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The Human Cytomegalovirus Major Immediate-Early Enhancer Determines the Efficiency of Immediate-Early Gene Transcription and Viral Replication in Permissive Cells at Low Multiplicity of Infection

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To determine the effect of the human cytomegalovirus (CMV) major immediate-early (MIE) enhancer or promoter on the efficiency of viral replication in permissive human cells, we constructed recombinant viruses with their human MIE promoter, enhancer, and promoter plus enhancer replaced with the murine CMV components. After a low multiplicity of infection (MOI) (0.01 PFU/cell), recombinant human CMV with the murine CMV promoter replicated like the wild type but recombinant virus with the murine enhancer replicated less efficiently. Immediate-early (IE) viral protein pIE72 (UL123), early viral protein (UL44), and viral DNA synthesis were significantly decreased. The effect of the human CMV enhancer substitution with the murine CMV enhancer was also demonstrated in different cell types by using recombinant virus with the UL127 promoter, driving the expression of green fluorescent protein (GFP). After an MOI of 1, GFP expression was high with the human CMV enhancer and significantly lower with the murine CMV enhancer. Even though at a high MOI (10 PFU/cell), the murine CMV enhancer was as efficient as the human CMV enhancer for the transcription of IE genes in human foreskin fibroblast cells, at lower MOIs, the murine CMV enhancer was less efficient. Proximal and distal chimeras of the human and murine enhancers also replicated less efficiently at a low MOI and expressed lower levels of GFP from the UL127 promoter. These experiments demonstrate that the entire human CMV enhancer has evolved for the efficient expression of the viral IE and early genes in human cells. Possible functions of the human CMV enhancer and promoter at a low MOI are discussed.

Human cytomegalovirus (CMV) is a member of the betaherpesvirus family. Gastrointestinal diseases and diseases such as pneumonitis, hepatitis, and retinitis are related to human CMV infection (7, 17). Human CMV can be reactivated from latency in immunocompromised individuals (26, 27). In cell culture, the host cell range of human CMV is narrow (40). Human CMV replicates productively in terminally differentiated cells such as fibroblast, epithelial, and endothelial cells and in monocyte-derived macrophages (20, 21, 39, 46).

During productive infection, CMV genes are expressed in a temporal cascade, designated immediate-early (IE), early, or late. The human CMV IE genes include the major IE genes (MIE) UL123 and UL122 (IE1 and IE2, respectively) and other auxiliary genes (9, 42). The human CMV IE genes are thought to play a critical role in the efficiency of replication. The messenger RNAs of the IE1 and IE2 genes are generated by the alternative splicing of a single precursor RNA. The proteins encoded by IE1 and IE2 have important roles in the regulation of subsequent viral gene expression. The IE2 gene is essential for early viral gene expression and viral replication (29). The IE2 protein also acts as an autorepressor, repressing transcription of the IE1 and IE2 genes by binding the *cis* repression sequence (CRS) between positions −13 and −1 relative to the transcription start site at position +1 of the MIE promoter (6, 24, 37). The IE1 gene is necessary for efficient replication after a low multiplicity of infection (MOI) (11, 14, 34).

The region upstream of the human CMV MIE promoter is divided into three regions, the modulator, the unique region, and the enhancer (33). The enhancer is also divided into a distal and a proximal enhancer. The distal enhancer is necessary for efficient IE gene expression and viral replication at a low MOI (31). Deletion of the modulator has no effect on viral replication in various cell types in cell culture (32). The unique region has no effect on transcription from the MIE promoter, but it does affect transcription from the UL127 promoter (28). While the murine CMV enhancer is bidirectional for the expression of the ie1 and ie3 genes and the ie2 gene, the human CMV enhancer is unidirectional for the expression of the IE1 and IE2 genes (19). The human CMV IE1 and IE2 genes are the functional homologues of the murine CMV ie1 and ie3 genes, respectively (41, 42). The murine CMV ie2 gene is not related to a human CMV gene, but it is the positional homologue of the human CMV UL127 gene. Both human and murine CMVs have very strong enhancers for the expression of MIE genes. The human CMV enhancer has four 18-bp repeat elements containing an NF-κB or rel binding site, five 19-bp repeat elements containing a CREB or ATF binding site, two AP-1 binding sites, and multiple SP-1 sites (33). The murine CMV enhancer contains six NF-κB or rel binding sites, one CREB or ATF binding site, and at least seven AP-1 binding sites (10). The different *cis*-acting elements act individually and synergistically to stabilize the RNA polymerase II transcription initiation complex on the promoter.

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Betaherpesviruses have distinct species and cell type specificities. That is, human CMV cannot replicate in murine cells and murine CMV cannot replicate in human cells (22). Replication of recombinant virus with the rat CMV enhancer substituted by the murine CMV enhancer was deficient in the rat fibroblast, and the recombinant virus spread less efficiently to the salivary gland of the rat (38). In contrast, when the murine enhancer was substituted by the human CMV enhancer, the recombinant virus replicated in cell culture and in the mouse like the wild type (wt) (3, 15). While the murine CMV enhancer is not essential for replication in murine fibroblasts in culture, it is required for replication in murine macrophages (3) and for cytopathic effects and disease in the animal (15). From these studies, it was unclear why CMV enhancers were of equal function in one case and not in another. It was also unclear whether CMV enhancers affect the efficiency of viral replication or the cell type specificity of replication.

To determine the effect of the human CMV enhancer on the replication of the virus, we constructed recombinant viruses with the human CMV enhancer, promoter, and enhancer plus promoter substituted with the murine CMV components. We also constructed recombinant viruses with chimeras of the human and murine CMV enhancer components. Although the human CMV recombinant virus containing the murine CMV enhancer functions in human foreskin fibroblast (HFF) cells as efficiently as the human CMV enhancer at a high MOI (10 PFU/cell), the murine CMV enhancer functions inefficiently for IE gene expression at lower MOIs. Substitution of the human CMV promoter with the murine CMV promoter had little to no significant effect. At a low MOI, an enhancer from murine CMV is not functionally equivalent to the human CMV enhancer. The proximal and distal components of the human CMV enhancer affect the efficiency of viral replication at a low MOI. These data indicate that the human CMV enhancer has evolved to have components for the successful replication of the virus in human cells. Reasons why the murine CMV enhancer is not the functional equivalent of the human CMV enhancer in human cells are discussed.

MATERIALS AND METHODS

Cells and virus. Primary HFF cells were maintained in Eagle's minimal essential medium (Mediatec, Herndon, Va.) supplemented with 10% newborn bovine serum (Sigma, St. Louis, Mo.), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2 as described previously (44). Virus titers were determined by standard plaque assays on HFF cells as described previously (32). After 1 h of virus adsorption, cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, and medium was added. Virus titers in the cells and extracellular fluid were determined between 1 and 10 days (d) postinfection (p.i.). An inverted direct scope (model SZX12 research stereo microscope, 0.5× lens objective; Olympus America, Inc., Melville, N.Y.) was used to compare plaque sizes.

THP-1 cells were grown in RPMI 1640 (Mediatec) supplemented with 10% fetal calf serum (HyClone, Logan, Utah), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2. Only low-passage THP-1 cells were used. To differentiate THP-1 cells, growth medium was supplemented with 20 nM phorbol 12-myristate 13-acetate (PMA; Sigma) plus 50 μM hydrocortisone.

Enzymes. Restriction endonucleases were purchased from New England Biolabs, Inc. (Beverly, Mass.). T4 DNA ligase and polymerase were obtained from Roche Applied Science (Indianapolis, Ind.). High-fidelity *Taq* DNA polymerase was purchased from Invitrogen (Carlsbad, Calif.). RNasin and RNase-free DNase were purchased from Promega (Madison, Wis.). These enzymes were used according to the manufacturers' instructions.

Plasmid construction. Plasmid pKS+MIE−583/+78, containing the human CMV Towne DNA from positions −583 to +78 of the MIE enhancer and promoter relative to the +1 transcription start site, was used. Cassettes that contained the human CMV enhancer plus the murine CMV promoter, the murine CMV enhancer plus the human CMV promoter, and the murine CMV enhancer plus the murine CMV promoter were constructed as follows. The murine CMV promoter from positions −60 to −13 relative to the transcription start site, the murine CMV enhancer from −1272 to −60, and the murine CMV enhancer plus promoter from −1272 to −13 were amplified from plasmid pSV11CAT (a gift from U. Koszinowski) by PCR with the following sets of primer pairs: 5′-CGACGCTGAGCTCGCCTCTTATACCC-3′ with 5′-AGGG TCAACCATTCTATTGGCTG-3′ and 5′-AGGGATATCCGTGCCAATATG G-3′ with 5′-AGGACTAGTAAAGCCAAACGCCCCC-3′. All sequences amplified by PCR were confirmed by automated dideoxynucleotide sequencing (University of Iowa DNA core). The PCR products were digested by the restriction endonucleases *Sac*I and *Hin*cII, *Eco*RV and *Spe*I, and *Sac*I and *Spe*I, respectively; cloned into pKS+MIE−583/+78 at the corresponding restriction endonuclease sites; and named pdlMGFP(CAT)dlSpeI-*Spe*I+mP (murine promoter), pdlMCATdlSpeI-*Spe*I+mE (murine enhancer), and pdlMGFP(CAT)dlSpeI-*Spe*I+mE/P (murine enhancer plus promoter), respectively. Cassettes that contained the chimera enhancers of human CMV distal plus murine CMV proximal and murine CMV distal plus human CMV proximal were constructed as follows. The human CMV distal enhancer from positions −300 to −580 and the human CMV proximal enhancer from positions −78 to −300 were amplified from plasmid pKS+MIE−583/+78 by PCR with the following sets of primer pairs: 5′-CCTTAATTAAGCCATTTACCGTCATTGACGTCA-3′ with 5′-TG GCCATTGCATACGTTGTATC-3′ and 5′-CCTTAATTAACCGCCTGGCAT TATGCCCAGTAC-3′, with 5′-TAATACGACTCACTATAGGG-3′, respectively. The PCR products were digested with restriction endonucleases *Spe*I-*Pac*I and *Pac*I-*Eag*I, respectively, and cloned into pdlGFP(CAT)dlSpeI-*Spe*I+mE at the corresponding restriction endonuclease sites. To construct the shuttle vectors, each *Spe*I-to-*Eag*I DNA fragment was cloned into pdlMGFPdlSpeI-*Spe*I or pdlMCATdlSpeI-*Spe*I. Plasmid pdlMGFPdlSpeI-*Spe*I or pdlMCATdlSpeI-*Spe*I was constructed by deleting the *Spe*I-*Spe*I fragment of plasmid pdlMCAT−694/−583 as described previously (P. E. Lashmit, J. L. Meier, and M. F. Stinski, unpublished data).

Recombinant viruses. Recombinant viruses were isolated by the method of Greaves et al. (13) described previously (32). The parental virus RdlMSVgpt was used as described previously (32). wt viruses RdlM−582/−1108Egfp and RdlMCAT116 were previously reported (31). HFF cells were transfected with either 5 or 10 μg of each shuttle vector plus RdlMSVgpt DNA by the calcium phosphate precipitation method of Graham and van der Eb (12). After approximately 10 d, virus in the extracellular fluid was harvested, diluted either 1:20 or 1:100, and used to infect Lesch-Nyhan human fibroblasts (Coriell Cell Repository, Camden, N.J.). Selection of recombinant viruses was done in medium containing 50 μg of 6-thioguanine (Sigma)/ml. Viral plaques were transferred to HFF cells in 12-well plates. To isolate the recombinant viruses, green fluorescent protein (GFP) expression was detected by using a UV microscope (CX41 educational microscope) with a digital camera (DP12 digital camera; Olympus) or chloramphenicol acetyltransferase (CAT) assays were performed. Recombinant viruses were plaque purified twice.

Southern blot analysis. Culture medium containing virus was subjected to low-speed centrifugation to pellet particulate material and high-speed centrifugation to pellet the virus as described previously (45). After solubilization of the viral envelope with 1% Sarkosyl and digestion of the viral proteins with 200 μg of proteinase K per ml in 0.1% sodium dodecyl sulfate (SDS), viral DNAs were phenol-chloroform extracted and ethanol precipitated. Viral DNAs were digested with the restriction endonucleases *Blp*I and *Spe*I or *Pac*I and *Spe*I and then subjected to 1.0% agarose gel electrophoresis as described previously (32, 48). Southern blot analysis was done as described previously (32). Probes 1, 2, and 3 (Fig. 1) or probes 1 and 2 (see Fig. 7) were generated by labeling with [^{32}P]dCTP (Amersham Pharmacia Biotech, Piscataway, N.J.) DNA fragments *Blp*I-*Sac*I, *Eag*I-*Blp*I, and *Blp*I-*Spe*I, respectively, by using Ready-To-Go DNA labeling beads.

DNA replication assay. After infection with an MOI of 0.01, cells were collected 5 and 9 d p.i. Viral DNA was isolated as described above. Lambda DNA (2 μg) was added to each sample after cell lysis but before proteolysis and phenol-chloroform extraction to control for sample-to-sample variation in processing, endonuclease digestion, and loading. Viral genomes were digested with endonuclease *Hin*dIII, fractionated in a 0.6% agarose gel, and subjected to Southern blot analysis as described previously (31). The 1.6-kbp *Bam*HI-*Hin*dIII fragment of plasmid p1.6 (T probe) was used to probe human CMV genomic termini containing the long terminal repeat or long inverted repeat as described previously (31). Lambda DNA was labeled with [^{32}P]dCTP as described above and was used for probing lambda DNA fragments.

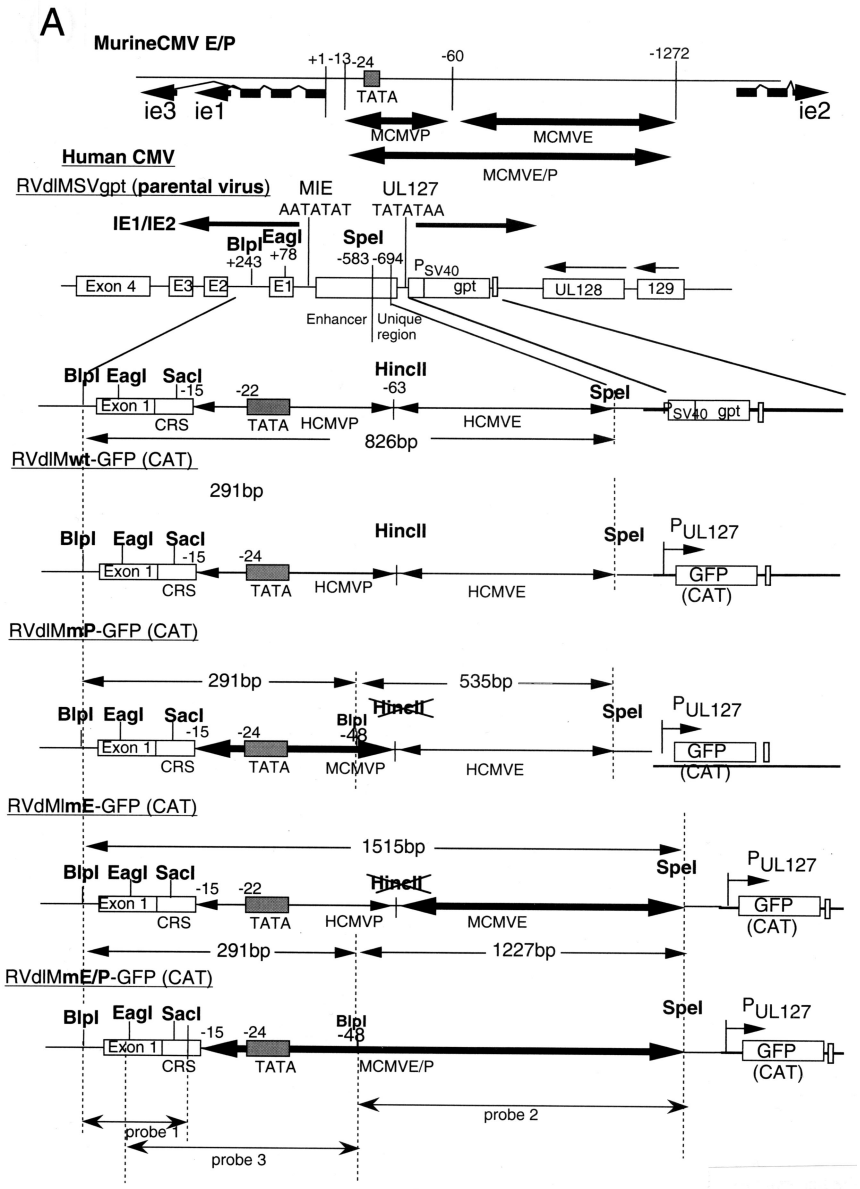


FIG. 1. Structural analysis of recombinant viruses. (A) Schematic diagram of parental and recombinant viruses. All recombinant viruses containing GFP or CAT were derived from RVdlMSVgpt (parental virus). The murine CMV promoter (MCMVP), enhancer (MCMVE), and enhancer plus promoter (MCMVE/P) are designated with a bold arrow. The HCMV promoter (HCMVP), enhancer (HCMVE), and enhancer plus promoter are designated with a regular arrow. The viral DNA fragment sizes of all recombinant viruses digested with the restriction endonucleases *Blp*I and *Spe*I are indicated. ^{32}P-Labeled probes for Southern blot hybridization to confirm the recombinations are shown at the bottom as probes 1, 2, and 3. Shuttle vector construction and viral DNA transfection of HFF cells are described in Materials and Methods. (B) Southern blot analysis of the parental virus and the recombinant viruses. Viral DNAs were digested with restriction endonucleases *Blp*I and *Spe*I, fractionated by electrophoresis in 1.0% agarose, and subjected to hybridization with ^{32}P-labeled probe 1, 2, or 3. Standard molecular size markers are indicated to the left in base pairs. Lanes: 1, RVdlMSVgpt (parental virus); 2, RVdlMmP-GFP (mP-CAT); 3, RVdlMmE-GFP (mE-CAT); 4, RVdlMmE/P-GFP (mE/P-CAT); 5, RVdlMmP-CAT (mP-GFP); 6, RVdlMmE-CAT (mE-GFP); 7, RVdlMmE/P-CAT (mE/P-GFP).

Western blot analysis. HFF cells were collected at 7 and 10 d p.i., fractionated by SDS–9% polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes as described previously (35). To detect the pIE72 protein encoded by IE1 (UL123) and the p52 protein encoded by UL44, monoclonal antibodies 810 (Chemicon, Temecula, Calif.) and M0854 (Dako, Carpinteria, Calif.) were used, respectively. The enhanced chemiluminescence detection reagents (Pierce, Rockford, Ill.) and secondary horseradish peroxidase-labeled anti-mouse immunoglobulin G antibody (Amersham) were used according to the manufacturers' instructions.

Flow cytometric analysis. THP-1 cells (5×10^5) were infected at an MOI of 1 with wt or recombinant virus containing *gfp*. The cells were induced with PMA and hydrocortisone to differentiate them as described above. Cells were collected 3 d p.i., washed with PBS, and suspended in 1 ml of PBS containing 1% paraformaldehyde. GFP-expressing cells were separated from GFP-negative cells by FACScan flow cytometry (Becton Dickinson, Franklin Lakes, N.J.) with a 515- to 545-nm-pore-size filter (FL1) and a 564- to 606-nm-pore-size filter (FL2). Cell fluorescent intensities for GFP-expressing cells were measured with CellQuest software (Becton Dickinson) and calculated with FlowJo version 3.4 software (Tree Star, Inc., San Carlos, Calif.). The mean of the fluorescent intensities was determined after three independent experiments.

RNase protection assay. Antisense actin, IE1, and CAT riboprobes have been described previously (16, 24, 32). Cytoplasmic RNA was harvested at various

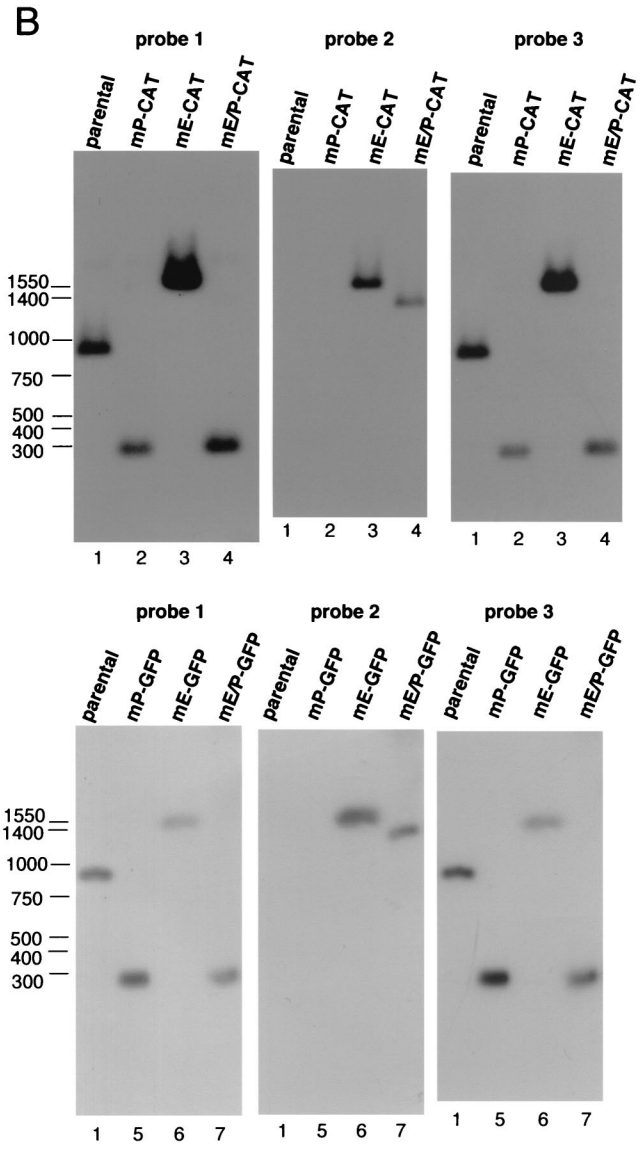


FIG. 1—*Continued.*

times after infection with an MOI of approximately 5 or 0.01 PFU/cell as described previously (47). Protein synthesis was inhibited with 200 μg of cycloheximide (Sigma)/ml added to the medium 30 min before infection and maintained throughout the infection. Twenty micrograms of RNA was hybridized to ^{32}P-labeled antisense actin, IE1, and CAT probes at room temperature overnight before digestion with RNase T1 (100 U) as described previously (23). The protected RNA fragments were subjected to electrophoresis in denaturing 6% polyacrylamide gels, followed by autoradiography on Hyperfilm MP (Amersham).

Multiplex real-time RT-PCR and PCR. For the detection of RNA, whole-cell RNA was isolated for 6 h p.i. according to the method of Chomczynski and Sacchi (8). Superscript II RNase H-negative reverse transcriptase (RT; Invitrogen) was used according to the manufacturer's directions to generate cDNA from 2 μg of RNA and 250 ng of random hexamers (Invitrogen) in a final volume of 20 μl. Samples were heat inactivated at 70°C for 15 min. For the detection of DNA, whole cells in 35-mm-diameter plates were harvested at 4 h p.i. in 400 μl of PCR lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.001% Triton X-100, 0.001% SDS) containing 50 μg of proteinase K/ml and incubated at 55°C for 100 min. The proteinase K was inactivated at 95°C for 20 min. Multiplex real-time PCR was performed for the simultaneous detection of probes containing 6-carboxyfluorescein (FAM) and VIC reporter fluorophores as described previously (30). Amplifications were performed in a final volume of 25 μl of the Platinum quantitative PCR SuperMix-UDG cocktail (Invitrogen). Each reaction mixture contained 2 μl of the first-strand cDNA or DNA, 5 mM $MgCl_2$, 500 nM concentrations of each MIE primer or gB primer, 250 nM MIE probe or gB probe, 6.25 nM concentrations of each 18S primer, and 50 nM 18S probe. MIE primer sequences were designed as described previously (30). Human CMV gB primer sequences were designed as 5′-GGCGAGGACAACGAAATCC-3′ and 5′-TG AGGCTGGGAAGCTGACAT-3′. The MIE reporter probe was designed as described previously (30). The gB reporter probe was designed as 5′-FAM-TT GGGCAACCACCGCACTGAGG-tetramethyl rhodamine (TAMRA)-3′ (IDT, Coralville, Iowa). Cellular 18S rRNA served as the endogenous control. The primers used to amplify the intronless ribosomal 18S cDNA and the VIC-labeled probe used for detecting this amplicon were obtained from PE Applied Biosystems (Branchburg, N.J.). Thermal cycling conditions were an initial 50°C for 2 min and 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The relative quantitations of MIE RNA and gB DNA were done as described previously (30).

RESULTS

Recombinant virus isolation and Southern blot analyses. The human CMV enhancer in the context of the viral genome is unidirectional for the expression of the IE1 and IE2 genes. In contrast, the murine CMV enhancer is bidirectional for the expression of either ie1 and ie3 or ie2 (Fig. 1A). The human CMV enhancer complements the murine CMV enhancer in the context of the murine CMV genome (3, 15). The recombinant virus replicates like wt murine CMV in cell culture and in the host (3, 15). In contrast, the murine CMV enhancer could not efficiently complement the rat CMV enhancer (38). The recombinant rat CMV replicated less efficiently at a low MOI in cell culture and in the host. To determine the role of the human CMV enhancer or promoter in the context of the viral genome in human cells, we constructed recombinant viruses with the human CMV enhancer, promoter, and enhancer plus promoter substituted with the murine CMV components (Fig. 1A). To select recombinant viruses from the parental virus (RVdlMSVgpt), the UL127 gene was replaced with the GFP gene or the CAT gene inserted downstream of the early UL127 promoter. For efficient expression, the region between positions −694 and −583 relative to the transcription start site of the MIE promoter was deleted because this region has a suppressive effect on the transcription from the UL127 promoter (28). All recombinant viruses contained the CRS between positions −1 and −13 of the human CMV MIE promoter. The IE86 protein binds the CRS and negatively autoregulates transcription from the MIE promoter (6, 24, 37). The murine CMV promoter and enhancer were defined as the regions relative to the transcription start site between positions −60 and −13 and between positions −1272 and −60, respectively (15).

All recombinant viral DNAs were digested with the restriction endonucleases *Blp*I and *Spe*I, fractionated by electrophoresis in 1% agarose gels, and immobilized for Southern blot hybridization with either probes 1, 2, or 3 (Fig. 1A) as described in Materials and Methods. Figure 1B demonstrates that the recombinant viruses had the appropriate DNA fragment sizes as predicted in Fig. 1A.

Growth kinetics of the recombinant viruses. To determine the effect of the murine CMV promoter, enhancer, or enhancer plus promoter on human CMV replication in HFF cells, the viral growth properties were analyzed. We were unable to detect any significant differences in the viral growth

properties after a high MOI (5 PFU/cell). After infection at a low MOI (0.01 PFU/cell), recombinant viruses with the murine CMV enhancer (mE) and enhancer plus promoter (mE/P) replicated less efficiently than the wt. Recombinant viruses with the murine CMV promoter (mP) replicated to the same level as the wt (Fig. 2A) or the parental virus (data not shown). There was an approximately 100-fold difference in the viral titers between the wt and the mE or mE/P at 5 to 10 d p.i. (Fig. 2A). The plaque size of recombinant viruses with the mE or mE/P was smaller than that of the wt or the mP (Fig. 2B). We concluded that the murine CMV enhancer can substitute for the human CMV enhancer, but the recombinant virus replicates less efficiently in human cells at a low MOI.

Effect on viral gene expression and DNA synthesis. To determine if the growth defect of recombinant viruses with the murine enhancer was due to a lower level of viral gene expression and genome amplification, we infected HFF cells at a low MOI (0.01 PFU/cell). Equal protein amounts were fractionated by SDS-polyacrylamide gel electrophoresis and Western blot analysis with monoclonal antibodies against viral pIE72 (UL123) or p52 (UL44) protein, respectively, as described in Materials and Methods. The protein levels of pIE72 and p52

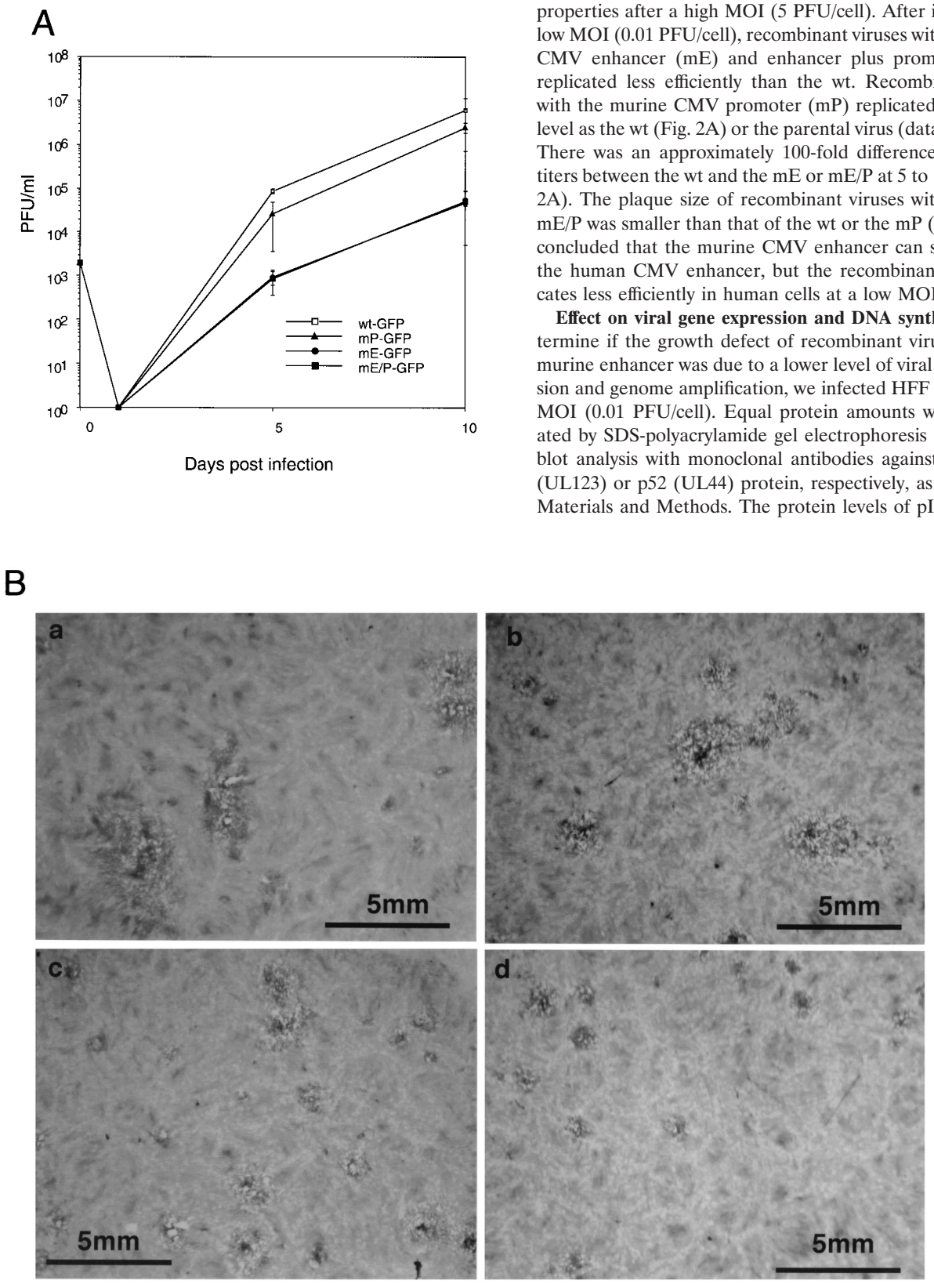


FIG. 2. Growth kinetics of wt and recombinant viruses. (A) Multistep growth curve of wt and recombinant viruses at an MOI of 0.01. HFF cells were used for growth and the plaque assay of recombinant viruses wt-GFP, mP-GFP, mE-GFP, and mE/P-GFP as described in Materials and Methods. Four independent assays were used to determine the mean and the standard error. (B) Plaque size of wt and recombinant viruses. All plaques were generated from inoculums 5 d p.i. (a) wt-GFP; (b) mP-GFP; (c) mE-GFP; (d) mE/P-GFP. Magnification, ×0.5.

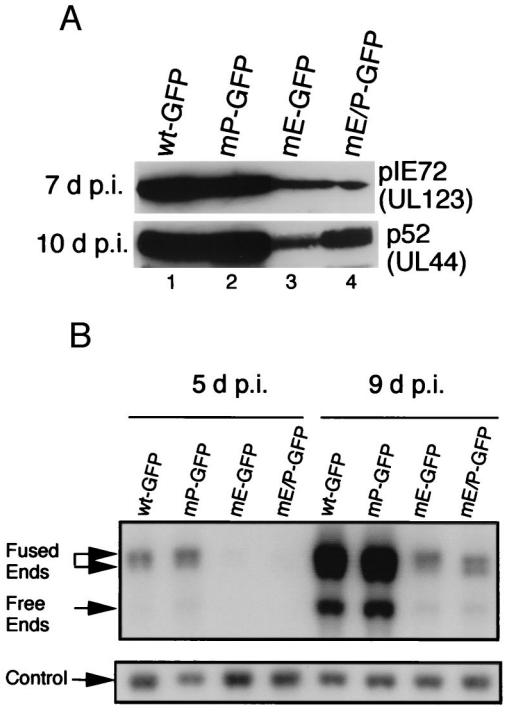


FIG. 3. Early viral protein and DNA synthesis with wt and recombinant viruses. (A) Western blot of IE pIE72 (UL123) and early p52 (UL44) proteins after infection with wt or recombinant viruses at an MOI of 0.01. pIE72 (UL123) and p52 (UL44) were detected 7 and 10 d p.i. with monoclonal antibodies 810 and M0854, respectively, as described in Materials and Methods. (B) Analysis of viral DNA synthesis after infection of HFF cells with wt and recombinant viruses at an MOI of 0.01. DNAs from infected HFF cells were isolated 5 and 9 d p.i., digested with the restriction endonuclease *Hin*dIII, and subjected to Southern blot hybridization with either a ^{32}P-labeled T probe or a lambda probe as described in Materials and Methods. Lambda DNA served as an internal control. Arrows designate the viral DNA fused ends (17.2 and 13.0 kb), free ends (9.7 kb), and the internal lambda DNA control.

were similar for the wt and the mP at 7 and 10 d p.i. In contrast, the levels of pIE72 and p52 were significantly lower for the mE and mE/P (Fig. 3A).

Since p52 is necessary for viral DNA synthesis (1, 36), DNA from an equal number of infected cells was collected and the viral DNA was quantitated by Southern blot hybridization with a T probe as described in Materials and Methods. After 5 or 9 d p.i., the viral DNAs of wt- and mP-infected cells were similar in relative amount. In contrast, the viral DNAs of mE- and mE/P-infected cells were significantly lower (Fig. 3B). Lambda DNA served as a loading control as described in Materials and Methods. We concluded that recombinant viruses with the human CMV enhancer substituted by the murine CMV enhancer enter the early phase of viral gene expression and viral DNA replication at a slower rate after a low MOI.

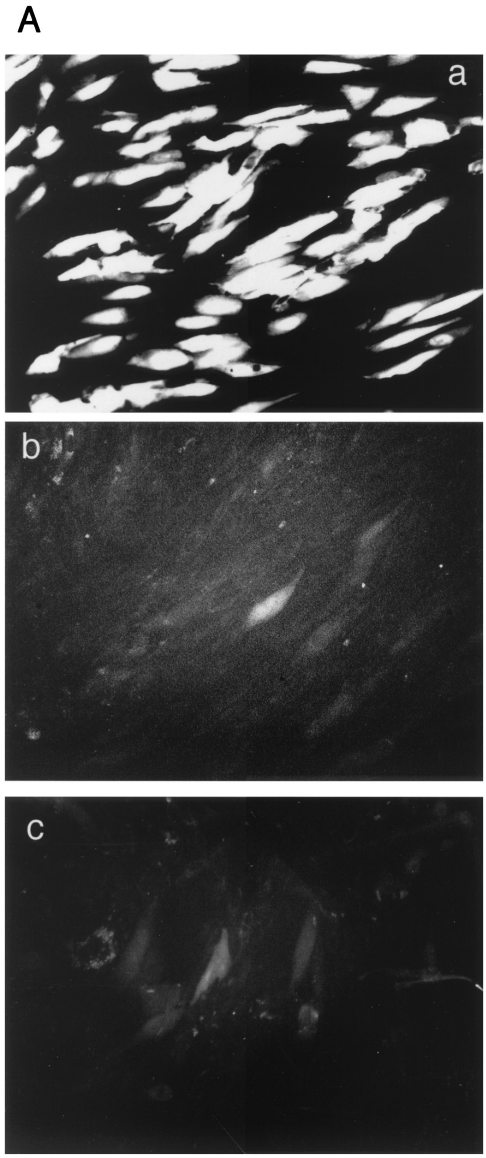
Gene expression in different cell types. To determine the effect of different cell types infected with the recombinant viruses, we used the UL127 promoter, driving the expression of GFP, as described in Materials and Methods. The recombinant viruses had the suppressive region upstream of the UL127 promoter between positions −694 and −583 deleted as described previously (28). Since significant differences were not detected between the wt and the mP, we compared the wt with the mE and mE/P. Figure 4A shows the relative intensities of GFP expression in HFF cells after an MOI of 1 PFU/cell. GFP expression was highest with the wt virus. With the mE and mE/P, there was a low level of GFP expression in most cells, with a few cells demonstrating a slightly higher level of GFP expression. Figure 4B shows the relative intensities of GFP expression in monocytic THP-1 cells differentiated for 3 d with PMA and hydrocortisone as described in Materials and Methods. Fluorescence-activated cell sorter analysis detected the highest relative amount of GFP expression in the wt and lower amounts in the mE and mE/P (Fig. 4B).

These data indicate that substitution of the human CMV enhancer with the murine CMV enhancer affected the efficiency of GFP expression from the UL127 promoter in different human cell types.

Effect of enhancer substitution on IE transcription. To determine the effect of enhancer substitution on gene transcription, we infected cells at an either high (approximately 5 PFU/cell) or low (0.01 PFU/cell) MOI and assayed for IE transcripts by an RNase protection assay. Since the mE and mE/P were similar in previous experiments, only the mE was compared to the wt. The RNase protection assay was performed with antisense IE1 or UL127-CAT riboprobes as described in Materials and Methods. After a high MOI and in the presence of cycloheximide, no difference in the level of IE1 RNA was detected for the wt, mP, or mE (Fig. 5A). Similar results were obtained with the antisense IE2 riboprobe (data not shown). With the wt and the mP, UL127-CAT was not detected because the UL127 promoter is an early viral promoter in this context, as described previously (28). In contrast, there was bidirectional IE transcription with the mE in the presence of cycloheximide, as expected. However, the IE CAT RNA level with recombinant virus mE was 3.5-fold lower than the IE1 RNA level. Whether this reflects differences in the degree of bidirectional transcription or the stability of the RNAs requires further investigation.

After a low MOI at 24 h p.i., no difference in the level of IE1 RNA was detected for the wt and the mP. In contrast, IE1 RNA from the mE was significantly reduced (Fig. 5B). IE2 RNA is difficult to detect by an RNase protection assay after a low MOI with the wt or with the recombinant viruses. To further compare the effect of enhancer substitution on IE transcription, we infected cells with the wt or the mE at MOIs of 10, 1, 0.1, and 0.01, isolated total cell RNAs at 6 h p.i., and assayed for IE transcripts by multiplex real-time RT-PCR. We also assayed in parallel for input viral DNAs by multiplex real-time PCR. The amount of IE RNA or viral DNA was comparable to that of the wt at an MOI of 10 as described in Materials and Methods.

The relative amounts of input viral DNAs were similar between the wt and the mE, with MOIs of 10, 1, and 0.1 (Fig. 6A). With an MOI of 10, there was no significant difference in the level of IE RNA detected. With MOIs of 1 to 0.01, the IE RNA levels of the wt were three- to sevenfold higher than those of the mE (Fig. 6B). At a high MOI (10 PFU/cell), the murine CMV enhancer was as efficient as the human CMV enhancer for transcription of the IE genes in HFF cells. At lower MOIs (1 to 0.01 PFU/cell), the murine CMV enhancer



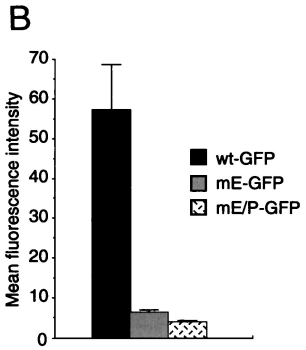


FIG. 4. GFP expression from the UL127 promoter after infection of different cell types with wt and recombinant viruses. (A) GFP expression of HFF cells at 3 d p.i. at an MOI of 1 detected by fluorescence microscopy. (a) wt-GFP; (b) mE-GFP; (c) mE/P-GFP. Magnification, ×20. (B) GFP expression of THP-1 cells at 3 d p.i. at an MOI of 1. Differentiation of THP-1 cells was induced with 20 nM PMA plus 50 μM hydrocortisone. Mean fluorescence intensities were determined with a FACScan flow cytometer. Three independent experiments were used to determine the mean and the standard error.

was less efficient than the human CMV enhancer for IE gene transcription in HFF cells.

Human CMV and murine CMV enhancer chimeras. The distal enhancer of human CMV affects IE gene expression and viral replication (31). The distal enhancer was defined as the region between positions −583 and −300, and the proximal enhancer was defined as the region between positions −300 and −63, relative to the MIE promoter transcription start site. To determine if the distal or proximal enhancer of human CMV had more of an effect on viral replication after a low MOI, we constructed human CMV and murine CMV chimeras of the proximal and distal enhancers. Figure 7A is a diagram of the recombinant viruses. Recombinant viral DNAs were digested with restriction endonucleases *Pac*I and *Spe*I, fractionated by electrophoresis in 1% agarose gels, and immobilized for Southern blot hybridization with either probe 1 or 2 (Fig. 7A) as described in Materials and Methods. Figure 7B shows a Southern blot demonstrating that the recombinant viruses were isolated. The recombinant virus containing the proximal human CMV (HCMV) enhancer is referred to as RVdlMmE+HCMVproximal-GFP(CAT) (mE+HCMV proximal), and the recombinant virus containing the distal human CMV enhancer is referred to as RVdlMmE+HCMVdistal-GFP(CAT) (mE+HCMV distal). mE+HCMVproximal contains the human CMV sequence from positions +1 to −300 and the murine CMV sequence upstream of position −256. mE+HCMV distal contains the murine CMV sequence between positions −63 and −256 and the human CMV sequence upstream of position −256 (Fig. 7A).

Growth kinetics of the recombinant viruses containing human and murine CMV enhancer chimeras. To determine if one component of the human CMV enhancer determines the efficiency of viral replication at a low MOI of 0.01, the growth of wt-CAT virus was compared to that of mE-CAT, mE+HCMVproximal-CAT, and mE+HCMVdistal-CAT. There was an approximately 100-fold difference in the viral titers between the wt-CAT virus and mE-CAT, mE+HCMVproximal-CAT, or mE+HCMVdistal-CAT at 5 and 10 d p.i. (Fig. 8A). In addition, wt-CAT virus plaque size was approximately twice the size of that in mE-CAT, mE+HCMVproximal-CAT, or mE+HCMVdistal-CAT (Fig. 8B). The murine CMV proximal or distal components of the enhancer cannot substitute for the human CMV enhancer components. These data indicate that both the proximal and distal components of the human CMV enhancer are required for the efficient replication of the virus after a low MOI of human cells.

Effect of the proximal and distal human and murine CMV enhancer chimeras on early viral gene expression. To determine the effect of the proximal or distal human CMV and murine CMV enhancer chimeras on early viral gene expression, we examined the recombinant viruses with the UL127 promoter driving the expression of GFP. HFF cells were infected at an MOI of 1 with wt-GFP, mE-GFP, mE+HCMVproximal-GFP, or mE+HCMVdistal-GFP. Figure 9 shows that the relative intensities of GFP are high with wt-GFP and

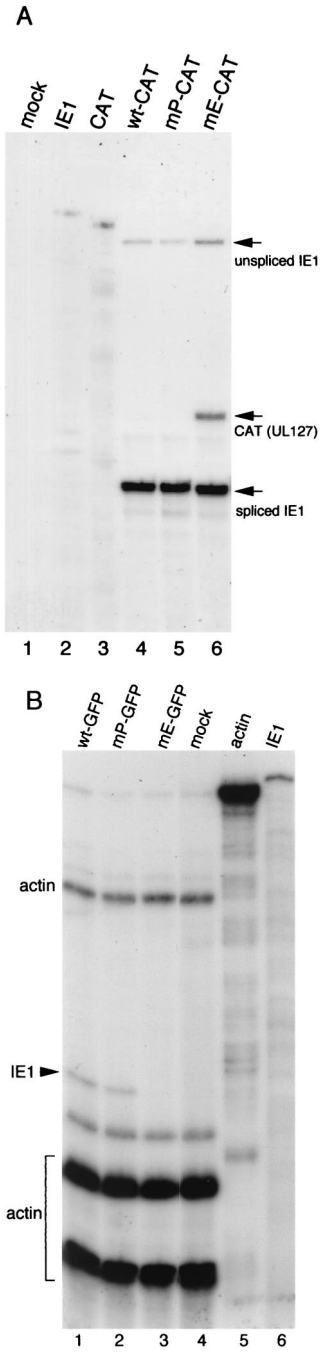


FIG. 5. Steady-state mRNA levels transcribed from the MIE promoter or the UL127-CAT promoter. Cytoplasmic RNA was analyzed by an RNase protection assay at 6 (A) or 24 (B) h p.i. as described in Materials and Methods. (A) IE1 and UL127-CAT mRNA after a high MOI (approximately 5 PFU/cell) with cycloheximide. Probes used in lanes 2 and 3 lacked RNase T_1. (B) IE1 mRNA after a low MOI (0.01 PFU/cell). Actin mRNA was used as a loading control. Probes used in lanes 5 and 6 lacked RNase T_1.

low with mE+HCMVproximal-GFP, mE+HCMVdistal-GFP, or mE-GFP. These data indicate that both the proximal and distal components of the human CMV enhancer determine the efficiency of early HCMV gene expression at a low MOI.

DISCUSSION

CMV IE gene expression is essential for viral replication. Human CMVs with the IE1 gene deleted replicate poorly at a low MOI of human fibroblasts (11, 14, 34). Human CMVs with the IE2 gene deleted and murine CMV with the homologue of IE2 (ie3) deleted fail to express early viral genes and fail to replicate viral DNA (2, 29). The enhancers upstream of these IE genes influence the probability of IE gene expression. The CMV enhancers are responsive to cellular transcription factors and to virion-associated glycoproteins and proteins (5, 18, 25, 50, 51). The impact of the virion-associated glycoproteins and proteins is greater at a high MOI when more noninfectious and infectious particles are present.

To determine the role of the human CMV enhancer in human cells, we isolated recombinant human CMVs with the promoter, the enhancer, or the enhancer plus promoter substituted with the murine CMV components. When we substituted the human CMV promoter with the murine CMV promoter, there was no difference in the expression of IE (pIE72) or early (p52) genes or in viral DNA synthesis for 5 to 7 d at a low MOI. Recombinant viruses with the murine CMV promoter replicated to the same level as the wt virus. The recombinant viruses containing the murine CMV promoter had the wt sequence for the transcription start site (position +1), initiator-like sequence (+1 to +7), and CRS (−1 to −15) and differed with the murine CMV sequence only between positions −48 and −16. Although the substitution with the murine CMV sequence between positions −48 and −16 disrupted the expression of the UL126 gene that encodes a viral protein designated ORF94 or genes antisense to UL126, deletion of UL126 from human CMV was reported to have no effect on viral replication in human fibroblasts (49).

When we substituted the human CMV enhancer with the murine CMV enhancer, there was a significant difference in IE (pIE72) and early (p52) gene expression and in viral DNA synthesis relative to those of the wt. Human CMV recombinant viruses with the murine CMV enhancer replicated slower and spread less efficiently to adjacent cells at a low MOI. Although IE transcription with the recombinant viruses was similar at high MOIs (5 to 10 PFU/cell), differences were detected at MOIs of 1 or less. These data indicate that the human CMV enhancer functions more efficiently in human CMV-infected human cells.

Cis-acting sites in the enhancer and transcription factors available in the cell determine the effects of the enhancer on the MIE promoter. Human and murine CMV enhancers have duplications of multiple transcription factor binding sites. While the human CMV enhancer has five CREB or ATF *cis*-acting sites, the murine CMV enhancer has only one. In contrast, the murine CMV enhancer has seven AP-1 *cis*-acting sites and the human CMV enhancer has only two. Both viral enhancers have multiple NF-κB and RAR-RXR *cis*-acting sites (4, 10, 33, 38, 43). The two enhancers were of equal strength after a high MOI of the human fibroblast, but not after a low MOI. Less IE transcription with the murine CMV enhancer after a low MOI could be due to the absence of a binding site(s) for an activated transcription factor. These data suggest that the human CMV enhancer has evolved to have

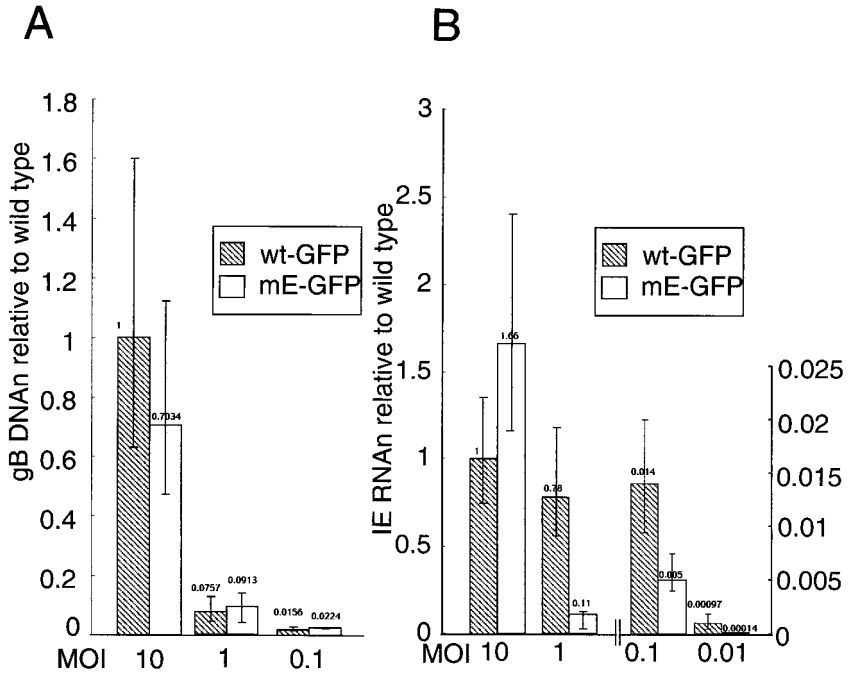


FIG. 6. Effect of different MOIs on IE transcription of wt-GFP and mE-GFP. Total cell RNAs and DNAs were isolated in parallel at 6 and 4 h p.i., respectively, and analyzed by multiplex real-time PCR as described in Materials and Methods. gB DNA or IE RNA was normalized to a internal ribosomal DNA or RNA control (respectively). (A) gB DNAs normalized (DNAn) relative to the wt with an MOI of 10, 1, or 0.1. (B) IE RNAs normalized (RNAn) relative to the wt with an MOI of 10, 1, 0.1 or 0.01.

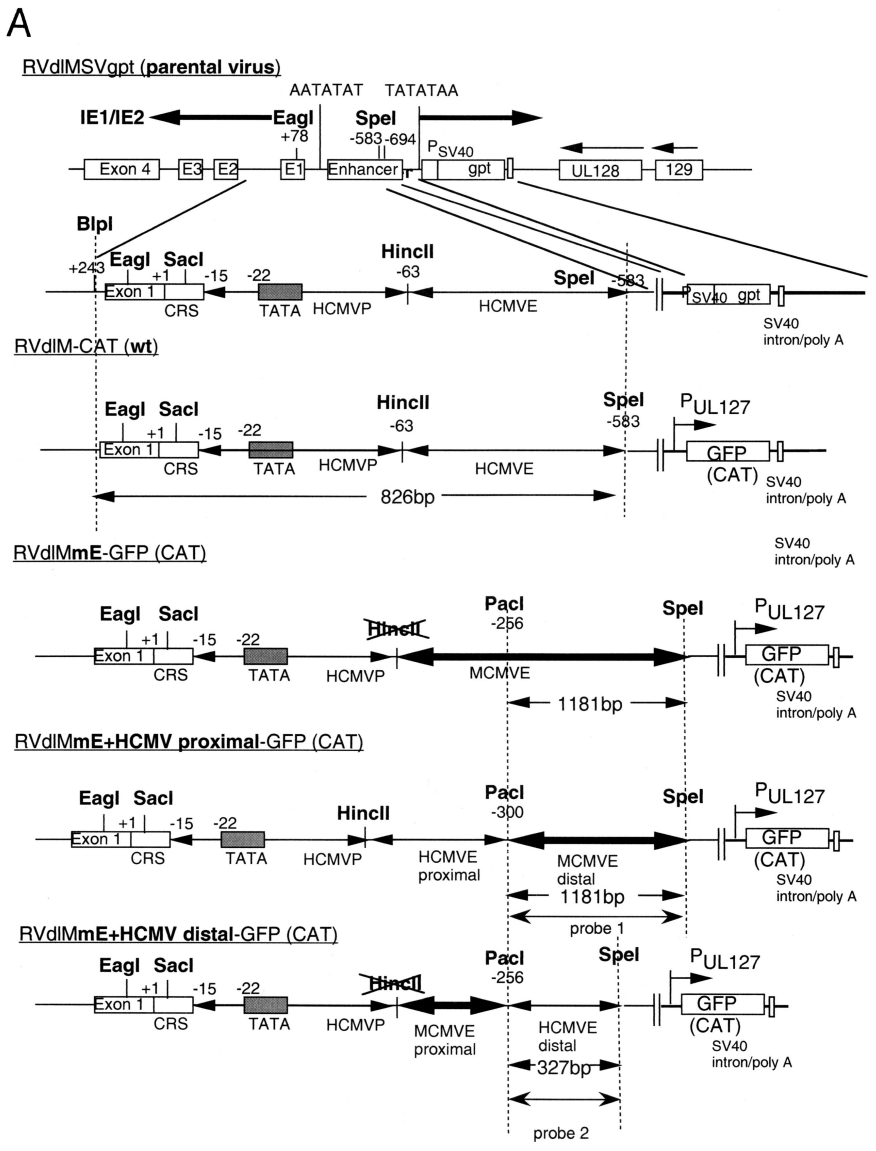
components to ensure the replication of the virus after a low MOI of human cells.

Substitution of the rat CMV enhancer with the murine CMV enhancer also reduced the efficiency of viral replication and spread in cell culture as well as in the rat (38). The rat CMV (English strain) enhancer has fewer *cis*-acting sites for eukaryotic transcription factor binding than the murine CMV enhancer (38). The rat CMV enhancer has only three AP-1 *cis*-acting sites and one NF-κB site. In contrast, the murine CMV enhancer has seven AP-1 sites and at least 12 NF-κB sites. If one accepts the model that the strength of an enhancer is determined by the number and type of transcription factor binding sites, then IE transcription with the murine CMV enhancer substitution should be greater at a low MOI. However, wt rat CMV replicated more efficiently in rat cells and in the rat than recombinant rat CMV containing the murine CMV enhancer. It is possible that the CMV enhancers have unrecognized *cis*-acting elements that contribute to downstream transcription in cells of different species. In general, the murine CMV enhancer in the context of the human or rat CMV genome is less efficient in initiating IE transcription in human or rat cells at a low MOI. Taken together, these experiments suggest that the number and type of *cis*-acting transcription factor binding sites may not be the only determinant for efficient replication at a low MOI.

Substitution of the murine CMV enhancer with the human CMV enhancer did not affect the replication efficiency of murine CMV in cell culture or in the mouse (15). The human CMV enhancer did not add negative or positive effects on transcription from the MIE promoter at a high or low MOI. These enhancers were considered to be equal in function. In contrast, the murine CMV enhancer substituted for the enhancer of either human or rat CMV was not paralogous. Whether or not the murine CMV enhancer has a negative element for human and rat CMV replication remains to be determined. Alternatively, the human and rat CMV enhancers have a positive element that may be species specific for human and rat cells, respectively.

The region between positions −580 and −300 of the human CMV enhancer, which contains the Ets, SRE, CREB or ATF, NF-κB, and SP1 *cis*-acting sites, affects the replication efficiency of human CMV at a low MOI (31). When the region was deleted, recombinant human CMVs expressed a lower level of IE genes, including the IE1 and IE2 genes in the large

FIG. 7. Structural analysis of recombinant viruses with human CMV and murine CMV enhancer chimeras. (A) Schematic diagram of recombinant viruses. All recombinant viruses containing GFP or CAT were derived from RVdlMSVgpt (parental virus). Murine CMV components are designated with a bold arrow, and human CMV components are designated with a regular arrow (see the legend to Fig. 1A). The viral DNA fragment size of all recombinant viruses digested with restriction endonucleases *Blp*I and *Spe*I or *Pac*I and *Spe*I are indicated. ^{32}P-Labeled probes for Southern blot hybridization to confirm the recombinations are shown at the bottom as probes 1 and 2. Shuttle vector construction and viral DNA transfection of HFF cells are described in Materials and Methods. (B) Southern blot analysis of the wt and recombinant viruses. Viral DNAs were digested with restriction endonucleases, fractionated by electrophoresis in 1.0% agarose, and subjected to hybridization with ^{32}P-labeled probe 1 or 2. Standard molecular size markers are indicated in base pairs to the left of the two groups of gels.


A
RVdlMSVgpt (parental virus)
AATATAT TATATAA
IE1/IE2
EagI
+78
SpeI
-583-694
PSV40
Exon 4
E3
E2
E1
Enhancer
gpt
UL128
129
BlpI
+243
EagI SacI
+1
-15
-22
Exon 1
CRS
TATA
HCMVP
HincII
-63
HCMVE
SpeI
-583
PSV40 gpt
SV40
intron/poly A
RVdlM-CAT (wt)
EagI SacI
HincII
-63
SpeI
-583
PUL127
GFP
(CAT)
826bp
RVdlMmE-GFP (CAT)
PacI
-256
MCMVE
1181bp
RVdlMmE+HCMV proximal-GFP (CAT)
-300
HCMVE
proximal
MCMVE
distal
probe 1
RVdlMmE+HCMV distal-GFP (CAT)
MCMVE
proximal
HCMVE
distal
327bp
probe 2
B
parental
mE+HCMV
proximal-CAT
mE+HCMV
distal-CAT
mE+HCMV
proximal-GFP
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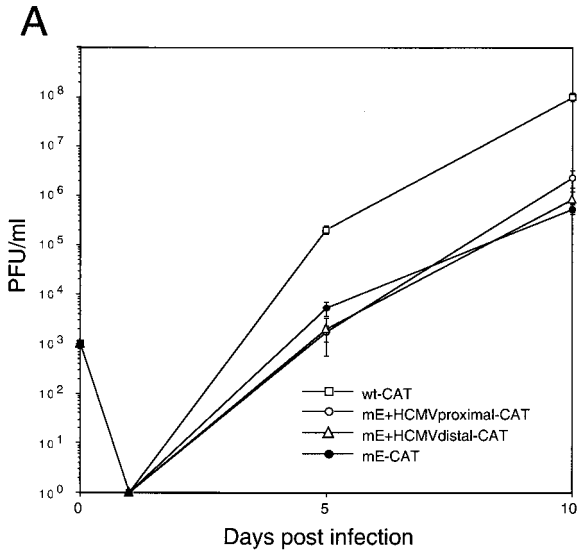

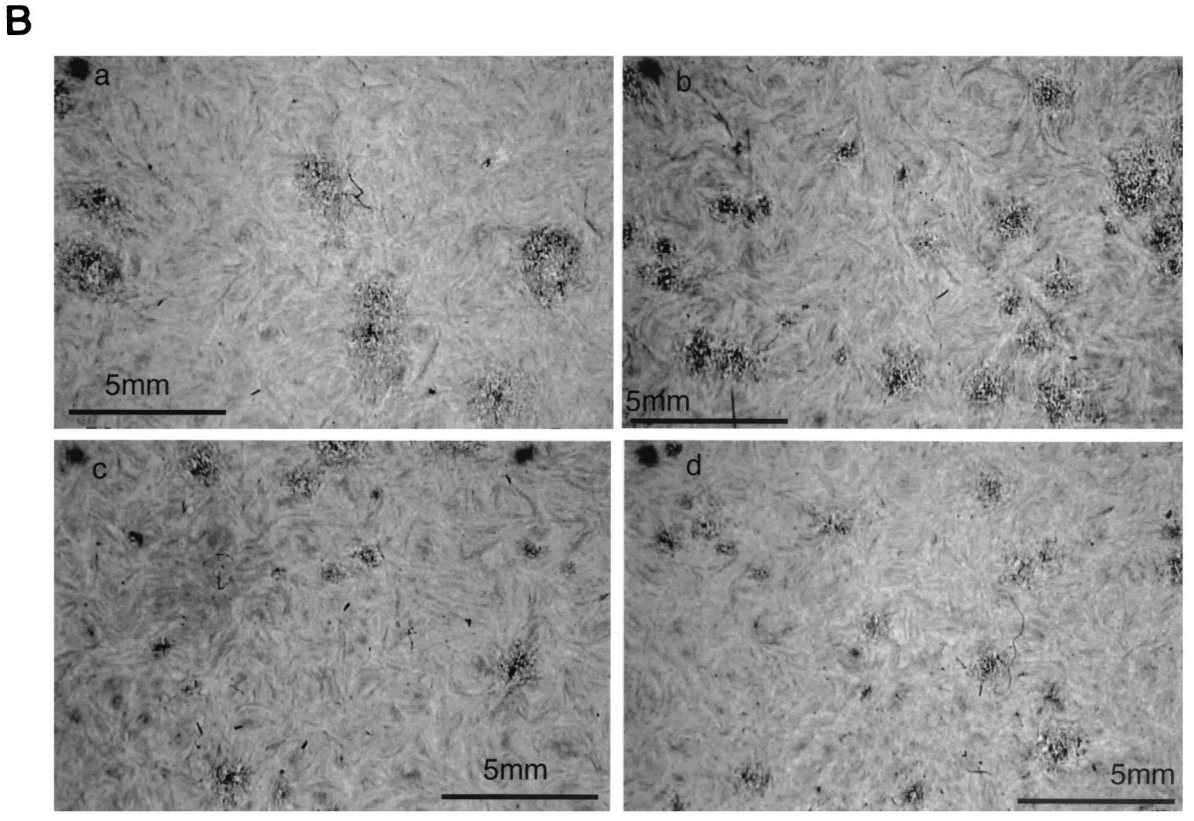

FIG. 8. Growth kinetics of the recombinant wt or human CMV and murine CMV enhancer chimeras. (A) Multistep growth curve of wt-CAT, mE+HCMVproximal-CAT, mE+HCMVdistal-CAT, or mE-CAT recombinant viruses at an MOI of 0.01. DNA was isolated in parallel and shown to be of equal input by multiplex real-time PCR as described in the legend to Fig. 6. HFF cells were used for growth and the plaque assay of the recombinant viruses as described in Materials and Methods. Three independent assays were used to determine the mean and the standard error. (B) Plaque sizes of parental and recombinant viruses. All plaques were generated from inoculums at 11 d p.i. (a) wt-CAT; (b) mE+HCMVproximal-CAT; (c) mE+HCMVdistal-CAT; (d) mE-CAT.

unique component of the viral genome as well as the IE US3 gene in the short unique component (31). Since IE genes separated by a distance of approximately 20 kbp were affected by this deletion, it is possible that the region between positions −580 and −300 has a global influence on the efficiency of transcription at a low MOI. We constructed recombinant viruses with the chimeras of human and murine CMV enhancers. Recombinant viruses with the proximal human CMV enhancer and a distal murine CMV enhancer or the distal human CMV enhancer and the proximal murine CMV enhancer replicated to lower levels than the wt virus and expressed lower levels of GFP driven by the UL127 early viral promoter. These data indicate that both the proximal and distal human CMV enhancer components are necessary for efficient early viral gene expression and viral replication after a low MOI.

Betaherpesviruses have cell type and species specificities for productive replication. While species specificity can be determined at the cell entry level, some CMVs enter cells of a

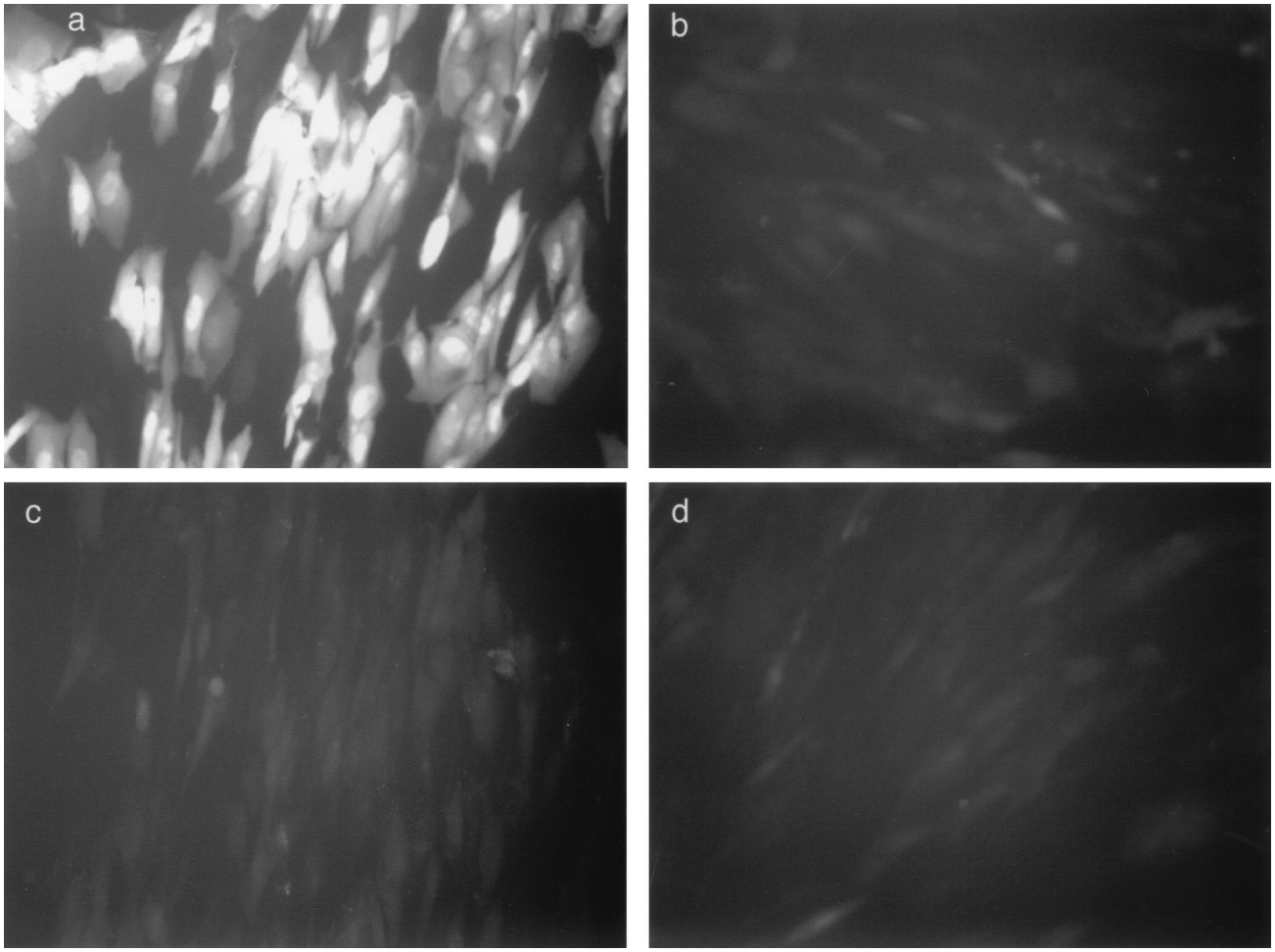

FIG. 9. Effect of proximal or distal human CMV and murine CMV enhancer chimeras on GFP expression from the UL127 promoter. HFF cells were infected at an MOI of 1, and GFP expression was detected by fluorescence microscopy at 3 d p.i. (a) wt-GFP; (b) mE+HCMVproximal-GFP; (c) mE+HCMVdistal-GFP; (d) mE-GFP. Magnification, ×20.

different species but fail to replicate their viral DNA and to produce infectious virus. The human CMV enhancers contain *cis*-acting elements for efficient transcription centers in human cells. Further investigation is necessary to determine whether a unique element in the human CMV enhancer affects the efficiency of IE gene transcription and replication of the virus at a low MOI.

ACKNOWLEDGMENTS

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