

The Major Immediate-Early Gene *ie3* of Mouse Cytomegalovirus Is Essential for Viral Growth†

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The significance of the major immediate-early gene *ie3* of mouse cytomegalovirus (MCMV) and that of the corresponding *ie2* gene of human cytomegalovirus to viral replication are not known. To investigate the function of the MCMV IE3 regulatory protein, we generated two different MCMV recombinants that contained a large deletion in the IE3 open reading frame (ORF). The mutant genomes were constructed by the bacterial artificial chromosome mutagenesis technique, and MCMV *ie3* deletion mutants were reconstituted on a mouse fibroblast cell line that expresses the MCMV major immediate-early genes. The *ie3* deletion mutants failed to replicate on normal mouse fibroblasts even when a high multiplicity of infection was used. The replication defect was rescued when the IE3 protein was provided in *trans* by a complementing cell line. A revertant virus in which the IE3 ORF was restored was able to replicate with wild-type kinetics in normal mouse fibroblasts, providing evidence that the defective growth phenotype of the *ie3* mutants was due to disruption of the *ie3* gene. To characterize the point of restriction in viral replication that is controlled by *ie3*, we analyzed the pattern of expression of selective early (β) and late (γ) genes. While we could detect transcripts for the immediate-early gene *ie1* in cells infected with the *ie3* mutants, we failed to detect transcripts for representative β and γ genes. These data demonstrate that the MCMV transactivator IE3 plays an indispensable role during viral replication in tissue culture, implicating a similar role for the human CMV *ie2* gene product. To our knowledge, the *ie3* deletion mutants represent the first MCMV recombinants isolated that contain a disruption of an essential gene.

Gene expression during the lytic replication cycle of cytomegalovirus (CMV) is, as in all herpesviruses, regulated in a cascade fashion (27). Viral gene expression starts with the transcription of immediate-early (IE or α) genes immediately after infection. Transcription of IE genes is carried out by the cellular RNA polymerase II and is not dependent on *de novo* synthesis of viral proteins. Viral transactivator proteins that are synthesized during the IE phase activate transcription of early (β) genes and give rise to a more extended gene expression program during the early phase of the replication cycle. Expression of late (γ) genes occurs after the onset of the viral DNA replication.

The structural organizations of the major IE gene regions of mouse and human CMV (MCMV and HCMV, respectively) show remarkable similarity (31). A complex regulatory sequence, the major IE enhancer promoter (MIEP), controls transcription of the IE genes. Five exons are encoded downstream of the MIEP. The first three exons are spliced to either exon 4, generating the *ie1* transcript, or to exon 5, generating the *ie2* transcript. In HCMV, the *ie1* transcript is translated into the acidic 72-kDa IE1 phosphoprotein. The HCMV *ie2* transcript gives rise to the 86-kDa IE2 phosphoprotein. The corresponding IE transcripts of MCMV encode the 89-kDa

acidic IE1 phosphoprotein pp89 (15, 16) and the 88-kDa IE3 protein (24).

The functions of the HCMV IE proteins have been well analyzed during recent years. Both of the major HCMV IE proteins are involved in regulation of viral gene expression. It has been suggested that the IE1 protein augments its own expression by positive autoregulation of the MIEP (6, 32, 33). IE1 also has a costimulatory function in the activation of viral early promoters (reviewed in 31 and 27). More recently, it has been shown that IE1 mediates the disruption of nuclear structures, the promyelocytic leukemia protein (PML)-associated nuclear bodies or nuclear domains 10 (ND10), probably in order to generate a favorable environment for replication of the HCMV genome (1, 2, 13, 17). The HCMV IE2-p86 protein is a potent transactivator of HCMV early promoters and of heterologous viral as well as cellular promoters (reviewed in 31 and 27). It is believed that the IE2-p86 protein is the key regulatory protein that governs early and most likely also late gene expression of HCMV. In addition, IE2 down-regulates transcription from its own promoter by binding to the *cis*-repression signal (*crs*) target site near the transcription start site of *ie1/ie2*, thereby mediating autoregulation of its own expression (21, 22, 36). Recent studies suggest that the IE2 protein is also involved in blocking the cell cycle of infected cells (35). In contrast to the thorough functional characterization of the isolated HCMV IE2 protein that was mostly done by transient transfection assays, little is known about its role in the context of the viral infection. An HCMV mutant virus with a deletion of the *ie2* gene is not available yet.

Although the functions of the MCMV IE3 protein are not as well analyzed as those of its HCMV counterpart, it is nevertheless clear that the MCMV IE3 protein plays a similar role for replication of MCMV as the HCMV IE2 protein does for HCMV. Namely, it activates MCMV early promoters and is

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able to repress transcription from the MCMV MIEP (5, 24). This functional equivalence is also reflected in the conservation of the amino acid sequences between the MCMV IE3 and the HCMV IE2 proteins. It is assumed that CMV IE proteins have an important role not only for initiation of the lytic replication cycle but probably also during reactivation of CMV from latency. This aspect will presumably be studied best with MCMV mutants in the mouse model. There is indeed evidence for episodes of *ie1* transcription during latency of MCMV (12, 18, 19). It is more interesting, however, that the occurrence of *ie3* transcripts during induced reactivation was often associated with more extended gene transcription of MCMV and with virus recurrence (20). The availability of an MCMV *ie3* mutant offers the possibility to study the function of the IE3 protein for growth of MCMV in tissue culture as well as its role in pathogenesis of the MCMV infection *in vivo*.

Here we report on the generation and characterization of MCMV *ie3* mutants. The *ie3*-deficient mutants did not replicate in normal mouse fibroblasts, but growth could be restored by a complementing cell line that provided the IE3 protein *in trans*. Our data show an essential regulatory function of the IE3 protein during the lytic replication cycle of MCMV.

MATERIALS AND METHODS

Cells and viruses. Mouse NIH 3T3 cells (ATCC CRL1658) were grown in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum. Primary mouse embryonic fibroblasts were prepared from BALB/c.BYJ mice and grown in Dulbecco's modified Eagle medium with 10% fetal calf serum. The bacterial artificial chromosome (BAC)-derived MCMV strain MW97.01 (34), which we refer to as parental MCMV in this study, was propagated on NIH 3T3 cells. The *ie3*-deficient mutants were grown on the complementing cell line NIH 3T3-Bam25.

Construction of NIH 3T3-Bam25 cells. NIH 3T3-Bam25 cells were derived from NIH 3T3 cells by cotransfecting pBam25 (14) and pPUR (Clontech, Palo Alto, Calif.), a plasmid containing the puromycin resistance gene, using the calcium phosphate technique (10) and selecting cells in medium containing puromycin (Sigma) at 5 μ g/ml. Plasmid pBam25 contains a 10.6-kbp *Bam*HI fragment of the MCMV genome (nucleotides [nt] 176,441 to 187,035 [30]) and encodes the MCMV *ie1* and *ie3* genes under control of the authentic MCMV enhancer *ie1/ie3* promoter (14). Cultures were re-fed every 3 to 5 days. Single colonies were picked using cloning cylinders and analyzed for IE1 expression by indirect immunofluorescence using monoclonal antibody Cromo 101 (kindly provided by S. Jonjic). Reverse transcription-PCR (RT-PCR) using *ie1*- and *ie3*-specific primers was carried out to confirm the presence of the *ie1* and *ie3* transcripts in NIH 3T3-Bam25 cells. Several cell lines were obtained. For the purpose of this study, we primarily used clone 23 and confirmed our results with clone 18.

Viral growth curves. Monolayers of NIH 3T3 cells or NIH 3T3-Bam25 cells in 24-well dishes were infected at a multiplicity of infection (MOI) of 2 (for single-cycle growth curves) or 0.05 PFU/cell (for multicycle growth curves) with the different MCMV recombinants. After a 1-h adsorption period, cells were washed three times with phosphate-buffered saline and fed with fresh medium. At different time points after infection, the supernatants of three separate cultures were harvested, cleared of cellular debris, frozen, and thawed. Viral titers were determined by standard plaque assays on NIH 3T3-Bam25 cells.

Plasmid construction. The recombination plasmid pSTKSie3 was constructed to delete the *ie3* gene from the BAC plasmid pSM3fr. Briefly, plasmid pSL301 (Invitrogen, Carlsbad, Calif.) was modified by insertion of an oligonucleotide adapter providing *Mun*I, *Hind*III, and *Nsi*I sites (forward, 5'-agc tgc aat tgc gaa gct tgg atg cat cc-3'; reverse, 5'-aat tgg atg cat cca agc ttc gca att gc-3') into the *Mun*I/*Hind*III digested vector. A 3.1-kbp *Nsi*I/*Pst*I fragment (nt 175,044 to 178,117 of MCMV [30]) was isolated from plasmid *Hind*III K (7) and cloned into the *Nsi*I site of the vector resulting in plasmid pCBie3. A 3.2-kbp *Hind*III/*Mun*I fragment (equivalent to MCMV nt 179,510 to 182,682 [30]) was excised from pp89UC (24) and integrated between the *Hind*III and *Mun*I sites of pCBie3, resulting in plasmid pp89.4. The complete insert was then transferred as a 6.3-kbp *Nsi*I/*Mun*I fragment into the shuttle plasmid pST76KS11, a derivative of pST76KSacB (4) that encodes the negative selection marker *sacB* (9).

For insertion of the green fluorescent protein (GFP) marker into the MCMV BAC plasmid, the recombination plasmid pST76KS-GFP was generated. Plasmid pUCH3L, comprising the MCMV *Hind*III L fragment (7), was digested with *Hpa*I, which released a 79-bp fragment (nt 184,236 to 184,315 [30]). An oligonucleotide adapter (forward, 5'-ggg atg cat tag ttt aaa cgg cgc gcc-3'; reverse, 5'-ggc ggc cgg ttt aaa cta atg cat ccc-3') was inserted that provided the restriction enzyme sites *Nsi*I and *Asc*I, resulting in plasmid pHMM5. The polylinker of plasmid pEGFP-C1 (Clontech) was removed by digestion with *Bam*HI and *Bgl*II

followed by religation. Then a 1.6-kbp *Nsi*I/*Mlu*I fragment comprising the HCMV MIEP, the GFP open reading frame (ORF), and the simian virus 40 polyadenylation signal was excised from the modified pEGFP-C1 plasmid and inserted between the *Nsi*I and *Asc*I sites of pHMM5. A 7.2-kbp *Msc*I/*Bam*HI fragment was excised and transferred to shuttle plasmid pST76KSacB (4).

For construction of a revertant virus genome, recombination plasmid pST76KSie3rev was made as follows; plasmid pp89.4 was digested with *Nsi*I and *Hind*III, and a 5.7-kbp *Nsi*I/*Hind*III K fragment (MCMV nt 175,044 to 180,728 [30]) representing the genomic MCMV *ie3* sequence was inserted. Then the complete *Nsi*I/*Mun*I insert (equivalent to MCMV nt 175,044 to 182,682 [30]) was transferred to the shuttle vector pST76KS11.

BAC mutagenesis and reconstitution of MCMV mutants. Recombination between the shuttle plasmids and the MCMV BAC plasmid pSM3fr (34) was performed by a two-step replacement procedure in the *Escherichia coli* strain CBTS as first described by O'Connor et al. (28) utilizing the recently described modifications (3, 4, 34). Recombinant viruses were reconstituted by transfection of the BAC plasmids into murine embryonic fibroblasts, NIH 3T3, or the complementing cell lines using the calcium phosphate transfection method.

Viral nucleic acid isolation and analysis. Preparation of total DNA from infected cells, restriction enzyme analysis, and gel electrophoresis was essentially done as described previously (3). MCMV BAC plasmids were isolated from 400-ml *E. coli* cultures by using Nucleobond PC 500 columns (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer.

RT-PCR. NIH 3T3 cells or NIH 3T3-Bam25 cells were infected with the different recombination viruses at an MOI of 0.5 PFU/cell. For selective expression of IE transcripts, the cultures were incubated for 30 min prior to infection to 13 h postinfection (p.i.) in the presence of cycloheximide (100 μ g/ml; Sigma). Total RNA was isolated at the indicated time points after infection by using the RNazol B method (Tel-Test, Inc., Friendswood, Tex.) according to the manufacturer's protocol. RNA samples were treated with RNase-free DNase I for 15 min at room temperature, and the DNase was inactivated at 65°C for 15 min. The RNA was reverse transcribed using oligo(dT) primers at 42°C for 50 min, and reactions were terminated by heating at 70°C for 15 min. The reverse transcribed products were treated with RNase H for 20 min at 37°C and amplified using specific primers. Primers *ie1*-R (5'-tac agg aca aca gaa cgc tc-3') and *ie1/ie3*-F (5'-cct cga gtc tgg aac cga aa-3') were used to amplify a 188-bp product within the *ie1* gene, primers *ie3*-R (5'-tgt gag gca gta gtt ata cc-3') and *ie1/ie3*-F were used to amplify a 299-bp fragment within the *ie3* gene, primers *gB*-R (5'-aga atg tca cgt cgc act gg-3') and *gB*-F (5'-gca cgt cgt agg taa at gc-3') were used to amplify a 509-bp region within the *gB* gene, and primers *HPRT*-R (5'-aga ttc aac ttg cgc tca tct gat gc-3') and *HPRT*-F (5'-tgg gat aac cga gaa ctt tgg-3') were used to amplify a 163-bp product within the hypoxanthine phosphoribosyltransferase (*HPRT*) cellular gene. These primer sets have been previously described (18, 19). Primers *M54C* (5'-cga gtt cgt tca cgt ttc cac ag-3') and *M54NC* (5'-gat atg caa gaa gag gta tat cg-3') were designed to amplify a 660-bp product within the MCMV *M54* gene. Primers *M115C* (5'-atc ttg atc tgg tgg ctg act ga-3') and *M115NC* (5'-gac ctc acc acc gta tca tgg tta-3') were designed to amplify a 679-bp product within the *M115* gene. PCRs were performed under the following conditions: 1 cycle at 94°C for 3 min; 30 cycles of 1 min at 94°C, 1 min at the corresponding annealing temperature, and 1 min at 72°C; and 1 cycle at 72°C for 10 min. Annealing temperatures were as follows: 51°C for the *M115*-specific primers, 58°C for the *ie1*, *ie3*, *gB*, and *HPRT* primers, and 60°C for the *M54*-specific primers. The presence of introns in the viral *ie1* and *ie3* genes and the cellular *HPRT* gene made it possible to distinguish the correct amplified RNA from contaminant viral or cellular DNA by its size. In the case of the *gB*, *M54*, and *M115* genes lacking introns, amplicates derived from RNA and DNA could not be distinguished by size. Control reactions carried out in the absence of reverse transcriptase were used to assess the specific detection of RNA. Amplified products were separated on a 1% agarose gel and visualized by ethidium bromide staining.

RESULTS

Construction of MCMV genomes with a deletion in the IE3 ORF. All functions described so far for the MCMV IE3 protein have been deduced from data that were obtained with transient transfection experiments (24). To examine the function of IE3 during the replication cycle of MCMV, we generated MCMV mutants with a deletion in the IE3 ORF. Construction of the mutant genomes was performed by using the recently established BAC mutagenesis procedure (25, 34). The MCMV BAC plasmid pSM3fr (34; Fig. 1, line 1) represents the parental genome that was used to construct the *ie3* deletion genomes. pSM3fr contains the complete MCMV genome cloned into a BAC vector. After transfection into permissive cells, it gives rise to recombinant MCMV whose growth properties are indistinguishable from wild-type (wt) MCMV (34). In order to disrupt the IE3 ORF, a 1.4-kbp deletion was in-

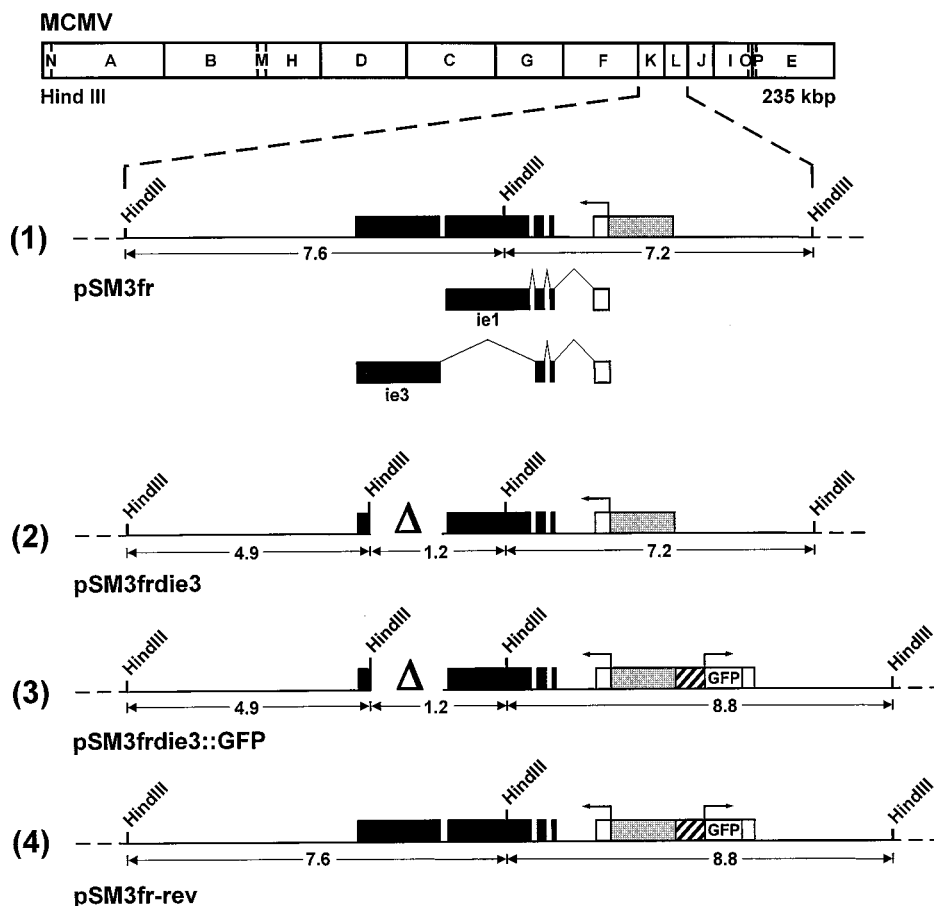


FIG. 1. Construction of *ie3*-deficient MCMV BAC genomes. The *Hind*III map of the MCMV genome is shown at the top. The expanded map of the *Hind*III K and L fragments represents the major IE gene region of MCMV. Coding exons are shown in black, and the first noncoding exon of the *ie1/ie3* transcription unit is depicted as an open rectangle. The gray box marks the MCMV enhancer *ie1/ie3* promoter. The structure of the parental BAC plasmid pSM3fr is indicated below line 1 of the expanded map. Starting with the parental MCMV BAC plasmid pSM3fr (line 1), the other BAC plasmids pSM3frdie3 (line 2), pSM3frdie3::GFP (line 3), and pSM3fr-rev (line 4) were generated by successive rounds of homologous recombination in *E. coli* as described in Materials and Methods. The deletion in the fifth exon of the *ie3* gene is marked by the delta (Δ). The cross-hatched box in front of the GFP ORF represents the HCMV MIEP.

roduced into the cloned MCMV genome by making use of the recombination procedures in *E. coli* as described in Materials and Methods. The deletion (nt 178,117 to 179,510 of the MCMV genome [30]) removed almost entirely the fifth exon of the MCMV *ie1/ie3* transcription unit (see Fig. 1, line 2). Thus, the MCMV genome of BAC plasmid pSM3frdie3 was unable to encode the IE3 protein. The position of the deletion in the BAC plasmid pSM3frdie3 was tagged with a *Hind*III restriction enzyme site (Fig. 1, line 2) in order to facilitate the characterization of the mutant genome. In a second step, the green fluorescent protein (GFP) reporter gene under control of the HCMV MIEP was introduced into the *ie3*-deficient genome, resulting in BAC plasmid pSM3frdie3::GFP (Fig. 1, line 3). The GFP expression cassette was inserted in front of the *ie2* gene (26) since it was previously shown that foreign genes can be inserted at this location without affecting the growth of the recombinant MCMV (23). Insertion of the GFP gene was performed with the intention to follow replication of the *ie3*-deficient genome in transfected cells by monitoring GFP expression. Finally, we generated a revertant genome by restoring the IE3 ORF. The revertant genome pSM3fr-rev was made to test whether the phenotype of the *ie3* knock-out mutants was caused solely by disruption of the *ie3* gene. The revertant genome also contained the GFP gene and could therefore be

distinguished from the genome of the parental virus (Fig. 1, compare lines 1 and 4).

The structure of the BAC plasmids was analyzed by digestion of plasmid DNA with restriction enzyme *Hind*III followed by agarose gel electrophoresis (Fig. 2). The 7.6-kbp *Hind*III K fragment of the parental BAC plasmid pSM3fr was missing in the BAC plasmid pSM3frdie3 and was replaced by two new fragments of 1.2 and 4.9 kbp (Fig. 1, lines 1 and 2; Fig. 2, compare lanes 1 and 2). Insertion of the GFP expression cassette in BAC plasmid pSM3frdie3::GFP resulted in a shift of the 7.2-kbp *Hind*III L fragment to a new fragment of 8.8 kbp (Fig. 1, lines 2 and 3). Hence, the 7.2-kbp *Hind*III fragment disappeared in the DNA of BAC plasmid pSM3frdie3::GFP, and a new band of 8.8 kbp was observed (Fig. 2, lane 3). Restoration of the *ie3* gene led to the reappearance of the 7.6-kbp *Hind*III K fragment in the revertant BAC plasmid pSM3fr-rev (Fig. 2, lane 4). Additional characterization of the *ie3*-deficient BAC plasmids was performed by digestion with restriction enzymes *Eco*RI and *Nsi*I. The observed DNA patterns of the BAC plasmids were as expected (data not shown). These results show that the intended modifications were introduced in the MCMV BAC plasmids and that no adventitious deletions or rearrangements could be detected anywhere else in the cloned genomes.

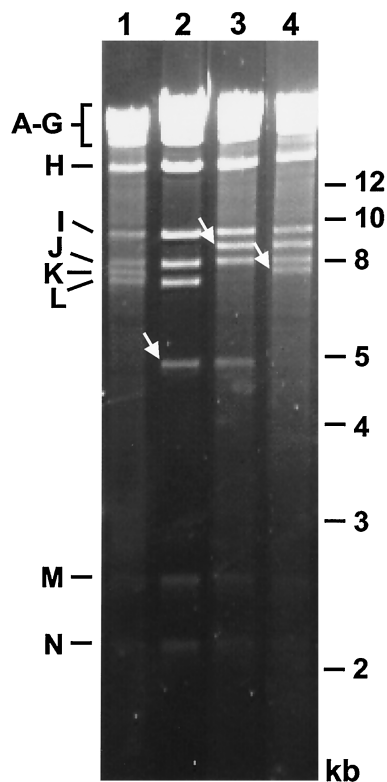


FIG. 2. Structural analysis of the *ie3*-deficient MCMV BAC genomes. Ethidium bromide-stained agarose gel of *Hind*III-digested BAC plasmids pSM3fr (lane 1), pSM3frdie3 (lane 2), pSM3frdie3::GFP (lane 3), and pSM3fr-rev (lane 4) after separation on a 0.7% agarose gel. The names of the MCMV *Hind*III fragments (7) and the sizes of the molecular-weight markers are shown in the left and right margin, respectively. New fragments in the BAC plasmids are marked with white arrows.

The *ie3* gene is essential for viral DNA infectivity. To test whether the *ie3* gene is essential for infectivity, the MCMV BAC plasmids were transfected into NIH 3T3 cells that are permissive for MCMV infection. The results of the experiments are shown in Table 1. Transfection of the parental BAC plasmid pSM3fr and of the revertant BAC plasmid pSM3fr-rev reproducibly resulted in the formation of plaques. Plaques occurred usually around day 4 or 5 posttransfection, and the infection spread rapidly throughout the monolayers. Cells harboring the revertant virus genome displayed a green fluorescence due to expression of GFP. Transfection of the *ie3*-deficient genomes pSM3frdie3 and pSM3frdie3::GFP into NIH 3T3 cells did not lead to plaque formation. Identical results were obtained after transfection of the BAC plasmids into mouse embryonic fibroblasts (data not shown). These results suggested that the *ie3* gene is essential for the lytic replication cycle of MCMV.

In a first attempt to prove that the failure of the *ie3*-deficient BAC plasmids to form plaques was due to the disrupted *ie3* gene, we performed rescue experiments by cotransfection of plasmid pBam25. pBam25 spans the deleted region and encodes the MCMV IE proteins IE1 and IE3 (15, 24). After cotransfection, pBam25 provides the missing IE3 protein in *trans*, to initiate the MCMV replication cycle. Recombination between the plasmid and the *ie3*-deficient genomes may eventually result in the reconstitution of replication-proficient genomes. The results of this experiment are shown in Table 1 (second line). The infectivity of BAC plasmids pSM3fr and

pSM3fr-rev was not influenced by cotransfection of plasmid pBam25. A few plaques appeared after cotransfection of the *ie3*-deficient BAC plasmids and pBam25. Typically, the plaques were first seen at 7 to 8 days posttransfection. After occurrence of the plaques, the infection spread rapidly throughout the tissue culture. Infected cells in the culture transfected with BAC plasmid pSM3frdie3::GFP displayed a green fluorescence. The reduced number of plaques as well as the delayed kinetics in plaque formation was consistent with the expectation that reconstitution of replication-proficient genomes by recombination with the complementing plasmid had to occur prior to plaque formation. Analysis of viral DNA obtained from these cultures showed that the reconstituted viruses had indeed acquired the *ie3* gene from the cotransfected plasmid (data not shown). Altogether, these experiments indicated that the *ie3*-deficient MCMV genomes cannot give rise to infectious virus in normal murine fibroblasts and that the replication-deficient genomes can be rescued by cotransfection of a complementing plasmid.

***trans*-complementation of viral DNA infectivity and reconstitution of MCMV *ie3* mutants.** Since the *ie3* gene seemed to be essential for replication of MCMV, a complementing cell line that provided the missing IE3 protein in *trans* was needed in order to reconstitute mutant viruses from the recombinant BAC plasmids. To this end, NIH 3T3 cells were transfected with plasmid pBam25 that encodes the MCMV IE genes *ie1* and *ie3*, and stable NIH 3T3-Bam25 cell lines were isolated as described in Materials and Methods.

The four different BAC plasmids were then transfected into one of the NIH 3T3-Bam25 cell lines. Plaques appeared around 5 to 7 days posttransfection, and the infection spread throughout the culture. To analyze the genome structure of the reconstituted mutants, viral DNA was isolated from infected NIH 3T3-Bam25 cells and subjected to restriction enzyme digestion. Fig. 3 shows the DNA fragment profiles after *Hind*III digestion of the four different MCMV genomes. The DNA patterns of the viral genomes were identical to those of the corresponding BAC plasmids (compare Fig. 2 and 3), confirming that the viral mutants were reconstituted from these BAC plasmids and that the viral genomes did not change during replication in the complementing cell line. These data demonstrate that the cell line was able to support replication of the *ie3*-deleted genomes and growth of the *ie3*-deficient MCMV mutants.

Growth analysis of the *ie3* mutants. The possibility to propagate the *ie3* mutants on the complementing cell line allowed us to prepare viral stocks. Thus, we could then perform infection experiments with the mutant viruses and test whether the *ie3* gene is definitely required for growth of MCMV in normal mouse fibroblasts. When NIH 3T3 cells were infected with a

TABLE 1. Plaque formation on NIH 3T3 cells after transfection of the MCMV BAC plasmids in the absence or presence of pBam25^a

Transfection condition	Plaque formation after transfection of BAC plasmid:			
	pSM3fr	pSM3frdie3	pSM3frdie3::GFP	pSM3fr-rev
BAC plasmid only	+++	-	-	+++
Cotransfection of BAC plasmid and pBam25 ^b	+++	+	+	++

^a Scoring of the transfections was as follows: +++, 10–20 plaques; ++, 5–10 plaques; +, 1–5 plaques; -, no plaques.

^b Plasmid pBam25 spans the deletion in the *ie3*-deficient BAC plasmids and encodes the MCMV IE genes *ie1* and *ie3*.

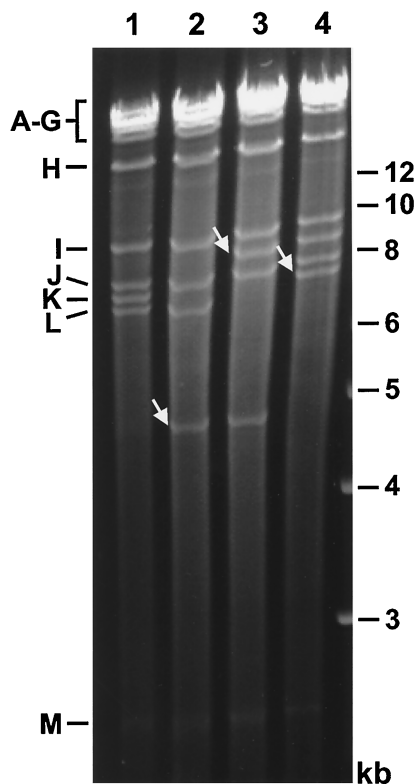


FIG. 3. Structural analysis of the genomes of the MCMV *ie3* mutants. DNA isolated from NIH 3T3-Bam25 cells infected with the parental MCMV (lane 1), the *ie3*-deficient mutants MCMVdie3 (lane 2), and MCMVdie3::GFP (lane 3), or the revertant virus MCMVrev (lane 4) was subjected to *Hind*III digestion, separated on a 0.7% agarose gel, and stained with ethidium bromide. Size markers are shown in the right margin, and the names of the *Hind*III fragments (7) are indicated in the left margin. New fragments in the genomes of the MCMV mutants are marked by white arrows.

low MOI of 0.05 PFU/cell, the amount of virus that could be found in the supernatant of cultures infected with the *ie3* mutant dropped below the detection level by 1 day p.i. Even 1 week p.i., viral progeny was not obtained in these cultures (Fig. 4A). Cultures that were infected with the parental MCMV strain displayed a rapid increase in the viral titers. The growth kinetics of the revertant virus MCMVrev were comparable to those of the parental virus, demonstrating that reinsertion of the *ie3* gene led to a complete rescue of the growth phenotype (Fig. 4A). This experiment indicated that the *ie3* gene is essential for replication of the MCMV when a low-input dose is used. Still, it was possible that the requirement for *ie3* could be overcome by using a high MOI. To examine the growth dependence of the mutant viruses on the input dose, infection experiments were performed by using an MOI of 2. Again, no growth of the *ie3* mutants was observed when cells were infected under these conditions. In contrast, the viruses that encode the IE3 protein rapidly grew to high titers (Fig. 4B). We concluded from these experiments that the *ie3* gene is absolutely essential for growth of MCMV in normal fibroblast cells, regardless whether a low- or a high-input dose is used.

Growth analyses were next performed on the NIH 3T3-Bam25 cell line to examine the growth behavior of the *ie3* mutants and the capability of the complementing cell line to support replication of the mutants. When NIH 3T3-Bam25 cells were infected with the *ie3* mutants at a low MOI of 0.05 PFU/cell, virus production could be detected at 3 days p.i., and

a rise of the virus titers was observed on days 4 to 5 p.i. (Fig. 4C). The increase in the titers of the *ie3* mutants was reduced in comparison to the titers of the parental virus. Maximal titers were obtained at day 5 p.i. with values of about 4×10^4 to 1×10^5 PFU/ml, while the *ie3*-expressing viruses achieved titers of about 4.2×10^6 PFU/ml. Similar observations were made when the NIH 3T3-Bam25 cells were infected at an MOI of 2 (Fig. 4D). Virus progeny was found 2 and 3 days p.i., but there was little further increase of the titers after day 3 p.i. The titers that were achieved with the parental and revertant viruses were about 1 to 1.5 orders of magnitude higher than those obtained with the *ie3* mutants. The difference between the titers of parental MCMV and *ie3* viruses was already seen on days 2 and 3 p.i. (Fig. 4D). Altogether, these data clearly indicate that the complementing cell line was able to support growth of the *ie3* deletion mutants, although the growth behavior of the mutants was altered in comparison to wt virus.

The *ie3* gene product is essential for early gene expression. Next, we asked at which stage the viral gene expression was blocked when cells were infected with the *ie3* mutants that are unable to express the regulatory protein IE3. Expression of representative viral genes was analyzed by RT-PCR using RNA that was isolated from cells infected with the *ie3* deletion mutant MCMVdie3::GFP and, for comparison, RNA that was with the parental MCMV. First, we tested whether the IE genes were transcribed when cells were infected in the presence of cycloheximide, i.e., in the absence of de novo protein synthesis. Transcripts arising from the *ie1* and *ie3* genes were

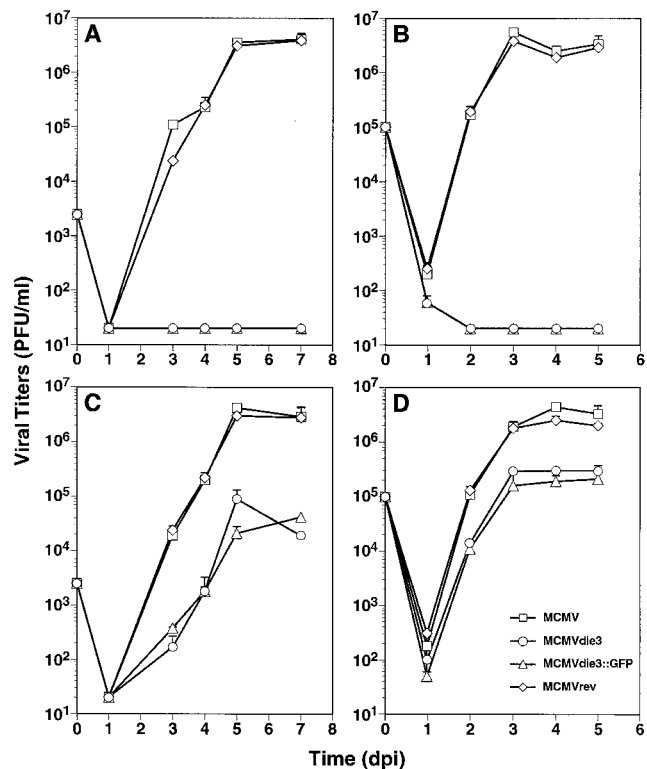


FIG. 4. Growth curve analysis of the MCMV *ie3* mutants. NIH 3T3 (A, B) or NIH 3T3-Bam25 (C, D) cells were infected at an MOI of 0.05 (A, C) or 2 PFU per cell (B, D) with the parental MCMV, MCMVdie3, and MCMVdie3::GFP and the revertant MCMVrev. At the indicated time points after infection (days p.i. [dpi]), supernatants from the infected cultures were harvested and titered on monolayers of NIH 3T3-Bam25 cells. The limit of detection was 20 PFU/ml. Error bars indicate the standard deviation from three separate cultures.

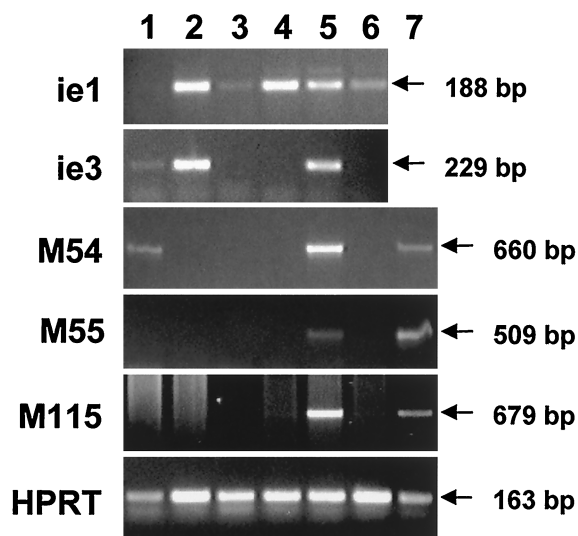


FIG. 5. Detection of viral transcripts after infection with an MCMV *ie3* mutant. NIH 3T3 (lanes 1 through 6) or NIH 3T3-Bam25 (lane 7) cells were infected at an MOI of 0.5 PFU per cell with parental MCMV (lanes 1, 2, and 5) or MCMVdie3::GFP (lanes 3, 4, 6, and 7) in the presence (lanes 2 and 4) or absence (lanes 1, 3, and 5 to 7) of cycloheximide. Whole-cell RNA was harvested at 13 h p.i. (lanes 1 through 4) or 20 h p.i. (lanes 5 through 7), treated with DNase, and reverse transcribed using oligo(dT). PCRs were performed using primer sets specific for *ie1*, *ie3*, M54, M55, M115, and HPRT as described in Materials and Methods. Amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. Sizes of the amplified products are indicated by arrows. Specific PCR-amplified products were not detected in control reactions in which the reverse transcriptase was not added during the RNA reverse transcription reaction (data not shown).

detected in cells infected with the parental virus (Fig. 5, lane 2). In cells infected with the *ie3* mutant MCMVdie3::GFP, only *ie1* transcripts could be detected (Fig. 5, lane 4). As expected, transcripts of IE genes accumulated under this condition of infection because transcription of IE genes was performed by the transcription machinery of the cell, and de novo synthesis of viral proteins was not required. Transcription of early and late genes did not occur since viral transactivator proteins that were required for early and late gene expression were not synthesized in the presence of cycloheximide. The data clearly indicate that the *ie3* mutant did not synthesize an *ie3* transcript (Fig. 5, lane 4). This result confirmed that the *ie3* gene had been disrupted in the *ie3* mutant and that the mutant was therefore unable to encode the regulatory IE3 protein.

Next, we examined the viral gene expression at 13 h p.i., a time point during the early phase of the infection cycle well before the onset of viral DNA replication (14; Fig. 5, lanes 1 and 3). In addition to the *ie1* and *ie3* transcripts, we found transcripts of the early gene M54, encoding the viral DNA polymerase (8) and a small amount of the transcript encoding the glycoprotein B (M55 [29, 30]) in MCMV-infected cells (Fig. 5, lane 1). In cells infected with the *ie3* mutant, viral gene expression was restricted to the *ie1* gene (Fig. 5, lane 3). The level of *ie1* transcripts in cells infected with the *ie3* mutant seemed higher than in cells infected with the parental virus (Fig. 5, compare lanes 1 and 3). This might indicate that feedback regulation of IE gene expression by the IE3 protein that leads to reduced levels of *ie1* transcripts in MCMV-infected cells during the early phase (Fig. 5, lane 1) cannot occur in cells infected with the *ie3* mutant and results in enhanced expression of the *ie1* gene (Fig. 5, lane 3). Transcripts of early genes could not be detected in MCMVdie3::GFP-infected cells by 13 h p.i.

(Fig. 5, lane 3). This result indicated that either activation of early gene transcription was completely impossible in cells infected with the *ie3* mutants or the time course of the viral gene expression program was delayed.

To distinguish between these possibilities, RNA isolated in the late phase of the infection cycle (20 h p.i.) was analyzed. RNA from MCMV-infected cells contained transcripts of the late gene M115 encoding glycoprotein L (37), in addition to the early and IE transcripts that were already detected at the earlier time point. Again, in RNA isolated from cells infected with the *ie3* mutant, only transcripts arising from the *ie1* gene could be detected (Fig. 5, lane 6). Thus, gene expression in MCMVdie3::GFP-infected cells was always restricted to the IE gene *ie1*, irrespective of whether the cells were infected in the presence or absence of CH and at which time point p.i. the infected cells were analyzed (Fig. 5, compare lanes 3, 4, and 6). Accordingly, the protein encoded by the *ie3* gene must exert a key function in activation of early gene expression.

To test directly whether *ie3* is important for activating early genes, we analyzed RNA isolated from the complementing NIH 3T3-Bam25 cell line that had been infected with the *ie3* mutant. Since the complementing cell line encodes the missing protein, the expression of early and late genes should be restored if the protein mediates the proposed regulatory function. The results of the experiment revealed the same profile of early and late viral transcripts in MCMVdie3::GFP-infected NIH 3T3-Bam25 cells as in MCMV-infected NIH 3T3 cells (Fig. 5, compare lanes 7 and 5). We concluded from these experiments that the complementing cell line provided a sufficient amount of the transactivating protein to achieve activation of early and late gene expression and to substitute for the lack of *ie3* expression by the *ie3* mutant. In summary, the data indicate that the protein encoded by the *ie3* gene plays an essential role for the activation of the viral gene expression program.

DISCUSSION

In this study, we report on the generation of MCMV *ie3*-deficient mutants. Disruption of the *ie3* gene on the cloned MCMV genome was achieved by utilizing the recently established BAC mutagenesis procedure. Transfection of *ie3*-deficient genomes into permissive cells did not result in plaque formation, indicating that the genomes were replication deficient. Infectious viruses could be reconstituted by transfection of the *ie3*-deficient genomes into a cell line that provided the missing IE protein *in trans*. The *ie3* mutants could not grow on normal non-complementing cells, indicating the essential function of the *ie3*-encoded protein. Transcript analysis in cells infected with an *ie3* mutant showed that early and late genes were not activated. Altogether, these data provided direct evidence for an essential regulatory role of *ie3* for replication of MCMV.

Construction of MCMV *ie3*-deficient mutants by the BAC technique. The mutant MCMV genomes were constructed by site-directed mutagenesis of the cloned MCMV genomes in *E. coli* (25, 34). This technique might be especially useful for mutagenesis of essential genes since construction of the mutant genome is completely independent of the ability of the corresponding mutant virus to grow in cell culture. Thus, we can first manipulate any gene of interest on the cloned genome, and, in a second separate step, we can examine the phenotypic consequences of the manipulation, e.g., whether the deleted gene is essential or nonessential.

The mutant BAC plasmids isolated from bacterial cultures were of clonal origin. Therefore, after transfection of the BAC

plasmids into the complementing cell line, we got mutant viruses only, and no selection against parental virus was required. We consider this a major advantage of our technique in comparison to conventional recombination techniques in complementing cell lines, because selection and isolation of mutant viruses might be quite cumbersome if the mutant has an impaired growth potential, in comparison to the wt virus.

Furthermore, we demonstrated that consecutive rounds of mutagenesis can be performed on the cloned MCMV genome without the need to reconstitute viral intermediates. We showed that intermediate steps can lead to replication-deficient genomes and that eventually a revertant replication-competent genome can be reconstituted. The GFP marker inserted into the revertant genome allowed us to differentiate between the parental and the revertant viruses. Rescue of the growth potential by reinsertion of the *ie3* gene in the revertant genome confirmed that the observed growth deficit of the *ie3* mutants was indeed due to the disruption of the *ie3* gene and excluded the possibility that any other mutation that may have been accidentally introduced somewhere else in the genome might have been responsible for the phenotype. To our knowledge, this is the first report on the generation and complementation of an MCMV mutant with a disruption of an essential gene.

Properties of the complementing cell line. The MCMV *ie3* mutants could be reconstituted and propagated on NIH 3T3-Bam25 cell lines. Neither the successful generation of the complementing cell line nor the fact that the cell line was able to support growth of the *ie3* mutants seems to be trivial. For example, several IE proteins of other herpesviruses turned out to be toxic for cells (11). Accordingly, construction of cell lines expressing such IE proteins was difficult. Since it has been reported that the HCMV IE2 protein, which is homologous to the MCMV IE3 protein, is able to block the cell cycle in transfected cells (35), one could expect that generation of MCMV IE3-expressing cell lines may be rather complicated. As is observed with coexpressing *ie1* and *ie2* of HCMV (A. Angulo and P. Ghazal, unpublished results), we did not encounter any problems in generating the NIH 3T3-Bam25 cell lines that express both of the MCMV major IE genes. Also, we reported before on the successful generation of a similar cell line that encodes the MCMV IE1 and IE3 proteins (5). However, this particular cell line failed to complement the *ie3* mutants. The reason for this is unclear at present but may be the result of inappropriate expression or modification of the MCMV major IE proteins.

The IE3 protein expressed by the complementing cell line was sufficient to allow growth of the *ie3* mutants. However, the titers of the *ie3* mutants obtained on the cell line did not reach those levels which were achieved with the parental and revertant viruses. The titers of the different viruses were determined on the complementing cell lines, and we have to consider that the efficiency of plaque formation of the *ie3* mutants might be lower than that of the parental virus. Although we do not have any indication for a reduced efficiency of plaque formation, we cannot completely rule out that the titers of the *ie3* mutants were underestimated. But even if the input titers were underestimated, the results indicate that the *ie3* mutants grow to lower final titers. There are several possible explanations for the altered growth kinetics of the *ie3* mutants on the complementing cell line. First, the amount of the IE3 protein in the cell line might not be as high as that during infection with wt viruses. Second, the amount of IE3 required may vary during the infection cycle. There is indeed evidence that the IE3 protein autoregulates expression from its own promoter (24). Though the IE genes in the complementing cell line were expressed from their authentic promoter, it is not clear

whether correct transcriptional regulation is maintained when the viral genes are integrated into the cellular chromatin. There is also evidence for posttranslational modification of the MCMV IE3 protein during the replication cycle, most likely by phosphorylation (24). We do not know whether appropriate modification of IE3 occurs in the complementing cell line. Finally, the copy number of the viral genomes goes up during replication, whereas the number of integrated IE genes in the cellular genomes remains constant. The lower titers of the *ie3* mutants in the complementing cell line can be easily explained if a certain amount of IE3 protein is required per viral genome. In this case, IE3 will become limiting in the cell line when the viral copy number increases. Accordingly, virus production will already cease at lower titers. This will not happen when IE3 is expressed from the wt genomes since the copy number of the *ie3* gene increases coordinately with the increase of the viral genomes. Additional experiments are required to explain the limited growth of the *ie3* mutants in the complementing cell line.

The *ie3* gene is essential for viral growth, and the *ie3* encoded protein is a key regulator for early gene expression. We provide several lines of evidence that the MCMV *ie3* gene is essential for viral growth. (i) MCMV BAC genomes with a large deletion in the ORF encoding the IE3 protein were unable to generate viral progeny. (ii) Viral infectivity could be restored in *cis* by cotransfection of a plasmid spanning the deleted region and in *trans* by transfection of the BAC plasmids into a complementing cell line that provided the missing IE protein. (iii) The *ie3* mutants that were reconstituted on the complementing cell line were unable to grow on normal fibroblasts either at low or high MOI.

During the IE phase of the infection cycle MCMV expresses at least two proteins that are encoded by the major IE region, namely the 89-kDa protein pp89 and the 88-kDa protein IE3. Due to disruption of exon 5 of the *ie1/ie3* transcription unit, it is clear that the *ie3* mutants are unable to express the IE3 protein. The *ie1* gene is not affected by the deletion, and the RNA analyses indicated that the *ie3* mutants express *ie1* transcripts in infected cells irrespective of whether infection occurred in the presence or absence of cycloheximide. Though enhanced expression of the IE1 protein might occur in cells infected with the *ie3* mutants because the lack of IE3 protein might lead to a failure in autoregulation of IE transcription (24), we consider it unlikely that the observed growth phenotype is due to altered IE1 expression. This belief is supported by the fact that disruption of the *ie1* gene does not result in a lethal phenotype (25 and unpublished data). The most likely explanation for the growth defect of the *ie3* mutants is their inability to synthesize the IE3 protein. This conclusion is further supported (i) by the observation that the complementing cell line that provides the IE3 protein in *trans* allows growth of the *ie3* mutants and (ii) by the fact that repair of the IE3 ORF in the revertant virus rescued the growth potential.

The restricted viral gene expression profile displayed by the *ie3* mutants is explained best by the absence of the regulatory function of the IE3 protein. Transcription of viral genes was confined to *ie1*, which can occur in the absence of viral regulatory proteins. Though our RNA analyses were limited to the important early genes encoding the viral DNA polymerase and the glycoprotein B and we cannot completely rule out that some early genes might be activated in the absence of the IE3 protein, it seems that gene expression by the *ie3* mutants is blocked at the IE stage of the infection cycle. Accordingly, the IE3 protein has a key regulatory function for activation of viral gene expression, i.e., for the switch from α to β gene expression. Further studies are required to investigate whether the

IE3 protein is just needed to initiate early gene activation or is required throughout the replication cycle in order to maintain viral gene expression.

The observation of the important regulatory function of the IE3 protein has two implications. If the counterpart of the MCMV IE3 protein in HCMV, IE2, possesses a similar key regulatory function, it may be possible to combat the HCMV infection by developing and using therapeutic compounds that interfere with this function of the HCMV IE2 protein. Second, gene activation by CMV IE proteins might represent a bottleneck not only during initiation of the lytic replication cycle but also during reactivation of CMV from latency. Indeed, data from Reddehase et al. suggest that the regulatory function of the IE3 protein is also pivotal during reactivation of MCMV (20). Again, this promises to offer a point of intervention at which to inhibit recurrence of CMV and to control the CMV infection at a very early stage.

In conclusion, we have shown that the *ie3* gene plays a key role for activation of MCMV gene expression. Given the many similarities between the MCMV IE3 and the HCMV IE2 protein, the data of our experiments predict a comparable essential role of the IE2 protein for gene expression of HCMV during the lytic replication cycle. The precise mechanism(s) and functional significance of the major IE transactivator of CMV in promoting lytic replication in vitro and in vivo are topics that remain to be explored.

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