JOURNAL OF VIROLOGY, Feb. 1997, p. 1246–1255 Vol. 71, No. 2
0022-538X/97/$04.00+0


# Effect of a Modulator Deletion on Transcription of the Human Cytomegalovirus Major Immediate-Early Genes in Infected Undifferentiated and Differentiated Cells

JEFFERY L. MEIER[1] AND MARK F. STINSKI[2]*

*Departments of Internal Medicine[1] and Microbiology,[2] University of Iowa College of Medicine, Iowa City, Iowa 52242*

Received 11 September 1996/Accepted 25 October 1996

**Differentiation-dependent expression of the human cytomegalovirus (HCMV) major immediate-early (MIE) genes, encoding IE1 and IE2, may partly govern virus replication in monocytic THP-1 and embryonal carcinoma (Tera-2) cells. The modulator of the MIE promoter was shown previously in transient transfection assays to repress transcription from promoter segments in undifferentiated THP-1 and Tera-2 cells but not in differentiated cells. To determine the biological importance of these findings, we constructed a recombinant HCMV (rΔMSVgpt) without a modulator. In comparison to wild-type (WT) virus, rΔMSVgpt exhibits a slight delay in growth in human fibroblasts, but there is no appreciable change in IE1 and IE2 transcription. Moreover, there is no appreciable change in the early/late kinetics of transcription of RNAs colinear with the predicted UL128 coding region, which is adjacent to the modulator, although the size distribution and abundance of these RNAs are altered. In infected undifferentiated THP-1 and Tera-2 cells, WT and rΔMSVgpt viruses produce minimal but comparable amounts of IE1 RNAs. The genomes of both viruses are detectable in similar amounts within these undifferentiated cells. Induction of cellular differentiation before infection overcomes the block in MIE gene transcription. WT and rΔMSVgpt infections of differentiated THP-1 cells produce similar levels of IE1 and IE2 RNAs. Thus, differentiation-dependent control of MIE gene transcription involves regulatory mechanisms other than the modulator. Possible alternative functions of the modulator are discussed.**

Human cytomegalovirus (HCMV) causes serious illness and sometimes death in persons with immature or compromised immune systems. The virus replicates productively in the endothelial cell, fibroblast, epithelial cell, smooth muscle cell, neuron, glial cell, and macrophage (49, 55, 56, 70, 71). Cellular factors governing HCMV replication may partly determine cell tropism. For instance, in certain cell types, e.g., lymphocytes and polymorphonuclear leukocytes (55, 56), only a restricted or abortive lytic HCMV infection occurs. Also, embryonal carcinoma (Tera-2) cells (12, 13, 29, 42), members of the monocyte lineage (25, 27, 30, 34, 39, 54, 57, 64, 67, 69), and neurons (47) are cell types in which an advanced level of cellular differentiation is needed for a fully productive HCMV infection. Hence, the terminally differentiated macrophage permits productive viral replication, while the relatively undifferentiated granulocyte-macrophage progenitor and blood monocyte can support only HCMV latency (9, 25, 27, 30, 34, 39, 63, 64, 67, 69).

Expression of the HCMV major immediate-early (MIE) genes encoding IE1 and IE2 appears to be critical for productive or lytic viral replication (26, 40, 45). The regulated expression of these genes may partly determine cell tropism, since such a relationship exists for murine cytomegalovirus (4). This association is also evident in HCMV-infected Tera-2 (29, 42) and primary or THP-1 monocytic (27, 39, 41, 64, 67, 69) cells, in which MIE gene transcription and virus replication are similarly regulated in a differentiation-dependent manner.

The control of MIE promoter transcriptional activity involves interplay between both positive and negative *cis*-acting regulatory elements (reviewed in references 41, 58, and 61). For instance, the MIE promoter has a complex enhancer containing binding sites for several different cellular factors (NF-κB, CREB/ATF, AP1, retinoic acid receptors, etc.) that are capable of stimulating transcription. Many of these stimulatory factors are reduced in amount or function within undifferentiated or certain resting or unstimulated cells (1, 11, 17, 19, 24, 44, 50). Enhancer activity can also be diminished by negative *cis*-acting elements.

One negative *cis*-acting region is located upstream of the MIE enhancer between base positions −750 and −1140 relative to the transcription start site of the MIE promoter (Fig. 1A). Several published reports indicate that this upstream region represses transcription from MIE promoter segments in transient transfection or in vitro transcription systems but that such repression is dependent on cell type and cellular state of differentiation (23, 33, 43, 51, 59). For example, this region represses transcription in undifferentiated Tera-2 (23, 28, 43, 51) and monocytic (e.g., THP-1) cells (23, 33, 59), but cannot do so after these cells have undergone differentiation or stimulation. Yet in other cell types, such as B lymphocytes and fibroblasts, the same *cis*-acting upstream region was reported to operate differently to stimulate MIE promoter activity (33, 43). Because of this mixed regulatory capacity, the upstream region was designated the modulator (42, 43). Some investigators have proposed that the modulator might contribute to the regulation of cell tropism and/or HCMV latency through its modulation of MIE gene expression (41, 53).

The mechanism(s) by which the modulator regulates the MIE promoter activity in in vitro and transfection studies is not understood. Cellular transcription factor YY1 (10, 21, 46, 52) is implicated as a repressor of MIE promoter activity in undifferentiated Tera-2 and monocytic THP-1 cells but not in differentiated cellular counterparts (28, 32, 54). This cellular factor binds in vitro to at least one site in the modulator and to a

* Corresponding author. Fax: (319) 335-9006. E-mail: CMDMFS@WEEG.VAXA.UIOWA.EDU.

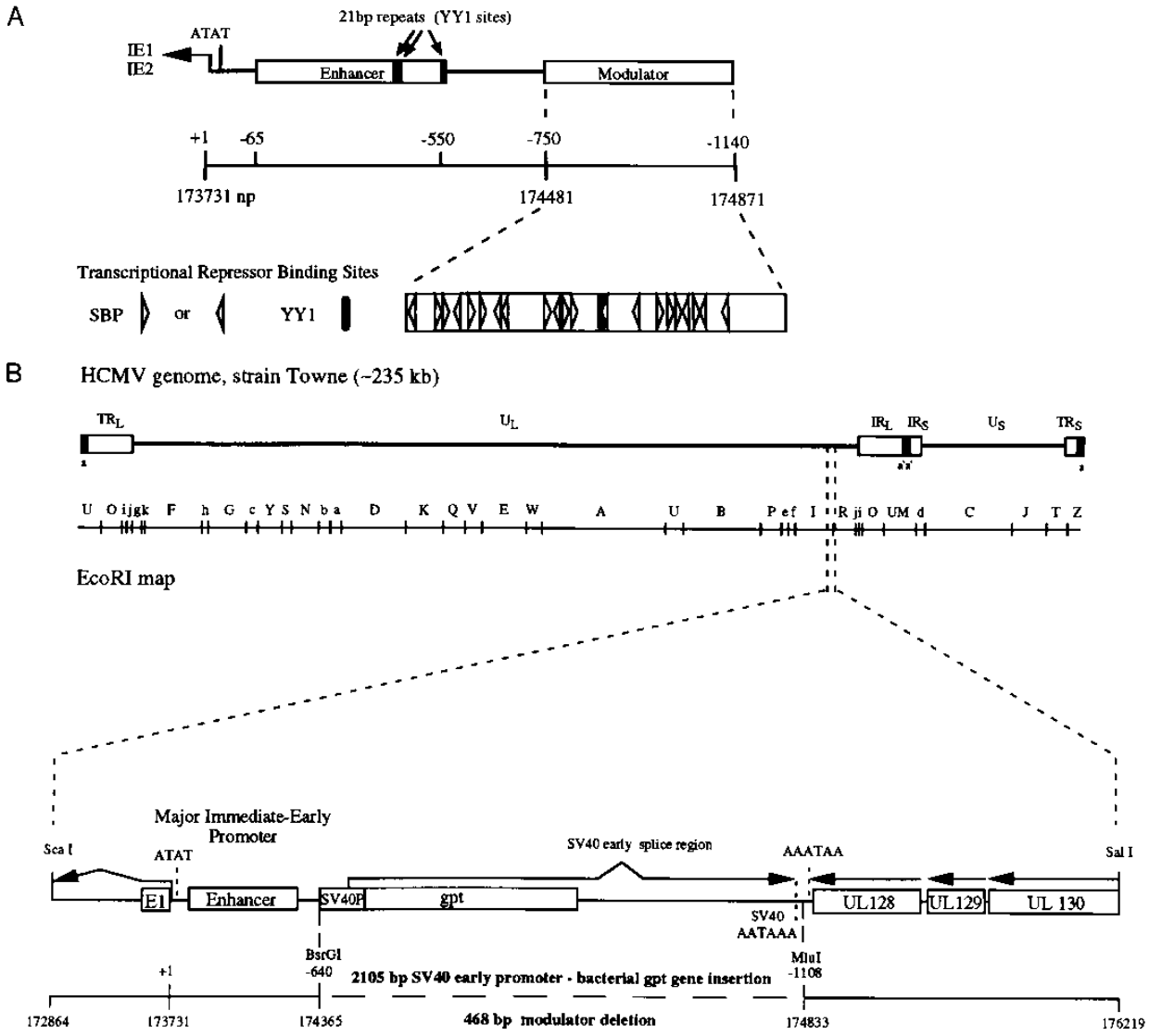



FIG. 1. (A) Schematic diagram of the structural organization of the HCMV MIE promoter. The MIE promoter is composed of proximal promoter (+1/−64), enhancer (boxed, −65/−550), unique region (−501/−749), and modulator (boxed, −750/−1140). Numerical base positions of these components relative to RNA start (horizontal arrow) site, and in reference to nucleotide positions in the genome of the HCMV strain AD169 (6), are depicted. The arrows point to locations of 21-bp repeats (YY1 sites) in the enhancer. The exploded view of the modulator shows positions of consensus recognition motifs of cellular transcriptional repressors, SBP (66) and YY1 (32). IE1 and IE2 represent the predominant MIE gene products. (B) Construction of rΔMSVgpt. A schematic diagram of HCMV genome (strain Towne) and its *Eco*RI map are shown, as well as an exploded view of a portion of unique long ($U_L$) genomic segment (nt 172864 to 176219) which was modified for subsequent use in homologous recombination. The modification consists of a 468-bp modulator deletion (−640/−1108) filled with an SV40 early transcription unit and bacterial *gpt* ORF (2,105-bp insertion). The SV40 early transcription unit is comprised of enhancerless minimal promoter (−138/+57), splice region, and polyadenylation signal (5′-AATAAA-3′). Also depicted are locations of predicted UL128, UL129, and UL130 ORFs (open boxes); exon 1 (open box, E1) of IE1 and IE2; SV40 early promoter (SV40P; open box); directionality of transcription units (solid arrows), which is presumptive for UL128, UL129, and UL130; and the putative polyadenylation signal (3′-AAATAA-5′) of UL128 and/or other upstream ORFs.

site within each of three 21-bp motifs (21-bp repeats) located in the distal one-third of the MIE enhancer (Fig. 1A) (32). Also dispersed throughout the modulator are 21 copies of a TRTCG motif (16 TATCG and 5 TGTCG copies) that is bound in vitro by an uncharacterized cellular protein(s) (23, 66). Protein complex formation on a TATCG-containing sequence coincides with repression of MIE promoter activity in the undifferentiated Tera-2 and THP-1 cells (23). The TRTCG motif possesses silencer activity for the HCMV immediate-early (IE) US3 promoter, as well as in some heterologous promoter constructs (5, 66). In these cases, silencing occurs in diverse cell types and variably involves additional sufficiently spaced TRTCG-like elements, other flanking sequences, or both. While the silencer binding protein(s) (SBP) that binds the TRTCG motif remains uncharacterized, there are *Drosophila* nuclear proteins, BEAF-32A and -32B, that bind to a related palindromic site, $CGATAN_1TATCG$ (72). The BEAF-32 palindrome functions in *Drosophila* chromatin as an insulator to block the transcriptional effects of distal enhancers (72). BEAF-32 binding requires TATCG motifs that need not be configured as a prototypic palindrome (72). Whether the *Drosophila* BEAF paradigm is applicable to modulator function is untested. Last, the mechanism(s) by which the modulator stimulates MIE promoter activity in certain transiently transfected cell types, such as human fibroblasts, is unknown.

In this study, we determined the biological importance of the

modulator in regulating MIE gene transcription in both infected undifferentiated and differentiated cells, using a recombinant HCMV without a modulator. We show that a modulator deletion does not appreciably alter MIE gene transcription in infected human fibroblasts, differentiated THP-1 cells, or undifferentiated Tera-2 and THP-1 cells. These findings indicate that differentiation-dependent control of MIE gene transcription involves regulatory mechanisms other than the modulator. Other potential functions of the modulator are discussed.

## MATERIALS AND METHODS

**Cells and virus.** Primary human foreskin fibroblast (HFF) monolayers were grown in Eagle's minimal essential medium supplemented with 10% newborn bovine serum (Sigma, St. Louis, Mo.). THP-1 (American Type Culture Collection, Rockville, Bethesda, Md.) and Tera-2 embryonal carcinoma (American Type Culture Collection) cells were grown in RPMI and McCoy's 5a media, respectively, containing 10% fetal bovine serum (JRH Bio Sciences, Lenexa, Kans.) and 2 mM glutamine. Only low-passage-number THP-1 and Tera-2 cells were used for study. All cell culture media contained streptomycin (100 μg/ml) and penicillin (100 U/ml). Differentiation of THP-1 and Tera-2 cells was achieved with supplementation of growth media with 20 nM phorbol 12-myristate 13-acetate (PMA; Sigma) plus 50 μM hydrocortisone for 1 day and 10 μM retinoic acid (Sigma) for 4 days, respectively.

HFF monolayers were used to propagate HCMV strain Towne (multiplicity of infection [MOI] of $\sim 10^{-6}$ PFU/cell), producing high titers of cell-free virus (60). Recombinant virus was propagated similarly, although it was also grown in the presence of mycophenolic acid (10 μg/ml) and xanthine (50 μg/ml). High-titer recombinant virus stocks were prepared from virus grown for at least one passage in the absence of mycophenolic acid and xanthine. Cell-free virus was passed through a 0.45-μm-pore-size filter. Virus adsorption was carried out for 1 to 2 h.

Single-step viral growth curves (MOI of 1 PFU/cell) were performed on low-passage-number HFF monolayers grown in six-well culture plates. Input virus titer was determined prior to and at the time of inoculation (0 days postinfection [dpi]) by counting viral plaques grown in HFF cells. The addition of human gamma globulin (200 μg/ml) to the growth medium at 1 dpi produced results that were comparable to those obtained by growth of plaques under agarose. At the indicated times postinfection, cells were scraped into 1/5 volume of overlying medium, adjusted with 1:1 volume of newborn bovine serum, and stored at −80°C. In parallel, samples of all time points were thawed, sonicated, centrifuged (1,000 × *g* for 5 min), and titered on HFF cells, using the plaque assay method described above.

**Plasmids.** The derivation of pMSDT-E has been detailed previously (65). pIE1EM and pM contain *Bam*HI-*Sal*I (HCMV AD169 nucleotides [nt] 170970 to 176219 [6]) and *Bgl*II-*Sal*I (nt 171444 to 176219) HCMV (strain Towne) genomic fragments of pMSDT-E, respectively, which were inserted at corresponding sites of pGEM-4Z (Promega, Madison, Wis.); the *Bgl*II end (blunted with T4 polymerase) was joined to the *Sma*I site of pGEM-4Z. To construct pΔMSVgpt, the *Bsr*GI-*Mlu*I segment (nt 174365 to 174833) of pM was replaced with a synthetic duplex oligonucleotide (5′-GTACATCTAGATCTAAGCTTG GATCCA-3′) containing *Bgl*II, *Hin*dIII, and *Bam*HI sites, as well as compatible *Bsr*GI and *Mlu*I ends. A *Bgl*II-*Hin*dIII fragment containing the minimal simian virus 40 (SV40) early promoter (−138/+57) of plasmid pGL-promoter (Promega) and a *Hin*dIII-*Bam*HI fragment of pMSG (Pharmacia, Piscataway, N.J.) which contains the guanine phosphoribosyltransferase (*gpt*) open reading frame (ORF), SV40 early splice region, and SV40 early polyadenylation signal were transferred to corresponding sites in the oligonucleotide insert. The *Sca*I-*Sal*I fragment (nt 172864 to 176219) of this mutated pM vector was subcloned into *Sma*I and *Sal*I sites of pGEM-4Z to yield pΔMSVgpt.

**HCMV recombination.** Recombinant virus rΔMSVgpt was constructed using a modification of the procedures described by Vieira et al. (68) and Greaves et al. (16). Subconfluent HFF cells (75 $cm^2$) were transfected with pΔMSVgpt (10 μg) by the DNA-calcium phosphate coprecipitation method of Graham and van der Eb (15) and 24 h later were infected with HCMV Towne strain (MOI of 1 PFU/cell). At 10 dpi, cells were scraped into 2 ml of growth medium containing 1:1 volume of newborn bovine serum and sonicated, and a 100-μl aliquot was used to infect HFF cells (75 $cm^2$). Mycophenolic acid (10 μg/ml) and xanthine (50 μg/ml) were added to the growth medium to select for rΔMSVgpt virus. Virus was harvested as before at 10 to 14 dpi, and the enrichment cycle was repeated twice. HCMV plaque isolation (MOI of $10^{-5}$ to $10^{-6}$ PFU/cell) was carried out on HFF monolayers grown under 0.5% agarose; mycophenolic acid and xanthine were present at this and all subsequent steps. Plaques of all sizes were harvested and transferred to HFF cells grown in a 48-well tissue culture flask. Once infected wells reached 100% cytopathic effect, the growth medium containing cell-free virus was removed and stored at −80°C. Infected cells were washed with phosphate-buffered saline (PBS), incubated at 50°C for 2 to 3 h in 200 μl of 20 mM Tris-HCl (pH 8.0)–1 mM EDTA–100 mM NaCl (STE) containing 0.05% sodium dodecyl sulfate (SDS), 0.05% Sarkosyl, and 20 μg of proteinase K per ml, phenol-chloroform/chloroform extracted, and DNA precipitated with ethanol-sodium acetate. Lyophilized DNA was resuspended in 50 μl of Tris-EDTA, boiled for 3 min and cooled on ice, the sample volume was evenly split, and samples were subjected in duplicate to dot blot hybridization (Schleicher & Schuell, Keene, N.H.) according to the manufacturer's instructions. One filter was hybridized to a *gpt*-specific $^{32}$P-labeled probe, and the other was hybridized to a modulator-specific probe. Probe preparation, hybridization, and wash conditions are described below. Several positive plaques (recombinant-to-wild-type ratio, 1:20) were identified. Of these plaques, two individual rΔMSVgpt isolates (r1 and r2) were identified, amplified, and subjected to two additional rounds of plaque isolation.

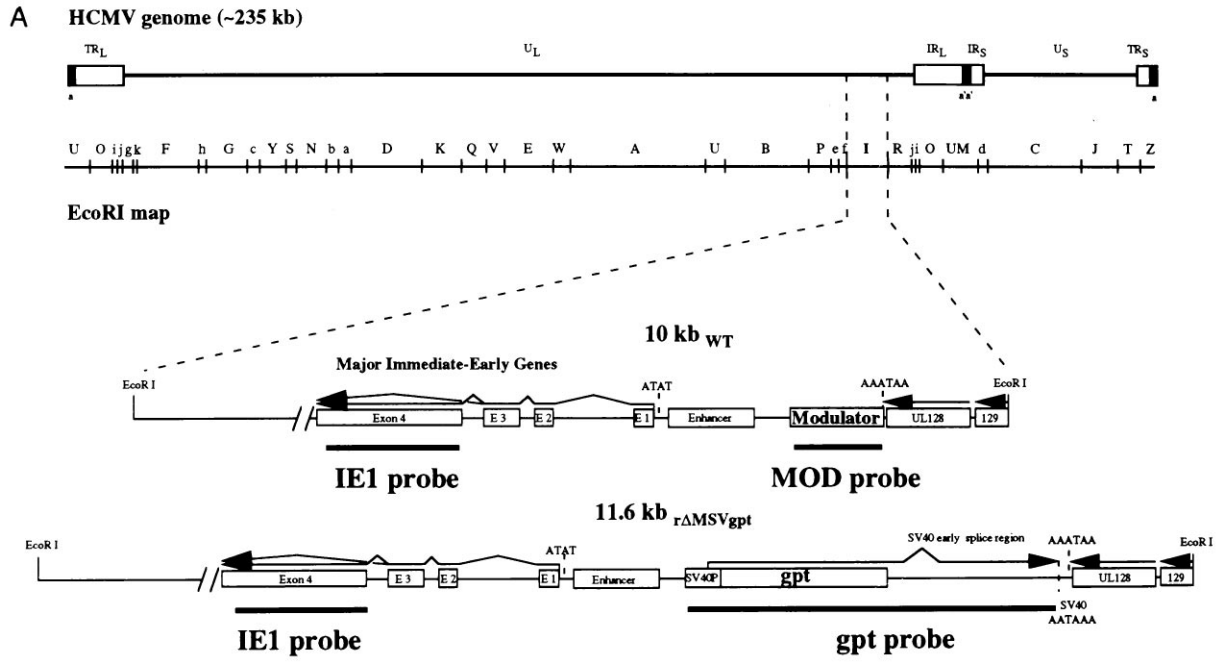
**RNA analysis.** Whole-cell RNA from uninfected and HCMV-infected HFF, THP-1, or Tera-2 cells was isolated by the method of Chomczynski and Sacchi (7). Viral IE, early, and late RNAs used in Northern blot analyses were isolated from infected cells under cycloheximide (CH; 200 μg/ml) treatment for 24 h, under phosphonoacetic acid (200 μg/ml) treatment for 48 h, or with no treatment for 48 h, respectively (31). Northern blot analysis of RNA fractionated on a 6% formaldehyde–1.5% agarose gel was performed as described previously (37) except that the hybridization solution contained 10% dextran sulfate, 1× SSPE (0.18 M NaCl, 10 mM $NaH_2PO_4$, 1 mM EDTA [pH 7.7]), 10% SDS, and 100 μg of sheared salmon sperm DNA per ml. Hybridization was carried out at 68°C for 4 to 12 h; a high-stringency wash was performed at 60 to 63°C in 0.1× SSPE–0.1% SDS. IE1, IE2, and UL128 RNAs were hybridized to *Acc*I-*Acc*I (nt 170953 to 172366), *Xho*I-*Sca*I (nt 170232 to 170683), and *Pst*I-*Xho*I (nt 174872 to 175184) duplex DNA fragments, respectively, which were gel purified and multiprime $^{32}$P labeled (Amersham, Arlington Heights, Ill.). IE1- and UL128-specific fragments were taken from pIE1EM; the IE2-specific fragment was obtained from pSVCS. The US3-specific probe was derived as a *Hin*dIII-*Bam*HI fragment of plasmid pMal-C2 (2a). The actin probe represents a fragment of human actin excised from a pBR322-based plasmid (20) by using *Pst*I. Stripping of probes from blots was achieved by boiling the Nytran in 0.2% SDS.

The RNase protection assay was adapted from the method of Bordonaro et al. (3). Cells were infected in the absence or presence (120 μg/ml) of CH (31), and whole-cell RNA was prepared at the indicated times postinfection. Antisense IE1 (22), IE2 (35), and actin (66) $^{32}$P-labeled riboprobes were generated as described previously. RNA-RNA hybridization was performed at 52°C overnight, and hybrids were digested with 150 U of $T_1$ RNase (Boehringer Mannheim, Indianapolis, Ind.) at 30°C for 1 h. Protected products were analyzed on 6% polyacrylamide-urea gels.

**Genome analysis.** Culture medium containing cell-free HCMV was spun at 1,000 × *g* for 10 min at room temperature. Supernatant virus was centrifuged at 55,000 × *g* for 1 h at 4°C. The virus pellet was resuspended in STE, adjusted to 0.05% SDS–0.05% Sarkosyl–200 μg of proteinase K per ml, and incubated at 50°C for 2 to 3 h. Released viral DNA was subjected to phenol-chloroform/chloroform extraction, ethanol-sodium acetate precipitation, and digestion with *Eco*RI, *Pst*I, or *Bam*HI. DNA fragments were fractionated on a 0.5% agarose gel, stained with ethidium bromide, and then subjected to Southern blot analysis and autoradiography (2). Hybridization, wash, and probe removal conditions were the same as those used for Northern blotting. IE1-, modulator-, and *gpt*-specific probes were generated from multiprime $^{32}$P-labeled *Acc*I-*Acc*I (nt 170953 to 172366), *Bsr*GI-*Mlu*I (nt 174371 to 174836), and *gpt* cassette DNA fragments, respectively. Each fragment was gel purified prior to radiolabeling.

**Quantitative competitive PCR (QC-PCR).** THP-1 or Tera-2 cells were washed three times in serum-free medium at the completion of virus adsorption and then placed in growth medium. Wild-type (WT)-, rΔMSVgpt-, and mock-infected cells ($10^6$) were processed in parallel at the indicated times after infection. Cells were washed in PBS and resuspended in 50 μl of ice-cold buffer A (10 mM HEPES [pH 8.0], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol) (8) containing 0.1% Triton X-100 (27). Pipetting up and down several times ensured thorough cell lysis as monitored by light microscopy. Pelleted nuclei were resuspended in 25 μl of PCR lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.001% Triton X-100, 0.0001% SDS) containing 20 μg of proteinase K per ml and incubated at 55°C for 100 min. Proteinase K was inactivated by incubation at 95°C for 20 min. Each sample was divided into 5-μl portions and analyzed in the absence or presence of the indicated amounts of a 1,765-bp fragment of IE1 cDNA. PCR amplification of IE1 DNA was performed with primers IEP3C and IE4BII, described by Kondo et al. (27). The predicted sizes of the unspliced and spliced PCR products are 387 and 217 bp, respectively. PCR amplification was done for 35 cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 2 min.

**Immunofluorescence.** Uninfected and HCMV-infected cells ($10^5$), which had either been grown on glass coverslips or centrifuged onto glass slides, were fixed in methanol for 5 min and soaked 5 min in 0.3% Triton X-100 in PBS (9). Undifferentiated Tera-2 cells were subjected to study at passages 2 to 3, since the proportion of infected cells expressing IE1 or IE2 increased with greater number of passages. THP-1 cells were used at passages 5 to 10. Cells were blocked with 20% goat serum in PBS at room temperature for 15 min. Anti-IE1/IE2 murine monoclonal antibody MAB810 (1:100 dilution; Chemicon, Temucula, Calif.) was added, and the mixture was incubated at room temperature for 60 min. Cells were thoroughly rinsed with PBS and incubated for 1 h at room temperature with fluorescein isothiocyanate-conjugated goat anti-murine $F(ab')_2$ (1:1,000 dilution in PBS; DAKO, Carpinteria, Calif.). After a thorough PBS rinse, fluorescent cells were imaged and photomicrographs were obtained with an Olympus BH-2 fluorescence microscope. Antibody MAB810 recognizes a peptide sequence in



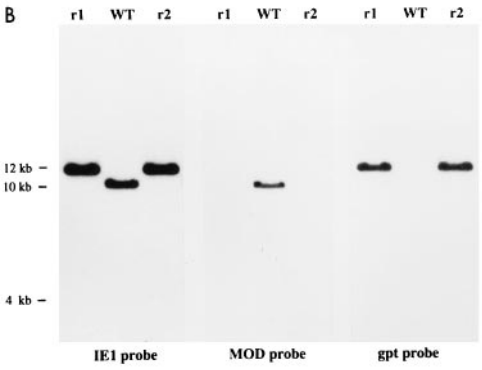




FIG. 2. Structural analysis of rΔMSVgpt. (A) Schematic diagram of the HCMV (strain Towne) genome and its *Eco*RI map, as well as an exploded view contrasting *Eco*RI I fragments of WT and rΔMSVgpt viruses and indicating locations in exon 4 of IE1, modulator, and SV40-*gpt* cassettes targeted by IE1, MOD, and *gpt* probes (thick bars), respectively. (B) Genomic DNAs of WT virus and rΔMSVgpt isolates r1 and r2 were subjected to *Eco*RI digestion, agarose gel fractionation, and Southern blot analysis with a series of $^{32}$P-labeled probes as described in Materials and Methods. The same Southern blot was serially hybridized to IE1, MOD, and *gpt* probes as indicated (see Materials and Methods). Positions of accompanying DNA size standards are given.

exon 2 of both IE1 and IE2 proteins (36). IE1/IE2 antigen was serially examined at 1, 2, 3, 5, 7, 9, 11, and 13 dpi in undifferentiated THP-1 cells and at 0.2, 1, 5, and 7 dpi in undifferentiated Tera-2 cells. HCMV-infected HFF cells (MOI of 1) at 4 h postinfection (hpi) served as a strong positive control, whereas mock-infected cells provided a negative control, which were analyzed in parallel with study specimens.

## RESULTS

**Construction of an HCMV lacking the modulator.** To ascertain the modulator's function in regulating MIE gene expression in HCMV-infected undifferentiated and differentiated cells, we removed the modulator from HCMV strain Towne by using a homologous recombination strategy described previously (16, 68). As shown schematically in Fig. 1, the recombinant virus rΔMSVgpt was constructed by replacement of the modulator (468-bp deletion) with an SV40 early kinetic class transcription unit containing the bacterial *gpt* ORF (2,105-bp insertion). This allowed for dominant selection of rΔMSVgpt growth in the presence of mycophenolic acid and xanthine. Other structural features of rΔMSVgpt include preservation of the putative polyadenylation signal of the UL128 transcription unit, which lies adjacent to the modulator, and use of the minimal SV40 early promoter (−138/+57) that lacks its enhancer (72-bp repeats). This SV40 promoter segment has negligible basal transcriptional activity (14, 18) and has no negative *cis*-acting elements.

Two rΔMSVgpt isolates, r1 and r2, were randomly chosen for purification and study. Comparison of WT and rΔMSVgpt (isolates r1 and r2) genomes by *Eco*RI, *Pst*I, or *Bam*HI endonuclease digestion revealed only the expected restriction fragment length polymorphisms (RFLP), even after several passages of the virus in the absence of selective pressure (data not shown). Southern blot analyses with a series of $^{32}$P-labeled probes confirmed the intended structural change, as exemplified in the analysis of the *Eco*RI RFLP (Fig. 2). This mutation was stable since probes corresponding to either exon 4 of IE1 or the *gpt* gene detected only the expected 11.6-kbp *Eco*RI I genomic fragment. The failure of $^{32}$P-labeled pGEM-4Z to hybridize to rΔMSVgpt DNA indicated that vector backbone was not incorporated into the genome during recombination (data not shown).

The growth of both r1 and r2 isolates was assessed in HFF cells. Recombinant virus stocks were prepared in the absence of mycophenolic acid so as to obtain a high virus titer and to eliminate any potential effect of carryover drug. Single-step growth curves (MOI of 1 PFU/cell) performed on newly confluent HFF monolayers are shown in Fig. 3. They reveal that rΔMSVgpt and WT viruses have similar growth kinetics, although both r1 and r2 produce slightly lower (1 log or less) titers of virus at initial times of infection (2 and 3 dpi). The modest delay in rΔMSVgpt growth was also evident in growth curves generated by using subconfluent HFF cells of another

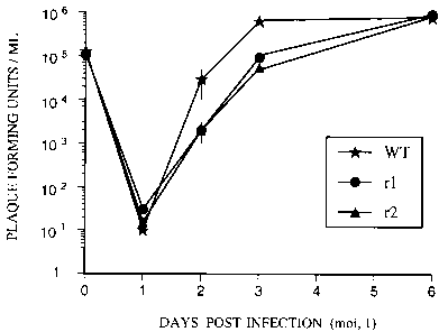



FIG. 3. Single-step growth curves of WT and rΔMSVgpt infections of HFF cells. HFF cells were infected in parallel with WT and rΔMSVgpt (isolates r1 and r2) viruses at an MOI of 1 PFU/cell (MOI, 1). On the indicated days postinfection, virus was harvested and PFU/milliliter was determined as detailed in Materials and Methods. Virus titer at day 0 is that of input inoculum. Each growth curve represents the average of two independent experiments; error bars represent the range but are too small to be seen at all time points.

source or a higher MOI (5 to 10 PFU/cell) (data not shown). The plaque size of rΔMSVgpt was generally smaller than that of WT virus (data not shown).

**MIE gene transcription in infected HFF cells.** We examined both temporal regulation and amounts of MIE RNAs in rΔMSVgpt-infected HFF cells. HFF monolayers were infected (MOI of 1 PFU/cell) in parallel with WT and rΔMSVgpt (r2) viruses. Total cellular RNA was isolated at 2, 4, 8, and 12 hpi and subjected to Northern blot analyses using a series of $^{32}$P-radiolabeled probes. As shown in Fig. 4, IE2 RNA was detected at 4 hpi and was reduced in amount at 8 and 12 hpi in both r2 and WT infections. The quantities of IE2 RNA made by the various viruses were similar when we controlled for differences in inoculum, as revealed by analysis of HCMV US3 RNA, which is expressed with kinetics closely matching those of IE2. Likewise, the temporal regulation and amount of IE1 RNA were not appreciably changed by the insertion/deletion mutation. The other rΔMSVgpt isolate, r1, exhibits a similar pattern of IE1 and IE2 transcription (data not shown).

We also determined whether the rΔMSVgpt MIE promoter met criteria for IE transcriptional kinetics. In these studies,

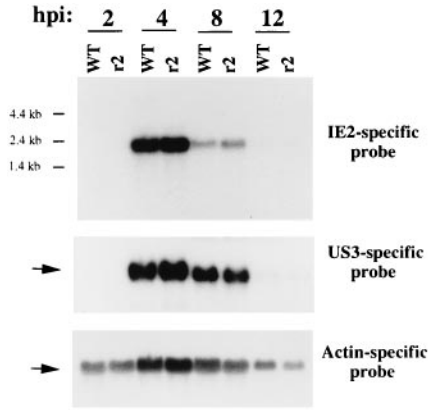



FIG. 4. Time course analysis of MIE RNAs in WT- and rΔMSVgpt-infected HFF cells. In parallel, total cellular RNA was harvested at 2, 4, 8, and 12 hpi of WT- and rΔMSVgpt (r2)-infected HFF cells (MOI of 1) and subjected to Northern blot analyses (see Materials and Methods); 10 μg of RNA was applied to each lane. The same Northern blot was serially hybridized to IE2-, US3-, and actin-specific $^{32}$P-labeled probes as indicated (details in Materials and Methods). Positions of US3 and actin RNAs (denoted by arrows) and sizes of selected RNA markers are shown.

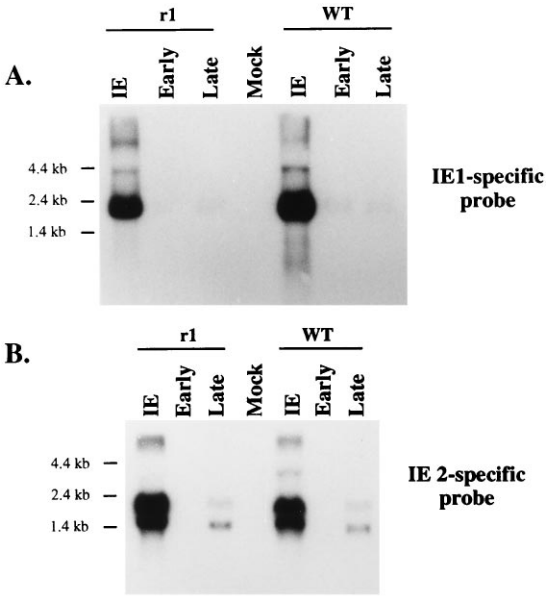



FIG. 5. Analysis of MIE RNAs in the IE, early, and late stages of WT and rΔMSVgpt infections of HFF cells. Viral IE (CH, 24 h), early (phosphonoacetic acid, 48 h), and late (untreated, 48 h) transcripts in total cellular RNA were prepared (Materials and Methods), in parallel, from uninfected (Mock) and WT- and rΔMSVgpt (r1)-infected HFF cells (MOI of 3 to 5 PFU/cell). These RNAs were subjected to Northern blot analyses as indicated above, and the same Northern blot was serially hybridized to IE1-specific (A) and IE2-specific (B) $^{32}$P-labeled probes. RNA integrity was verified by ethidium staining and an actin-specific probe (not shown). Positions and sizes of accompanying RNA markers are given.

HFF cells were infected with rΔMSVgpt (r1) or WT virus under conditions described in Materials and Methods that define viral RNAs as IE, early, or late transcripts. Northern blot analysis of these RNAs with an IE1-specific probe is shown in Fig. 5A. Only a very small amount of IE1 (1.95 kb) RNA was detected in the early and late stages of both r1 and WT infections (lanes Early and Late). As expected, IE1 RNA of both viruses accumulated greatly when protein synthesis was inhibited (lanes IE). Under this same condition, there was also an abundance of IE2 (2.25 and 1.7 kb) RNAs of both viruses, as determined by hybridization with an IE2-specific probe (Fig. 5B, lanes IE). However, these IE2 transcripts were undetectable at early times of infection with either virus (lanes Early). At late times of infection, r1 and WT viruses produced similar amounts of 1.5-kb IE2 RNAs which arise from the viral late promoter, and both viruses expressed low but comparable amounts of the 2.25-kb IE2 RNAs which originate from the MIE promoter (Fig. 5B).

These data suggest that removal of the modulator from the virus produces little to no change in temporal regulation and amount of IE1 and IE2 transcription in infected HFF cells.

**Effect of modulator deletion on adjoining UL128 transcription unit.** To determine whether the modulator regulates the neighboring predicted UL128 ORF (Fig. 1B) (6), we analyzed RNAs produced from this region throughout lytic infection of HFF cells. A Northern blot analysis of RNAs obtained at IE, early, and late times of WT or rΔMSVgpt (r1) infection is shown in Fig. 6A. A probe corresponding to the UL128 ORF detected several transcripts that differ in size. Their expression is dependent on protein synthesis and increases concomitantly with virus replication, although DNA synthesis is not required. This pattern of expression is typical of an early/late kinetic class transcription unit and persists in the modulator's absence.

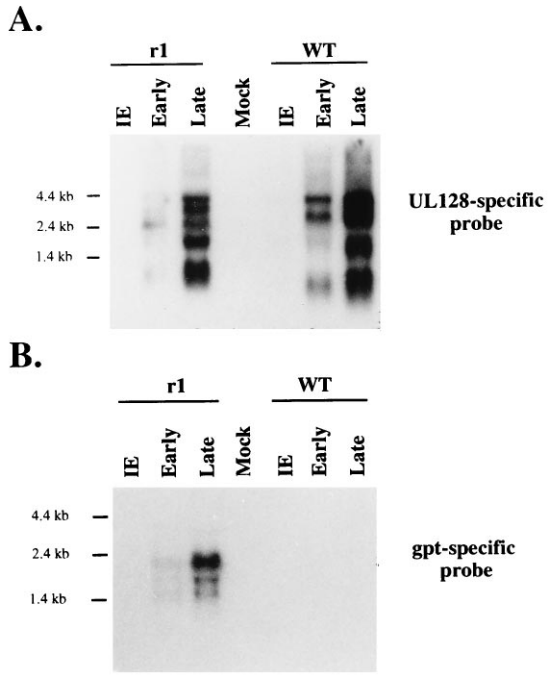



FIG. 6. Analysis of UL128 and SV40-*gpt* RNAs. Total cellular RNA from uninfected (Mock) and WT- and r1-infected HFF cells in the IE, early, or late stage of infection was subjected to Northern blot analysis as described for Fig. 5 except that the blots were hybridized to either a UL128-specific (A) or *gpt*-specific (B) $^{32}$P-labeled probe (Materials and Methods). Autoradiographic exposures were for 3 days. The blot in panel A represents a rehybridization of the blot in Fig. 5. In panel B, the RNAs were of equivalent integrity among lanes as determined by ethidium bromide staining and hybridization with IE1- and UL128-specific probes (not shown). Positions of accompanying RNA size markers are depicted.

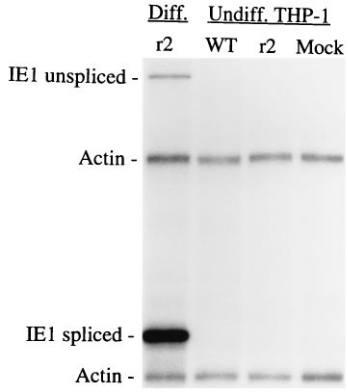



FIG. 7. Dependency on differentiation for IE1 transcription in infected THP-1 cells. Total cellular RNAs (20 μg) isolated from WT-, r2-, and mock-infected (MOI of 5) THP-1 cells at 1 dpi were subjected to RNase protection analysis of unspliced (280 nt) and spliced (145 nt) IE1 RNAs and cellular actin RNA (220 nt) (see Materials and Methods). RNase protection conditions were optimized for IE1 RNA detection, and they protected actin RNA of the expected size but also reproducibly generated a shorter actin RNA by-product. Differentiated THP-1 cells (Diff.) were produced by stimulation of undifferentiated THP-1 cells (Undiff.) with PMA (20 nM) and hydrocortisone (50 μM) for 1 day prior to and during infection. Autoradiographic exposure was for 7 days. Positions of protected unspliced and spliced IE1 and actin RNAs are depicted.

However, the size distribution and abundance of RNAs colinear with UL128 varied somewhat between WT and r1 viruses in the early and late stages of infection. These RNAs were polyadenylated (data not shown), suggesting that the consensus polyadenylation signal located immediately downstream of the UL128 ORF (Fig. 1B) is functional, although 3′ processing or termination of these RNAs may be affected by the nearby insertion/deletion.

We also examined whether the activity of the minimal SV40 early promoter was affected by its insertion 140 bp upstream of the MIE enhancer (base position −640 [Fig. 1]). As shown in Fig. 6B, *gpt* RNA transcribed from the minimal SV40 early promoter of r1 virus was only detectable in early and late stages of infection. The greater amount of *gpt* RNA late in infection likely reflects amplification of viral template. These transcriptional kinetics are comparable to those previously reported for an early SV40 promoter located elsewhere in the HCMV genome but well away (>10 kbp) from IE kinetic class promoters (62). Thus, the SV40 promoter appears to retain early transcriptional kinetics despite being immediately upstream of the MIE enhancer.

**MIE gene transcription in infected undifferentiated and differentiated monocytic THP-1 cells.** Previous studies have determined that infected undifferentiated THP-1 cells are nonpermissive for HCMV replication and rarely (≤0.06% of cells) produce IE1 protein, whereas prior differentiation of THP-1 cells allows both a permissive infection and the expression of IE1 protein (67, 69). The presence of IE1 protein in these infected cells was assessed in situ with an IE1-specific monoclonal antibody. We used a similar approach to determine whether a modulator deletion affects production of either IE1 or IE2 protein in infected THP-1 cells (see Materials and Methods). We detected almost no IE1 or IE2 protein antigen in r2-infected undifferentiated THP-1 cells (MOI of 10), as less than 0.01% of infected low-passage-number cells contained these viral antigens when serially examined by indirect immunofluorescence for 13 dpi (data not shown; see Materials and Methods). This result matched that of a WT infection. Neither r2- nor WT-infected cells produced infectious progeny. In THP-1 cells infected after differentiation with PMA (20 nM) plus hydrocortisone (50 μM), IE1 or IE2 antigen was detected in 5 to 10% of cells at 2 dpi, and infectious progeny was also eventually produced (data not shown). WT and r2 infections did not differ in the proportion of differentiated THP-1 cells containing IE1 or IE2 antigen (data not shown).

To determine whether a modulator deletion affected MIE gene transcription in these infected THP-1 cells, we examined IE1 RNA production by using an RNase protection assay. As shown in Fig. 7, both unspliced and spliced IE1 RNAs are readily detected in infected differentiated THP-1 cells at 1 dpi (MOI of 5). In contrast, these RNAs were not detected in either WT- or r2-infected undifferentiated THP-1 cells. We investigated whether this finding could reflect inefficient viral entry into undifferentiated THP-1 cells. Nuclei of WT- and r2-infected undifferentiated THP-1 cells (MOI of 10) were isolated and subjected to QC-PCR analysis of HCMV genomes as described by Kondo et al. (27). On the basis of results shown in Fig. 8A, we estimated that there is an average of 0.7 to 1.0 viral genome equivalent per nucleus of both WT- and r2-infected cells at 1 dpi. It is possible that not all viral genomes were inside the nucleus since some of them could conceivably be attached to residual cytoskeleton.

Since both WT and r2 viruses penetrate undifferentiated THP-1 cells, we treated these infected cells with CH so as to increase the amount of RNA produced from viral IE promoters. Spliced IE1 RNA was detected in infected undifferentiated THP-1 cells in low relative amounts. Unspliced and spliced IE1 RNAs were found in infected differentiated THP-1 and HFF cells in high relative amounts. IE1 RNA levels did not differ between WT and r2 infections of either undifferentiated

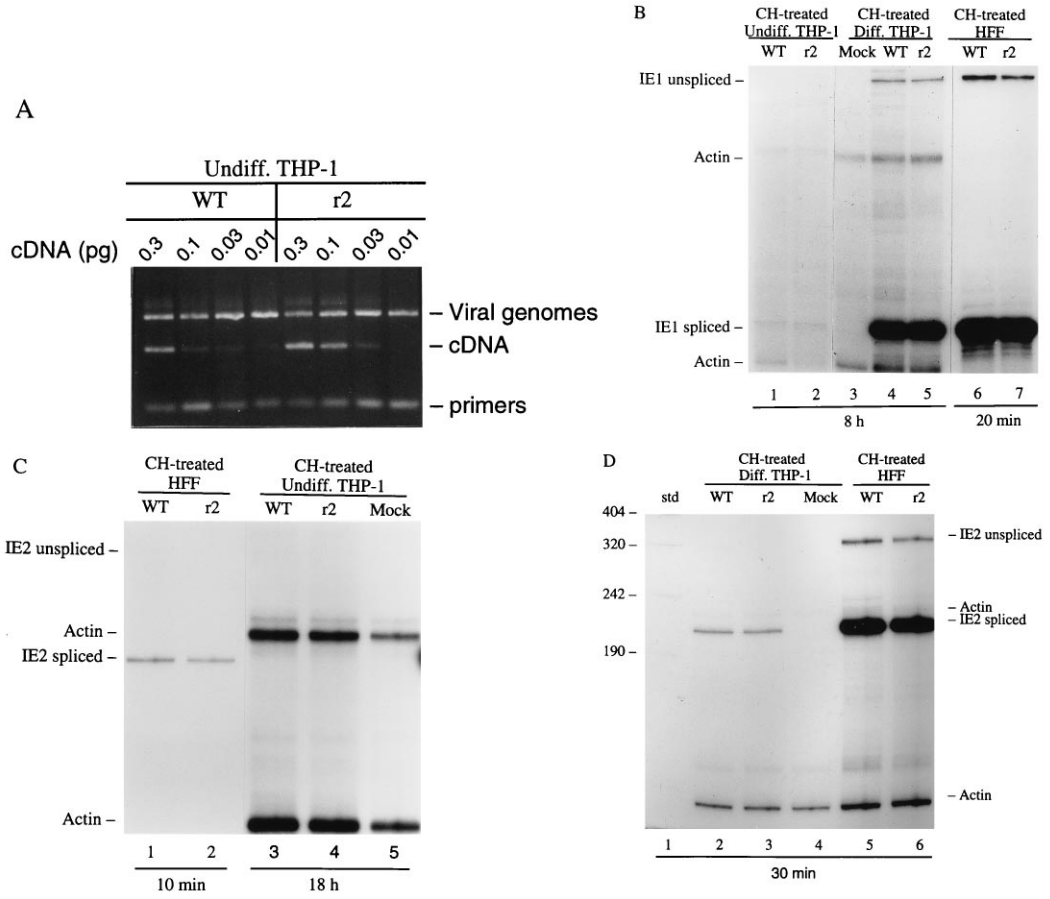



FIG. 8. (A) HCMV entry into undifferentiated THP-1 cells. Nuclei prepared from WT- and r2-infected (MOI of 10) undifferentiated (Undiff.) THP-1 cells at 1 dpi were subjected to QC-PCR that amplifies a region spanning intron 3 of the HCMV IE1 gene (see Materials and Methods); all steps were done in parallel. Amplification of viral genomes and IE1 cDNA generates 387- and 217-bp products, respectively. Each QC-PCR mixture contained $2 \times 10^5$ nuclei and the indicated amount (0.3, 0.1, 0.03, or 0.01 pg) of the 1,765-bp IE1 cDNA. QC-PCR products were fractionated on an agarose gel containing ethidium bromide. This QC-PCR analysis served as a control for WT and r2 MOI in panel C. (B) IE1 transcription in CH-treated undifferentiated and differentiated THP-1 cells. WT and r2 infections (MOI of 10) were carried out for 8 h in CH-treated (120 μg/ml) undifferentiated (Undiff.; lanes 1 and 2) and differentiated (Diff.; lanes 4 and 5) THP-1 cells and HFF cells (lanes 6 and 7). Uninfected (Mock) differentiated THP-1 cells (lane 3) served as a negative control. Total cellular RNA (20 μg) was isolated for RNase protection analysis of unspliced and spliced IE1 and actin RNAs as described for Fig. 7. All steps were done in parallel, and all samples were fractionated on the same denaturing gel. Autoradiographic exposures were for 8 h (lanes 1 to 5) or 20 min (lanes 6 and 7) so as to allow resolution of IE1 RNAs. Positions of protected unspliced and spliced IE1 and actin RNAs are shown. (C) IE2 transcription in CH-treated undifferentiated THP-1 cells. Total cellular RNA (20 μg) was isolated from CH-treated WT- and r2-infected HFF (lanes 1 and 2) and WT-, r2-, and mock-infected (MOI of 10) undifferentiated THP-1 (Undiff.; lanes 3 to 5) cells at 8 hpi and was subjected to RNase protection analysis of unspliced (324 nt) and spliced (210 nt) IE2 and actin (230 and 122 nt) RNAs. As a control for WT and r2 MOI, HCMV genome entry into cells was examined with QC-PCR analysis, and this result is shown in panel A. All steps were done in parallel, and all samples were fractionated on the same denaturing gel. Autoradiographic exposures were for 10 min (lanes 1 and 2) or 18 h (lanes 3 to 5) so as to optimize comparison of IE2 RNAs. Positions of protected unspliced and spliced IE2 and actin RNAs are shown. (D) IE2 transcription in CH-treated differentiated THP-1 cells. WT-, r2-, and mock-infected differentiated THP-1 (Diff., lanes 2 to 4) and WT- and r2-infected HFF (lanes 5 and 6) cells were prepared for RNase protection of IE2 and actin RNAs as described for panel C. Autoradiographic exposure was for 30 min. Positions of protected unspliced and spliced IE2 and actin RNAs are shown. Sizes of nucleotide standards (std; lane 1) are depicted.

THP-1, differentiated THP-1, or HFF cells (Fig. 8B). We did not detect IE2 RNA in WT- or r2-infected undifferentiated THP-1 cells that were CH treated (Fig. 8C). In contrast, IE2 RNAs were detectable in infected CH-treated differentiated THP-1 (Fig. 8D) and HFF (Fig. 8C and D) cells. When normalized for MOI or nucleus-associated viral genomes, WT and r2 infections of either cell type did not differ in the amounts of IE2 RNAs produced.

Our findings suggest that MIE gene transcription in infected monocytic THP-1 cells is dependent on cellular differentiation and that deletion of the modulator does not appreciably alter this dependency.

**MIE gene transcription in infected undifferentiated embryonal Tera-2 cells.** The modulator's negative *cis*-acting effect on MIE promoter activity was demonstrated repeatedly in transfection studies using undifferentiated Tera-2 cells (23, 28, 43, 51). We therefore examined whether a modulator deletion affected MIE gene transcription during infection of these cells. Consistent with previous reports (29, 42), we rarely found WT-infected undifferentiated Tera-2 cells of very low passage number expressing IE1 or IE2 protein antigen, as determined by in situ indirect immunofluorescence analyses conducted throughout a 7-day infection (MOI of 10) (data not shown; see Materials and Methods). However, these antigens were detectable in 80 to 90% of retinoic acid-differentiated Tera-2 cells at 1 dpi (data not shown). The proportion of undifferentiated and differentiated Tera-2 cells containing IE1 or IE2 antigen did not differ between WT and r2 infections (data not shown). To determine if WT and r2 viruses are capable of penetrating undifferentiated Tera-2 cells, we subjected nuclei of infected cells to QC-PCR analysis of viral genomes at 3 dpi. Figure 9A reveals that WT and r2 viruses are equivalent in ability to

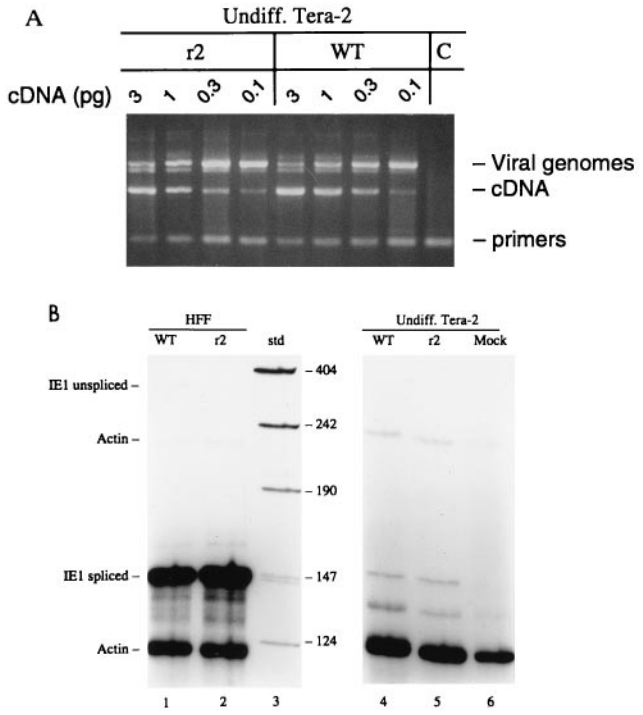



FIG. 9. (A) HCMV entry into undifferentiated Tera-2 cells. Nuclei prepared from WT- and r2-infected (MOI of 10) undifferentiated (Undiff.) Tera-2 cells at 3 dpi were subjected to QC-PCR, using the approach described for Fig. 8A. Each QC-PCR mixture contained $2 \times 10^5$ nuclei and the indicated amount (3.0, 1.0, 0.3, or 0.1 pg) of the 1,765-bp IE1 cDNA. C denotes a control QC-PCR mixture containing $2 \times 10^5$ uninfected nuclei and no IE1 cDNA. All steps were performed in parallel. (B) IE1 transcription in undifferentiated (Undiff.) Tera-2 cells. Total cellular RNA (20 μg) was isolated from WT- and r2-infected HFF (lanes 1 and 2) and WT-, r2-, and mock-infected undifferentiated (Undiff.) Tera-2 (lanes 4 to 6) cells at 8 hpi and subjected to RNase protection of unspliced and spliced IE1 and actin RNAs as described for Fig. 8B. Undifferentiated Tera-2 cells were infected at passage 3. All steps were done in parallel, and all samples were fractionated on the same denaturing gel. Sizes of nucleotide standards (std; lane 3) are depicted. Autoradiographic exposure was for 18 h.

penetrate these cells. We also examined whether some IE1 transcription might take place in infected undifferentiated Tera-2 cells not receiving CH treatment. As shown in Fig. 9B, a small amount of spliced IE1 RNA was detected by RNase protection assay in 20 μg of total cellular RNA isolated at 8 hpi. There was no difference between WT and r2 infections in the relative amounts of IE1 RNAs produced at 8 hpi. In parallel infections of HFF cells, the amounts of IE1 RNAs generated in WT and r2 infections did not appreciably differ at 8 hpi, thus demonstrating equivalence of WT and r2 MOIs in undifferentiated Tera-2 cells.

These data further support the notion that IE1 transcription is repressed in infected undifferentiated Tera-2 cells and indicate that a modulator deletion cannot reduce this repression.

## DISCUSSION

Previous studies addressing modulator function used transfection (23, 28, 43, 51, 59) or in vitro transcription (33) methods in which the modulator's *cis*-acting effects on MIE promoter segments were measured. These studies revealed that the modulator could either repress or stimulate transcription depending on cell type or state of cellular differentiation. The modulator repressed transcription in undifferentiated Tera-2 (5- to 10-fold) (23, 28, 43, 51) and THP-1 (3- to 4-fold) (23, 59) cells but not in differentiated cellular counterparts. This repression decreased successively as sizes of 5′ modulator truncation deletions increased (23). In contrast, the modulator stimulated transcription three- to fivefold in HFF cells (33, 43). Whether these findings reflected biologically important regulatory processes awaited the generation of a genetically engineered virus.

We describe here a recombinant HCMV (rΔMSVgpt) without a modulator of the MIE promoter and provide an analysis of MIE gene expression in infected HFF, THP-1, and Tera-2 cells. Three considerations support the validity of using rΔMSVgpt to explore modulator function. First, a thorough RFLP analysis of the rΔMSVgpt genome reveals only the expected deletion/insertion mutation, which is not deleterious to genome stability yet is sufficient to eliminate the modulator. Second, the random choice of two rΔMSVgpt isolates for study partly controls for unwanted, inapparent genomic mutations that could change the viral phenotype. Third, important steps are taken to diminish potential confounding variables. They include deletion of the modulator while preserving the putative UL128 polyadenylation signal and insertion of a selectable *gpt* gene in a manner which should not perturb MIE promoter function. The enhancerless minimal early SV40 promoter was chosen for expression of a bacterial *gpt* ORF because it has negligible basal transcriptional activity (14, 18) and is devoid of known negative *cis*-acting elements, including consensus binding sites for YY1 or SBP. Therefore, such an insertion ought not substitute functionally for the modulator.

In HFF cells, both rΔMSVgpt isolates grow nearly as well as WT virus (Fig. 3), but a slight delay in their growth is consistently evident from growth curves (MOI of 1 or 10) and plaque size. Whether the modest growth difference is a direct consequence of the modulator's absence or a result of an alteration in another determinant(s) of virus growth is unclear. This difference is apparently not the result of defective regulation of the MIE genes, as both temporal regulation and amounts of IE1 and IE2 RNAs are comparable to those of WT virus when normalized for MOI, genome input, or expression of another IE RNA (US3) (Fig. 4, 5, 8B to D, and 9B). We do not dismiss the possibility that a difference in MIE gene transcription could be demonstrable under other circumstances, such as a very low MOI, dissimilar fibroblast growth conditions, or different assay methods.

A modulator deletion does not increase MIE gene transcription in infected undifferentiated THP-1 (Fig. 8B and C) and Tera-2 (Fig. 9B) cells. WT and rΔMSVgpt viruses are equally capable of penetrating these cells and in localizing to nuclei preparations (Fig. 8A and 9A). These two viruses produce only minimal but equivalent relative amounts of IE1 RNAs in such a setting, supporting the notion that IE1 transcription is markedly repressed in both WT and rΔMSVgpt infections. Our finding of IE1 protein being detectable in situ in only a rare cell (e.g., <0.01% of the total population) among an infected population of IE1-nonexpressing undifferentiated cells is consistent with findings of other investigators (29, 42, 67, 69). These results suggest that such exceptional cells are unlikely to contribute appreciably to the IE1 RNA detectable from the total cell population. Nonetheless, we cannot completely discount the possibility that a modulator deletion could alter IE1 transcription in a subpopulation of undifferentiated cells. Both IE1 and IE2 RNAs are more abundant in infected differentiated THP-1 (Fig. 7, 8B, and 8D) and Tera-2 (data not shown) cells, and their amounts do not appear to differ among WT and rΔMSVgpt infections.

The modulator could be playing an accessory role in regulating MIE promoter activity. For instance, transcription from a MIE promoter lacking a modulator may be adequately re-

pressed in infected undifferentiated cells owing to a redundancy in negative *cis*-acting elements located elsewhere in the MIE promoter, such as the 21-bp repeats. Redundancy of regulatory elements is believed to explain discordance between in vivo and in vitro findings with regard to the regulation of some other promoters, e.g., regulation of the adenovirus major late promoter (48). Further studies are required to determine whether the modulator serves an accessory function in repressing MIE gene expression in infected undifferentiated THP-1 and Tera-2 cells.

The modulator's function could possibly be something other than for regulation of MIE promoter activity. For example, a 393-bp hypothetical UL127 ORF extends the length of the modulator but in a direction opposite that of the neighboring MIE genes (6). The amino acid structure of UL127 is totally conserved among HCMV Towne and AD169 isolates, is not found in other herpesviruses, and lacks any substantive homology to proteins entered in the National Center for Biotechnology Information database (38). Remarkably, a TATATAA motif matching the sequence of the TATA box used by the opposing MIE promoter is positioned upstream of the UL127 ORF. Whether UL127 is expressed remains to be determined.

The modulator's *cis*-acting elements could possibly have a role in regulating UL127 or adjoining hypothetical ORFs, like UL128. This prospect is intriguing since the modulator contains 16 copies of a TATCG motif, which insulates *Drosophila* genes from the effects of distal enhancers when configured as $CGATAN_1TATCG$, although other configurations are possibly functional (72). In light of this consideration, it is of interest that the SV40 promoter retains early transcriptional kinetics (Fig. 6B) despite proximity to the MIE enhancer and ablation of the modulator, which removes all TATCG motifs. Also, the early/late transcriptional kinetics of UL128 appear to be unaltered by a modulator deletion in infected HFF cells (Fig. 6A). Other *cis*-acting mechanisms may protect these upstream genes from the effects of the MIE enhancer.

Some change in size distribution and/or abundance of UL128 transcripts is appreciated in rΔMSVgpt-infected HFF cells and may reflect a change in 3′ processing or termination of these RNAs since the insertion/deletion lies 15 bp downstream of the consensus polyadenylation signal. Alternatively, the mutation could be disrupting transcription or processing of ORFs that may overlap the modulator. Whether a modulator deletion alters the amount or function of products of UL128 or other overlapping ORFs cannot be ascertained because they remain uncharacterized. Thus, it is unknown if a potential abnormality of this kind could result in a delay of rΔMSVgpt growth in fibroblasts. Last, the modulator has features reminiscent of origins of replication, i.e., the possession of palindromic structure, many potential binding sites for cellular transcription factors, and an enrichment in A/T bases, inviting speculation of its candidacy as an episome origin of replication in viral latency.

Although the modulator's function remains unclear, our findings add to an understanding of the biological role of this complicated region of the virus. These findings also reveal that regulatory mechanisms other than the modulator play an important role in the differentiation-dependent control of MIE gene transcription.

## ACKNOWLEDGMENTS

We thank members of the laboratory for helpful discussions of this work and Philip Lashmit for his assistance in preparation of the manuscript. We are grateful to Marty Stoltzfus for critical reading of the manuscript.

This work was supported by grants AI-13562 and HL37121 from the National Institutes of Health. J.L.M. is a recipient of the Burroughs Wellcome Young Investigator Award of the Infectious Disease Society.

## REFERENCES

1. **Angulo, A., C. Suto, M. F. Boehm, R. A. Heyman, and P. Ghazal.** 1995. Retinoid activation of retinoic acid receptors but not of retinoid X receptors promotes cellular differentiation and replication of human cytomegalovirus in embryonal cells. J. Virol. **69:**3831–3837.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology, vol. 1, p. 2.9.1–2.9.5. John Wiley & Sons, New York, N.Y.

2a. **Bissell, J. E., and M. F. Stinski.** Unpublished data.

3. **Bordonaro, M., C. F. Saccomanno, and J. L. Nordstrom.** 1994. An improved T1/A ribonuclease protection assay. BioTechniques **16:**428–430.
4. **Cavanaugh, V., R. Stenberg, T. Staley, H. Virgin, M. MacDonald, S. Paetzold, H. Farrell, W. Rawlinson, and A. Campbell.** 1996. Murine cytomegalovirus with a deletion of genes spanning *Hin*dIII-J and -I displays altered cell and tissue tropism. J. Virol. **70:**1365–1374.
5. **Chan, Y., W. Tseng, and G. Hayward.** 1996. Two distinct upstream regulatory domains containing multicopy cellular transcription factor binding sites provide basal repression and inducible enhancer characteristics to the immediate-early IES (US3) promoter from human cytomegalovirus. J. Virol. **70:**5312–5328.
6. **Chee, M. S., S. Bankier, S. Beck, R. Bohni, C. R. Brown, T. Horsnell, C. A. Hutchisno III, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154:**125–169.
7. **Chomcynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
8. **Dignam, J., R. Lebovitz, and R. Roeder.** 1983. Accurate transcription by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
9. **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.
10. **Flanagan, J. R., K. G. Becker, D. L. Ennist, S. L. Gleason, P. H. Driggers, B.-Z. Levi, E. Apella, and K. Ozato.** 1992. Cloning of a negative transcription factor that binds to the upstream conserved region of Moloney murine leukemia virus. Mol. Cell. Biol. **12:**38–42.
11. **Ghazal, P., C. DeMattei, E. Giulietti, S. A. Kliewer, K. Umesono, and R. M. Evans.** 1992. Retinoic acid receptors initiate induction of the cytomegalovirus enhancer in embryonal cells. Proc. Natl. Acad. Sci. USA **89:**7630–7634.
12. **Gonczol, E., P. W. Andrews, and S. A. Plotkin.** 1985. Cytomegalovirus infection of human teratocarcinoma cells. J. Gen. Virol. **66:**509–515.
13. **Gonczol, E., P. W. Andrews, and S. A. Plotkin.** 1984. Cytomegalovirus replicates in differentiated but not in undifferentiated human embryonal carcinoma cells. Science **224:**159–161.
14. **Gorman, M. C., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
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., J. M. Brown, J. Vieira, and E. S. Mocarski.** 1995. Selectable insertion and deletion mutagenesis of the human cytomegalovirus genome using the E. coli guanosine phosphoribosyl transferase (gpt) gene. J. Gen. Virol. **76:**2151–2160.
17. **Griffin, G. E., K. Leung, T. M. Folks, S. Kunkel, and G. J. Nabel.** 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-kappa B. Nature (London) **339:**70–73.
18. **Gruss, P., R. Dhar, and G. Khoury.** 1981. Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. USA **78:**943–947.
19. **Gupta, S., D. Campbell, B. Derijard, and R. J. Davis.** 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science **267:**389–393.
20. **Hanukoglu, I., N. Tanese, and E. Fuchs.** 1983. Complementary DNA sequence of a human cytoplasmic actin interspecies divergence of 3′ noncoding regions. J. Mol. Biol. **163:**673–678.
21. **Hariharan, N., D. E. Kelly, and R. P. Perry.** 1991. δ, a transcription factor that binds to downstream elements in several polymerase II promoters, is a functionally versatile zinc finger protein. Proc. Natl. Acad. Sci. USA **88:**9799–9803.
22. **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.
23. **Huang, T. H., T. Oka, T. Asai, T. Okada, B. W. Merrills, R. H. Gerston, R. H. Witson, and K. Itakura.** 1996. Repression by a differentiation-specific factor of the human cytomegalovirus enhancer. Nucleic Acids Res. **24:**1695–1701.
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. **Iskenderian, A. C., L. Huang, A. Reilly, R. M. Stenberg, and D. G. Anders.** 1996. Four of eleven loci required for transient complementation of human cytomegalovirus DNA replication cooperate to activate expression of replication genes. J. Virol. **70:**383–392.
27. **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.
28. **Kothari, S., J. Baillie, J. G. Sissons, and J. H. Sinclair.** 1991. The 21bp repeat element of the human cytomegalovirus major immediate early enhancer is a negative regulator of gene expression in undifferentiated cells. Nucleic Acids Res. **19:**1767–1771.
29. **LaFemina, R., and G. S. Hayward.** 1986. Constitutive and retinoic acid-inducible expression of cytomegalovirus immediate-early genes in human teratocarcinoma cells. J. Virol. **58:**434–440.
30. **Lathey, J. L., and S. A. Spector.** 1991. Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages. J. Virol. **65:**6371–6375.
31. **Leatham, M. P., P. R. Witte, and M. F. Stinski.** 1991. Alternate promoter selection within a human cytomegalovirus immediate-early and early transcription unit (UL119-115) defines true late transcripts containing open reading frames for putative viral glycoproteins. J. Virol. **65:**6144–6153.
32. **Liu, R., J. Baillie, J. G. Sissons, and J. H. Sinclair.** 1994. The transcription factor YY1 binds to negative regulatory elements in the human cytomegalovirus major immediate early enhancer/promoter and mediates repression in non-permissive cells. Nucleic Acids Res. **22:**2453–2459.
33. **Lubon, H., P. Ghazal, L. Hennighausen, C. Reynolds-Kohler, C. Lockshin, and J. Nelson.** 1989. Cell-specific activity of the modulator region in the human cytomegalovirus major immediate-early gene. Mol. Cell. Biol. **9:**1342–1345.
34. **Maciejewski, J. P., E. E. Bruening, R. E. Donahue, E. S. Mocarski, N. S. Young, and J. S. St. Jeor.** 1992. Infection of hematopoietic progenitor cells by human cytomegalovirus. Blood **80:**170–178.
35. **Malone, C. L., D. H. Vesole, and M. F. Stinski.** 1990. Transactivation of a human cytomegalovirus early promoter by gene products from the immediate-early gene IE2 and augmentation by IE1: mutational analysis of the viral proteins. J. Virol. **64:**1498–1505.
36. **Mazeron, M. C., G. Jahn, and B. Plachter.** 1992. Monoclonal antibody E-13 (McAb 810) to human cytomegalovirus recognizes an antigen encoded by exon 2 of the major immediate-early gene. J. Gen. Virol. **73:**2699–2703.
37. **Meier, J. L., R. P. Holman, K. D. Croen, J. E. Smialek, and S. E. Straus.** 1993. Varicella-zoster transcription in human trigeminal ganglia. Virology **193:**193–200.
38. **Meier, J. L., and M. F. Stinski.** Unpublished data.
39. **Minton, E. J., C. Tysoe, J. H. Sinclair, and J. G. Sissons.** 1994. Human cytomegalovirus infection of the monocyte/macrophage lineage in bone marrow. J. Virol. **68:**4017–4021.
40. **Mocarski, E. S., G. Kemble, J. Lyle, and R. F. Greaves.** 1996. A deletion mutant in the human cytomegalovirus gene encoding IE1 491aa is replication defective due to a failure in autoregulation. Proc. Natl. Acad. Sci. USA **93:**11321–11326.
41. **Nelson, J., K. Fish, C. Ibanez, A. Depto, P. Ghazal, A. Moses, and R. Jupp.** 1993. Dependence of cytomegalovirus replication on monocyte differentiation, p. 77–86. *In* S. Michelson and S. A. Plotkin (ed.), Multidisciplinary approach to understanding cytomegalovirus disease. Elsevier Science, New York, N.Y.
42. **Nelson, J. A., and M. Groudine.** 1986. Transcriptional regulation of the human cytomegalovirus major immediate-early gene is associated with induction of DNase I-hypersensitive sites. Mol. Cell. Biol. **6:**452–461.
43. **Nelson, J. A., C. Reynolds-Kohler, and B. Smith.** 1987. Negative and positive regulation by a short segment in the 5′-flanking region of the human cytomegalovirus major immediate-early gene. Mol. Cell. Biol. **7:**4125–4129.
44. **Pankov, R., N. Neznanov, A. Umezawa, and R. G. Oshima.** 1994. AP-1, ETS, and transcriptional silencers regulate retinoic acid-dependent induction of keratin 18 in embryonic cells. Mol. Cell. Biol. **14:**7744–7757.
45. **Pari, G. S., and D. G. Anders.** 1993. Eleven loci encoding *trans*-acting factors are required for transient complementation of human cytomegalovirus ori-Lyt-dependent DNA replication. J. Virol. **67:**6979–6988.
46. **Park, K., and M. L. Atchison.** 1991. Isolation of a candidate repressor/activator. NF-E1 (YY1, δ), that binds to the immunoglobulin κ 3′ enhancer and the immunoglobulin heavy-chain μE1 site. Proc. Natl. Acad. Sci. USA **88:**9804–9808.
47. **Poland, S. D., L. L. Bambrick, G. A. Dekaban, and G. P. A. Rice.** 1994. The extent of human cytomegalovirus replication in primary neurons is dependent on host cell differentiation. J. Infect. Dis. **170:**1267–1271.
48. **Reach, M., L. E. Babiss, and C. S. H. Young.** 1990. The upstream factor-binding site is not essential for activation of transcription from the adenovirus major late promoter. J. Virol. **64:**5851–5860.
49. **Schmidbauer, M., H. Budka, W. Ulrich, and P. Ambros.** 1989. Cytomegalovirus (CMV) disease of the brain in AIDS and connatal infection: a comparative study by histology, immunocytochemistry, and in situ DNA hybridization. Acta Neuropathol. (Berlin) **79:**286–293.
50. **Shattock, R. J., J. S. Frieland, and G. E. Griffin.** 1993. Release of human immunodeficiency virus by the THP-1 cells and human macrophages is regulated by cellular adherence and activation. J. Virol. **67:**3569–3575.
51. **Shelbourn, S. L., S. K. Kothari, J. G. P. Sissons, and J. H. Sinclair.** 1989. Repression of human cytomegalovirus gene expression associated with a novel immediate early regulatory region binding factor. Nucleic Acids Res. **17:**9165–9171.
52. **Shi, Y., E. Seto, L.-S. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GLI-kruppel-related protein, and relief of repression by adenovirus E1A protein. Cell **67:**377–388.
53. **Sinclair, J., and P. J. G. Sissons.** 1994. Human cytomegalovirus: pathogenesis and models of latency, p. 249–258. *In* E. K. Wagner (ed.), Seminars in virology. Herpesvirus latency, vol. 5. Academic Press, London, United Kingdom.
54. **Sinclair, J. H., J. Baillie, L. A. Bryant, J. A. Taylor-Wiedeman, and J. G. Sissons.** 1992. Repression of human cytomegalovirus major immediate early gene expression in a monocytic cell line. J. Gen. Virol. **73:**433–435.
55. **Sinzger, C., A. Grefte, B. Plachter, A. S. H. Gouw, T. Hauw 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.
56. **Sinzger, C., B. Plachter, A. Grefte, T. Houwe The, and G. Jahn.** 1996. Tissue macrophages are infected by human cytomegalovirus. J. Infect. Dis. **173:**240–245.
57. **Soderberg, C., S. Larsson, S. Bergstedt-Lindqvist, and E. Moller.** 1993. Definition of a subset of human peripheral blood mononuclear cells that are permissive to human cytomegalovirus infection. J. Virol. **67:**3166–3175.
58. **Stamminger, T., and B. Fleckenstein.** 1990. Immediate-early transcription regulation of human cytomegalovirus. Curr. Top. Microbiol. Immunol. **54:**3–19.
59. **Stein, J., H. Volk, C. Liebenthal, D. H. Kruger, and S. Prosch.** 1993. Tumour necrosis factor α stimulates the activity of the human cytomegalovirus major immediate early enhancer/promoter in immature monocytic cells. J. Gen. Virol. **74:**2333–2338.
60. **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.
61. **Stinski, M. F., M. P. Macias, C. L. Malone, A. R. Thrower, and L. Huang.** 1993. Regulation of transcription from the cytomegalovirus immediate early promoter by cellular and viral proteins, p. 3–12. *In* S. Michelson and S. A. Plotkin (ed.), Multidisplinary approach to understanding cytomegalovirus disease. Elsevier Science, New York, N.Y.
62. **Takekoshi, M., F. Maeda-Takekoshi, S. Ihara, S. Sakuma, and Y. Watanabe.** 1991. Site-specific stable insertion in the human cytomegalovirus genome of a foreign gene under control of the SV40 promoter. Gene **101:**209–213.
63. **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.
64. **Taylor-Wiedeman, J. A., J. G. P. Sissons, and J. H. Sinclair.** 1994. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. J. Virol. **68:**1597–1604.
65. **Thomsen, D. R., and M. F. Stinski.** 1981. Cloning of the human cytomegalovirus genome as XbaI fragments. Gene **16:**207–216.
66. **Thrower, A. R., G. C. Bullock, J. E. Bissell, and M. F. Stinski.** 1996. Regulation of a human cytomegalovirus immediate early gene (US3) by a silencer/enhancer combination. J. Virol. **70:**91–100.
67. **Turtinen, L. W., and B. J. Seufzer.** 1994. Selective permissiveness of TPA differentiated THP-1 myelomonocytic cells for human cytomegalovirus AD169 and Towne. Microb. Pathog. **16:**373–378.
68. **Vieira, J., H. E. Farrell, W. D. Rawlinson, and E. S. Mocarski.** 1994. Genes in the *Hin*dIII J fragment of the murine cytomegalovirus genome are dispensable for growth in cultured cells: insertion mutagenesis with a *lacZ/gpt* cassette. J. Virol. **68:**4837–4846.
69. **Weinshenker, B. G., S. Wilton, and G. P. A. Rice.** 1988. Phorbol ester-induced differentiation permits productive human cytomegalovirus infection in a monocytic line. Immunology **140:**1625–1631.
70. **Wiley, C. A., and J. A. Nelson.** 1988. Role of human immunodeficiency virus and cytomegalovirus in AIDS encephalitis. Am. J. Pathol. **133:**73–81.
71. **Wiley, C. A., R. D. Schrier, F. J. Denaro, J. A. Nelson, P. W. Lampert, and M. B. Oldstone.** 1986. Localization of cytomegalovirus proteins and genome during fulminant central nervous system infection in an AIDS patient. J. Neuropathol. Exp. Neurol. **45:**127–139.
72. **Zhao, K., C. M. Hart, and U. K. Laemmli.** 1995. Visualization of chromosomal domains with boundry element-associated factor BEAF-32. Cell **81:**879–889.