JOURNAL OF VIROLOGY, Dec. 2004, p. 12788–12799 Vol. 78, No. 23
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.23.12788–12799.2004

Role of the Proximal Enhancer of the Major Immediate-Early Promoter in Human Cytomegalovirus Replication

Hiroki Isomura,[1,2] Tatsuya Tsurumi,[2] and Mark F. Stinski[1]*

Department of Microbiology, Carver College of Medicine, University of Iowa, Iowa City, Iowa,[1] and Division of Virology, Aichi Cancer, Center Research Institute, Chikusa-ku, Nagoya, Japan[2]

Received 16 July 2004/Accepted 19 July 2004

The human cytomegalovirus (CMV) enhancer has a distal component (positions −550 to −300) and a proximal component (−300 to −39) relative to the transcription start site (+1) of the major immediate-early (MIE) promoter. Without the distal enhancer, human CMV replicates slower and has a small-plaque phenotype. We determined the sequence requirements of the proximal enhancer by making 5′-end deletions to positions −223, −173, −116, −67, and −39. Even though recombinant virus with the proximal enhancer deleted to −39 has the minimal TATA box-containing MIE promoter element, it cannot replicate independently in human fibroblast cells. Recombinant virus with a deletion to −67 has an Sp-1 transcription factor binding site which may represent a minimal enhancer element for recombinant virus replication in human fibroblast cells. Although recombinant virus with a deletion to −223 replicates to titers at least 100-fold less than that of the wild-type virus, it replicates to titers 8-fold higher than that of recombinant virus with a deletion to −173 and 20-fold higher than that of virus with a deletion to −67. Recombinant virus with a deletion to −173 replicates more efficiently than that with a deletion to −116. There was a direct correlation between the level of infectious virus replication and time after infection, amount of MIE gene transcription, MIE and early viral protein synthesis, and viral DNA synthesis. The extent of the proximal enhancer determines the efficiency of viral replication.

Although infection by human cytomegalovirus (HCMV), a member of the betaherpesvirus family, occurs in most individuals, it is usually asymptomatic. HCMV is reactivated under immunosuppressive conditions causing pneumonitis, hepatitis, retinitis, and gastrointestinal diseases (5, 21). The virus replicates productively in terminally differentiated cells, such as fibroblasts, epithelial cells, and endothelial cells, and in monocyte-derived macrophages (11, 12, 25, 35, 50, 51, 56). HCMV can be latent in $CD34^+$ hematopoietic progenitor cells, monocytes, and $CD34^+$-derived dendritic cells from healthy seropositive individuals (19, 32, 33). The mechanism underlying maintenance of the latent viral genome and the switch between the latent and lytic forms of CMV infection remains unclear.

After primary infection or reactivation from latency, the immediate-early (IE) genes of CMV play a key role in determining the efficiency of viral replication. HCMV IE genes include the major IE genes (MIE) UL123 and UL122 (IE1 and IE2, respectively) and auxiliary IE genes TRS1/IRS1, UL36-38, and US3 (8, 53). Alternative splicing of a precursor generates the messenger RNAs of the IE1 and IE2 genes. The pIE72 and pIE86 proteins encoded by the IE1 and IE2 genes, respectively, have important roles in the regulation of subsequent viral gene expression. The IE1 gene is necessary for efficient viral replication after low multiplicity of infection (MOI) (13, 16, 43). The IE2 gene product is essential for early viral gene expression and autoregulates transcription of the IE1 and IE2 genes (7, 36, 38, 46). Together, the IE1 and IE2 gene products determine the efficiency of viral replication.

The human CMV MIE enhancer-containing promoter regulates the level of MIE gene expression. The region upstream of the human CMV MIE promoter is divided into three regions: the modulator, the unique region, and the enhancer (reviewed in references 42 and 54). The modulator has no effect on MIE transcription and viral replication in diverse types of cells in culture (41). The unique region also has no effect on transcription from the MIE promoter, but one or more *cis*-acting elements in the unique region repress transcription from the divergent early viral UL127 promoter (34, 37). The human CMV enhancer between positions −550 and −39 relative to the transcription start site at +1 has four 18-bp repeat elements containing consensus NF-κB/rel binding sites, five 19-bp repeat elements containing consensus or near-consensus CREB/activating transcription factor (ATF) binding sites, and three 21-bp repeats containing YY1, Est.2 repressor factor, and Sp-1 sites. In addition, there are four 16-bp repeats of unknown function. There are three liganded retinoic acid receptor sites, two AP-1 binding sites, multiple SP-1 binding sites, one Ets site, and one serum response factor (reviewed in references 42 and 54). The enhancer is divided into a distal component and a proximal component (40). The distal enhancer between positions −550 and −300 is not required at high MOI, but it is required for efficient IE gene expression and viral replication at low MOI (40). UV-inactivated human CMV at a high viral-particle-to-cell ratio can rescue the recombinant virus with the distal enhancer deleted. Without the distal enhancer, the recombinant virus replicates slower and has a small-plaque phenotype in human fibroblast (HF) cells (39, 40). In addition, a recombinant human CMV has a small-

* Corresponding author. Mailing address: Department of Microbiology, Carver College of Medicine, 3-772 BSB, University of Iowa, Iowa City, IA 52242. Phone: (319) 335-7792. Fax: (319) 335-9006. E-mail: mark-stinski@uiowa.edu.

plaque phenotype when the enhancer is replaced by the entire murine CMV enhancer (28). Proximal and distal chimeras of the human and murine CMV enhancers replicate less efficiently at low MOI and have a small-plaque phenotype (28). These findings indicate that both the distal enhancer and the proximal enhancer of human CMV are required for efficient replication of the virus. The influence of the distal enhancer on the proximal enhancer is presently not understood.

It was inferred from transient transfection experiments that the 18-bp repeat containing a NF-κB/rel binding site and the 19-bp repeat containing a CREB/ATF binding site were the key elements for efficient viral replication found in the distal and proximal enhancers (24, 47, 48, 52). The putative role of the NF-κB sites was questioned when mutation of these sites in all 18-bp repeats in the human CMV enhancer replacing its equivalent in the murine CMV enhancer failed to alter viral replication at high or low MOI (3). In addition, mutation of all five CREB/ATF sites in the human CMV enhancer of the intact viral genome had no effect on viral replication in cell culture at high or low MOI (31).

Here we have determined the minimal sequence of the proximal enhancer for human CMV replication in human fibroblast cells and the effect of upstream proximal enhancer sequences on the efficiency of viral replication in the absence of the distal enhancer. Recombinant human CMV bacterial artificial chromosome (BAC) with the entire enhancer deleted to position −39 or the proximal enhancer deleted to positions −67, −116, −173, and −223 relative to the transcription start site of the MIE promoter were isolated and studied.

MATERIALS AND METHODS

Cells and virus. Primary human foreskin fibroblast (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 (55). NF-κB dominant negative cells designated IκBαM and LXSN control cells were kindly provided by C. Benedict (3). The virus titer of wild-type (wt) human CMV Towne and recombinant viruses was determined by standard plaque assays on HFF cells as described previously (41). Recombinant viruses without the distal enhancer had the small-plaque-size phenotype as described previously (28). The titers of recombinant viruses without the distal enhancer were normalized to wt virus by the amount of viral DNA in HFF cells prior to viral replication at 4 h postinfection (p.i.). The titers of the recombinant viruses were adjusted by dilution with growth medium. The viral DNA input of wt and recombinant viruses was determined after infecting HFF cells in 35- or 60-mm-diameter plates in triplicate and harvesting the cells at 4 h p.i. in PCR lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.001% Triton X-100, and 0.001% sodium dodecyl sulfate [SDS]) containing 50 μg of proteinase K/ml. The mixture was incubated at 55°C for 100 min, and the proteinase K was inactivated at 95°C for 10 min. The relative amount of input viral DNA was estimated either by semiquantitative PCR using the primer pairs of pFUS14 (5′-TTGCAGGCCACGA ACAACGT-3′) and pRUS14 (5′-GTCTACGGATTGCTGACGCT-3′) or by multiplex real-time PCR as described below.

Enzymes. Restriction endonucleases were purchased from New England Biolabs Inc. (Beverly, Mass.). An expanded high-fidelity PCR system was purchased from Invitrogen (Carlsbad, Calif.) or Roche (Mannheim, Germany). RNasin and RNase-free DNase were purchased from Promega (Madison, Wisc.) and Takara (Tokyo, Japan), respectively. The enzymes were used according to the manufacturers' instructions.

Mutagenesis of HCMV BAC. We used a rapid homologous recombination system in *Escherichia coli* expressing bacteriophage lambda recombination proteins exo, beta, and gam (provided by D. Court, National Institutes of Health, Bethesda, Md.) (60). BAC of human CMV Towne was obtained from F. Liu (University of California, Berkeley, Calif.) (10). The enhancers from positions −39, −67, −116, −173, and −223 to −636 relative to the transcription start site of +1 were deleted from wild-type Towne BACs. Double-stranded DNAs for recombination contained a kanamycin resistance gene flanked by the 34-bp minimal FLP recombination target (FRT) sites (5′-GAAGTTCCTATTCTCTAGAAAGTATA GGAACTTC-3′) and 70 bp of homologous viral DNA sequence. The forward primer −636EFRTFKanF, 5′-GGTTATATAGCATAAATCAATATTGGCTATTGG CCATTGCATACGTTGTATCTATATCATAATATGTACAGAAGTTCCTA TTCTCTAGAAAGTATAGGAACTTCCGATTTATTCAAC-3′, was used with the following reverse primers to generate deletions between positions −636 and −39, −67, −116, −173, or −223 in the enhancer: −39RFRTRKanR, 5′-ACA GCGTGGATGGCGTCTCCAGGCGATCTGACGGTTCACTAAACGAGCT CTCCTTATATAGACCTCCCACGAAGTTCCTATACTTTCTAGAGAATA GGAACTTCGCCAGTGTTACAACCA-3′; BAC-67RFRTRKanR, 5′-TGAC GGTTCACTAAACGAGCTCTGCTTATATAGACCTCCCACCGTACACGC CTACCGCCCATTTGCGTCAGAAGTTCCTATACTTTCTAGAGAATAGG AACTTCGCCAGTGTTACAACCA-3′; −116RFRTRKanR, 5′-ACGCCTAC CGCCCATTTGCGTCAATGGGGCGGAGTTGTTACGACATTTTGGAAA GTCCCGTTGATTTTGGGAAGTTCCTATACTTTCTAGAGAATAGGAA CTTCGCCAGTGTTACAACCA-3′; −173RFRTRKanR, 5′-CGTTGATTTTG GTGCCAAAACAAACTCCCATTGACGTCAATGGGGTGGAGACTTGGA AATCCCCGTGAGTGAAGTTCCTATACTTTCTAGAGAATAGGAACTT CGCCAGTGTTACAACCA-3′; and −223RFRTRKanR, 5′-CTTGGAAATCC CCGTGAGTCAAACCGCTATCCACGCCCATTGATGTACTGCCAAAAC CGCATCACCATGGGAAGTTCCTATACTTTCTAGAGAATAGGAACTT CGCCAGTGTTACAACCA-3′. Amplification by PCR was as follows: 1 cycle of denaturation at 94°C for 2 min; 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 5 min; 1 cycle of extension at 72°C for 7 min or at 94°C for 2 min; 30 cycles at 94°C for 2 min, at 55°C for 2 min, and at 72°C for 2 min; and 1 cycle at 72°C for 7 min. To remove residual template DNA, the PCR products were digested with DpnI at 37°C for l.5 h. The DNAs were phenol-chloroform extracted and precipitated with 95% ethanol. Approximately 100 ng of each DNA fragment was subjected to electroporation into competent *E. coli* DY380 containing human CMV Towne BAC. Electroporation was performed using a Bio-Rad Gene Pulser III (2.5 kV, 200 Ω, and 25 μF) or BTX ECM 830 electroporator (3 kV, 100 μs, 10 pulses) following the suggestion of W. Dunn and F. Liu (University of California) (10).

Bacteriophage-encoded recombination proteins for the homologous recombination were induced at 42°C for 15 min as described previously (60).

Excision of the kanamycin resistance gene. To delete the kanamycin resistance gene, the recombinant human CMV BAC was transformed into *E. coli* DH10B. Plasmid pCP20 (provided by G. Hahn, Max von Pettenkofer Institute, Munich, Germany) that expresses FLP and deletes kanamycin at the FRT sites was transformed into DH10B containing the recombinant human CMV BAC. Human CMV BAC without kanamycin was selected on Luria-Bertani plates containing ampicillin and chloramphenicol.

Southern blot analysis. Recombinant BACs were purified using the NucleoBond BAC Maxi kit (Macherey-Nagel, Duren, Germany). BACs were digested with restriction endonucleases BlpI and XhoI and then subjected to 1.0% agarose gel electrophoresis as described previously (59). Southern blot analysis was done as described previously (41). A probe (see Fig. 2) was generated by labeling BlpI-SacI DNA fragments using a Megaprime DNA labeling system (Amersham Pharmacia Biotech, Piscataway, N.J.) and [^{32}P]dCTP (Amersham Pharmacia Biotech).

PCR analysis. PCR analysis was performed using the primer pairs of HCMVF, 5′-CCCGGTGTCTTCTATGGAGGT-3′, and HCMVUL127R, 5′-GGTTATA TAGCATAAATCAATATTGGCTATTGG-3′. The PCR cycling program was 1 cycle of denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min 30 s; and 1 cycle of elongation at 72°C for 7 min. The DNA PCR product was cloned into a TA cloning vector (Invitrogen) and sequenced after recombination and excision (University of Iowa DNA Core and Aichi Cancer Center Research Institute Central Facility).

Recombinant virus isolation. HFF cells were transfected with either 1 or 3 μg of each recombinant BAC in the presence of 1 μg of plasmid pSVpp71 by the calcium phosphate precipitation method of Graham and Van der Eb (15). After 5 to 21 days, viral plaques appeared. After 5 to 7 days of 100% cytopathic effect (CPE), the extracellular fluid was harvested and used either undiluted or diluted 1:10 for infection of HFF cells. After 5 to 7 days of 100% CPE, the extracellular fluid-containing virus was stored at −80°C in 50% newborn calf serum until used.

Northern blot analysis. Cytoplasmic RNAs from mock-infected or HCMV-infected HFFs were isolated as described previously (6, 20). Ten to twenty micrograms of cytoplasmic RNA was subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and transferred to maximum-strength Hybond N+ membrane (Amersham). Northern blot analysis was performed as described previously (41). IE1 DNA was amplified by PCR using the primer pairs

of ex4F (5′-AAGCGGGAGATGTGGATGGC-3′) and ex4R (5′-GGGATAGT CGCGGGTACAGG-3′), and the DNA PCR product was cloned into TA cloning vector (Invitrogen). A radioactive probe was generated by labeling with [^{32}P]dCTP as described above. The gels were quantitated using the histogram method of Adobe Photoshop version 2.0.

Multiplex real-time reverse transcriptase PCR and PCR. For detection of low levels of IE RNA, whole-cell RNA was isolated at 24 h p.i. using TRI reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. SUPERSCRIPT II RNase H-negative reverse transcriptase (Invitrogen) was used according to the manufacturer's directions to generate first-strand 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 detection of viral DNA, cells in 60-mm-diameter plates were harvested at 4 h p.i. in triplicate with PCR lysis buffer containing 50 μg of proteinase K/ml as described above. Multiplex real-time PCR was performed for simultaneous detection of probes containing 6-carboxyfluorescein and VIC reporter fluorophores as described previously (39). Amplifications were performed in a final volume of 25 μl containing PLATINUM Quantitative PCR SUPERMIX-UDG cocktail (Invitrogen). Each reaction mixture contained 2 μg 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 primers and MIE reporter probe were designed as described previously (39). HCMV gB primers were designed as 5′-GGCGAGG ACAACGAAATCC-3′ and 5′-TGAGGCTGGGAAGCTGACAT-3′. The gB reporter probe was designed as 5′-6-carboxyfluorescein-TTGGGCAACCACCG CACTGAGG-tetramethyl rhodamine-3′ (IDT, Coralville, Iowa). Cellular 18S DNA or 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 of 95°C for 15 s and 60°C for 1 min. The quantitation of relative MIE RNA or gB DNA was done according to a standard curve analysis as described previously (39).

Western blot analysis. HFF cells were collected at 1, 2, and 3 days p.i., lysed in SDS-dissociation buffer, fractionated by electrophoresis in a 10% SDS-polyacrylamide gel, and blotted onto a polyvinylidene difluoride membrane as described previously (45). To detect the pIE72 and pIE86 proteins encoded by IE1 and IE2, respectively, the p52 protein encoded by UL44, or p36 protein encoded by cellular GAPDH, monoclonal antibody NEA-9221 (Perkin-Elmer, Boston, Mass.), M0854 (Dako, Carpinteria, Calif.), or MAB374 (Chemicon, Temucula, Calif.), respectively, was used. Anti-pGAPDH antibody was used to prove equal protein loading. Enhanced chemiluminescence detection reagents (Amersham) and the secondary horseradish peroxidase-labeled anti-mouse immunoglobulin G (IgG) antibody (Zymed, San Francisco, Calif.) were used according to the manufacturers' instructions. Images were processed by LumiVision PRO (Aisin/Taitec, Tokyo, Japan) with a cooled charge-coupled device camera and assembled with an Apple G4 computer using Adobe Photoshop version 2.0.

Viral DNA replication assay. After infection with an equal viral DNA input equivalent to an MOI of approximately 1, cells were collected at 2, 3, and 4 days p.i. Cells in triplicate 35-mm-diameter plates in were suspended in lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS) containing 20 μg of RNaseA/ml and 50 μg of proteinase K/ml. After incubation at 55°C for 10 min and 95°C for 10 min, the DNA was phenol and chloroform extracted and precipitated with ethanol. 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 HindIII, fractionated in a 0.6% agarose gel, and subjected to Southern blot analysis as described previously (28). The 1.6-kbp BamHI-HindIII fragment of plasmid p1.6 (T probe) was used to probe human CMV genomic termini containing long inverted repeats as described previously (40).

EMSA. All probes and competitor DNAs were purchased from Invitrogen. The sequences for Sp-1-60, Sp-1-60mut, Sp-1-70, and Sp-1-70mut probe or competitor were as follows: SpI-60, 5′-TGACGCAAATGGGCGGTAGGCGTG T-3′ (sense) and 5′-CGTACACGCCTACCGCCCATTTGC-3′ (antisense); SpI-60mut, 5′-TGACGCAAATTTTCTTTAGGCGTGT-3′ (sense) and 5′-CGTAC ACGCCTAAAGAAAATTTGC-3′ (antisense); SpI-70, 5′-GTCGTAATAACC CCGCCCCGTTGAC-3′ (sense) and 5′-TGCGTCAACGGGGCGGGGTTAT TAC-3′ (antisense); and SpI-70mut, 5′-GTCGTAATAATTTTGTTTTGTTGA C-3′ (sense) and 5′-TGCGTCAACAAAACAAAATTATTAC-3′ (antisense). The probes and nonradioactive competitor DNAs were denatured at 95°C and annealed gradually by cooling down to room temperature (RT). Probes were prepared by 3′-end labeling using the Klenow fragment of the *E. coli* DNA polymerase I and [^{32}P]dGTP (Amersham). Unincorporated deoxynucleoside triphosphates were removed by Chromaspin+TE-10 columns (Clontech, Palo Alto, Calif.). Electrophoretic mobility shift assay (EMSA) with nuclear extracts was performed essentially as described previously (23), with minor modifications. Two micrograms of nuclear extract from HeLa cells was preincubated with nonradioactive competitor DNA at 50- or 200-fold molar excess relative to the probe in the presence of 2 μg of poly(dI-dC) and buffer I (20 mM HEPES [pH 7.9] containing 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.01% Nonidet P-40, and 9% glycerol) at RT for 15 min. Then, 176 fmol of radioactive probe was added to the reaction mixture and incubated at RT for 15 min. The DNA-protein complexes were separated from the free probe by electrophoresis in a 5% nondenaturing polyacrylamide gel in 0.5× TAE (20 mM Tris-acetate [pH 7.2] containing 1.0 mM EDTA) at 4°C. The gels were dried and exposed to Hyperfilm MP (Amersham). In antibody supershift experiments, 2 μg of nuclear extract was incubated with 2 μg of poly(dI-dC) DNA and buffer I in the presence or absence of nonradioactive DNA at room temperature for 15 min. Ten micrograms of anti-IgG control antibody (Zymed, San Francisco, Ca.) or anti-Sp-1 polyclonal antibody sc-59x (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) was added to the reaction mixture at RT for 30 min before 176 fmol of probe (50,000 cpm) was added. The reaction mixture was allowed to incubate at RT for 15 min. Electrophoresis was performed as described above.

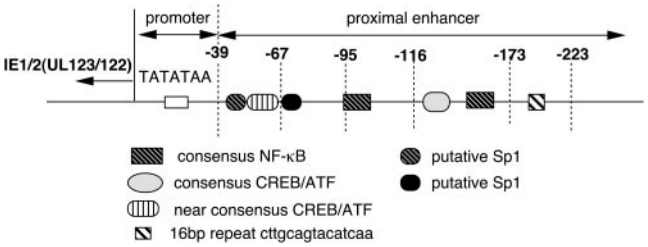


FIG. 1. Schematic representation of selected *cis*-acting elements in the proximal enhancer of human CMV upstream of the IE1 and IE2 (UL123 and UL122, respectively) genes. The promoter-containing TATA box and the proximal enhancer are defined as the regions between positions −39 and +1 and between positions −39 and −300, respectively. Consensus, near-consensus, and putative binding sites for the various *cis*-acting elements of the proximal enhancer are shown.

RESULTS

Recombinant virus isolation. Even though the enhancers of human and murine CMV exist in the same position in their respective viral genomes and the human CMV enhancer can substitute for the murine CMV enhancer, the murine CMV enhancer is not functionally equivalent to the human CMV enhancer. Murine CMV distal or proximal enhancer components cannot restore efficient human CMV replication (28). The region of the human CMV enhancer between positions −223 and −39 relative to the transcription start site of +1 has two 18-bp repeats containing consensus NF-κB/rel binding sites, two 19-bp repeats containing one consensus, one near-consensus CREB/ATF binding site, and one 16-bp repeat. There are also two putative Sp-1 binding sites approximately located at positions −60 and −70 (Fig. 1). To determine the role of the human CMV proximal enhancer, we constructed recombinant human CMV BAC DNAs with the distal enhancer deleted and the proximal enhancer deleted to positions −223, −173, −116, −67, and −39. To demonstrate that the phenotype of the recombinant viruses was due to the mutation in the enhancer and not to a spontaneous mutation in another area of the human CMV Towne BAC clone, we separately prepared and characterized two independent isolates for each mutation and found no difference in the phenotype.

Since a drug resistance gene is necessary for selecting the recombinant BAC from wt, we constructed the recombinant BACs to contain the kanamycin resistance (Kan^r) gene. To avoid the possibility that the Kan^r gene would affect the enhancer function, we constructed the recombinant BACs with flanking FRT sequences. The Kan^r gene was excised from −39+FRT+Kan (Fig. 2B) by FLP-mediated recombination to give −39+FRT. After excision of the Kan^r gene, only 34 bp of FRT was left in the MIE enhancer (Fig. 2A). Insertion of the 34 bp of FRT into the wt enhancer at position −68 or −78 had no effect on viral DNA synthesis after 8 days at low MOI relative to the wt control (data not shown). All recombinant BACs were digested with the restriction endonucleases BlpI and XhoI, fractionated by agarose gel electrophoresis, and further analyzed by Southern blot hybridization as described in Materials and Methods. The Kan^r gene contains an XhoI site, and the region of UL128 also has an XhoI site. After excision of the Kan^r gene, the DNA fragments digested by BlpI and XhoI were approximately the same size (Fig. 2B). Therefore, PCR analysis with the primer pairs of UL127R and HCMVF was used to distinguish between the presence and absence of the Kan^r gene. Figure 2C demonstrates that the recombinant viruses had the appropriate DNA fragment sizes as predicted in Fig. 2B. PCR analysis revealed the correct size of the DNA fragments after excision of the Kan^r gene (Fig. 2D).

HFF cells were transfected with these recombinant human CMV BACs by the calcium phosphate precipitation method as described in Materials and Methods. Because the human CMV IE2 gene product is toxic to cells, it was not possible to construct stable cell lines expressing the human CMV IE2 gene. We were able to isolate recombinant viruses with the enhancer deleted to positions −223, −173, −116, and −67 but not to position −39. These results indicated that the minimal enhancer sequence for viral growth in HFF cells is between positions −39 and −67. During the isolation of the recombinant viruses, the CPE was significantly more delayed with deletion to −67 than with deletion to −223, suggesting that the sequence upstream of −67 is important for the function of the proximal enhancer.

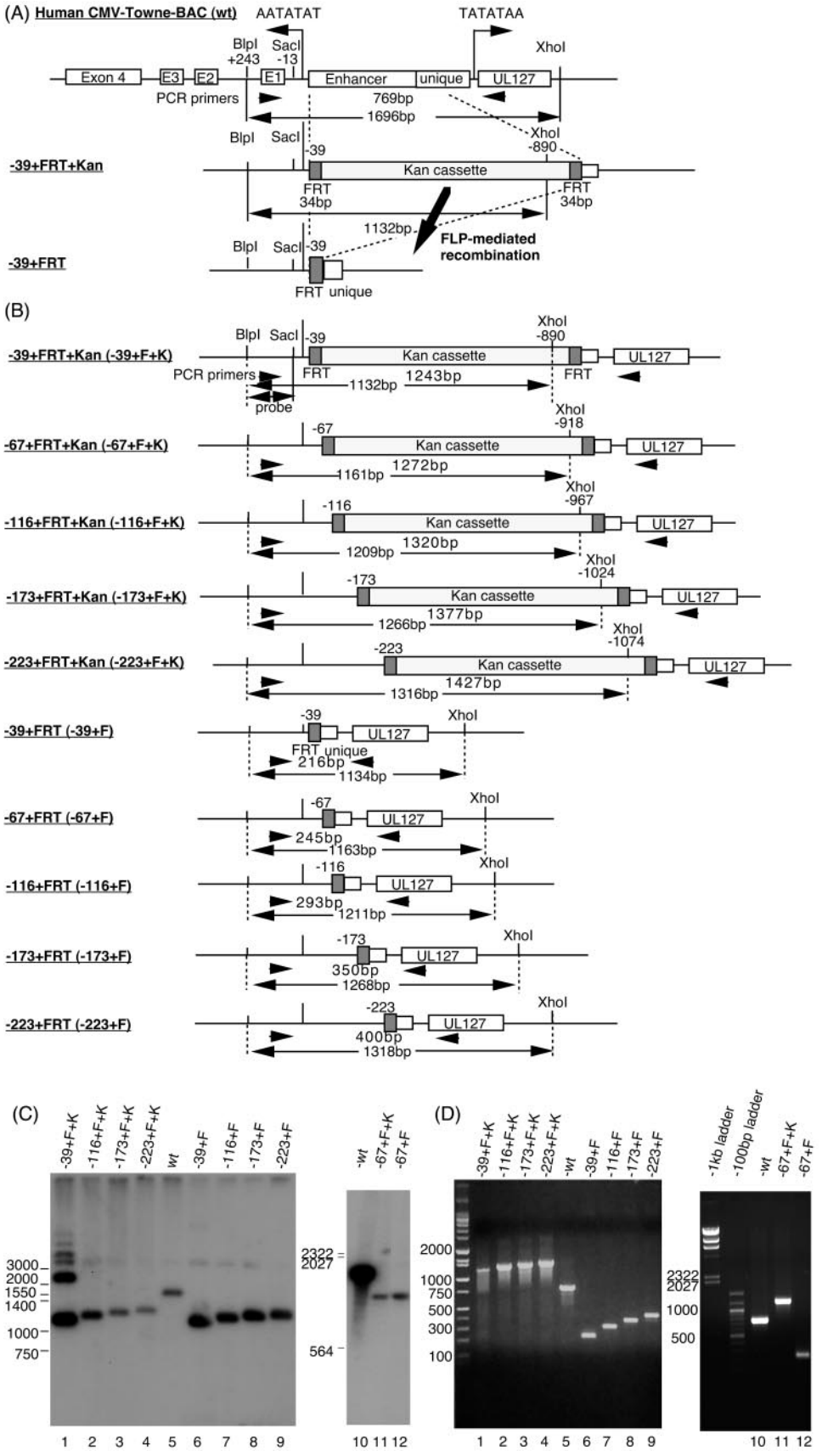
Effect of proximal enhancer deletions on IE gene transcription. Since the level of recombinant viral DNA replication without the Kan^r gene and with the FRT in the wt enhancer was similar (data not shown), we analyzed in detail the recombinant viruses without the Kan^r gene in the enhancer. To determine the effect of the proximal enhancer on IE1 gene transcription, Northern blot analysis was performed. The amount of viral DNA input for the various recombinant viruses was determined at 4 h p.i. by semiquantitative PCR assay as described in Materials and Methods. After infection with an equal viral DNA input equivalent to an MOI of approximately 1 for wild-type and various recombinant viruses, RNAs were harvested at 6 and 24 h p.i. and subjected to electrophoresis. Ethidium bromide staining of 28S and 18S rRNA confirmed that equal amounts of RNA were loaded in each lane (Fig. 3). Steady-state IE1 RNA from the wt virus was detected at 6 and 24 h p.i. Although a low level of IE1 RNA from −223+F (Fig. 2B) was detected at 24 h p.i., IE1 RNAs from −173+F and −116+F were not detected by Northern blot analysis (Fig. 3). The steady-state level of IE1 RNA was determined by histogram analysis as described in Materials and Methods and was found to be approximately eightfold higher at 24 h p.i. with the wt than with recombinant virus −223+F. These data confirmed that recombinant viruses without the distal enhancer have lower levels of MIE transcription.

Since Northern blot analysis was not sensitive enough to detect the IE RNAs from the recombinant viruses without the distal enhancer, we used multiplex real-time PCR as described in Materials and Methods. HFF cells were infected with the wt and the recombinant viruses −223+F, −173+F, −116+F, and −67+F. Total cellular RNA was harvested at 24 h p.i. and assayed for MIE RNA. We also assayed in parallel for input viral DNAs at 4 h p.i. relative to cellular ribosomal 18S DNA as described in Materials and Methods. Multiplex real-time PCR indicated that viral DNA levels in infected cells after recombinant virus infection differed by a factor of less than 1.4. MIE RNA levels of the recombinant viruses decreased approximately 2-fold for −223+F, 8-fold for −173+F, 14-fold for −116+F, and 33-fold for −67+F, relative to the wt levels (Fig. 4). These results indicated that the extent of the proximal enhancer of human CMV determines the level of MIE transcription from the MIE promoter.

Effect of proximal enhancer deletion on IE and early viral gene expression. To determine the effect of deletions of the proximal enhancer on expression of IE and early (E) viral proteins, HFF cells were infected with −223+F, −173+F, and −116+F as well as the wt virus at an equal viral DNA input equivalent to an MOI of approximately 1. Infected cells were harvested at 1, 2, and 3 days p.i., and equal amounts of protein were fractionated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with monoclonal antibodies against viral proteins pIE72 (UL123), pIE86 (UL122), and p52 (UL44) or cellular p36 GAPDH as described in Materials and Methods. The protein levels of pIE72, pIE86, and p52 of the recombinant viruses were dramatically decreased compared to the wt levels at 1, 2, and 3 days p.i. (Fig. 5A). The viral protein level of pIE72 was lower for recombinant virus −116+F than for −223+F and −173+F at 2 days p.i. (Fig. 5A). p36 GAPDH served as a protein loading control. The viral protein levels did not closely parallel the MIE RNA levels. These results may reflect differences in the assays in which real-time PCR is more quantitative, and Western blot is more qualitative.

Since p52 is necessary for viral DNA replication, DNA from an equal number of infected cells was collected, and the viral DNA was quantified by Southern blot hybridization with a T probe. Lambda DNA served as a sample processing and loading control as described in Materials and Methods. At 2 days p.i., the viral DNA levels of −173+F and −223+F in infected cells were lower than wt levels, and the viral DNA levels of −116+F were lower than those of −173+F and −223+F (Fig. 5B). For recombinant viruses −116+F and −67+F, a longer incubation period was necessary to detect the viral DNAs. The deletion between positions −67 and −116 did not have a major effect on the rate of viral DNA synthesis (Fig. 5C). Taken together, these findings indicated that the rate of viral DNA synthesis was determined by the extent of the deletion of the proximal enhancer.

Growth of the recombinant viruses. To determine the effect of the proximal enhancer deletions on the rate of infectious virus production, HFF cells were infected in triplicate with the wt, −223+F, −173+F, and −67+F at an equal viral DNA



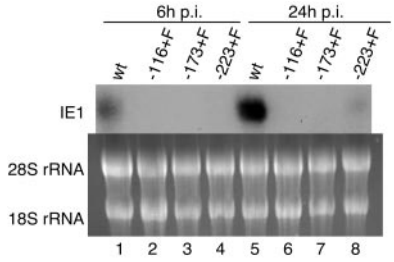


FIG. 3. Viral IE gene transcription with the wild type and the recombinant viruses. Steady-state IE1 mRNA levels from the MIE promoter after infection with an equal viral DNA input equivalent to an MOI of approximately 1. Cytoplasmic RNAs were analyzed with an IE1 probe by Northern blotting at 6 and 24 h p.i. as described in Materials and Methods. 28S and 18S rRNA served as controls for an equal amount of RNA loading. Lanes: 1 and 5, wt; 2 and 6, −116+F; 3 and 7, −173+F; 4 and 8, −223+F; 1 to 4, 6 h p.i.; 5 to 8, 24 h p.i.

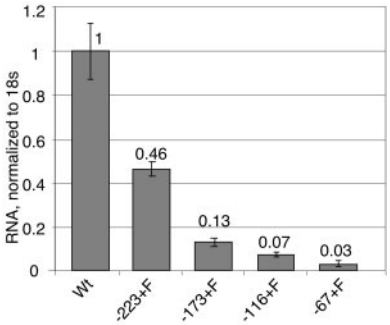


FIG. 4. Effect of deletion of the proximal enhancer on IE transcription assayed by multiplex real-time reverse transcriptase PCR. HFF cells were infected with wt virus, −223+F, −173+F, −116+F, and −67+F at an equal viral DNA input equivalent to an MOI of approximately 1. Total DNAs and RNAs were isolated in parallel at 4 and 24 h p.i, respectively, and analyzed by multiplex real-time PCR as described in Materials and Methods. The assay was done in quadruplicate, and the standard error of the mean was determined. IE RNAs were normalized to 18S RNA and to wt RNA.

input equivalent to an MOI of approximately 1. After lysis of the cells in the extracellular fluid, the total infectious virus level was assayed on HFF cells at 1, 5, and 10 days p.i. as described in Materials and Methods.

As expected, wt virus produced a large-plaque phenotype and reached titers at least 100-fold greater than those of the recombinant viruses without the distal enhancer (Fig. 6). All recombinant viruses lacking the distal enhancer produced a small-plaque phenotype of approximately similar size (data not shown). Recombinant virus −223+F replicated to titers 8- and 20-fold higher at 10 days p.i. than −173+F and −67+F, respectively (Fig. 6). Recombinant virus −67+F replicated to lower titers than −173+F (Fig. 6). Differences in the rate of replication between −116+F and −67+F were not detected within 10 days p.i. (data not shown). These data indicate that there are sequence elements in the proximal enhancer of human CMV upstream of position −116 that affect the efficiency of infectious virus replication.

Recombinant viruses −116+F and −173+F. Human CMV activates NF-κB activity within the first hour after infection, and then NF-κB activity is repressed (3, 9, 61). The recombinant virus −116+F has one consensus NF-κB binding site, and the recombinant virus −173+F has two (Fig. 1). We compared viral DNA synthesis in dermal HF cells with a dominant negative mutant of the inhibitor of NF-κBalpha (IκBαM) with control dermal HF cells (LXSN) (3). IκBαM inhibits activation of the NF-κB pathway. After infection with the wt, −173+F, or −116+F at an equal virus DNA input equivalent to an MOI of approximately 1, DNA from an equal number of infected cells was collected at 2, 3, and 4 days p.i. Southern blotting was performed to quantify the viral DNA using a T probe as described in Materials and Methods. At 3 days p.i., there was no difference between wt viral DNA levels in IκBαM cells and those in control cells (Fig. 7). Viral DNA replication of −173+F and −116+F was higher in the dermal HF control cells than in IκBαM cells (Fig. 7). −173+F viral DNA was present in higher amounts than that of −116+F in both cell types. While −116+F DNA replication was detected in the dermal HF control cells at 4 days p.i., longer exposures of the autoradiogram were required to detect −116+F DNA in IκBαM cells. These data indicate that an inhibitor of the NF-κB pathway did not prevent viral DNA synthesis by recombinant virus −173+F or −116+F, but the level of viral DNA synthesis was lower in the cells that inhibit the NF-κB pathway. Differences in recombinant viral DNA levels between the control cells and the IκBαM cells require further investigation.

Recombinant virus −67+F. While the HCMV BAC DNA from virus −39+F, which has the TATA box-containing promoter, could not replicate, the HCMV BAC DNA of virus −67+F could replicate. These results suggest that a minimal element of HCMV replication is between positions −67 and −39. The recombinant virus −67+F contains a putative Sp-1

FIG. 2. Structural analysis of recombinant human CMV BACs. (A) Schematic arrangement of the recombination of the recombinant BAC −39+FRT+Kan and −39+FRT. The entire enhancer was replaced with the Kan^r gene, and then the Kan^r gene was excised by FLP-mediated recombination. Only 34 bp of the FRT sequence is left after excision. (B) Diagram of the recombinant BACs with and without the Kan^r gene. All recombinant BACs were constructed using the PCR-based rapid recombination system, and the Kan^r gene was excised as described above. The viral DNA fragment sizes of all recombinant viruses digested with restriction endonucleases BlpI and XhoI are indicated. Southern blot hybridization was with the ^{32}P-labeled probe designated in the diagram. PCR was performed using primers designated in the diagram. (C) Southern blot analysis of the wt and recombinant BACs of −39+F, −116+F, −173+F, −223+F, and −67+F with and without the Kan^r gene (K). Viral DNAs were digested with restriction endonucleases BlpI and XhoI, fractionated by electrophoresis in 1.0% agarose, and subjected to hybridization with a ^{32}P-labeled probe. Standard molecular size markers are indicated to the left in numbers of base pairs. Lanes: 1, −39+F+K; 2, −116+F+K; 3, −173+F+K; 4, −223+F+K; 5, wt; 6, −39+F; 7, −116+F; 8, −173+F; 9, −223+F; 10, wt; 11, −67+F+K; 12, −67+F. (D) PCR analysis of the wt and the recombinant BACs. The products were amplified using the primers shown above and fractionated by electrophoresis in 1.0% agarose, stained with ethidium bromide, and sequenced as described in Materials and Methods. Lanes: 1, −39+F+K; 2, −116+F+K; 3, −173+F+K; 4, −223+F+K; 5, wt; 6, −39+F; 7, −116+F; 8, −173+F; 9, −223+F; 10, wt; 11, −67+F+K; 12, −67+F.

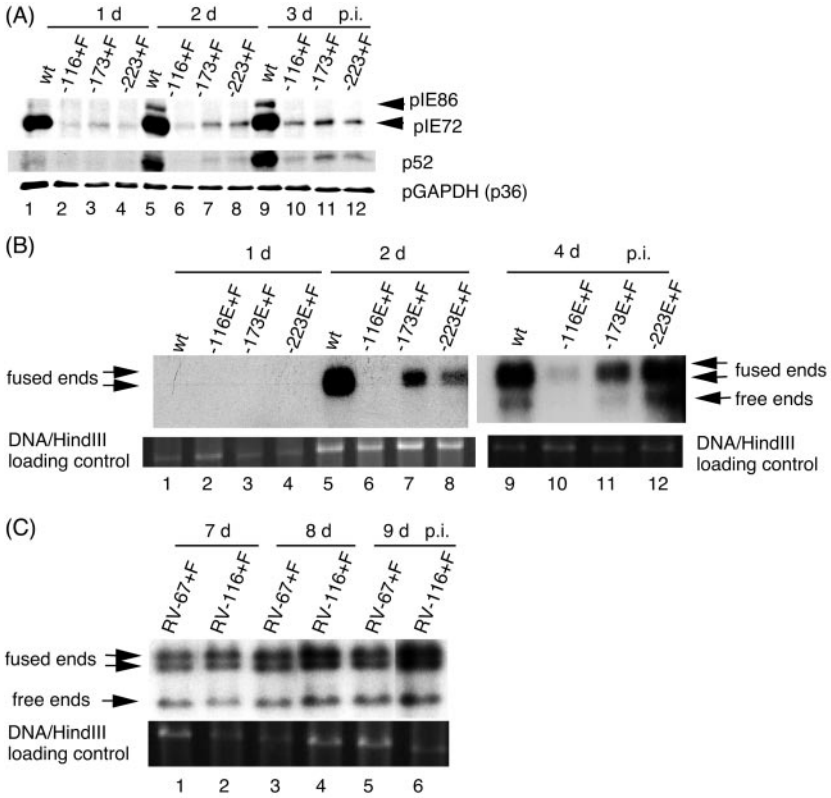


FIG. 5. Effect of proximal enhancer deletion on viral IE and early gene expression. HFF cells were infected with an equal viral DNA input equivalent to an MOI of approximately 1 and analyzed for viral protein or viral DNA. (A) Western blot of immediate-early pIE72 (UL123), pIE86 (UL122), and early p52 (UL44) proteins after infection with wt or recombinant viruses at an equivalent viral DNA input. pIE72 and pIE86 and p52 were analyzed 1, 2, and 3 days (d) p.i. with monoclonal antibodies NEA-9221 and M0854, respectively, as described in Materials and Methods. Lanes: 1, 5, and 9, wt; 2, 6, and 10, −116+F; 3, 7 and 11, −173+F; 4, 8, and 12, −223+F; 1 to 4, 1 day p.i.; 5 to 8, 2 days p.i.; 9 to 12, 3 days p.i. Anti-pGAPDH(p36) antibody was used to show equal protein loading. (B) Analyses of viral DNA synthesis after infection of HFF cells with wt and recombinant viruses at an equal viral DNA input equivalent to an MOI of approximately 1. DNAs from infected HFF cells were isolated 1, 2, and 4 days p.i., digested with restriction endonuclease HindIII, and subjected to Southern blot hybridization with ^{32}P-labeled T probe as described in Materials and Methods. Lambda DNA served as a sample processing and loading control. Arrows designate the viral DNA fused ends at 17.2 and 13.0 kb, free ends at 9.7 kb, and internal lambda DNA control. Lanes: 1, 5, and 9, wt; 2, 6, and 10, −116+F; 3, 7, and 11, −173+F; 4, 8, and 12, −223+F; 1 to 4, 1 day p.i.; 5 to 8, 2 days p.i.; 9 to 12, 4 days p.i. (C) Analysis of viral DNA synthesis after infection of HFF cells with recombinant virus −67+F and −116+F. Infection and viral DNA analysis were as described above. Lanes: 1, 3, and 5, −67+F; 2, 4, and 6, −116+F; 1 and 2, 7 days p.i.; 3 and 4, 8 days p.i.; 5 and 6, 9 days p.i.

binding site between positions −57 and −52 (Fig. 1). To determine whether the putative Sp-1 binding site is bound by Sp-1, an EMSA using a probe between positions −67 and −40 was prepared. We also prepared a probe containing an upstream putative Sp-1 site between positions −88 and −61. These probes were designated −60 and −70, respectively. Figure 8A demonstrates the DNA sequences of the putative Sp-1 sites and the mutations in the sites. Lanes 1 and 5 of Fig. 8B demonstrate that complex X and complex Y were detected using the probe of the respective sites plus nuclear extract. The complexes were reduced using 50- or 200-fold molecular excess of nonradioactive competitors (Fig. 8B, lanes 2, 3, 6, and 7). The mutated Sp-1 sites were unable to compete at 200-fold molar excess for DNA complexes X and Y (Fig. 8B, lanes 4 and 8). To determine whether the cellular protein(s) binding to the DNA elements approximately located at position −60 or −70 included Sp-1, a supershift assay with polyclonal antibody against Sp-1 was performed. Rabbit polyclonal antibody against Sp-1 (Fig. 8C, lanes 1, 2, 4, and 5), but not control IgG

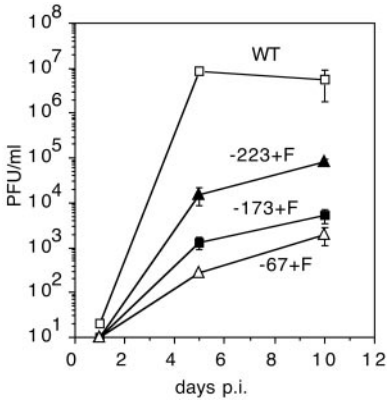


FIG. 6. Growth of recombinant viruses without the distal enhancer and with deletions in the proximal enhancer. A multistep growth curve was determined after infection with wt and recombinant viruses at an equal viral DNA input equivalent to an MOI of approximately 1 as described in Materials and Methods. Three independent assays were used to determine the mean and the standard error.

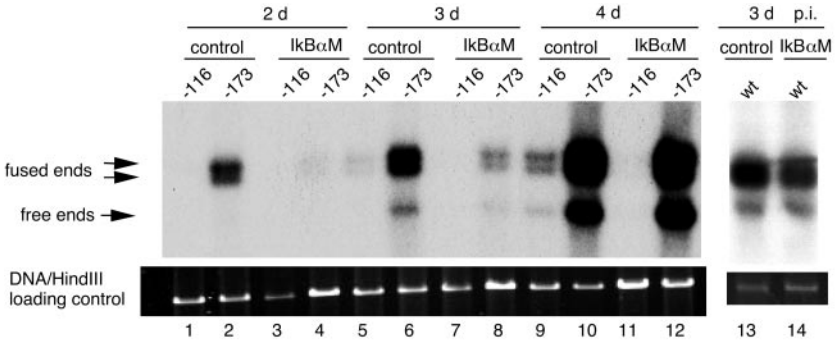


FIG. 7. Replication of recombinant viruses −173+F and −116+F in dermal fibroblast control cells and NF-κB dominant negative dermal fibroblast. Cells were infected with the wt, −173+F, or −116+F at an equal viral DNA input equivalent to an MOI of approximately 1. DNAs were isolated 2, 3, and 4 days (d) p.i., digested with restriction endonuclease HindIII, and subjected to Southern blot hybridization with ^{32}P-labeled T probe as described in Materials and Methods. Lambda DNA served as a sample processing and loading control. The arrows designate the viral DNA fused ends at 17.2 and 13.0 kb, free ends at 9.7 kb, and internal lambda DNA control.

(Fig. 8C, lanes 3 and 6), caused complex X to supershift. The antibodies had no effect on complex Y. These data suggest that Sp-1 binding at approximate position −60 could meet a minimal requirement of the proximal enhancer for recombinant virus −67+F growth in HFF cells. The −60 probe is the proximal enhancer sequence from position −67 to −40 and contains the partial 19-bp repeat with the sequence 5′-TGACGC AA-3′ between positions −67 and −60, which is not a consensus CREB/ATF site. Whether another *cis*-acting element effects transcription from the MIE promoter in this region requires further investigation.

DISCUSSION

CMV MIE gene expression is essential for viral replication (38). The enhancer upstream of the MIE genes is thought to influence the efficiency of transcription. Since viral IE genes are expressed in the absence of de novo protein synthesis, the enhancers are responsive to cellular transcription factors. In addition, UV-inactivated human CMV can rescue recombinant viruses without the distal enhancer (39). The human CMV envelope glycoproteins and tegument proteins may affect the level of cellular transcription factors in the cell as follows. Viral glycoproteins increase cellular transcription factors NF-κB and Sp-1 (61). Phosphatidylinositol 3-kinase-mediated signaling is activated by viral glycoprotein gB interacting with the epidermal growth factor receptor which in turn activates cellular transcription factors (58). Tegument protein pp71 activates transcription by inhibiting cellular repressors. The hDaxx-histone deacetylase repressor complex is inactivated by the tegument protein pp71 binding to hDaxx (22, 27). E2F transcription factors and movement of the cell cycle toward G_1/S is activated by pp71 inducing degradation of tumor suppressor protein Rb (29, 30). Cellular signaling through the phospholipase C pathway is activated by CMV G-protein-coupled receptors, which activate cellular transcription factors (17, 57). Human CMV also delivers to the cell virion-associated mRNAs that could code for factors that affect the level of cellular transcription factors (4). These different virion-associated components for the activation of cellular transcription factors are assumed to impact the activity of the human CMV enhancer.

While the CMV enhancers have multiple transcription factor binding sites, it is unknown whether one type of site functions more efficiently than another site in a given cell type. Even though the MIE enhancers of CMVs are positional homologues, CMV enhancers are not functionally equivalent. For example, the human CMV enhancer can substitute for the murine CMV enhancer for replication in mouse embryo fibroblast cells in culture and the host (2, 18). In contrast, the murine CMV enhancer cannot fully substitute for the human CMV enhancer for replication in human fibroblast cells in culture (28). However, the simian CMV enhancer can fully substitute for the human CMV enhancer, whereas the enhancer from another human betaherpesvirus, human herpesvirus 6, cannot substitute (H. Isomura and M. F. Stinski, unpublished data). In addition, the murine CMV enhancer is dispensable for replication in cell culture but essential for replication in the host (14). As demonstrated in the present report, enhancerless human CMV (−39+F) with the TATA box-containing promoter element did not replicate in cell culture. Unlike murine CMV, the IE2 gene of human CMV cannot be expressed in cells to rescue recombinant virus −39+F because of the effects of pIE86 protein on the cell.

The distal enhancer between positions −550 and −300 determines efficient human CMV replication and a normal plaque phenotype (39, 40). The distal enhancer of murine CMV cannot substitute for the human CMV distal enhancer. Likewise, the murine CMV proximal enhancer cannot fully substitute for the human CMV proximal enhancer (28). All of the recombinant viruses described in this report lack the distal enhancer, replicated slowly in HFF cells, and had a small-plaque phenotype. These studies indicate that both the distal and proximal enhancers of human CMV are specific and necessary for efficient viral replication in cell culture.

To characterize the proximal enhancer, we made 5′-end deletions in the viral genome using human CMV BAC and the red/gam recombination system described by Yu et al. (60). In this study, the region between positions −300 and −223 of the proximal enhancer was not analyzed. This region contains two 16-bp repeat elements, one consensus Ap-1 site, and one 18-bp repeat containing a consensus NF-κB binding site (42, 54). These elements are repeated between positions −223 and −39, and consequently, we focused on this region. A retinoic

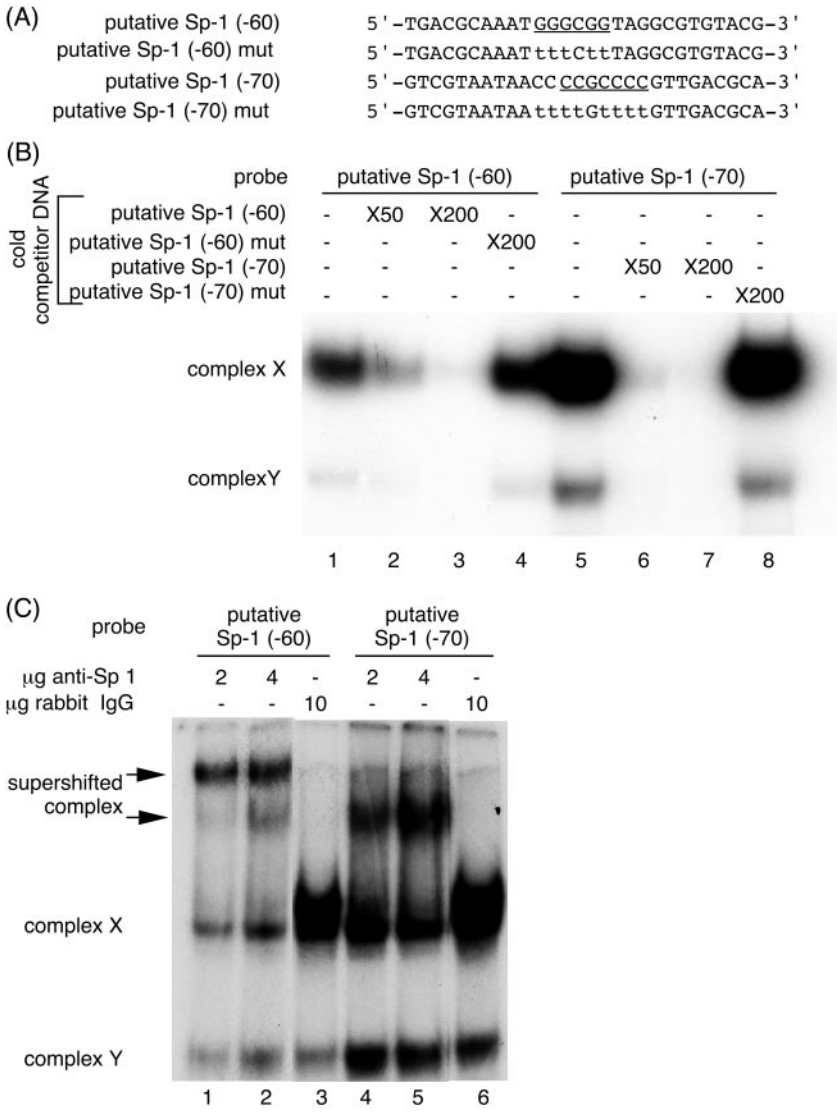


FIG. 8. Binding of the Sp-1 transcription factor to the two putative Sp-1 binding sites. (A) Sequences of probes and competitors of human CMV putative and mutant Sp-1 binding sites around positions −60 and −70 (Fig. 1). The consensus Sp-1 binding sites are underlined. Probes and competitors were generated as described in Materials and Methods. (B) Autoradiogram of EMSA and competition assay using nuclear extract. Lanes: 1, putative Sp1 probe around position −60 plus nuclear extract; 2 and 3, putative Sp-1 probe around position −60 plus nuclear extract in the presence of a 50- or 200-fold molar excess of nonradioactive Sp-1(-60) DNA, respectively; 4, putative Sp-1 probe around position −60 plus nuclear extract in the presence of a 200-fold molar excess of nonradioactive Sp-1 mutant competitors; 5, putative Sp-1 probe around position −70 plus nuclear extract; 6 and 7, putative Sp-1 probe around position −70 plus nuclear extract in the presence of 50- and 200-fold molar excess of nonradioactive Sp-1(-70) DNA, respectively; 8, putative Sp-1 probe around position −70 plus nuclear extract in the presence of a 200-fold molar excess of nonradioactive Sp-1(-70) mutant DNA. The specific complexes X and Y are indicated. Complexes are resolved on a 5% nondenaturing polyacrylamide gel. Free probe is at the bottom of the gel, which is not shown. (C) Supershift assay of the DNA-protein complex with anti-Sp-1 antibody. Lanes: 1 and 2, putative Sp-1 probe around position −60 plus nuclear extract with 2 and 4 μg of anti-Sp-1 antibodies, respectively; 3, putative Sp-1 probe around position −60 plus nuclear extract with 10 μg of anti-rabbit IgG; 4 and 5, putative Sp-1 probe around position −70 plus nuclear extract with 2 and 4 μg of anti-Sp-1 antibodies, respectively; 6, putative Sp-1 probe around position −70 plus nuclear extract with 10 μg of anti-rabbit IgG.

acid receptor site between positions −300 and −223 is not repeated downstream of position −223. However, recent evidence suggested that the retinoic acid receptor sites in the enhancer in the context of the viral genome did not have a significant effect on activation of transcription from the MIE promoter in cell culture (40).

Recombinant human CMVs with the proximal enhancer extending to position −223 replicated titers approximately 100- to 1,000-fold lower than those of wild-type virus. Northern blot analysis detected an at least eightfold higher level of steady-state IE1 RNA from wt than from recombinant virus −223+F. Real-time reverse transcriptase PCR assay detected significantly higher levels of steady-state MIE RNA from recombinant virus −223+F than from −173+F, −116+F, and −67+F at 24 h p.i. There was a significant reduction in the level of viral IE proteins pIE86 and pIE72 and early viral protein p52 as

detected by Western blotting. There was also a significant reduction in viral DNA synthesis. Recombinant virus −223+F replicated to an approximately 10-fold higher titer of virus than recombinant virus −173+F at 5 or 10 days p.i. A 16-bp repeat with a putative NF-1 binding site is located between positions −223 and −173. However, a positive effect of the 16-bp repeat element on the minimal MIE promoter was not detected in transient transfection assays. In contrast, a positive effect on downstream gene expression was detected for the 18-, 19-, and 21-bp repeats placed upstream of a minimal human CMV MIE promoter (24, 52). The effect of the 16-bp repeat on transcription from the proximal-enhancer-containing MIE promoter is being investigated.

The region in the proximal enhancer between positions −173 and −116 contains one 18-bp repeat with a consensus NF-κB site and one 19-bp repeat with a consensus CREB/ATF site. There was a higher level of MIE transcription at 24 h p.i. with recombinant virus −173+F than with −116+F. The MIE viral proteins and the early viral protein p52 were at higher levels for recombinant virus −173+F. Viral DNA synthesis, which is also an indicator of early viral gene expression, was also significantly higher with recombinant virus −173+F than with −116+F at 2 to 4 days p.i. In addition, viral DNA synthesis in either IκBαM cells that inhibit the NF-κB pathway or control cells was also significantly higher with recombinant virus −173+F than with −116+F. NF-κB was not required for viral replication of recombinant virus −173+F or −116+F. Since recombinant virus −173+F had an additional 19-bp repeat containing a consensus CREB/ATF element and an 18-bp repeat containing a consensus NF-κB element, these elements may have contributed to the higher level of MIE transcription detected with −173+F. Inhibition of NF-κB in IκBαM human fibroblast cells had no effect on the replication kinetics of human CMV containing the entire wild-type enhancer. Even though infection with human CMV induces high levels of NF-κB within the first hour after infection, activation of NF-κB does not appear to be required for wild-type human CMV replication in fibroblast cells (3, 14). In addition, murine CMV with the enhancer substituted by the human CMV enhancer containing mutated NF-κB binding sites replicated like the wild-type virus in mouse fibroblast cells (3, 14).

When the CREB/ATF binding sites were mutated in the entire enhancer in the presence of other transcription factor DNA binding sites, there was no significant effect on the level of human CMV MIE gene transcription in HFF cells at high or low MOI (31). However, the CREB/ATF sites were responsive to forskolin, which induces cyclic AMP- and protein kinase A-mediated phosphorylation of CREB/ATF and allows for CBP or p300 to promote transcription (44, 49). The level of response was proportional to the number of wild-type CREB/ATF binding sites present in the enhancer (31). Therefore, it is likely that other transcription factor binding sites in the enhancer can compensate for mutated CREB/ATF sites in HFF cells. The multiple CREB/ATF sites could be important in other cell types.

The efficiency of MIE transcription is reflected in the level of viral DNA synthesis and infectious virus production. While significant viral DNA synthesis was detected within 3 to 4 days p.i. with recombinant virus −173+F, similar levels of viral DNA synthesis with recombinant viruses −116+F and −67+F required 7 days p.i. The slow rate of viral DNA synthesis with recombinant virus −116+F correlated with the low levels of MIE protein and early p52 protein at 2 to 3 days p.i. Although the region between positions −116 and −67 slightly increased the level of MIE transcription from the MIE promoter at 24 h p.i., differences in the level of viral DNA synthesis were small and not detected until 8 to 9 days p.i.

While recombinant virus −39+F could not produce infectious viruses in HFF cells, −67+F could. The region between positions −67 and −39 contains an Sp-1 binding site and a partial 19-bp repeat with a nonconsensus CREB/ATF site. Since the viral Sp-1 binding site between positions −67 and −39 is consensus and was found to bind Sp-1 in this report, this cellular transcription factor may be important for MIE transcription from the proximal enhancer. Human CMV infection up-regulates Sp-1 binding activity early after infection, and this activity is due to the virion-associated envelope glycoproteins (61). It is likely that the Sp-1 binding site between positions −67 and −39 is a minimal element required for MIE transcription.

The proximal enhancer between positions −223 and −67 influences an approximately 100-fold difference in the production of infectious virus at 5 and 10 days p.i. The region contains one 16-bp repeat, one 19-bp repeat, and two 18-bp repeats with putative NF-1 and consensus CREB/ATF and NF-κB DNA binding sites, respectively. In addition, there are one AP-1 site and one Sp-1 site in this region. While the Sp-1 site between positions −67 and −39 may play a role for the minimal enhancer, the NF-1, AP-1, Sp-1, CREB/ATF, and NF-κB sites may cumulatively affect the activity of the proximal enhancer. How these elements cooperate synergistically to affect the level of MIE transcription from the proximal enhancer and whether one or more elements are of greater importance in a given cell type are not known.

The proximal enhancer presumably interacts with the distal enhancer, because there is an additional 100-fold increase in the rate of infectious virus production when the distal enhancer is present. How the distal enhancer impacts the activity of the proximal enhancer is not known. With human CMV, a portion of the viral DNA is detected adjacent to the nuclear domain 10 (ND10) early after infection (1, 26). How viral DNA is targeted to a specific area after entry into the nucleus is unclear. It is possible that a specific attachment of the enhancer to the nuclear matrix may facilitate MIE transcription. The position of the cellular transcription factor binding sites in the enhancer may also be critical for transcription of the MIE genes if the viral genome is associated with cellular nuclear matrix and ND10. Both the distal and proximal enhancers of human CMV are required for the efficient replication of the virus. UV-inactivated human CMV significantly enhances transcription from the MIE promoter with an upstream proximal enhancer (39). The elements in the proximal enhancer that respond to the virion-associated proteins, glycoproteins, or mRNAs require further investigation.

ACKNOWLEDGMENTS

We thank members of the laboratory and Jeffery Meier for critical reading of the manuscript.

This work was supported by grant AI-13562 from the National Institutes of Health (to M.F.S.) and grants 14021138 and 12470073 from the

Ministry of Education, Science, Sports, and Culture and Technology of Japan (to T.T.).

REFERENCES

1. **Ahn, J.-H., W.-J. Jang, and G. S. Hayward.** 1999. The human cytomegalovirus IE2 and UL112–113 proteins accumulate in viral DNA replication compartments that initiate from the periphery of promyelocytic leukemia protein-associated nuclear bodies (PODs or ND10). J. Virol. **73:**10458–10471.
2. **Angulo, A., M. Messerle, U. H. Koszinowski, and P. Ghazal.** 1998. Enhancer requirement for murine cytomegalovirus growth and genetic complementation by the human cytomegalovirus enhancer. J. Virol. **72:**8502–8509.
3. **Benedict, C. A., A. Angulo, G. Patterson, S. Ha, H. Huang, M. Messerle, C. F. Ware, and P. Ghazal.** 2004. Neutrality of the canonical NF-κB-dependent pathway for human and murine cytomegalovirus transcription and replication in vitro. J. Virol. **78:**741–750.
4. **Bresnahan, W. A., and T. Shenk.** 2000. Subset of viral transcripts packaged within human cytomegalovirus particles. Science **288:**2373–2376.
5. **Britt, W. J., and C. A. Alford.** 1996. Cytomegaloviruses, p. 2493–2523. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
6. **Chang, C.-P., C. L. Malone, and M. F. Stinski.** 1989. A human cytomegalovirus early gene has three inducible promoters that are regulated differentially at various times after infection. J. Virol. **63:**281–290.
7. **Cherrington, J. M., E. L. Khoury, and E. S. Mocarski.** 1991. Human cytomegalovirus *ie*2 negatively regulates α gene expression via a short target sequence near the transcription start site. J. Virol. **65:**887–896.
8. **Colberg-Poley, A. M.** 1996. Functional roles of immediate early proteins encoded by the human cytomegalovirus UL36–38, UL115–119, TRS1/IRS1 and US3 loci. Intervirology **39:**350–360.
9. **Compton, T., E. A. Kurt-Jones, K. W. Boehme, J. Belko, E. Latz, D. T. Golenbock, and R. W. Finberg.** 2003. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. J. Virol. **77:**4588–4596.
10. **Dunn, W., C. Chou, L. Hong, R. Hai, D. Patterson, V. Stolc, H. Zhu, and F. Liu.** 2003. Functional profiling of a human cytomegalovirus genome. Proc. Natl. Acad. Sci. USA **100:**14223–14228.
11. **Fish, K. N., W. Britt, and J. A. Nelson.** 1996. A novel mechanism for persistence of human cytomegalovirus in macrophages. J. Virol. **70:**1855–1862.
12. **Fish, K. N., A. S. Depto, A. V. Moses, W. Britt, and J. A. Nelson.** 1995. Growth kinetics of human cytomegalovirus are altered in monocyte-derived macrophages. J. Virol. **69:**3737–3743.
13. **Gawn, J. M., and R. F. Greaves.** 2002. Absence of IE1 p72 protein function during low-multiplicity infection by human cytomegalovirus results in a broad block to viral delayed-early gene expression. J. Virol. **76:**4441–4455.
14. **Ghazal, P., M. Messerle, K. Osborn, and A. Angulo.** 2003. An essential role of the enhancer for murine cytomegalovirus in vivo growth and pathogenesis. J. Virol. **77:**3217–3228.
15. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of adenovirus 5 DNA. Virology **52:**456–467.
16. **Greaves, R. F., and E. S. Mocarski.** 1998. Defective growth correlates with reduced accumulation of viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus *ie1* mutant. J. Virol. **72:**366–379.
17. **Gruijthuijsen, Y. K., P. Casarosa, S. J. F. Kaptein, J. L. V. Broers, R. Leurs, C. A. Bruggeman, M. J. Smit, and C. Vink.** 2002. The rat cytomegalovirus R33-encoded G protein-coupled receptor signals in a constitutive fashion. J. Virol. **76:**1328–1338.
18. **Grzimek, N. K. A., J. Podlech, H.-P. Steffens, R. Holtappels, S. Schmalz, and M. J. Reddehase.** 2000. In vivo replication of recombinant murine cytomegalovirus driven by the paralogous major immediate-early promoter-enhancer of human cytomegalovirus. J. Virol. **73:**5043–5055.
19. **Hahn, G., R. Jores, and E. S. Mocarski.** 1998. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. Proc. Natl. Acad. Sci. USA **95:**3937–3942.
20. **Hermiston, T. W., C. L. Malone, P. R. Witte, and M. F. Stinski.** 1987. Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. J. Virol. **61:**3214–3221.
21. **Ho, M.** 1991. Cytomegalovirus: biology and infection. Plenum Publishing Corp., New York, N.Y.
22. **Hofmann, H., H. Sindre, and T. Stamminger.** 2002. Functional interaction between the pp71 protein of human cytomegalovirus and the PML-interacting protein human Daxx. J. Virol. **76:**5769–5783.
23. **Huang, L., and M. F. Stinski.** 1995. Binding of cellular repressor protein or the IE2 protein to a *cis*-acting negative regulatory element upstream of a human cytomegalovirus early promoter. J. Virol. **69:**7612–7621.
24. **Hunninghake, G. W., M. M. Monick, B. Liu, and M. F. Stinski.** 1989. The promoter-regulatory region of the major immediate-early gene of human cytomegalovirus responds to T-lymphocyte stimulation and contains functional cyclic AMP-response elements. J. Virol. **63:**3026–3033.
25. **Ibanez, C. E., R. Schrier, P. Ghazal, C. Wiley, and J. A. Nelson.** 1991. Human cytomegalovirus productively infects primary differentiated macrophages. J. Virol. **65:**6581–6588.
26. **Ishov, A. M., R. M. Stenberg, and G. G. Maul.** 1997. Human cytomegalovirus immediate early interaction with host nuclear structures: definition of an immediate transcript environment. J. Cell Biol. **138:**5–16.
27. **Ishov, A. M., O. V. Vladimirova, and G. G. Maul.** 2002. Daxx-mediated accumulation of human cytomegalovirus tegument protein pp71 at ND10 facilitates initiation of viral infection at these nuclear domains. J. Virol. **76:**7705–7712.
28. **Isomura, H., and M. F. Stinski.** 2003. Effect of substitution of the human cytomegalovirus enhancer or promoter with the murine cytomegalovirus enhancer or promoter on replication in human fibroblast. J. Virol. **77:**3602–3614.
29. **Kalejta, R. F., and T. Shenk.** 2002. Manipulation of the cell cycle by human cytomegalovirus. Front. Biosci. **7:**295–306.
30. **Kalejta, R. F., and T. Shenk.** 2003. Proteasome-dependent, ubiquitin-independent degradation of the Rb family of tumor suppressors by the human cytomegalovirus pp71 protein. Proc. Natl. Acad. Sci. USA **100:**3263–3268.
31. **Keller, M. J., D. G. Wheeler, E. Cooper, and J. L. Meier.** 2003. Role of the human cytomegalovirus major immediate-early promoter's 19-base-pair-repeat cyclic AMP-response element in acutely infected cells. J. Virol. **77:**6666–6675.
32. **Kondo, K., H. Kaneshima, and E. S. Mocarski.** 1994. Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. Proc. Natl. Acad. Sci. USA **91:**11879–11883.
33. **Kondo, K., J. Xu, and E. S. Mocarski.** 1996. Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. Proc. Natl. Acad. Sci. USA **93:**11137–11142.
34. **Lashmit, P. E., C. A. Lundquist, J. L. Meier, and M. F. Stinski.** 2004. A cellular repressor inhibits human cytomegalovirus transcription from the UL127 promoter. J. Virol. **78:**5113–5123.
35. **Lathey, J. L., and S. A. Spector.** 1991. Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages. J. Virol. **65:**6371–6375.
36. **Liu, B., T. W. Hermiston, and M. F. Stinski.** 1991. A *cis*-acting element in the major immediate-early (IE) promoter of human cytomegalovirus is required for negative regulation by IE2. J. Virol. **65:**897–903.
37. **Lundquist, C. A., J. L. Meier, and M. F. Stinski.** 1999. A strong negative transcriptional regulatory region between the human cytomegalovirus UL127 gene and the major immediate-early enhancer. J. Virol. **73:**9039–9052.
38. **Marchini, A., H. Liu, and H. Ahu.** 2001. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. J. Virol. **75:**1870–1878.
39. **Meier, J. L., M. J. Keller, and J. J. McCoy.** 2002. Requirement of multiple *cis*-acting elements in the human cytomegalovirus major immediate-early distal enhancer for viral gene expression and replication. J. Virol. **76:**313–326.
40. **Meier, J. L., and J. A. Pruessner.** 2000. The human cytomegalovirus major immediate-early distal enhancer region is required for efficient viral replication and immediate-early expression. J. Virol. **74:**1602–1613.
41. **Meier, J. L., and M. F. Stinski.** 1997. Effect of a modulator deletion on transcription of the human cytomegalovirus major immediate-early genes in infected undifferentiated and differentiated cells. J. Virol. **71:**1246–1255.
42. **Meier, J. L., and M. F. Stinski.** 1996. Regulation of human cytomegalovirus immediate-early gene expression. Intervirology **39:**331–342.
43. **Mocarski, E.** 1996. Latency and regulatory functions in the IE1/IE2 locus of cytomegalovirus, abstr. S-16. 21st Herpesvirus Workshop, Northern Illinois University, DeKalb, Ill.
44. **Montminy, M.** 1997. Transcription regulation by cyclic AMP. Annu. Rev. Biochem. **66:**807–822.
45. **Murphy, E. A., D. N. Streblow, J. A. Nelson, and M. F. Stinski.** 2000. The human cytomegalovirus IE86 protein can block cell cycle progression after inducing transition into the S phase of permissive cells. J. Virol. **74:**7108–7118.
46. **Pizzorno, M. C., and G. S. Hayward.** 1990. The IE2 gene products of human cytomegalovirus specifically down-regulate expression from the major immediate-early promoter through a target located near the cap site. J. Virol. **64:**6154–6165.
47. **Prosch, S., K. Staak, J. Stein, C. Liebenthal, T. Stamminger, H. D. Volk, and D. H. Kruger.** 1995. Stimulation of the human cytomegalovirus IE enhancer/promoter in HL-60 cells by TNFalpha is mediated via induction of NF-kappaB. Virology **208:**197–206.
48. **Sambucetti, L. C., J. M. Cherrington, G. W. G. Wilkinson, and E. S. Mocarski.** 1989. NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J. **8:**4251–4258.
49. **Shaywitz, A. J., and M. E. Greenberg.** 1999. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu. Rev. Biochem. **68:**821–861.
50. **Sinzger, C., A. Grefte, B. Plachter, A. S. H. Gouw, T. H. The, and G. Jahn.** 1995. Fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells

are the major targets of human cytomegalovirus infection in lung and gastrointestinal tissues. J. Gen. Virol. **76:**741–750.
51. **Sinzger, C., B. Plachter, A. Grefte, A. S. H. Gouw, T. H. The, and G. Jahn.** 1996. Tissue macrophages are infected by human cytomegalovirus. J. Infect. Dis. **173:**240–245.
52. **Stamminger, T., and B. Fleckenstein.** 1990. Immediate-early transcription regulation of human cytomegalovirus, p. 3–19. *In* ●●●, Current topics in microbiology and immunology, vol. 54. Springer-Verlag, Berlin-Heidelberg, Germany.
53. **Stenberg, R. M.** 1996. The human cytomegalovirus major immediate-early gene. Intervirology **39:**343–349.
54. **Stinski, M. F.** 1999. Cytomegalovirus promoter for expression in mammalian cells, p. 211–233. *In* J. M. Fernandez and J. P. Hoeffler (ed.), Gene expression systems: using nature for the art of expression. Academic Press, San Diego, Calif.
55. **Stinski, M. F.** 1978. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J. Virol. **26:**686–701.
56. **Taylor-Wiedeman, J., J. G. Sissons, L. K. Borysiewicz, and J. H. Sinclair.** 1991. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. J. Gen. Virol. **72:**2059–2064.
57. **Waldhoer, M., T. N. Kledal, H. Farrell, and T. W. Schwartz.** 2002. Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities. J. Virol. **76:**8161–8168.
58. **Wang, D., W. Bresnahan, and T. Shenk.** 2003. Human cytomegalovirus encodes a highly specific RANTES decoy receptor, abstr. 1.52. 28th Intl. Herpesvirus Workshop, Madison, Wisc.
59. **Wathen, M. W., and M. F. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate-early, early, and late times after infection. J. Virol. **41:**462–477.
60. **Yu, D., H. M. Ellis, C.-E. Lee, N. A. Jenkins, N. G. Copeland, and D. L. Court.** 2000. An efficient recombination system for chromosome engineering in Escherichia coli. Proc. Natl. Acad. Sci. USA **97:**5978–5983.
61. **Yurochko, A. D., E.-S. Hwang, L. Rasmussen, S. Keay, L. Pereira, and E.-S. Huang.** 1997. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-κB during infection. J. Virol. **71:**5051–5059.