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*-regression 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
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 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/cByJ mice and grown in Dulbecco's modified Eagle medium with 10% fetal calf serum. The bacterial artificial chromosome (BAC)-derived MCMV strain MW9701 (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 pBluescriptII (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 μg/ml. Plasmid pBluescriptII contains a 10.6-kbp BamHI 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 an antibody (Cromby Croma 14) 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 calculated with a 0.05 PFU/cell (multiplicity of infection) plaque assay for 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 recombinant plasmid pSTKie3c was constructed to delete the ie3 gene from the BAC plasmid pSM3fr. Briefly, plasmid pSM3fr (Invitrogen, Carlsbad, Calif.) was modified by insertion of an oligonucleotide adapter providing Muni I, HindIII, and Nol sites (forward, 5′-agc tag gaa gta gtt atc cct cc-3′; reverse, 5′-aat tag atc gaa ctc aag gcg ttc-3′) into the Muni I/HindIII digested vector. A 3.1-kbp NsiI/PstI fragment (nt 175,044 to 178,117 of MCMV [30]) was cloned into the Nol site of the vector resulting in plasmid pCPliec. A 3.2-kbp HindIII/MuniI fragment (equivalent to MCMV nt 179,510 to 182,682 [30]) was excised from pBP9UC (24) and integrated between the HindIII and MuniI sites of pCPliec, resulting in plasmid pBP9. The complete insert was then transferred as a 6.3-kbp Nol/MuniI fragment into the shuttle plasmid pSKS11, a derivative of pSTK76KS112, by following a religation. Then a 1.6-kbp Nol/MuniI fragment comprising the MCMV IE1, the GFP open reading frame (ORF), and the simian virus 40 enhancer were amplified from the modified pGEFG-C1 plasmid and inserted between the Nol and AscI sites of pPHMM5. A 2.7-kbp MekUmb/HindIII fragment was excised and transferred to shuttle plasmid pST76KasacB (4).

For construction of a revertant virus genome, recombinant plasmid pST76Ksie3v was made as follows; plasmid pPhp9 was digested with NolI and HindIII, yielding a 5.7-kbp NsiI/HindIII fragment (equivalent to MCMV nt 175,510 to 182,682 [30]) representing the genomic MCMV ie3 sequence was inserted. Then the Nol/MuniI insert (equivalent to MCMV nt 175,044 to 182,682 [30]) was transferred to the shuttle vector pST76KsK11.

For construction between the shuttle plasmids and the MCMV BAC plasmid pSM3fr (34) was performed by a two-step replacement procedure in the Escherichia coli strain C8070 (12) 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 manufacturer’s protocol. RNA samples were treated with RNase-free DNase I for 15 min at 37°C before the presence of cyclophamine (100 μM) and cycloheximide (100 μM). 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. DNA was using oligo(dT) primers at 42°C for 45 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 ic1-R (5′-tag aca gag gaa gac ggc tc-3′) and ic1e3-F (5′-ctc cgt tgc aat ccg aa-3′) were used to amplify a 188-bp product within the ie1 gene, primers ic3-R (5′-tgt gag cga gta gta aca cc-3′) and ic3e3-F were used to amplify a 299-bp fragment within the ie3 gene, primers gB-R (5′-aga ttc cgt gga gaa gac gac ggc tc-3′) and gB-F (5′-gag aac aag gaa gac ggc tc-3′) were used to amplify a 509-bp region within the gB gene, and primers HPRT-R (5′-ata ctc tgt gcc tca tct gcg ctc-3′) and HPRT-F (5′-ttg gat aac agg cca gaa ctt tgt tgg-3′) were used to amplify a 163-bp product within the hypoxanthine phosphoribosyltransferase (HPRT) cellular gene. The primers were designed previously (18, 19).

Primers M54C (5′-cgt gta tgt gcc tca tct cag ag-3′) and M54NC (5′-cgt gag cga gta gta ctc-3′) were designed to amplify a 660-bp product within the MCMV M54 gene. Primers M115C (5′-atc tgt tgc cta tgt cag act ga-3′) and M115NC (5′-gac ctc acc aca gta gtc tga-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 complementary viral or cellular DNA by size. In the absence of gB, and M115 genes lacking introns, amplificates 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-
introduced 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 HindIII 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). Insertion of the GFP expression cassette in BAC plasmid pSM3frdie3::GFP resulted in a shift of the 7.2-kbp HindIII fragment to a new fragment of 8.8 kbp (Fig. 1, lines 2 and 3). Hence, the 7.2-kbp HindIII 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 HindIII K fragment in the revertant BAC plasmid pSM3fr-rev (Fig. 2, lane 4).

FIG. 1. Construction of ie3-deficient MCMV BAC genomes. The HindIII map of the MCMV genome is shown at the top. The expanded map of the HindIII 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 ie1 and ie3 transcripts 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.

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 HindIII followed by agarose gel electrophoresis (Fig. 2). The 7.6-kbp HindIII 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 HindIII L fragment to a new fragment of 8.8 kbp (Fig. 1, lines 2 and 3). Hence, the 7.2-kbp HindIII 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 HindIII 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 EcoRI and NsiI. 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.
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 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 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

![Fig. 2. Structural analysis of the *ie3*-deficient MCMV BAC genomes. Ethidium bromide-stained agarose gel of HindIII-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 HindIII 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.](image-url)
low MOI of 0.05 PFU/cell, the amount of virus that could be found in the supernatant of cultures infected with the \textit{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 MCMV\textit{rev} were comparable to those of the parental virus, demonstrating that reinsertion of the \textit{ie3} gene led to a complete rescue of the growth phenotype (Fig. 4A). This experiment indicated that the \textit{ie3} gene is essential for replication of the MCMV when a low-input dose is used. Still, it was possible that the requirement for \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 $4 \times 10^4$ to $1 \times 10^5$ PFU/ml, while the \textit{ie3}-expressing viruses achieved titers of about $4.2 \times 10^6$ 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 \textit{ie3} mutants. The difference between the titers of parental MCMV and \textit{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 \textit{ie3} deletion mutants, although the growth behavior of the mutants was altered in comparison to wt virus.

The \textit{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 \textit{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 \textit{ie3} deletion mutant MCMV\textit{die3::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 \textit{ie1} and \textit{ie3} genes were

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3}
\caption{Structural analysis of the genomes of the MCMV \textit{ie3} mutants. DNA isolated from NIH 3T3-Bam25 cells infected with the parental MCMV (lane 1), the \textit{ie3}-deficient mutants MCMV\textit{die3} (lane 2), and MCMV\textit{die3::GFP} (lane 3), or the revertant virus MCMV\textit{rev} (lane 4) was subjected to \textit{HindIII} 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 \textit{HindIII} fragments (7) are indicated in the left margin. New fragments in the genomes of the MCMV mutants are marked by white arrows.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4}
\caption{Growth curve analysis of the MCMV \textit{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, MCMV\textit{die3}, and MCMV\textit{die3::GFP} and the revertant MCMV\textit{rev}. 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.}
\end{figure}
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-proficient 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 postranslational 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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