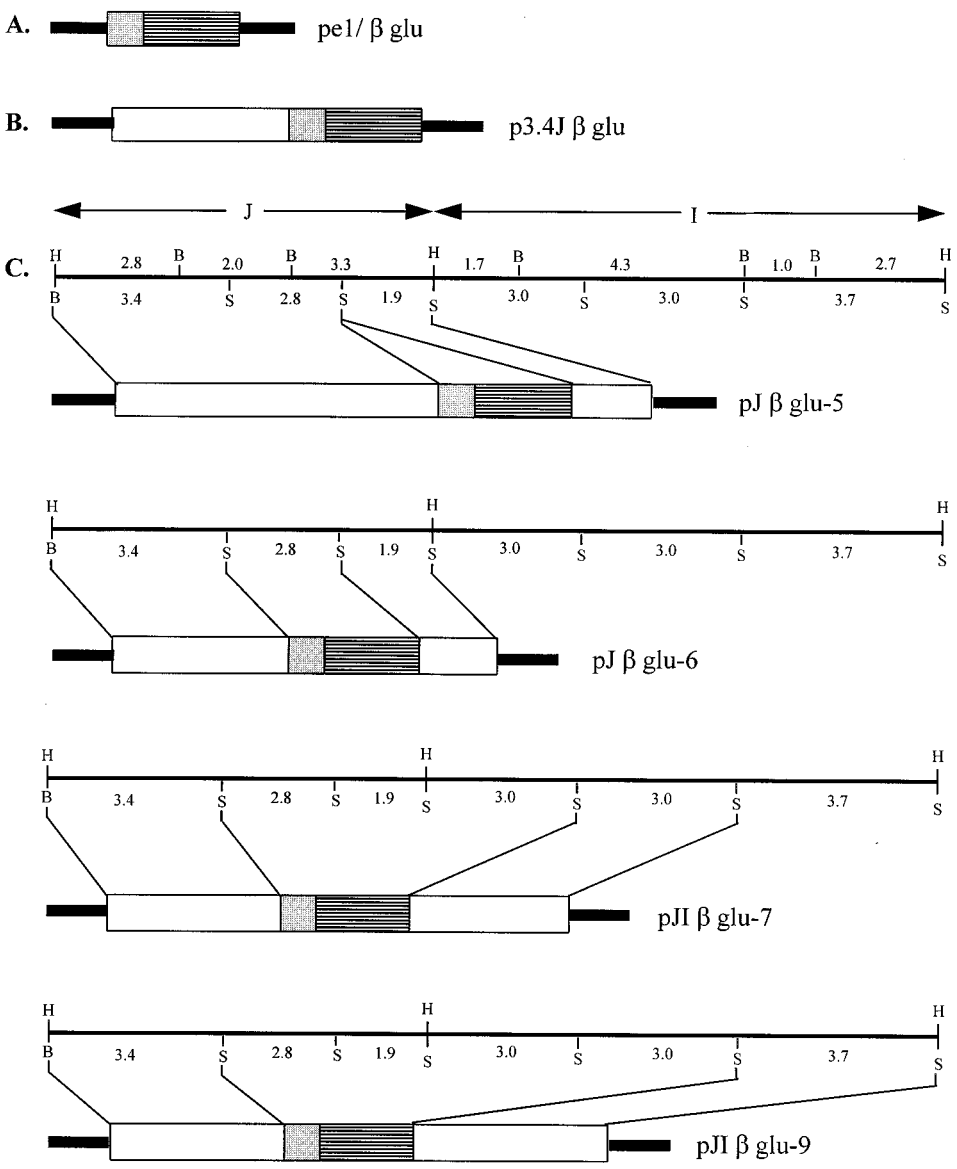

FIG. 1. Plasmids used to generate MCMV recombinant viruses. (A) Plasmid pe1/βglu carries an *Sst*I-to-*Nco*I fragment (0.2 kb) from the MCMV e1 promoter (shaded box) coupled to the *Nco*I-to-*Sst*I fragment (2.2 kb) of the β-glu gene (striped rectangle). This 2.4-kb e1/β-glu cassette was cloned into the *Sst*I site of the plasmid vector pGEM-7Z (solid rectangles). (B) Plasmid p3.4Jβglu contains the first 3.4 kb of MCMV *Hin*dIII-J sequences (open rectangle) coupled to the e1/β-glu cassette. The solid rectangles represent vector sequences of plasmid pGEM-4Z. (C) Organization of recombination plasmids. The thin horizontal lines represent an expansion of the MCMV *Hin*dIII J and I fragments (indicated by opposing arrows), showing restriction endonuclease sites for *Bgl*II (B), *Hin*dIII (H), and *Sst*I (S), as well as the distance between these sites (indicated in kilobases). The vertically slanted lines illustrate those MCMV sequences (open rectangles) used to construct the indicated recombination plasmids. Vector sequences of pGEM-4Z are shown as solid rectangles. Recombination plasmid pJβglu-5 carries the e1/β-glu cassette inserted at the second *Sst*I site in *Hin*dIII-J. The remaining recombination plasmids were constructed by using p3.4Jβglu opened at the 3′ end of the β-glu gene.

postinfection. For early transcription, phosphonoacetic acid was added to the culture medium at a concentration of 250 μg/ml at the time of virus infection for 24 h prior to total RNA isolation. For late RNA (L RNA) isolation, total RNA was harvested from cultures at 24 h after infection. The Qiagen RNeasy kit for total RNA isolation (Qiagen, Chatsworth, Calif.) was used to harvest RNA from infected cells as described by the manufacturer. Northern blot analysis was as described by Maniatis et al. (38). The Boehringer Mannheim Genius system was used for hybridization and detection as described for Southern blots.

IE1 and IE2 RNAs were detected by using an MCMV *Hin*dIII-L probe prepared from the gel-purified 7.3-kb fragment following *Hin*dIII digestion of this MCMV genomic clone. The early RNAs (E RNAs) were detected by using a probe of the e1 gene, prepared from the 1.4-kb *Nco*I-to-*Eco*RI fragment derived from the 5.5-kb *Pst*I fragment of MCMV *Hin*dIII-F. The L RNAs were detected by using a probe of the glycoprotein B (gB) gene, prepared from the 3.5-kb *Bam*HI-to-*Pvu*II fragment derived from plasmid pLig3.5 (58) (kindly provided by Ulrich Koszinowski, University of Heidelberg, Heidelberg, Germany).

Intensities of the 2.75-kb IE1 RNA transcript, the 2.60-kb e1 transcript, and the 3.30-kb gB transcript were estimated by densitometric scanning of autoradiographs. A Sun UNIX-SPARC station 5 densitometer (Sun Microsystems), equipped with an OmniMedia (Torrance, Calif.) 12cx-XRS scanner, was used to scan and analyze autoradiographs. The Bio-Image (Ann Arbor, Mich.) whole-band analyzer program was used to quantitate the integrated intensities of these transcripts.

RESULTS

Generation of recombinant MCMV. Using the recombination plasmids illustrated in Fig. 1C in cotransfections with MCMV DNA, four MCMV mutants were generated: RV5, RV6, RV7, and RV9 (Fig. 2). RV5 was generated by using the recombination plasmid pJβglu-5 and contains an insertion mutation in which the e1/β-glu cassette was inserted at the second *Sst*I site within *Hin*dIII-J. RV6 was constructed by using the

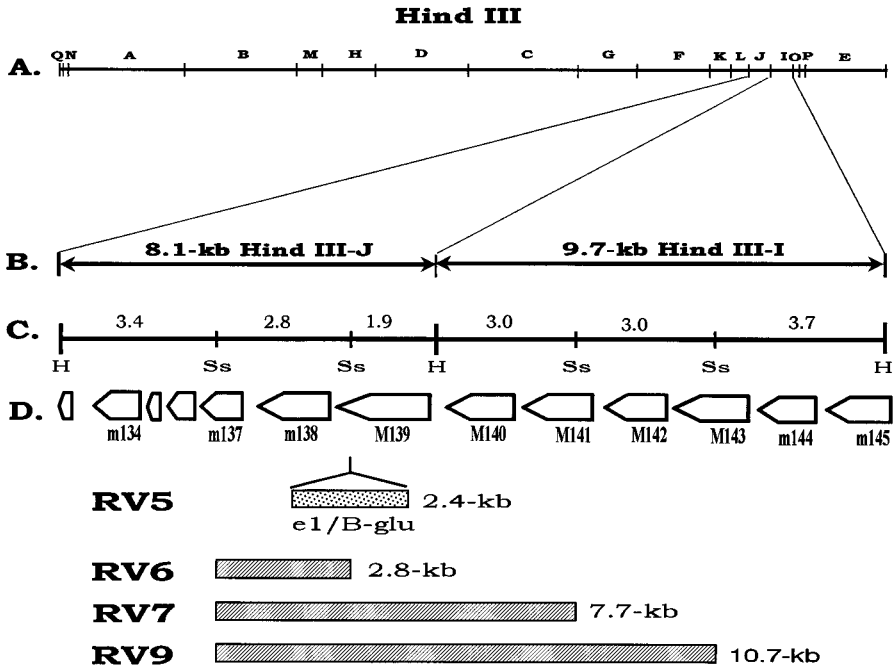

FIG. 2. Insertion and deletion mutants of MCMV *Hin*dIII-J and -I. (A) *Hin*dIII restriction map of the WT Smith strain MCMV genome, which is 230 kb in length. (B) Expansion of the *Hin*dIII-J and -I regions, showing the locations of *Hin*dIII (H) and *Sst*I (Ss) sites and fragment sizes in kilobase pairs. (C) Potential ORFs within *Hin*dIII-J and -I. ORFs assigned to *Hin*dIII-J have been published (65). The M prefix denotes ORFs with sequence homology to HCMV; the m prefix denotes sequence unique to MCMV. (D) Insertion and deletion mutants of MCMV *Hin*dIII-J and -I. The numbers following the rectangles indicate the size of the e1/β-glu cassette (stippled rectangle) or of the deletions (hatched rectangles) in kilobase pairs.

recombination plasmid pJβglu-6 and contains a deletion mutation in which 2.8 kb of viral sequences within *Hin*dIII-J were deleted and replaced with the e1/β-glu cassette. RV7 was generated with the recombination plasmid pJIβglu-7 and contains a deletion mutation missing 7.7 kb of MCMV sequences, 4.7 kb from *Hin*dIII-J and 3.0 kb from *Hin*dIII-I. RV9 is a deletion mutant, constructed by using the recombination plasmid pJIβglu-9, which lacks 10.7 kb of MCMV sequences, 4.7 kb from *Hin*dIII-J and 6.0 kb from *Hin*dIII-I.

Each RV was subjected to at least five rounds of plaque purification before a high-titer virus stock was prepared. Southern blot analyses were then conducted with DNA isolated from RV-infected NIH 3T3 cells to (i) verify each targeted mutation to the MCMV genome and (ii) determine if any contaminating WT virus was present in the RV stocks. Figure 3 illustrates the results of these Southern blot analyses using probes to *Hin*dIII-J, *Hin*dIII-I, and the β-glu gene. For all four mutants, the sizes of the recombinant bands were exactly those predicted from the *Hin*dIII and *Bgl*II restriction maps of the *Hin*dIII-J and -I regions, verifying that the recombination events occurred properly. In addition, overexposures of the Southern blots of RV5, RV6, and RV7 failed to reveal the presence of WT bands, indicating that the stocks of these mutant viruses were pure and free of any WT MCMV. In contrast, Southern blot analysis of RV9 clearly revealed the presence of WT bands in addition to the recombinant bands. It was evident from these blots that approximately one-half of the viral DNA isolated from cells infected with RV9 was WT MCMV DNA. Three independent attempts were made to further purify the RV9 stock but were unsuccessful. This finding suggested that RV9 is a defective mutant whose replication requires the presence of complementing WT MCMV. Because the isolation of a pure RV9 stock was not possible, this mutant was not included in subsequent experiments described below.

Identification of ORFs within *Hin*dIII-I. ORFs within *Hin*dIII-J were as reported previously (65). Analyses of potential protein-coding regions within MCMV *Hin*dIII-I identified six ORFs (Fig. 2D), four of which (M140, M141, M142, and M143) have homology with the US22 gene family of HCMV (67) and human herpesvirus 6 (47). The data are consistent with independent transcription data showing that the three largest transcripts mapped to MCMV *Hin*dIII-J initiate in *Hin*dIII-I and are transcribed in a leftward direction (62, 65).

In vitro growth of MCMV mutants in NIH 3T3 fibroblasts. To assess whether MCMV *Hin*dIII-J or -I sequences have a role in the growth of virus in cell culture, the growth rates of WT and mutant viruses were determined in murine NIH 3T3 fibroblasts. Figure 4 represents multistep growth curves of the MCMV mutants in these cells. The results demonstrate that the growth rate of each RV in NIH 3T3 fibroblasts was similar to that of WT MCMV, confirming that the mutations introduced into these viruses do not negatively affect MCMV replication in cultured fibroblasts.

In vivo growth of MCMV mutants. To assess the ability of recombinant MCMV to replicate in vivo, the growth of each mutant virus was determined in the salivary glands, spleens, livers, and lungs of infected mice. In the salivary glands (Fig. 5), the replication of RV5 was significantly less than that of WT MCMV, reaching a titer at 14 days postinoculation that was 2 log units lower than that of WT virus. The replication of RV6 in the salivary glands was even lower than that of RV5, reaching levels that were 4 log units less than that observed for WT virus. And most dramatically, RV7 could not be detected by standard plaque assay of salivary gland homogenates.

To determine if the growth of these mutant viruses in the salivary gland was perhaps delayed, virus titers in this organ were determined at 2, 4, and 6 weeks after infection. The results at 2 weeks postinoculation were similar to those shown

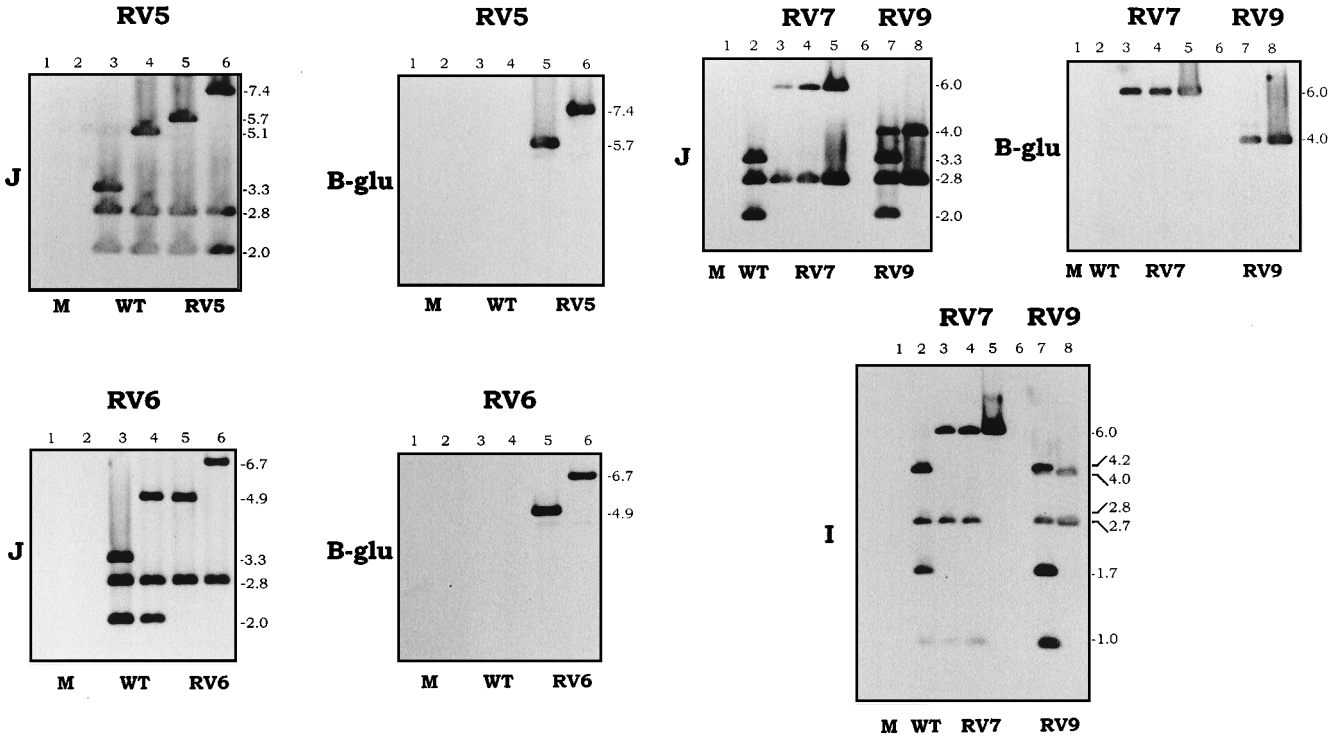

FIG. 3. Southern blot analyses of recombinant MCMV. DNA was isolated from NIH 3T3 cells that were mock-infected (M) or infected with WT virus or the indicated RV for 24 h. DNA probes of MCMV *Hin*dIII-J (J), *Hin*dIII-I (I), and the β-glu gene were used. For RV5 and RV6, the odd-numbered lanes indicate digestion with *Hin*dIII and *Bgl*II, and the even-numbered lanes indicate digestion with *Bgl*II alone. For RV7 and RV9, the infected cell DNAs were digested with *Hin*dIII and *Bgl*II. For RV7, lanes 3 and 4 represent DNA isolated from cells infected with independent isolates of this mutant, and lane 5 represents the recombination plasmid used to generate RV7. RV9-infected cell DNA is represented in lane 7, and its recombination plasmid is represented in lane 8. Sizes of the DNA fragments are indicated in kilobase pairs.

in Fig. 5. By 6 weeks postinoculation, the titers for all viruses (WT and mutant) were no longer detectable by plaque assay, indicating that these mutants do not display delayed kinetics for salivary gland growth. Instead, it is evident that the genes deleted in these viruses are required for the efficient dissemination to or replication in the salivary gland.

In the spleens of infected mice, low levels of WT MCMV could be detected on days 7 (average of $10^{2.0}$ PFU/ml of homogenate) and 10 (average of $10^{1.1}$ PFU/ml). However, none of the mutant viruses could be detected in the spleen at any of the four time points examined. In the lungs, WT virus was detected only on day 10 postinoculation, at a titer of $10^{3.0}$ PFU/ml of homogenate. Again, none of the mutant viruses were detected by plaque assay of lung homogenates. In the livers of infected animals, neither WT nor mutant MCMV was detected in this experiment. Although the MCMV mutants did not replicate to detectable levels in the spleens, lungs, or livers of infected mice, the levels of parental, tissue culture-passaged WT virus detected in these organs were low, making any valid comparisons between WT and mutant MCMV growth in these tissues difficult.

Because tissue culture-passaged MCMV is greatly attenuated for growth in target organs (other than the salivary gland) in vivo (48), the relative in vivo attenuation of the mutant viruses compared with that of WT virus could not be determined in the above-described experiments using normal, immunocompetent mice. Therefore, growth of RV7 was compared with that of WT MCMV in immunodeficient CB17 SCID mice, which lack functional T and B lymphocytes. SCID mice are sensitive to extremely low levels of MCMV replication, as these animals succumb to 2 to 3 PFU of tissue culture-derived WT MCMV (49).

The results shown in Fig. 6 indicate that indeed, infection with parental WT MCMV resulted in 100% mortality by day 21 postinoculation. In contrast, infection with the same dose of RV7 produced no mortality in these immunodeficient mice. Because RV7 did not cause lethal infection over the period of

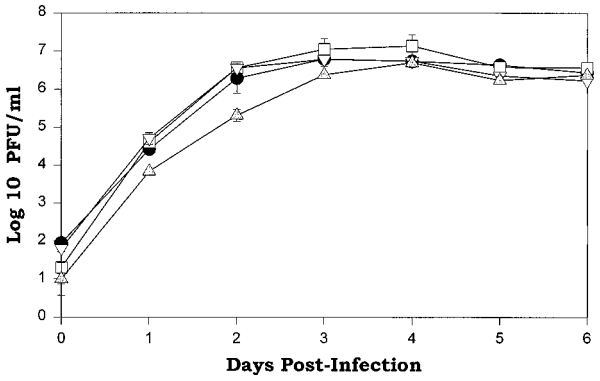

FIG. 4. In vitro growth of MCMV mutants in NIH 3T3 fibroblasts. Multistep growth curves of each RV were determined in NIH 3T3 fibroblasts as described in the Materials and Methods. Each datum point represents the average of two separate cultures, and the standard deviation of the geometric mean is indicated by the error bars. Error bars not evident indicate that the standard deviation was less than or equal to the height of the symbols. ●, WT MCMV; □, RV5; ▽, RV6; △, RV7.

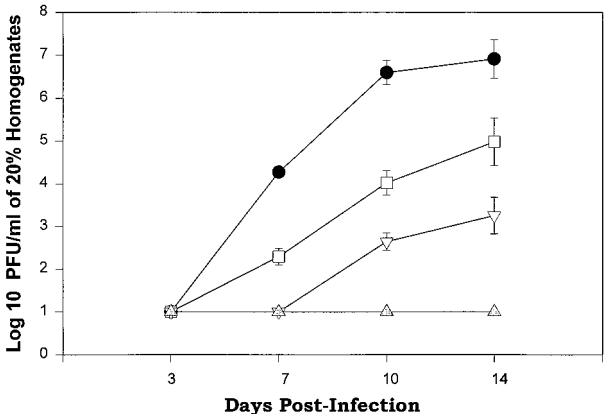

FIG. 5. Growth of MCMV mutants in the salivary glands of infected mice. At the indicated days postinoculation, submaxillary salivary glands were harvested and titered as described in Materials and Methods. Each datum point represents the average of three animals, and the standard deviation of the geometric mean is indicated by the error bars. Error bars not evident indicate that the standard deviation was less than or equal to the height of the symbols. ●, WT MCMV; □, RV5; ▽, RV6; △, RV7.

observation, two separate experiments were performed to quantitate virus in the spleens of SCID mice 10 days postinfection with WT MCMV or RV7. Infection with WT MCMV yielded spleen titers of $10^{2.9}$ ($\pm 10^{0.2}$ standard error of the mean) PFU/ml. However, titers of RV7 were undetectable (less than $10^{1.5}$ PFU/ml). Hence, RV7 MCMV is defective with respect to replication in its natural host.

Growth of RV7 in primary murine cell lines. Because RV7 failed to replicate to detectable levels in SCID mice, it was possible that this mutant is defective for growth in primary murine cells (as opposed to NIH 3T3 cells). To examine this possibility, the growth of RV7 was determined in primary embryo fibroblasts and bone marrow macrophages derived from BALB/c mice, the same strain or *H-2* haplotype that was used in the in vivo studies. In primary fibroblasts, RV7 grew as efficiently as WT virus (Fig. 7), indicating that this large deletion in the MCMV genome did not affect virus growth in primary fibroblasts. In macrophages differentiated from bone marrow cells, the growth of WT MCMV and that of RV7 were not significantly different (Fig. 8), indicating that the deletion also did not influence virus growth in primary macrophages. Collectively, these data demonstrate that the drastically reduced growth of RV7 in vivo was not the result of a defect for growth in primary murine cells per se.

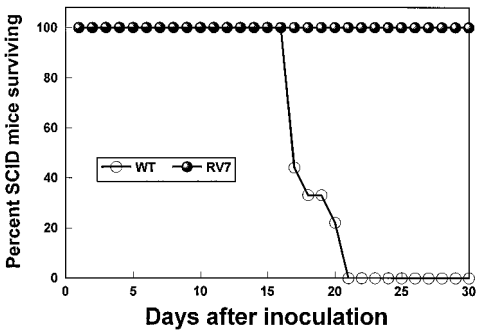

FIG. 6. Attenuation of RV7 in SCID mice. SCID mice were inoculated intraperitoneally with 7.8×10^5 PFU or either WT MCMV or RV7 and monitored daily for survival.

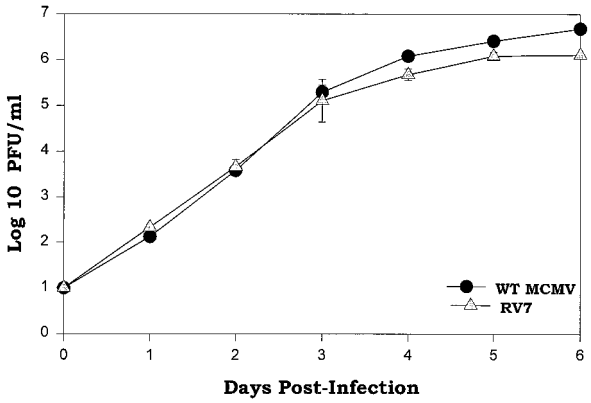

FIG. 7. Growth of RV7 in primary embryo fibroblasts. Multistep growth curves of WT MCMV and RV7 were determined in fibroblasts derived from 19-day-old BALB/c embryos as described in the legend to Fig. 4.

In vitro growth of MCMV mutants in IC-21 macrophages. During a natural MCMV infection, monocytes and macrophages play critical roles in viral pathogenesis, particularly in dissemination and latency (12, 33, 60). The state of macrophage differentiation clearly influences permissiveness for MCMV replication (6, 36, 46). Therefore, as a complement to the studies described above, growth of WT MCMV and that of mutants were compared in the fully permissive peritoneal macrophage cell line IC-21 (41). Figure 9A shows multistep growth curves of the mutant viruses in this macrophage cell line. RV5 and RV6 grew similarly to WT virus; however, the growth of RV7 in these cells was significantly reduced, by as much as 1,000-fold.

In this type of growth curve, the yield of virus at each time point depends on both the rate of virus replication and virus spread from cell to cell. The reduced growth rate of RV7 in IC-21 macrophages could be the result of a defect in virus replication, a defect in cell-to-cell spread, or defects in both replication and spread. To distinguish among these possibilities, a single-step growth curve of RV7 in these macrophages was determined (Fig. 9B). In this experiment, the cultures were infected with 5 PFU per cell to ensure that all of the cells were infected simultaneously such that effects of cell-to-cell spread are eliminated. Again, the growth of RV7 was lower than that of WT MCMV. The yield of WT virus was 100-fold greater than the input titer; the yield of RV7 was 32-fold greater than

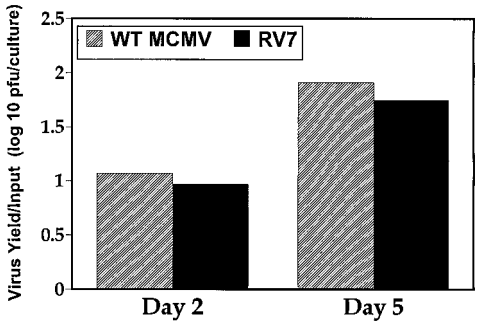

FIG. 8. Growth of WT MCMV and RV7 in bone marrow macrophages. Bone marrow macrophages were infected with either WT MCMV or RV7 and harvested at 0, 2, and 5 days postinfection as described in Materials and Methods. Data are presented as the $\log_{10}$ yield per input PFU, obtained by dividing the titer at 2 or 5 days by the titer at time zero. Data are the means of two experiments.

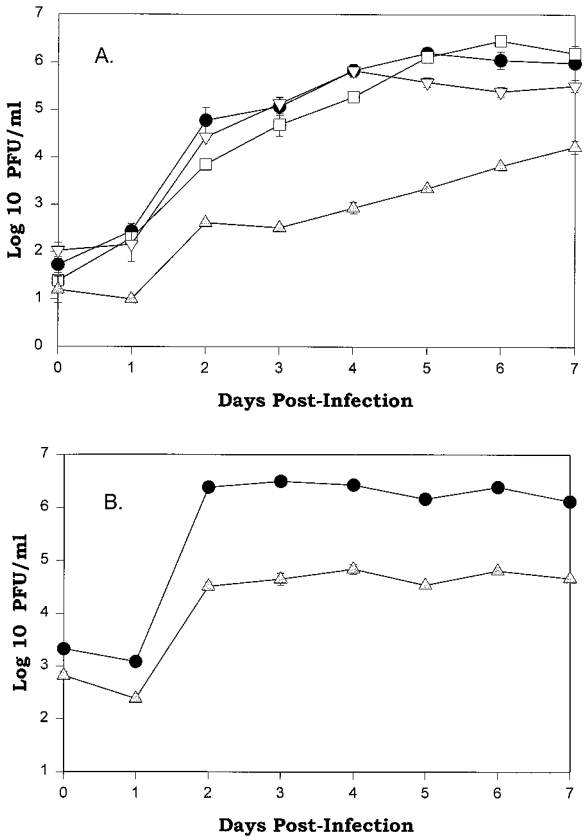

FIG. 9. In vitro growth of MCMV mutants in IC-21 macrophages. Multistep (A) and single-step (B) growth curves of each RV were determined in IC-21 macrophages as described in Materials and Methods. Each datum point is as described in the legend to Fig. 4. (A) ●, WT MCMV; □, RV5; ▽, RV6; △, RV7. (B) ●, WT MCMV; △, RV7.

the input titer. The reduction in RV7 growth compared with WT virus growth in this one-step growth curve was less than that observed in the previous multistep growth curve (Fig. 9A). These data indicate that the reduced growth of this mutant in IC-21 macrophages is due to a defect in both virus replication and cell-to-cell spread. In addition, this experiment demonstrates that the reduced growth of RV7 in these macrophages is not merely the result of a slower replication rate, as WT MCMV and RV7 reached their peak levels by 2 days postinfection and then remained at these levels for the duration of the experiment (Fig. 9B). It is noteworthy that RV7 grew comparable to WT virus in a simian virus 40-transformed fibroblast cell line (30).

Analysis of IE, E, and L gene expression in RV7-infected IC-21 cells. To further investigate the defect in RV7 replication in IC-21 macrophages, Northern blot analyses were conducted to determine at which stage of the replication cycle the block in growth occurred. Total RNA was isolated from infected NIH 3T3 and IC-21 cells under predominantly immediate-early, early, or late conditions and was hybridized to probes of known IE, E, or L genes, respectively (Fig. 10). Figure 10A is a Northern blot of IE RNAs isolated at 3 h postinfection and hybridized to a probe of the IE1 and IE2 genes (both contained within the *Hin*dIII L fragment). Levels of IE1 gene expression (represented by the 2.75-kb transcript) in RV7-infected IC-21 macrophages were much lower than levels observed in WT- or RV6-infected cells. Densitometric scanning of the autoradiograph revealed an eightfold reduction in the levels of the 2.75-kb IE1 transcript in RV7-infected cells compared with WT MCMV-infected cells (integrated intensities of 10.25 for RV7 and 82.67 for WT MCMV). In contrast, there were no significant differences in IE1 gene expression among WT and mutant viruses in infected NIH 3T3 cells (integrated intensities of 37.24, 32.56, and 37.96 for WT-, RV6-, and RV7-infected cells, respectively). This reduction in RV7 IE1 gene expression in IC-21 macrophages is consistent with the reduced growth of this mutant virus in these cells and suggests that a block in RV7 replication occurs at or before the immediate-early phase of virus replication.

Because subsequent virus gene expression in permissive cells is dependent on IE gene products, one consequence of reduced IE gene expression would be reduced E and L gene expression as well. Therefore, E and L gene expression was also investigated by Northern blot analyses in RV7-infected IC-21 macrophages. Total RNA was isolated from infected NIH 3T3 and IC-21 cells under early conditions and was hybridized to a probe of the e1 gene (Fig. 10B), which produces two transcripts of 2.6 and 3.0 kb. Expression of the 2.6-kb e1 transcript in RV7-infected IC-21 macrophages was reduced more than sixfold compared with expression in WT-infected cells (integrated intensities of 2.39 for RV7 and 14.62 for WT MCMV). In contrast to IC-21 cells, there were no significant differences in e1 expression between WT virus and RV7 in

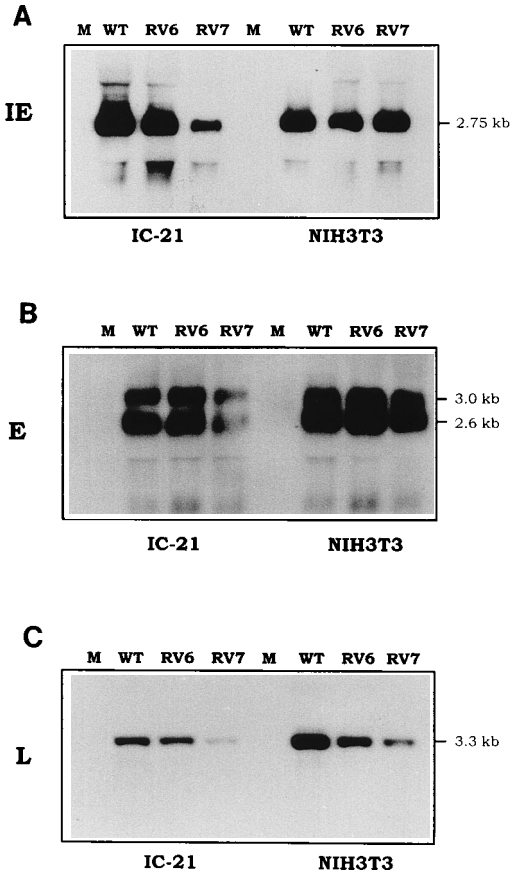

FIG. 10. Northern blot analyses of IE, E, and L RNAs from RV7-infected cells. Total RNA was isolated from infected cells under immediate-early, early, or late conditions and hybridized to probes of known IE, E, or L genes, respectively, as described in Materials and Methods. In all blots, 5 μg of total RNA per lane was analyzed. Sizes of the RNA transcripts are indicated on the right.

infected NIH 3T3 fibroblasts (integrated intensities of 24.41 for WT MCMV and 20.49 for RV7).

To examine L gene expression, total RNA was isolated at late times and hybridized to a probe of the gB gene (Fig. 10C). In IC-21 macrophages, the levels of gB transcripts (migrating at 3.3 kb) were more than 20-fold lower in RV7-infected cells than in WT-infected cells (integrated intensities of 0.27 for RV7 and 5.47 for WT MCMV). In NIH 3T3 fibroblasts, the levels of gB transcripts in RV7-infected cells were also reduced, but less than fivefold, compared with levels in WT-infected cells (integrated intensities of 2.44 for RV7 and 11.69 for WT MCMV). The reason for this reduction in RV7-infected NIH 3T3 cells is unknown but may be related to the smaller plaque size consistently observed with RV7 infection in these cells (data not shown). Nevertheless, the reduced L gene expression evident in RV7-infected IC-21 macrophages correlates with the significantly reduced levels of IE and E gene expression and suggests that poor replication of RV7 in these cells is due to inadequate IE gene expression.

Analysis of RV7 entry in macrophages. A possible explanation for the defect in RV7 replication and reduced IE gene expression in IC-21 macrophages is that RV7 does not enter these cells as efficiently as WT MCMV. Perhaps the defect lies upstream of IE gene expression, during the attachment and penetration phases of infection. To investigate this possibility, duplicate cultures of cells were infected simultaneously, and at 3 h postinfection, RNA was harvested from one culture for Northern blot analysis (of IE1 gene expression) and DNA was harvested from the other culture for Southern blot analysis (of input viral DNA) (Fig. 11). If RV7 does not enter IC-21 macrophages as efficiently as WT MCMV enters, then the Southern blot should reveal a band of lesser intensity in the DNA isolated from RV7-infected cells compared with that from WT-infected cells. On the contrary, the Southern blot (Fig. 11A) clearly demonstrates that RV7 entered these macrophages as efficiently as WT MCMV. Densitometric scanning of the autoradiograph revealed very similar intensities of the viral DNA present in RV7- and WT-infected cells at 3 h postinfection (integrated intensities of 10.12 for RV7 and 10.80 for WT MCMV). In NIH 3T3 cells, the entry of RV7 appeared to be slightly less than that of WT MCMV (integrated intensities of 13.06 for RV7 and 21.07 for WT MCMV), although this difference was less than twofold and is probably not significant.

Results of the simultaneous Northern blot (Fig. 11B) were similar to those shown in Fig. 9A. There was an eightfold reduction in the levels of the IE1 transcript present in RV7-infected IC-21 macrophages compared with WT-infected cells (integrated intensities of 1.99 for RV7 and 17.11 for WT MCMV). In NIH 3T3 fibroblasts, there was a slight reduction in the levels of IE1 transcripts in RV7-infected cells compared with WT-infected cells (integrated intensities of 5.33 for RV7 and 9.58 for WT MCMV). However, this difference is less than twofold and may reflect the slightly reduced entry of RV7 into these cells in this particular experiment (Fig. 11A). Collectively, these data demonstrate that the reduction in IE gene expression observed in IC-21 macrophages infected with RV7 is not the result of a defect in virus entry.

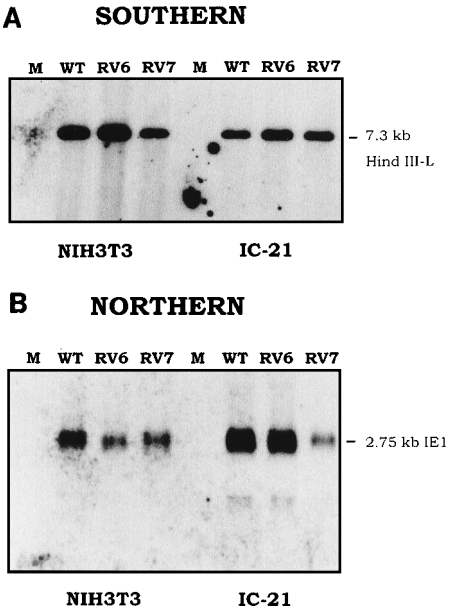

FIG. 11. In vitro analysis of RV7 entry into fibroblasts and macrophages. Cultures were mock infected (M) or were infected with WT virus or the indicated RV at a multiplicity of 4 PFU per cell. At 3 h postinfection, RNA was harvested from one culture for Northern blot analysis, and DNA was harvested from the other culture for Southern blot analysis. For the Southern blot, infected cell DNAs were digested with *Hin*dIII and hybridized to a probe of the MCMV *Hin*dIII L fragment. The Northern blot was also hybridized to a *Hin*dIII-L probe. Sizes of the MCMV *Hin*dIII L fragment and the IE1 RNA transcript are indicated on the right.

DISCUSSION

In this study, we generated MCMV mutants containing deletions of various sizes within the *Hin*dIII-J and -I regions. Analyses of these virus mutants confirm that *Hin*dIII-J is nonessential for replication in cell culture (65) and additionally extend the boundary of the nonessential region into the adjacent *Hin*dIII I fragment. Because RV9 could not be purified from WT virus, we speculate that the middle third of *Hin*dIII-I (map units 0.866 to 0.878) contains a gene(s) which is essential for virus replication. This region encodes two potential ORFs (M142 and M143), one or both of which may be essential for MCMV replication in vitro. An alternative hypothesis is that the region of *Hin*dIII-I unique to RV9 contains a gene whose function is duplicated in *Hin*dIII-J or the first third of *Hin*dIII-I, such that the deletion of both genes renders the virus replication defective.

The sizes of the deletions within the recombinant viruses were inversely correlated with the extent of replication of the mutants in salivary glands, suggesting that multiple genes may be involved in salivary gland growth. It appears from these data and those of others (9, 35, 40) that mutations throughout the *Hin*dIII-J and -I regions affect salivary gland growth, as optimal growth in this tissue appears to be exquisitely sensitive to mutations within this region. In addition, the mere presence of the β-glu gene may affect replication of MCMV in the salivary gland (60).

The failure of RV7 to replicate in vivo is not restricted to the salivary gland, as virus was also undetectable in the spleens of immunocompromised SCID mice. The attenuation of RV7 in SCID mice is highly significant, as these animals are extremely susceptible to MCMV infection (49). Thus, RV7 expresses a unique phenotype in that its replication is undetectable in target organs of both normal and immunodeficient mice. RV7 contains a wild-type *sgg1* gene, but the deletion removes sequences encoding several other MCMV gene products. These include the Fc receptor for immunoglobulin G encoded by ORF m138, an uncharacterized gene product from m137, and those produced from the three largest *Hin*dIII-J transcripts which originate in *Hin*dIII-I. Two additional ORFs in *Hin*dIII-I (M142 and M143) are also deleted in RV7.

RV7 failed to replicate to detectable levels in SCID mice despite the fact that RV7 grew comparably to WT virus in primary fibroblasts and bone marrow macrophages in vitro. The question remains as to the relevancy of these two cell types to MCMV growth in tissues, where the target cell types may be mesothelial cells, endothelial cells, dendritic cells, or tissue macrophages (60). There may indeed be low levels of RV7 replication in at least some cell type in the SCID mouse, and this low viral burden may persist in these animals or effectively be controlled by interferon or natural killer cells (66).

In contrast to bone marrow macrophages, RV7 grew poorly in the macrophage cell line IC-21. The inability of RV7 to replicate to wild-type levels in this cell line compared with the bone marrow macrophages is most likely related to differences in the state of differentiation of the two host cells. The IC-21 cell line is a simian virus 40-transformed peritoneal macrophage which displays properties of a differentiated macrophage, including high phagocytic activity and lysozyme and acid phosphatase production (41). More recent studies revealed that RV7 also replicates poorly compared with WT virus in peritoneal macrophages isolated from C57BL/6 mice (55a). Another difference between the bone marrow macrophages and IC-21 cells is haplotype. The $H\text{-}2^b$ haplotype of the IC-21 cells may confer a restriction on RV7 growth in macrophages, and this restriction is potentially absent from $H\text{-}2^d$ BALB/c bone marrow cells. It is unclear at this time if RV7 is unique among *Hin*dIII-J and -I mutants in its hampered ability to replicate in the IC-21 cell line. However, preliminary data indicate that MCMV *Hin*dIII-J mutants disrupted in ORF m134 (RQ401), m136 (RM874), or M139 (RM873) (40, 65) grow to high titers in IC-21 cells, as does RV6, in which m137 and m138 are deleted. Confirmation of efficient replication of RM873 (65) would imply that gene products regulating growth of RV7 in the IC-21 macrophage cell line are encoded in ORFs M140 and/or M141. These studies are currently in progress.

The poor growth of RV7 in the IC-21 macrophage cell line was at least partially due to a block in IE gene expression and, as expected, subsequent E and L gene expression. It is possible that *Hin*dIII-I encodes a virion structural protein, such as a tegument protein, which is delivered into the cell upon virus penetration and functions in concert with cellular factors to enhance immediate-early transcription in macrophages but is not necessary for efficient immediate-early transcription in fibroblasts. HCMV contains a virion-associated transactivator, the pp71 tegument protein encoded by the UL82 gene, which enhances transcription from the major IE promoters of both HCMV and MCMV by as much as 20-fold (37). An alternative possibility is that the *Hin*dIII-I sequence deleted in RV7 contains an enhancer region required for efficient immediate-early transcription in IC-21 macrophages. The major IE promoter of MCMV is located in the *Hin*dIII L fragment, approximately 11 kb to the left of *Hin*dIII-I.

The immediate-early phases of HCMV gene expression are differentially regulated in macrophages compared with fibroblasts (32). For example, a 1.4-kb mRNA from the major IE gene region of HCMV is not detectable during infection of human fibroblasts but is readily detected in infected human macrophages at immediate-early times. These results suggest that this IE gene product exhibits cell-type-specific expression and may be important in the regulation of HCMV infection in macrophages. Perhaps the mutation in RV7 deletes an MCMV gene product with a similar function and thereby blocks the course of subsequent viral gene expression in murine macrophages.

The failure of RV7 to replicate in target organs of the infected mouse and its greatly reduced ability to grow in a differentiated macrophage cell line strongly suggest that the nonessential regions of MCMV *Hin*dIII-J and -I deleted in this mutant play important roles in pathogenesis in vivo. Interactions between CMV and macrophages are viewed as critical to viral pathogenesis (3, 6, 12, 14, 26, 33, 53, 54, 60, 61). Peripheral blood monocytes function to disseminate virus during acute infection (12, 53, 60, 61), and monocytes and macrophages are the most likely cell types harboring latent CMV (6, 33, 60, 61). Because monocytes and macrophages play key roles in CMV dissemination, tissue tropism, and latency, viral gene products which regulate growth in this cell type are predicted to significantly affect MCMV pathogenesis in vivo.

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