

Enhancer Requirement for Murine Cytomegalovirus Growth and Genetic Complementation by the Human Cytomegalovirus Enhancer†

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Received 28 May 1998/Accepted 29 July 1998

The cytomegalovirus (CMV) enhancer is a highly complex regulatory region containing multiple elements that interact with a variety of host-encoded transcription factors. Many of these sequence elements are conserved among the different species strains of CMV, although the arrangement of the various elements and overall sequence composition of the CMV enhancers differ remarkably. To delineate the importance of this region to a productive infection and to explore the possibility of generating a murine CMV (MCMV) under the control of human CMV (HCMV) genetic elements, the MCMV enhancer was resected and replaced either with nonregulatory sequences or with paralogous sequences from HCMV. The effects of these various deletions and substitutions on viral growth in transfected or infected tissue-culture cells were evaluated. We found that mutations in MCMV that eliminate or substitute for the enhancer with nonregulatory sequences showed a severe deficiency in virus synthesis. This growth defect is effectively complemented by the homologous MCMV enhancer as well as the HCMV enhancer. In the latter case, the chimeric viruses (hybrid MCMV strains) containing the molecularly shuffled human enhancer exhibit infectious kinetics similar to that of parental wild-type and wild-type revertant MCMV. These results also show that open reading frames m124, m124.1, and m125 located within the enhancer region are nonessential for growth of MCMV in cells. Most importantly, we conclude that the enhancer of MCMV is required for optimal infection and that its diverged human counterpart can advantageously replace its role in promoting viral infectivity.

Members of the species-specific cytomegalovirus (CMV) family possess potent transcriptional enhancers upstream of their major immediate-early promoters (MIEPs) (6, 10, 40, 41). The MIEP is one of the first promoters to activate upon infection driving expression of key regulatory immediate-early (IE) proteins of the virus. In addition, stringent regulation of its activity *in vivo* implicates an important role for the MIEP in viral cell-type tropism (4, 5, 22). Thus, transcriptional regulation of the MIEP by the enhancer is believed to play a pivotal role in determining the outcome of a productive infection (13). The MIEP enhancers of the CMV family are highly complex and contain multiple arrays of interdigitating repeat and unique sequence elements (reviewed in reference 13). The arrangement of these regulatory modules and overall sequence composition of the enhancers differ considerably between the different species strains (6, 10, 40). The majority of cellular transcription factors known to interact with the enhancer elements are regulated through signal transduction pathways. In many instances, the same signal-regulated transcription factors have been shown to interact among the different CMV enhancers (e.g., references 2, 3, and 9 and references therein). It therefore appears that while these enhancers differ in primary sequence structure, their functional regulatory elements are highly related. In this connection, it is noteworthy that many of

the different species members of the CMV family also share many biological characteristics.

A limited number of mutations have been characterized in regions closely associated with the MIEP enhancer in both murine (MCMV) and human (HCMV) CMVs (7, 29, 30, 32, 34). In HCMV and MCMV, genetic disruption of the *ie1* open reading frames (ORFs) (whose transcripts are initiated from their MIEPs) have shown that while the *ie1* protein is nonessential for viral growth, it is necessary for optimal infection (15, 32, 34). Importantly, few mutations have been successfully constructed in the MIEP locus, and so far none have been generated in the enhancer region. In HCMV, a region immediately upstream of the enhancer encompassing the modulator region of the MIEP has been deleted and shown not to be essential for MIEP activity in infected differentiated and undifferentiated NT2 cells and human foreskin fibroblasts (30). It therefore appears that mutations in regions closely associated with MIEP enhancer are nonessential for determining a productive infection. However, it is likely that the lack of characterized mutants in the enhancer region occurs because of the difficulty in generating virus mutants with growth disadvantages. Recently, we (M.M. and U.K.) have reported a novel strategy for the genetic manipulation of the MCMV genome, based on the cloning of the infectious viral genome as a bacterial artificial chromosome (BAC) in *Escherichia coli* (32). This approach provides a useful system for the study of virus mutants with growth defects. To date, the predicted importance of the enhancer of CMV to infection remains untested.

In this study, we show by using the MCMV BAC system, that the enhancer of MCMV plays an important role in promoting a productive infection. We also show that wild-type growth

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† Publication no. 11682-IMM of The Scripps Research Institute.

characteristics can be completely restored to enhancerless virus, by linking in *cis* the homologous MCMV or heterologous HCMV enhancer. Moreover, the genetic complementation studies with the human enhancer provide direct evidence that the enhancer is not involved in restricting the species-specific host range of a CMV infection.

MATERIALS AND METHODS

Virus and cells. Murine NIH 3T3 fibroblasts (ATCC CRL1658) were propagated in Dulbecco's modified essential medium supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 µg of gentamicin per ml, and 10% calf serum. Mouse embryonic fibroblasts (MEFs), derived from the embryos of timed pregnant BALB/c.ByJ mice on day 19 of gestation, were cultured in the same medium as NIH 3T3 cells, except that 10% fetal bovine serum was used instead of calf serum. The Smith strain of MCMV, originally obtained from Ann Campbell (Eastern Virginia Medical School), was used as the parental wild-type MCMV in this study. Stocks of MCMV were prepared in NIH 3T3 cells, and titers were determined by standard plaque assay on NIH 3T3 cells.

Plasmid constructs. Recombinant plasmids were constructed according to established procedures (28). Plasmid pBam25 containing the sequences from 176441 to 187035 of the MCMV genome placed into the *Bam*HI site of pA-CYC184 has been described previously (named pAMB25 in reference 18). The construction of pBam25H was done as follows: primers dNN and BlpI were used to amplify a 616-bp fragment from the HCMV enhancer with pMIEP(-1114/+112)/CAT as a template (14). Primer dNN (5'-gcc ctt taa atg cat aaa ggc gcc cct gca tac gtt gta tcc ata tc-3') contains a *Not*I site, an *Nsi*I site, and a *Dra*I site adjacent to nucleotide -667 (relative to the *ie1/ie2* HCMV transcription start site) of the HCMV enhancer. Primer BlpI (5'-gta cgc tca gcc cgc cca ttg ggc tca atg ggc c-3') contains an *Esp*I site adjacent to nucleotide -52 (relative to the *ie1/ie2* HCMV transcription start site) of the HCMV enhancer. The resulting PCR fragment was digested with *Esp*I and *Dra*I and inserted into *Nde*I-*Esp*I-digested pON401 (with the *Nde*I site filled in [29]) to generate pON401H. A 2.7-kbp *Mlu*I fragment of pON401H was inserted into *Mlu*I-digested pBam25 to create pBam25H.

Plasmid pIE111H was used for construction of enhancer deletions. pIE111H was generated by cloning the *Eco*RI-*Hind*III fragment (nucleotides 177008 to 180728) from pIE111 (31) into pUC19 followed by insertion of the 6.6-kb *Hind*III fragment of pON401H. To construct a plasmid that contains a deletion from nucleotides -48 to -1191 of the MCMV enhancer, pIE111H was digested by *Esp*I and *Not*I, filled in with Klenow polymerase, and religated, resulting in plasmid pEnh17. The construction of plasmid pEnhLuc was done as follows: primer luc. (for 5'-cat cta ggt ctc att aag taa tca gtc act cgc cta ggc cc-3' containing an *Esp*I site) and primer luc. rev (5'-ggg cct agg tgc cgc ggc gtc gat att agg aca cat-3' containing a *Not*I site) were used to amplify a 770-bp fragment from the luciferase gene (corresponding to amino acids 196 to 450 of the luc [luciferase] ORF). The resulting PCR fragment was digested with *Esp*I and *Not*I and inserted into the *Esp*I-*Not*I-digested pIE111H.

Mutagenesis of the MCMV BAC plasmid by the two-step replacement strategy requires about 2.5 to 3 kb of homologous sequences on each side of the mutation (32, 37). To provide the required homologies, plasmid pUC-L (containing the MCMV *Hind*III L fragment cloned into pUC19) was digested with *Hpa*I and *Nco*I, and an adapter molecule containing *Eco*RI and *Sal*I sites (underlined) was inserted (forward, 5'-tag gga taa cag ggt aat gaa ttc att taa tac tag tgc cga cg-3'; reverse, 5'-cat ggc tgc aca cta gta tta aat gaa ttc att acc ctt tta tcc cta-3'), resulting in plasmid pUC-LO. To provide the homology on the other side of the enhancer, an *Spe*I site (MCMV nucleotide 178736) in plasmid pIE111 (31) was converted to a *Mun*I site by insertion of an oligonucleotide (5'-cta ggg caa ttg cc-3'). Next, a 3.9-kb *Mun*I fragment (nucleotides 178736 to 182682) was isolated and inserted into the *Eco*RI site of the adapter in plasmid pUC-LO, leading to plasmid pEnhDel. A 7.5-kb *Sal*I fragment from pEnhDel was transferred to the shuttle plasmid pMBO96 (37) and used to introduce the 1.6-kb *Mun*I-*Hpa*I deletion into the MCMV BAC plasmid pSM3 and to generate BAC plasmid pE1. The MCMV BAC plasmid pSM3 (32) contains the MCMV genome with BAC vector sequences replacing a nonessential region for replication in vitro (spanning nucleotides 209756 to 217934 within the *Hind*III E' fragment) of the viral genome. Therefore, ORFs m151 to m158 are completely or partially deleted in pSM3. To construct the shuttle plasmid for introduction of the *Esp*I-*Nde*I deletion of the MCMV enhancer into BAC plasmid pSM3, the 1.0-kb *Mlu*I fragment of pEnhDel was replaced with the 1.5-kb *Mlu*I fragment from plasmid pEnh17, and the 8-kb *Sal*I fragment was transferred to pMBO96. To introduce the luciferase stuffer mutation into the BAC plasmid pSM3, the 1.0-kb *Mlu*I fragment of pEnhDel was replaced with the 2.2-kb *Mlu*I fragment from plasmid pEnhLuc, and the 8.7-kb *Sal*I fragment was transferred to the shuttle plasmid pST76-A (37a).

BAC mutagenesis. Mutagenesis of the MCMV BAC plasmid pSM3 was performed as described previously (32). In brief, shuttle plasmids were electroporated into *E. coli* CBTS bacteria (20) that already contained the BAC plasmid pSM3. Note that *E. coli* CBTS is RecA⁺ at 30°C and virtually RecA⁻ at temperatures higher than 37°C (the CBTS strain was constructed by M. O'Connor, University of California Irvine). Transformants were selected at 30°C on Luria

broth (LB) plates containing chloramphenicol (12.5 µg/ml) and tetracycline (10 µg/ml) or chloramphenicol with ampicillin (50 µg/ml), using shuttle plasmid pMB097 or pST76-A, respectively. Clones that formed cointegrates were identified by streaking the bacteria on new LB plates followed by incubation at 43°C. To allow resolution of the cointegrates, clones were streaked on LB plates containing chloramphenicol only and incubated at 30°C. Bacteria were restreaked on LB plates with chloramphenicol to separate clones that contain resolved plasmids and clones that still contain cointegrates. Clones with resolved plasmids were identified by screening for the loss of the antibiotic marker of the shuttle plasmid. Usually, about 5 to 10% of the clones resolved the cointegrates. Finally, BAC plasmid DNA was isolated from 10-ml overnight cultures by the alkaline lysis procedure (28) and characterized by restriction enzyme digestion to identify BAC plasmids that received the mutation. Midi preparations of BAC plasmids were prepared from 100-ml *E. coli* cultures as described previously (28, 32).

Transfections and virus construction. To generate recombinant viruses, BAC plasmids were transfected, in the absence or presence of pBam25 or pBam25H, into NIH 3T3 cells (in six-well dishes) by the calcium phosphate precipitation technique essentially as described previously (28). Six hours posttransfection, cells were treated with glycerol (15% glycerol in HEPES-buffered saline) for 3 min as described previously (28). The progeny virus that replicates in these cultures was harvested when 100% cytopathic effect was observed in the cultures and then was used to infect fresh cells. Two independent recombinants of the hMCMV-ES (from two independent cotransfections of pE1 with pBam25H) hybrid and one recombinant MCMVrev (from a cotransfection of pE1 with pBam25) were subjected to three rounds of plaque purification before a higher-titer stock was prepared.

Viral infections. NIH 3T3 cells or MEFs were infected with wild-type MCMV or different MCMV recombinants at the different multiplicities of infection (MOIs) indicated in the figure legends. After a 1-h adsorption period, the virus inoculum was removed, the cultures were washed three times with phosphate-buffered saline, and fresh medium was added. At different times after infection, the supernatants of three independent cultures were harvested, frozen, and thawed, and infectious virus was quantitated by standard plaque assay on NIH 3T3 cells. For selective expression of IE transcripts, the cultures were incubated for 30 min prior to infection and up to 12 h postinfection in the presence of cycloheximide (100 µg/ml).

DNA and RNA analysis. Total cell DNA was isolated from infected NIH 3T3 cells (in 60-mm-diameter tissue culture dish) when 100% cytopathic effect was observed in the cultures. Briefly, infected cells were scraped, washed twice with phosphate-buffered saline, and lysed in 1 ml of a solution containing 10 mM Tris (pH 7.8), 10 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), and 250 µg of proteinase K per ml, and incubated at 55°C for 4 h. DNA was then purified by phenol-chloroform extraction and precipitated with ethanol. When DNA restriction enzyme patterns were analyzed, viral DNA fragments after restriction enzyme digestion were run on 0.5% agarose gels for approximately 18 h at 0.5 V/cm. DNA blotting was conducted as described previously (28). The HCMV enhancer-specific probe used was a 0.34-kbp *Spe*I-*Sna*BI fragment from pMIEP (-1145/+112)CAT. Whole-cell RNA was isolated from either uninfected or infected NIH 3T3 cells (in six-well dishes) by using RNazol B (Tel-Test, Inc., Friendswood, Tex.) according to the manufacturer's protocol. Northern blot analyses were performed as described in reference 28. Two to eight micrograms of RNA was separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde, transferred to a nylon membrane, and hybridized with a 1.6-kbp *Mlu*I-*Hind*III fragment from pON401 to specifically detect *ie1/ie3* transcripts (29). The probes used were isolated from agarose gels and then radiolabeled with [α -³²P]dATP by the random-primed labeling method (12).

Immunoblot analyses. NIH 3T3 cells (in six-well dishes) were infected with MCMV at an MOI of 0.5 PFU/cell. At different times after infection, samples were lysed in protein sample buffer, vortexed, and boiled for 5 min. The polypeptides of cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (8% polyacrylamide) and transferred to nitrocellulose filters. Filters were incubated with an *ie1*-specific monoclonal antibody, Cromax 101, and as a secondary antibody horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Amersham, Buckinghamshire, England) was used. Blots were developed with the Enhancer chemiluminescence system (Amersham) according to the manufacturer's protocol.

RESULTS

Generation of enhancerless MCMV genomes. To determine if the enhancer is essential for efficient viral growth in tissue culture cells, a series of recombinant MCMV genomes lacking enhancer sequences were constructed by using the recently described MCMV BAC system (32). In this system, the MCMV genome has been cloned as a BAC in *E. coli*, and viral progeny can be reconstituted by transfecting the MCMV BAC plasmid into eukaryotic cells permissive for MCMV. As a parental BAC plasmid for the generation of BACs carrying enhancer-

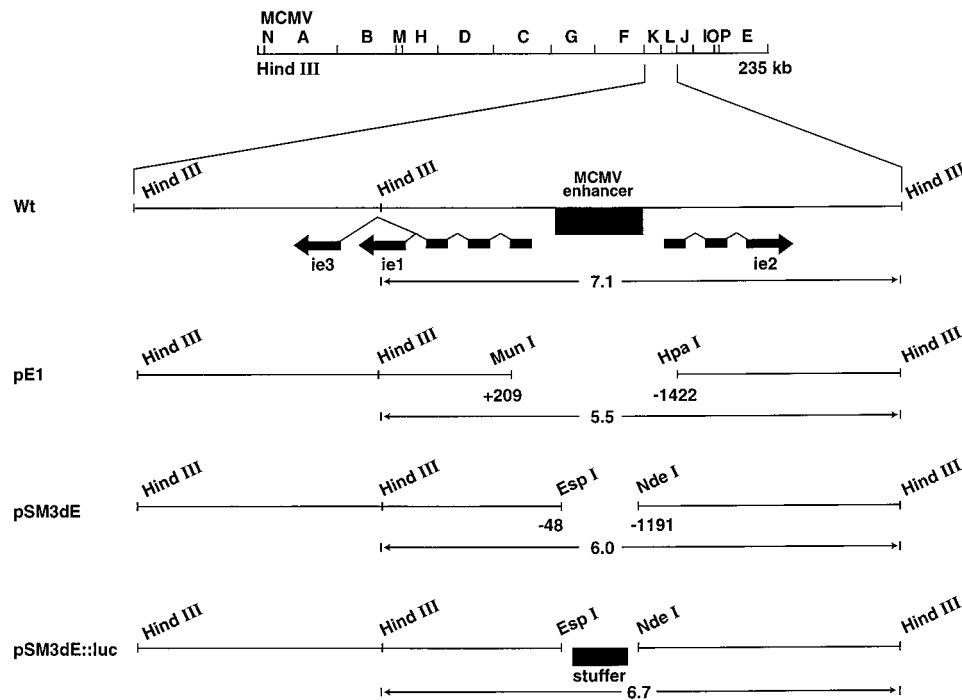


FIG. 1. Construction of enhancerless MCMV BAC genomes. The top line represents the *Hind*III map of the wild-type (Wt) MCMV genome, with the *Hind*III K and L regions expanded below to show the region containing the MIE genes (*ie*1 to *ie*3). The MCMV BAC plasmids pE1, pSM3dE, and pSM3dE::luc, shown below the wild-type MCMV genome, were generated by homologous recombination in *E. coli* with pSM3 as the parental MCMV BAC genome as indicated in Materials and Methods. A *Mun*I-*Hpa*I fragment (from +209 to -1421 relative to the *ie*1/*ie*3 MCMV transcription start site) in the *Hind*III L fragment of the wild-type MCMV genome was removed to generate pE1. In pSM3dE, an *Esp*I-*Nde*I fragment (from nucleotide sequences -48 to -1191 relative to the *ie*1/*ie*3 MCMV transcription start site) was deleted in the *Hind*III L fragment of the wild-type MCMV genome. The MCMV BAC plasmid pSM3dE::luc contains a 770-bp fragment from the luciferase gene replacing nucleotide sequences from -48 to -1191 (relative to the *ie*1/*ie*3 MCMV transcription start site) in the *Hind*III L fragment of the wild-type MCMV genome. The illustration is not drawn to scale.

less viral genomes, we used pSM3. The BAC plasmid pSM3 contains the MCMV genome with BAC vector sequences replacing a nonessential region for replication *in vitro* within the *Hind*III E' fragment at the right-terminal end of the viral genome (spanning nucleotides 209756 to 217934). A schematic representation of the various BAC recombinant genomes constructed is summarized in Fig. 1. In the first MCMV BAC plasmid, pE1, an *Mun*I-*Hpa*I fragment of approximately 1.6 kbp in the *Hind*III L fragment of parental BAC pSM3 is deleted. Deletion of this 1.6-kbp fragment, which encompasses nucleotide sequences from +209 to -1421 of the IE region, effectively removes the complete MIEP regulatory region and also disrupts the first exon of *ie*1/*ie*3. To generate a less extensive disruption of the MIEP in which only enhancer sequences have been removed, a 1.1-kbp *Esp*I-*Nde*I sequence (within the *Hind*III L fragment) extending from nucleotide positions -48 to -1191 of the MCMV MIEP was resected from pSM3 to construct the enhancerless BAC plasmid pSM3dE (see Fig. 1 and Materials and Methods for details). In addition, and to maintain the relative positions of the promoters flanking the MCMV enhancer, a BAC plasmid, pSM3dE::luc, that contains a stuffer fragment replacing the deleted enhancer segment was generated (Fig. 1). In pSM3dE::luc, an internal nonregulatory 770-bp fragment from the firefly luciferase reporter gene was inserted within nucleotides -48 and -1191 of the enhancer region of MCMV. To verify the structure of the recombinant BAC plasmids generated, their *Hind*III restriction patterns were analyzed. As shown in Fig. 2, in comparison with the parental BAC plasmid pSM3, the recombinant enhancerless MCMV BAC plasmids lack the wild-type 7.1-kbp *Hind*III L

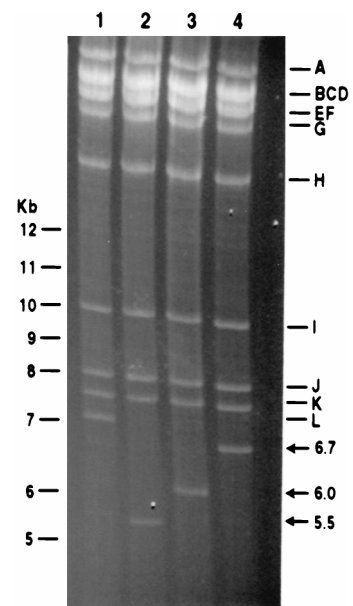


FIG. 2. Structural analysis of enhancerless MCMV BAC genomes. Ethidium bromide-stained agarose gels of *Hind*III-digested BAC plasmids pSM3 (1), pE1 (2), pSM3dE (3), and pSM3dE::luc (4) after separation on a 0.5% agarose gel. The *Hind*III fragment name (11) is indicated to the right of the set of lanes, and the size markers are shown at the left margin. The size of the new *Hind*III L fragments is indicated with an arrow at the right margin.

TABLE 1. Transfection of NIH 3T3 cells with pSM3 or recombinant MCMV BAC plasmids in the presence and absence of pBam25

Transfection condition ^a	No. of plaques (days to detection) in culture ^b			
	pSM3	pE1	pSM3dE	pSM3dE::luc
No pBam25	28 (5–9)	0	5 (8–24)	2 (7–26)
pBam25	25 (4–8)	30 (8–13)	50 (6–12)	32 (7–12)

^a pBam25, a plasmid expressing ie1 and ie3, carries a *Bam*HI fragment containing sequences from 176441 to 187035 of the MCMV genome.

^b Shown is the number of plaques first developed in the cultures. In parentheses is shown the number of days after transfection when plaques were initially detected and when cultures reached a complete cytopathic effect.

fragment, which instead is replaced by the expected 5.5-kbp fragment in pE1, 6.0-kbp fragment in pSM3dE, and 6.7-kbp fragment in pSM3dE:luc. In addition, comparison of the *Xba*I and *Eco*RI fragment profiles with those of parental BAC pSM3 indicated that pE1, pSM3dE, and pSM3dE:luc were free of any detectable deletions or insertions in other regions outside the *Hind*III L fragment of the viral genome (data not shown). These results demonstrate the successful deletion of the enhancer and its replacement by a stuffer fragment in the recombinant BAC plasmids pE1, pSM3dE, and pSM3dE:luc, respectively.

Requirement of the enhancer region for viral DNA infectivity. To test directly the significance of the enhancer to infection, recombinant MCMV BAC genomes either containing (pSM3) or lacking the MIEP enhancer region (pE1 and pSM3dE) were prepared from bacteria and transfected into NIH 3T3 cells, and viral DNA infectivity was assessed by plaque formation. Note that in pE1, the entire MIEP region has been deleted, whereas in pSM3dE, the MIEP is truncated at position –48 and therefore lacks exclusively enhancer sequences while retaining the wild-type MIEP core promoter (TATA box) element. The results from this set of experiments are shown in Table 1. As expected, numerous plaques developed in NIH 3T3 cells when transfected with the wild-type MCMV BAC plasmid, pSM3, and cultures reached a complete cytopathic effect within 9 days. In contrast, NIH 3T3 cells transfected with the recombinant MCMV genome containing the large 1.6-kbp deletion in the MIEP enhancer, pE1, failed to develop any plaques. These results suggest that elimination of the complete MIEP, including part of exon 1 of ie1/ie3, generates a replication-incompetent virus. When NIH 3T3 cells were transfected with pSM3dE, a minimal number of small plaques were produced, and cultures took more than twice as long (24 days) to reach cytopathic effect (Table 1). Similar results were achieved by transfecting the MCMV BAC plasmids into MEFs (data not shown). Altogether, the results of these experiments are consistent with the enhancer being important for optimal infectivity.

In pSM3dE, the 1.1-kbp *Esp*I-*Nde*I deletion of enhancer sequences (from nucleotide positions –48 to –1191 of the MCMV-MIEP) results in positioning the ie2 promoter-regulatory region immediately adjacent to the MIEP TATA box (31) (Fig. 1). Specifically, in this BAC plasmid, nucleotide position –48 of the MCMV-MIEP enhancer is immediately adjacent to nucleotide position –183 relative to the ie2 transcription start site. Thus, it is possible that the minimal infectivity detected for pSM3dE in permissive cells is due to partial compensation of MIEP activity by the MIEP TATA box alone or by regulatory elements from the ie2 promoter. In order to maintain an appropriate distance between the ie1 and ie2 promoters, nonregulatory DNA was inserted in place of the enhanc-

er. Accordingly, the resulting recombinant, named pSM3dE:luc, was next analyzed for DNA infectivity. As shown in Table 1, this recombinant when transfected in NIH 3T3 or MEF cells developed a minimal number of small plaques. These results therefore suggest that while there is an important requirement of the enhancer region for a productive infection, it is not absolutely essential.

Complementation of viral DNA infectivity of enhancerless MCMV by transfection of cells with an ie1/ie3 expression plasmid. In the next set of experiments, we sought to determine whether the defect in infectivity observed in the enhancer-deficient genomes is due to genetic alteration of the IE locus alone. For this purpose, we used a recombinant plasmid, pBam25, that expresses ie1/ie3 genes upon transfection in NIH 3T3 cells (Fig. 3 and data not shown). In the presence of pBam25, approximately equal numbers of plaques were developed for each enhancerless recombinant compared with reconstituted MCMV derived from pSM3 (Table 1). These results demonstrate that the failure of enhancerless recombinants to efficiently form plaques is due to a specific alteration of the IE locus.

To further establish that the only mutation introduced into the enhancerless MCMV recombinant exists in the specific IE region deleted, we sought to rescue pE1 by selecting for a revertant wild-type virus in the presence of cotransfected pBam25 (Fig. 3). In these experiments, new cell monolayers were infected with supernatants derived from pE1 and pBam25 cotransfected cells, and a revertant virus was subsequently purified by multiple rounds of plaque purification. Lane 2 in Fig. 4 shows that the DNA fragment profile following *Hind*III digestion for the revertant MCMV genome exhibited, as expected, the natural *Hind*III fragment of 7.1 kbp in place of the 5.5-kbp *Hind*III L fragment present in the enhancerless genome pE1 (compare lane 2 in Fig. 4 with lane 2 in Fig. 2). We next examined the growth kinetics of plaque-purified revertant virus compared with those of the parental wild-type MCMV. The revertant wild-type and parental wild-type viruses were found to have similar growth kinetics (Fig. 5). These results demonstrate that genetic ablation of the complete MIEP enhancer, including part of exon 1 of ie1/ie3, results in the inability of the virus to produce new progeny. In addition, these results support the conclusion that the enhancer of MCMV is important for effecting a productive viral infection, but it is not absolutely essential.

Genetic complementation and restoration of growth of enhancerless MCMV by the HCMV enhancer. Since the enhancer region of MCMV contains a number of putative ORFs (m124, m124.1, and m125) (38), it is possible that the loss of these MCMV-specific sequences may be responsible, in part, for the severely impaired ability of enhancerless MCMV to grow. In order to test this hypothesis and to explore the possibility of whether the human enhancer can rescue growth, we sought to construct a hybrid virus that substituted the MCMV enhancer for the HCMV enhancer. For these experiments, we first constructed a plasmid, pBam25H, containing the MCMV-MIE region in which sequences from –48 to –1191 of the MCMV enhancer were replaced by the paralogous sequences from –52 to –667 of the HCMV enhancer (Fig. 3 [see Materials and Methods for details]). We next tested the ability of pBam25H to complement *in trans* the growth defect of the enhancerless BAC plasmid (pE1) in transient cotransfection assays. In these experiments, cotransfection of pE1 and pBam25H in NIH 3T3 cells led to the development of plaques that took around 9 days to reach a complete cytopathic effect. Therefore, these results indicate that the severe deficiency of enhancerless MCMV to grow is not due to the loss of expression from one of the

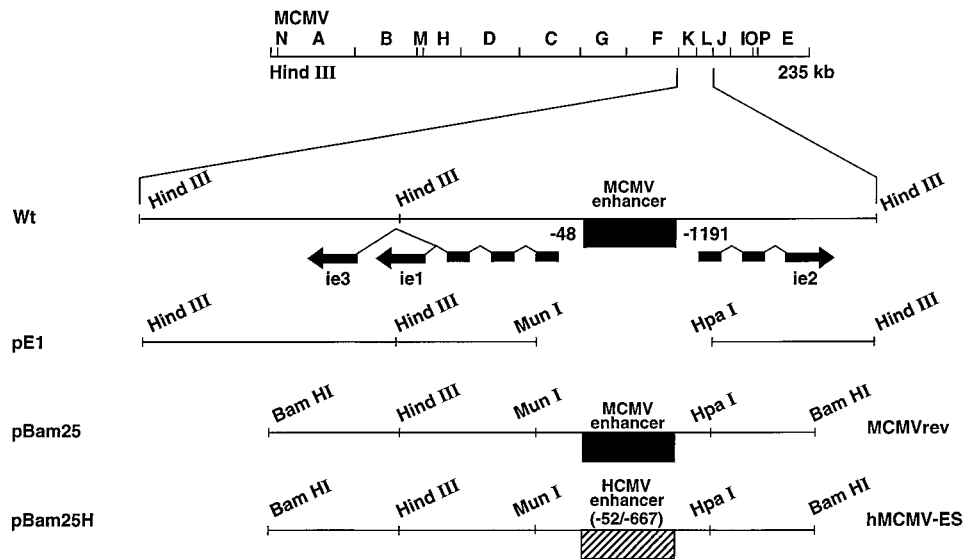


FIG. 3. Construction of hMCMV-ES mutants. The top line represents the *Hind*III map of wild-type MCMV genome, with the *Hind*III K and L regions expanded below to show the region containing the MIE genes (ie1 to ie3). In the MCMV BAC plasmid pE1, a *Mun*I-*Hpa*I fragment (from nucleotide sequences +209 to -1421 relative to the ie1/ie3 MCMV transcription start site) has been deleted in the *Hind*III L region of the wild-type MCMV genome. Recombinant viruses MCMVrev and hMCMV-ES were constructed by cotransfection of MCMV BAC plasmid pE1 with pBam25 or pBam25H, respectively, into NIH 3T3 cells. pBam25, a plasmid expressing ie1 and ie3, carries a *Bam*HI fragment containing sequences from 176441 to 187035 of the MCMV genome. pBam25H carries the *Bam*HI fragment (containing sequences from 176441 to 187035 of the MCMV genome) in which sequences from -48 to -1191 (relative to the ie1/ie3 MCMV transcription start site) have been replaced by sequences from -52 to -667 of the HCMV enhancer. The solid and hatched boxes represent the MCMV and HCMV enhancers, respectively. The diagram is not drawn to scale.

putative ORFs (m124-m125) that overlap the enhancer region and further suggest that the HCMV enhancer may complement an enhancerless MCMV.

Accordingly, we next attempted to isolate a recombinant genome containing the enhancer swap. For these experiments, new cell monolayers were infected with supernatants derived from two independent cotransfections of pE1 and pBam25H. From these transfer experiments, two independent hybrid strains, hMCMV-ES1 and hMCMV-ES2, were generated after three rounds of plaque purification.

In order to investigate the *cis* replacement of the MCMV enhancer by its human counterpart and confirm the integrity of the hybrid viruses, NIH 3T3 cells were infected with hMCMV-ES1 and hMCMV-ES2, and total cell DNA was isolated when cells showed a complete cytopathic effect. As expected, when the genomes of the two chimeric viruses were analyzed by *Hind*III digestion, the appropriate *Hind*III L fragment of 7.1 kbp present in wild-type MCMV was reduced to a 6.6-kb fragment as a result of the replacement of the MCMV enhancer by the HCMV enhancer (Fig. 4). Furthermore, these and other restriction enzyme digestion analyses of hMCMV-ES1 and hMCMV-ES2 in comparison with wild-type MCMV showed identical banding patterns outside the *Hind*III L region (Fig. 4 and data not shown). In addition, hybridization with a radiolabelled HCMV enhancer probe specifically detected the 6.6-kbp fragment in the two chimeric viruses but not any fragments in the wild-type MCMV and MCMVrev genomes (Fig. 4). To further examine the integrity of the ES strains, the HCMV enhancer region was sequenced. No changes were observed in sequences from -52 to -667 of the HCMV enhancer present in the two chimeric strains after a minimum of six passages in tissue culture (1). On the basis of the extensive restriction enzyme digest analyses, sequencing, and hybridization studies, we conclude that hMCMV ES-1 and hMCMV ES-2 contain the HCMV enhancer in the place of its murine paralog.

To determine whether the enhancer exchange altered the

ability of the chimeric viruses to grow in cell culture, growth analyses were performed at different MOIs. For these experiments, NIH 3T3 cells were infected with hMCMV-ES1 and hMCMV-ES2 at MOIs of 0.01 and 5, and viral titers in the supernatant of the cultures were determined at different times after infection and compared with that of the wild-type or revertant MCMV. Figure 5 shows that both hMCMV-ES1 and -2 strains exhibit growth properties remarkably similar to those of wild-type and revertant MCMV strains as determined by single-step (Fig. 5A) and multistep (Fig. 5B) growth analyses.

We next investigated to what extent expression of ie1/ie3 transcripts had been altered by the replacement of the MCMV enhancer with the human counterpart. In wild-type MCMV-

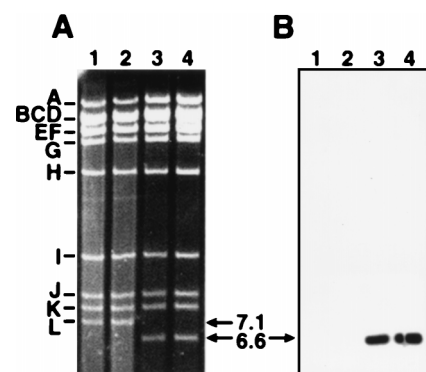


FIG. 4. Structural analysis of hMCMV mutants. DNA isolated from NIH 3T3 cells infected with wild-type MCMV (1), MCMVrev (2), hMCMV-ES1 (3), or hMCMV-ES2 (4) was subjected to *Hind*III digestion and separated on a 0.5% agarose gel. Bands were visualized with ethidium bromide (A) or transferred to nylon filters and hybridized to a 32 P-labeled 340-bp *Spe*I-*Sna*BI fragment from the HCMV enhancer (B). The *Hind*III fragment name (11) is indicated to the left set of lines, and the sizes of the natural and new *Hind*III L fragments for each virus are shown with an arrow.

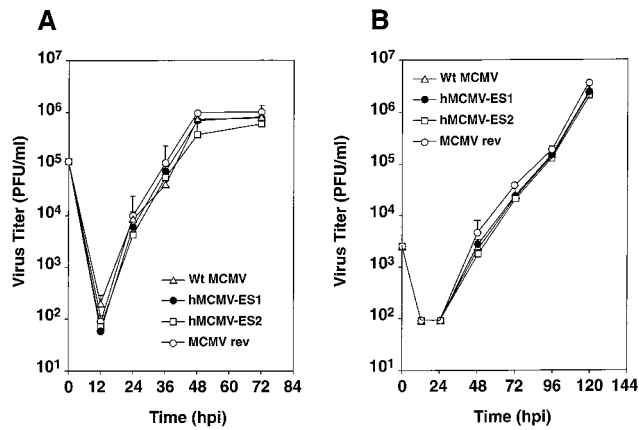


FIG. 5. Growth kinetics of hMCMV-ES mutants. Murine NIH 3T3 cells were infected at an MOI of 5 (A) or 0.01 PFU (B) per cell with wild-type (Wt) MCMV (Smith strain), MCMVrev, hMCMV-ES1, or hMCMV-ES2. At the indicated time points (hours) after infection (hpi) supernatants from the infected cultures were harvested, and titers were determined by plaque assay on NIH 3T3 cells. Each data point represents the average and standard deviation from three separate cultures.

infected cells, *ie1/ie3* expression is abundant at immediately and late times, but is reduced during early times of infection (39). Accordingly, NIH 3T3 cells were infected at an MOI of 0.5 with hMCMV-ES1 and hMCMV-ES2, and RNA was extracted at different times after infection and analyzed by Northern blotting with an *ie1/ie3*-specific probe. To control for RNA loading, Northern blots were also probed with a radiolabelled cDNA probe to GAPDH (Fig. 6). Figure 6 shows that the patterns observed for the major 2.75-kb *ie1/ie3* transcripts were quantitatively similar in cells infected by both strains of hMCMV and wild-type MCMV or MCMVrev-infected cells. These results indicate that the temporal expression pattern and appropriate levels of *ie1/ie3* RNA are synthesized upon infection with the enhancer swap strains. We next determined the expression levels of *ie1* protein in cells infected by these chimeric viruses. In MCMV-infected cells, the 89-kDa *ie1* protein can be initially detected at 1 h postinfection and is detectable during the whole replication cycle (19, 31). NIH 3T3 cells were infected with both isolates of hMCMV-ES, and wild-type or revertant MCMV and cell lysates were prepared at different times postinfection. Protein expression levels were monitored by Western blotting with the *ie1*-specific antibody. As shown in Fig. 7, at an MOI of 0.5 PFU/cell, temporal expression patterns and levels of *ie1* expression in cells infected with the two hMCMV-ES isolates were comparable to those in the revertant virus and wild-type MCMV-infected cells. Altogether, these results demonstrate that the human enhancer can efficiently substitute for the MCMV enhancer in tissue culture.

DISCUSSION

This study shows that the enhancer region of MCMV plays a critical role for efficiently effecting productive infection in tissue culture cells. We further demonstrate that genetically exchanging the MCMV enhancer with that from HCMV effectively produces wild-type growth characteristics. These results have a number of important implications for understanding and exploiting the role of the enhancer in the CMV infectious program.

Enhancer is important for MCMV growth. The results of this study clearly show that enhancer-deficient strains or re-

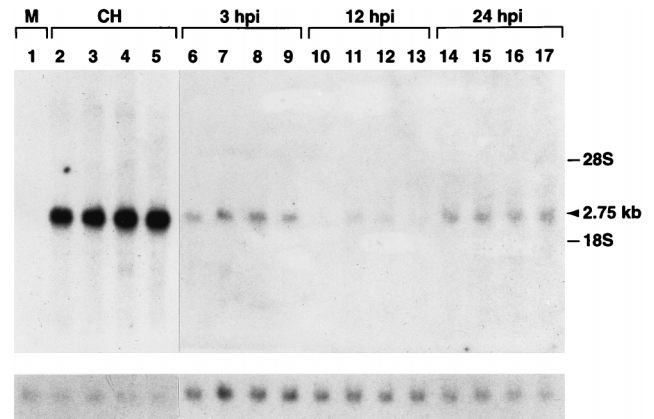


FIG. 6. Expression of RNA kinetics of *ie1/ie3* transcripts by hMCMV-ES mutants. NIH 3T3 cells were mock infected (lane 1) or infected at an MOI of 0.5 PFU/cell with wild-type MCMV (lanes 2, 6, 10, and 14), hMCMV-ES1 (lanes 3, 7, 11, and 15), hMCMV-ES2 (lanes 4, 8, 12, and 16), and MCMVrev (lanes 5, 9, 13, and 17). Whole-cell RNA was harvested at the indicated times after infection in the presence (lanes 2 to 5) or absence (lanes 1 and 6 to 17) of CH, separated on a formaldehyde-agarose gel, transferred to a nylon membrane, and probed with a 1.6-kb *MluI-HindIII* fragment from pON401 that was specific for *ie1* transcripts. The position of the 2.75-kb transcripts is indicated on the right by an arrow. The positions of the 18S and 28S rRNAs are shown. The blot was then hybridized with a ³²P-labeled GAPDH probe as an internal RNA control. The 1.4-kb GAPDH band detectable in all lanes is shown on the bottom panels.

combinant genomes of MCMV are severely compromised in their ability to synthesize virus in infected or transfected cells. Previous studies have indicated that enhancers play a number of distinct roles in a viral life cycle. The most important role served by a viral enhancer is in the initiation of the infectious program by which it acts as a potent transcriptional activator region for the viral IE genes. In agreement, our experiments showing that a related but distinct enhancer can efficiently substitute for the naturally occurring enhancer strongly suggest that a primary function of this region is to increase the efficiency of IE gene expression by serving as a potent activator of transcription. These enhancer swap experiments might also underscore the functional importance of the conserved regulatory elements within the HCMV and MCMV enhancers, although we predict that any enhancer could substitute, in part, for the CMV enhancer. Viral enhancers have also been implicated in the maintenance of an open chromatin structure prior

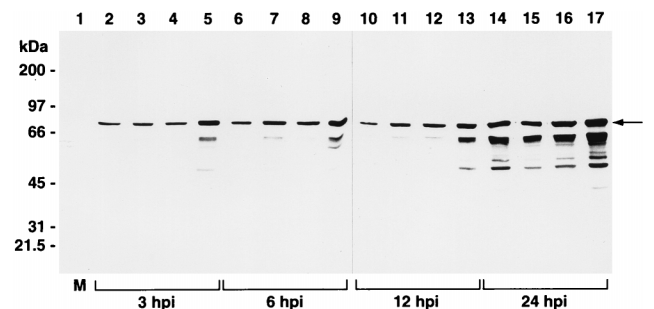


FIG. 7. Expression kinetics of *ie1* protein in hMCMV-ES-infected cells. NIH 3T3 cells were mock infected (lane 1) or infected at an MOI of 0.5 PFU/cell with wild-type MCMV (lanes 2, 6, 10, and 14), hMCMV-ES1 (lanes 3, 7, 11, and 15), hMCMV-ES2 (lanes 4, 8, 12, and 16), and MCMVrev (lanes 5, 9, 13, and 17). At the indicated times after adsorption, samples were lysed, subjected to SDS-PAGE analysis on 8% polyacrylamide gels, and reacted with an *ie1* monoclonal antibody. Molecular mass standards (M) appear on the left. The position of the 89-kDa protein is indicated by an arrowhead.

to and after the initiation of replication (8). In this connection, we note that in an infection of permissive cells by HCMV, marked DNase I hypersensitivity is observed in the enhancer region, indicating an open chromatin structure at early times of infection (35). In addition, viral enhancers may directly potentiate viral DNA replication (16, 17, 36) or may even be involved in localizing virion DNA to preferred nuclear compartments. It is conceivable that all of these possibilities may be responsible for the requirement of the enhancer for optimal MCMV infection. Whether the enhancer plays any direct role in promoting MCMV DNA replication or modifying chromatin structure remains to be determined.

Enhancer is not involved in the species restriction of CMV in tissue-culture cells. The CMV family is highly species specific. In this regard, the role of an enhancer in initiating the transcriptional program of a virus can, in principle, limit the virus to productively infect a specific host or cell type (23, 25, 27, 33). Previous *in vitro* and *in vivo* studies have indicated that the HCMV enhancer functions appropriately in mouse cells (4, 5, 22, 24), indicating that the strict species specificity of CMV is not at the level of the MIEP. In agreement with this notion, we provide in this study direct evidence that the enhancer is not responsible for the species-specific restriction in the ability of CMV to productively infect cells. At present, we cannot rule out the possibility that the species-specific enhancers may restrict *in vivo* growth of CMV, although the transgenic studies with the human enhancer indicate that this is unlikely to be the case (4, 5, 22). Indeed, our preliminary experiments indicate that hMCMV strains are capable of productively infecting the mouse (1).

The new hMCMV strains and future implications. In this study, we show that the HCMV enhancer can completely restore the growth deficiency of an enhancerless MCMV. Importantly, these chimeric (hMCMV-ES) strains represent an MCMV that is under the control of HCMV genetic elements and thus may provide the development of a new model for exploring the *in vivo* importance of select HCMV enhancer elements during acute, latent, and reactivated infections. In particular, mutations introduced in specific binding sites for transcription factors used by the HCMV enhancer will clarify the involvement of the various signal-regulated transcription factors in controlling aspects of viral pathogenesis and latency. Finally, the observation that viral growth can be inhibited by eliminating the enhancer might have important implications in the development of novel ligand-specific drugs for therapy against CMV. For instance, it is possible to generate molecules to recognize specific base pairs in the minor groove by using hairpin polyamides (42) and the major groove by using DNA-binding antibodies (26) or zinc finger chimeras (21).

ACKNOWLEDGMENTS

We thank Stipan Jonjic for providing monoclonal antibody Croma 101, Gyorgy Pósfai for plasmid pST₇₆-A, and Ed Mocarski for plasmid pON401. We thank Stefanie Eichler for excellent technical assistance in performing mutagenesis of the MCMV BAC plasmids. We thank Fátima García del Rey and Alison Razinski for technical support. We thank Kelly White for assistance in the preparation of the manuscript.

This work was supported by grants from the National Institutes of Health to P.G. (CA-66167 and AI-30627). P.G. is a Scholar of the Leukemia Society of America. A.A. was a fellow from the Ministerio de Educación y Ciencia (Spain).

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