JOURNAL OF VIROLOGY, Oct. 1994, p. 6589–6597
0022-538X/94/$04.00+0

Vol. 68, No. 10

Use of Recombinant Virus To Assess Human Cytomegalovirus Early and Late Promoters in the Context of the Viral Genome

CATHERINE P. KOHLER,[1] JULIE A. KERRY,[1] MICHAEL CARTER,[1] VIERA P. MUZITHRAS,[2] THOMAS R. JONES,[2] AND RICHARD M. STENBERG[1]*

Department of Microbiology and Immunology, Eastern Virginia Medical School, Norfolk, Virginia 23501,[1] and Molecular Biology Section, Medical Research Division, Lederle Laboratories, American Cyanamid Company, Pearl River, New York 10965[2]

Received 10 May 1994/Accepted 6 July 1994

We have developed a system to study human cytomegalovirus (HCMV) *cis*-acting promoter elements within the context of the viral genome. A recombinant HCMV (RV134) containing a marker gene (β-glucuronidase) was used to insert HCMV promoter-chloramphenicol acetyltransferase gene constructs into the viral genome between open reading frames US9 and US10. Using this system, we have studied the promoters for the early DNA polymerase gene (UL54), the early-late lower matrix phosphoprotein gene (pp65, UL83), and the true late 28-kDa structural phosphoprotein gene (pp28, UL99). Transient-expression assays demonstrated that the pp65 and pp28 promoters are activated earlier and to higher levels than typically observed with the endogenous gene. In contrast, insertion of these promoters into the viral genome resulted in kinetics which mimicked that of the endogenous genes. In addition, we have also tested a variant of the pp28 promoter (*d*24/26CAT) which is deleted from −609 to −41. This promoter behaved similarly to the wild-type pp28 promoter, indicating that sequences from −40 to +106 are sufficient for conferring true late kinetics. Taken together, these data demonstrate that the viral genome affords a level of regulation on HCMV gene expression that has been previously unrealized. Therefore, these experiments provide a model system for the analysis of *cis*-acting promoter regulatory elements in the context of the viral genome.

Human cytomegalovirus (HCMV) is an opportunistic pathogen that causes significant morbidity and mortality in immunocompromised or immunosuppressed individuals (reviewed in reference 1). HCMV causes numerous complications in patients with AIDS, with approximately 50% of such patients demonstrating HCMV infections of the brain at autopsy (30, 53). In addition, the virus causes numerous complications in the developing fetus via transplacental transmission and infection in utero (1, 29, 32). These complications range from relatively mild sequelae to severe complications such as congenital abnormalities. To date, little is known of the mechanisms that allow HCMV to replicate and cause disease in the infected individual.

Infection of permissive human cells with HCMV leads to an ordered sequential expression of viral genes (5, 27, 51). The first genes expressed after the virus infects a susceptible cell are the immediate-early (IE) genes (12, 21, 46, 48, 49, 52, 54). The IE proteins are important for the upregulation of viral and cellular gene expression as well as the autoregulation of IE gene expression (reviewed in reference 40). Expression of the IE genes is regulated by viral and cellular factors in a complex but as yet incompletely characterized manner (reviewed in reference 8). Early and late genes are thought to be regulated by IE proteins in association with cell transcription factors such as AP-1 (50), E2F (38), and the adenovirus major late transcription factor (19), as well as other uncharacterized cell proteins (17). Clearly, numerous regulatory influences impact on HCMV IE, early, and late gene expression, and the process is likely more complicated than once thought.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Eastern Virginia Medical School, P.O. Box 1980, 700 W. Olney Rd., Norfolk, VA 23501. Phone: (804) 446-5684. Fax: (804) 624-2255.

Several groups have expended considerable effort investigating the viral *trans*-acting factors and *cis*-acting elements as well as the cell proteins required for activation of HCMV early and late promoters (reviewed in references 37 and 40). However, all of these studies have employed transient assays to assess the mechanisms that regulate these promoters outside the context of the viral genome. While these approaches have provided useful information concerning viral and cellular proteins important for promoter activation, they are limited in addressing other aspects of viral gene expression. For example, with respect to our studies on an early-late gene, pp65 (6), and a true late gene, pp28 (7), we have found that the promoters for these genes behave in a promiscuous manner, i.e., they are activated earlier and to higher levels than typically observed for the endogenous gene. Clearly, additional constraints on viral gene expression exist, and alternative approaches will be necessary to more fully examine these complex levels of regulation.

Recently, Jones and coworkers (14, 15) demonstrated that a region of the unique short (U_S) component of the HCMV genome is dispensable for growth in cell culture. On the basis of these findings, we have developed a strategy to insert HCMV promoter-chloramphenicol acetyltransferase (CAT) gene constructs into the viral genome. The aim of these studies is to identify sequences responsible for regulating HCMV early and late gene expression. A recombinant virus, RV134, which expresses the β-glucuronidase gene under the control of the 2.7E promoter (15), was used to introduce HCMV promoter-CAT constructs into the transcriptionally barren intergenic region between the US9 and US10 open reading frames (13). The results demonstrated that after insertion into the viral genome, early and late HCMV promoters displayed accurate temporal expression, as opposed to expression in transient assays. These studies therefore provide a model system with

which to study *cis*-acting elements that regulate HCMV gene expression during a natural infection.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblast (HF) cells were used throughout these studies, and the conditions for their growth and infection have been described (44, 46). The Towne strain of HCMV has been described (44, 46). HCMV strain AD169 and its recombinant virus derivative RV134, containing the β-glucuronidase gene under the control of the 2.7E promoter, have been described (15). For all experiments, cells were infected with 5 to 20 PFU of virus per cell for 2 h, the inoculum was removed, and the cells were subsequently overlaid with minimal essential medium (MEM) containing 10% newborn calf serum. Infections in the presence of phosphonoacetic acid (PAA) were done as previously described (6).

Transient expression of viral promoters. The structures of the DNA polymerase (pol), pp65, and pp28 gene promoters have been described previously (6, 7, 40, 42). Analysis of viral promoters in transient assays was performed exactly as previously described (6, 42). Briefly, cells were transfected by the DEAE-dextran method and, 18 h after transfection, were superinfected with HCMV (Towne). At various times after infection, the cells were harvested and assayed for CAT activity as previously described (6, 42).

DNA sequence. The nucleotide sequence designation used throughout this study corresponds to the previously published work of Chee et al. (4). The nucleotide accession number is X17403.

Generation of recombinant virus expressing promoter-CAT constructs. To insert promoter-CAT constructs into the viral genome, the promoter-CAT plasmid of interest was digested with *Hin*dIII and *Bam*HI. This results in removal of the promoter-CAT construct as a cassette that can be cloned into a vector containing the appropriate flanking sequences. As a vector to allow recombination into the viral genome, we used pHXSB, a clone containing a 3,129-bp *Bam*HI (nucleotide [n] 197042)-*Sal*I (n200171) subfragment of *Hin*dIII X in the vector pT7-1 (U.S. Biochemical Corporation). This plasmid contains the coding regions for US8 through US11. The promoter-CAT construct of interest was blunt-end ligated into the unique *Apa*I site (n199021) located between US9 and US10. A plasmid in which the construct was oriented so that CAT was transcribed from right to left relative to the HCMV prototype arrangement (i.e., similar to the transcription of the neighboring US9 and US10 genes) was subsequently selected.

To generate recombinant virus, the recombination vector containing the promoter-CAT construct of interest was linearized at a unique restriction endonuclease site located within the multiple cloning site of pT7-1 and subsequently cotransfected with RV134 DNA as previously described (15). When the cultures reached 100% cytopathic effect (CPE), stocks of potential recombinant virus were harvested, frozen in aliquots, and titered for subsequent screening. To demonstrate the presence of CAT-expressing recombinants within the primary transfectant population, cells were infected with primary stocks of putative recombinant virus and, at 72 h after infection, were harvested for determination of CAT activity as previously described (6, 42).

Selection of white phenotype virus expressing CAT. To isolate virus that expressed a white phenotype in the presence of X-glu (5-bromo-4-chloro-3-indolyl-β-D-glucuronide; Biosynth AG), progeny virus from the primary transfection were plated on HF cells at 10 to 20 PFU per plate. Cells were overlaid with MEM containing 10% heat-inactivated fetal calf serum and 0.5% agarose and fed every 5 to 7 days. After 10 to 14 days, or when plaques became visible microscopically, plaques were picked directly to individual wells of 24-well culture dishes and incubated at 37°C until CPE occurred. At that time, cells were overlaid with MEM containing 10% fetal calf serum, 0.5% agarose, and 75 μg of X-glu per ml. Wells exhibiting a white or clear overlay after 3 days were considered to be negative for β-glucuronidase expression. From these wells, a plaque was picked and incubated for 60 min in 3 ml of MEM containing 10% newborn calf serum in order to liberate virus from the agarose plug. The suspension was divided, and 1 ml was inoculated onto each of three 100-mm dishes of HF cells. After 2 h, the cultures were overlaid with fresh medium and incubated until 100% CPE had occurred. Plates were harvested for virus stocks, CAT assay, and total-cell DNA. Individual isolates used in this study were RVpolCAT (*d*H1A6), RV65CAT(2B5), RV28CAT(328D6), and RV24/26CAT(A6).

Isolation of infected-cell DNA and Southern blot analysis. HF cells infected with recombinant viruses were harvested for total-cell DNA at 100% CPE. Cells were washed with Tris-buffered saline and gently lysed with 1 ml of 1% sodium dodecyl sulfate (SDS) in 30 mM Tris-HCl, pH 7.5, containing proteinase K (100 μg/ml), per 100 mm dish. The sample was incubated at room temperature overnight, mixed with 1 ml of 2× DNA extraction buffer (30 mM Tris-HCl [pH 7.5], 0.5 M NaCl, 2 mM EDTA), and subsequently extracted twice with an equal volume of phenol saturated with TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). The sample was extracted with chloroform and precipitated with 2 volumes of absolute ethanol. DNA was collected by centrifugation, dried, and resuspended in TE at 4°C overnight. Infected-cell DNAs were digested with *Hin*dIII and subjected to electrophoresis on 0.8% agarose gels in 1× TBE buffer. The gels were transferred to nitrocellulose and hybridized either to a probe for CAT or to the *Hin*dIII X fragment of AD169.

Assay of RVCAT-W growth in culture. Recombinant virus was assayed for progeny production by infecting HF cell monolayers at 0.01 PFU/cell. The medium was changed every 3 to 5 days until 100% CPE occurred. At that time, the medium was changed again, and 5 days later, the supernatants were harvested. All cultures were harvested at the same time after initial infection. Progeny virus was determined by plaque assay.

Isolation and analysis of infected-cell RNA. Total-cell RNA was isolated by our published approaches (41, 43, 45), and equal quantities of RNA were subjected to Northern (RNA blot) analysis. Blots were hybridized to radiolabeled probes for CAT or pp28 as previously described (6, 7).

RESULTS

Comparative analysis of early and late promoter-CAT constructs in transient-expression assays. Our studies on the pp28 and pp65 gene promoters had previously indicated that in transient assays, these promoters were activated earlier and to higher levels than the endogenous genes (6, 7). Normally, these promoters are activated with true late and early-late kinetics, respectively. To assess the kinetics of pol promoter expression and to directly compare this prototypical early gene with the pp65 and pp28 genes, the promoter-CAT constructs (Fig. 1A) were transfected into cells, superinfected with HCMV, and harvested for CAT assays at 24 and 72 h after infection. The data in Fig. 1B demonstrated that all of the promoters are activated with roughly the same kinetics, i.e., they are ex-

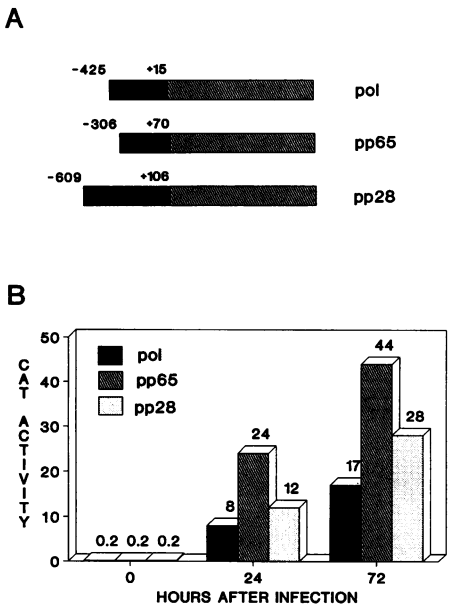


FIG. 1. (A) Structure of early and late promoter-CAT constructs. The sequences within the promoter-CAT constructs are indicated and have been previously described (6, 7, 17, 40). The CAT reporter gene is adjacent to the promoters and is represented by the hatched box. (B) Kinetics of transient promoter activity. HF cells were transfected with the indicated promoter-CAT constructs and subsequently superinfected 18 h later with 20 PFU of HCMV per cell. At the indicated times, cells were harvested for CAT assays. CAT activity is expressed as percent acetylation. Data from a single experiment of three replicate experiments are shown.

pressed at readily detectable levels at 24 h and these levels increase at 72 h after infection. For the pp65 and pp28 promoters, these data are inconsistent with the expression of the endogenous genes, as they show early kinetics similar to those of pol in transient assays.

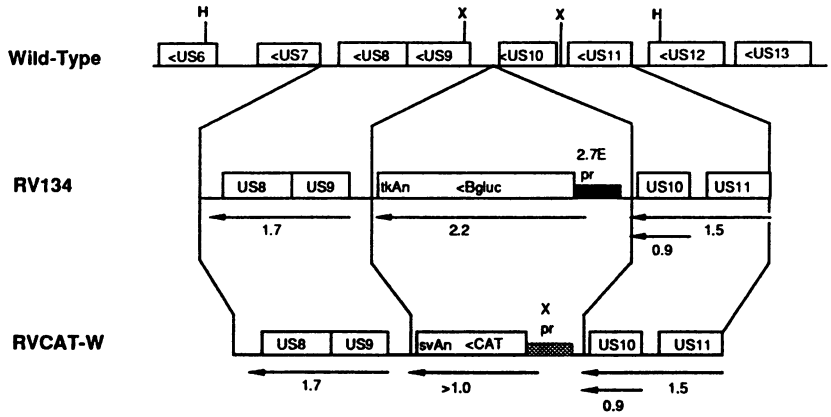
Strategy for generation of recombinant virus expressing promoter-reporter gene constructs. Since the pp65 and pp28 promoters are expressed in a promiscuous manner in transient assays, a system was developed for the insertion of these constructs into the viral genome. Previously, Jones and coworkers (15) demonstrated that transcription of an HCMV early promoter (2.7E) as well as a herpes simplex virus type 1 late glycoprotein promoter (gH) was regulated appropriately when the promoters were inserted into the transcriptionally barren US9-US10 intergenic region of the HCMV genome (13). In addition, recent studies with the herpes simplex virus system demonstrated that altering the genomic location of the UL38 promoter did not influence kinetic class (9). Therefore, we hypothesized that HCMV promoters (e.g., those for pol, pp65, and pp28) would be properly regulated when inserted into the US9-US10 intergenic region. Our strategy was to use as the parent virus recombinant HCMV RV134, which contains an insertion of the β-glucuronidase gene under the control of the 2.7E promoter in the aforementioned nonessential region between the US9 and US10 open reading frames (15). This mutant was shown to grow with kinetics similar to those of the wild-type strain AD169 and to generate blue plaques when grown in the presence of X-glu (15). The promoter-CAT constructs were engineered into pHXSB so that upon recombination into the parental RV134 genome, the 2.7E promoter and β-glucuronidase gene would be replaced by the promoter-CAT transcription unit (Fig. 2A). Accordingly, the proper recombination event would yield a virus (i.e., RVCAT-W in Fig. 2A) with a white-plaque phenotype when grown in the presence of X-glu. This CAT gene-containing virus would contain a slightly smaller DNA insertion than RV134 (Fig. 2B).

Recombinant virus was generated as described in Materials and Methods. Progeny virus from the primary transfection was titered and assayed for CAT activity. The presence of CAT activity within the infected-cell population in excess of that in uninfected controls indicated that a successful recombination had occurred. All of our primary virus populations expressed measurable CAT activity, and individual recombinants were selected at a frequency of approximately 1 to 2% (data not shown).

Characterization of recombinant virus. The CAT gene-containing recombinant virus was characterized by Southern blot hybridization analysis and growth studies. In each case, integration of the promoter-CAT transcription unit had occurred within the *Hin*dIII X DNA fragment, as indicated by a single hybridizing DNA fragment of the appropriate molecular weight (Fig. 3). The growth properties of the new recombinants were measured by multicycle virus yield studies following low-multiplicity infection. As demonstrated in Table 1, each of the new recombinants yield progeny virus whose abundance was similar to that of both parental RV134 and wild-type strain AD169 in two separate experiments. Taken together, the data indicate that the approach was successful with respect to generating the appropriate virus constructs.

Characterization of CAT expression from recombinant viruses. To assess the kinetics of expression from the promoter-CAT constructs within the context of the viral genome, HF cells were infected with the recombinant virus under study and harvested at various times after infection. In addition, cells were also infected in the presence of PAA, an inhibitor of viral DNA replication. The data in Fig. 4 demonstrated that each of the recombinant viruses expressed CAT in a manner consistent with its kinetic class. In RVpolCAT-infected cells (Fig. 4A), CAT was expressed at early times (24 to 48 h) as well as in the presence of PAA, and expression subsequently increased later in infection. In RV65CAT-infected cells (Fig. 4B), CAT was expressed at very low to undetectable levels early as well as in the presence of PAA, but expression increased dramatically at late times after infection. These results were consistent with the pattern of RNA expression for these two genes as previously described (6, 10, 22, 33). In RV28CAT-infected cells (Fig. 4C), CAT was expressed with true late kinetics, as expected for this late gene (7, 23, 26, 28), with low activity detected at 48 h and higher levels at 72 h, but not in the presence of PAA. These data validated our approach and demonstrated that an altered pattern of regulation occurs in transient assays compared with expression in the context of the viral genome.

We also assessed the levels of CAT RNA expressed by our recombinant viruses at 72 h in the presence or absence of PAA. These studies demonstrated that each virus expressed detectable levels of CAT RNA of the appropriate molecular mass and with the appropriate kinetics relative to the early-late transition (Fig. 5). RVpolCAT produced detectable CAT RNA in the presence of PAA and significantly higher levels in the absence of PAA. However, longer exposures were required to detect CAT mRNA expressed by RV65CAT in the presence of PAA (data not shown). In contrast, RV28CAT produced no detectable CAT mRNA in the presence of PAA. Therefore, CAT RNAs driven by these candidate promoters are expressed



	Net Increase In Genome Size	Genotype	Phenotype (X-gluc)
AD169	None	Gluc-/CAT-	White
RV134	2.83kb	Gluc+/CAT-	Blue
RVCAT-W	> 1.02kb (CAT)	Gluc-/CAT+	White
RVpolCAT	1.47kb	Gluc-/CAT+	White
RV65CAT	1.40kb	Gluc-/CAT+	White
RV28CAT	1.74kb	Gluc-/CAT+	White
RV24/26CAT	1.17kb	Gluc-/CAT+	White

FIG. 2. Generation of recombinant virus. The structure of the HCMV strain AD169 and RVCAT genomes is shown. The map shows the genomic organization and derivation of RV134 (15) and the RVCAT-W series of recombinant viruses. The first line is the expanded region of the wild-type genome from US6 through US13. The second line is the relevant region of the linearized plasmid constructed to make the RV134 recombinant virus. The black box is the 2.7E promoter (2.7E pr), and the herpes simplex virus type 1 thymidine kinase polyadenylation signal (tkAn) and the adjacent β-glucuronidase gene (Bgluc) are also shown. The third line is the relevant region of the linearized plasmid constructed to make the RVCAT-W recombinant virus series, which are derived from RV134. Vertical lines indicate boundaries of the HCMV sequences available for homologous recombination to generate the recombinant. The expected transcripts from this region are shown, and their sizes given in kilobases. The expected genotype and phenotype and the net change in the genome size of these recombinants, compared with wild-type AD169, are shown in the chart at the bottom of the figure. Abbreviations: X pr (shaded box), desired promoter-CAT sequence; svAn, simian virus 40 polyadenylation signal adjacent to the CAT gene; H, *Hin*dIII site; X, *Xho*I site; Gluc, β-glucuronidase expression.

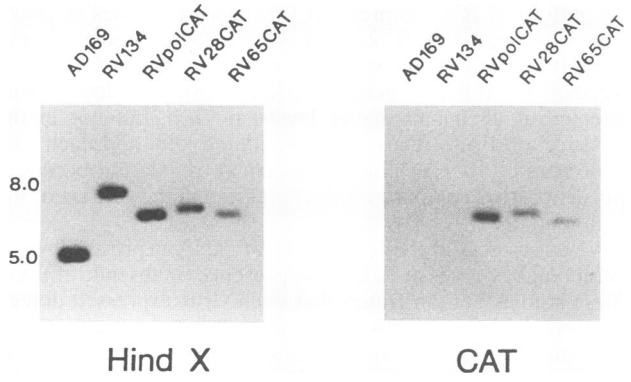


FIG. 3. Southern blot hybridization analysis of recombinant viruses. Total cellular DNA was isolated from cells infected with the indicated virus for 72 h, digested with *Hin*dIII, and subjected to Southern blot analysis. The blots were hybridized with a ^{32}P-labeled DNA probe generated from a linearized *Hin*dIII X clone or a CAT probe from pRSVCAT. The molecular sizes of the bands are indicated (in kilobases).

in a manner similar to that reported for the endogenous genes (6, 7, 10, 22, 23, 26, 28, 33).

Deletion analysis of the pp28 promoter. To investigate those sequences that determine kinetic class, the aforementioned approaches were used to insert a deleted variant of the pp28 promoter into the HCMV genome. This variant, *d*24/26CAT,

TABLE 1. Replication of RVCAT-W in human fibroblast cells[a]

Virus	Titer (PFU/ml)	
	Expt 1	Expt 2
AD169	2×10^7	3×10^6
RV134	1×10^7	5×10^6
RVpolCAT	7×10^6	6×10^6
RV65CAT	2×10^7	1×10^6
RV28CAT	1×10^7	2×10^7

[a] Human fibroblast (HF) cells were infected with 0.01 PFU of the indicated virus per cell and fed with fresh medium every fourth day until 100% CPE occurred. The medium was changed one final time, and the cells and supernatant were harvested 5 days later. Samples were frozen, stored at −85°C, and titered on HF cells.

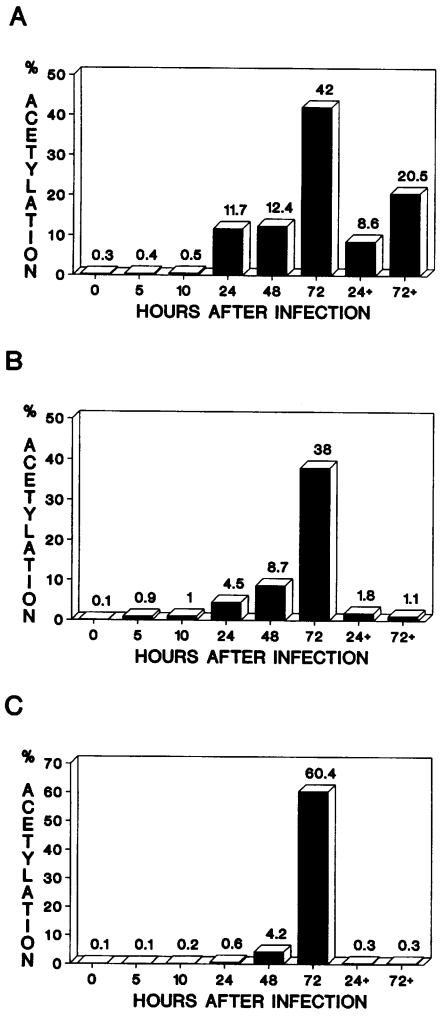


FIG. 4. Kinetics of CAT expression from recombinant viruses. HF cells were infected with 5 to 20 PFU per cell. Cells were harvested at the indicated times and subsequently assayed for CAT activity. The last two columns represent the kinetics of expression at the indicated times when the infection took place in the presence of PAA (200 μg/ml). Data are expressed as percent acetylation. (A) RVpolCAT; (B) RV65CAT; (C) RV28CAT.

represents a minimal construct that expresses readily detectable levels of CAT from the pp28 promoter in transient assays (7). Figure 6A represents a schematic of the pp28 promoter (p28dHCAT) as well as the *d*24/26CAT variant. IR4 is an inverted repeat located immediately 5′ of the pp28 TATA motif and has been shown to be important for the expression of this promoter in transient assays (7). Other sequences potentially involved in regulation of this promoter are also indicated.

A recombinant virus which contained *d*24/26CAT inserted into the viral genome was subsequently generated as described above for RV28CAT. Southern blot analysis demonstrated that insertion of *d*24/26CAT occurred at the appropriate locus, as a DNA fragment of the correct size was generated after digestion with *Hin*dIII (Fig. 6B). In addition, RV24/26CAT replicates in cells to levels equivalent to those of RV28CAT (data not shown).

Comparison of the kinetics of RV28CAT and RV24/26CAT.

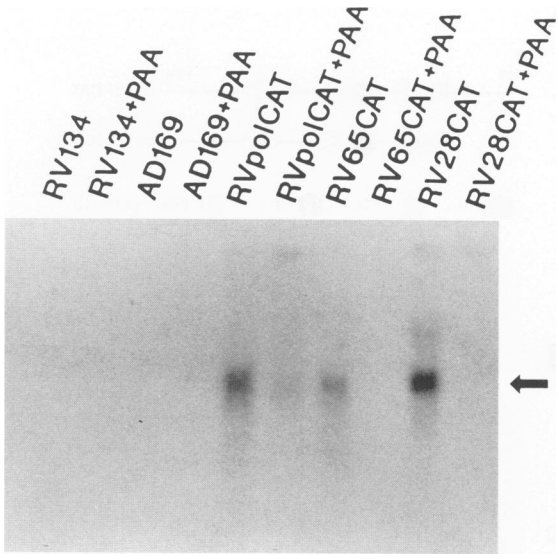


FIG. 5. Analysis of CAT RNA in recombinant virus-infected cells. Cells were infected with the indicated recombinant virus in the presence or absence of PAA (200 μg/ml), and total cellular RNA was isolated at 72 h after infection. An equal quantity of RNA (5 μg) was subjected to Northern blot analysis and hybridized to a CAT probe. The 1.2-kb CAT RNA is indicated by the arrow.

To assess the role of upstream pp28 promoter sequences in conferring late kinetics, CAT expression by RV28CAT was compared with expression from RV24/26CAT-infected cells. Cells were infected with the recombinant viruses and, at the indicated times, were harvested for CAT assay. Both viruses expressed CAT with similar kinetics, resulting in undetectable to low levels of CAT activity through 48 h followed by a dramatic increase at 72 h after infection (Fig. 7). Also, in RV24/26CAT-infected cells, expression of CAT was shown to be sensitive to PAA (data not shown). These data demonstrated that sequences from −40 to +106 within the pp28 promoter are responsible for conferring late kinetics.

Correlation of RNA expression to CAT levels. To demonstrate that the level of CAT activity reflected RNA expression from the CAT gene, we assessed CAT activity, template copy number, and RNA levels in RV28CAT- and RV24/26CAT-infected cells at 72 h after infection. CAT levels were approximately equal between the two viruses (Fig. 8A), and Southern blot analysis demonstrated that CAT DNA copy number was also approximately the same (data not shown). Analysis of CAT RNA in RV28CAT- and RV24/26CAT-infected cells demonstrated that the levels of RNA roughly reflected the relative levels of CAT activity. A comparison of pp28 RNA levels in RV28CAT- and RV24/26CAT-infected cells demonstrated that the infections were similar in terms of efficiency. Taken together, these data imply that these promoters behave similarly when measured in the context of the virus.

DISCUSSION

In this study, we have established an approach for inserting promoter-reporter gene constructs into the HCMV genome to study their expression in a more natural context. Promoters of HCMV genes representing three different kinetic classes were examined: the promoters for an early gene, pol (UL54); an

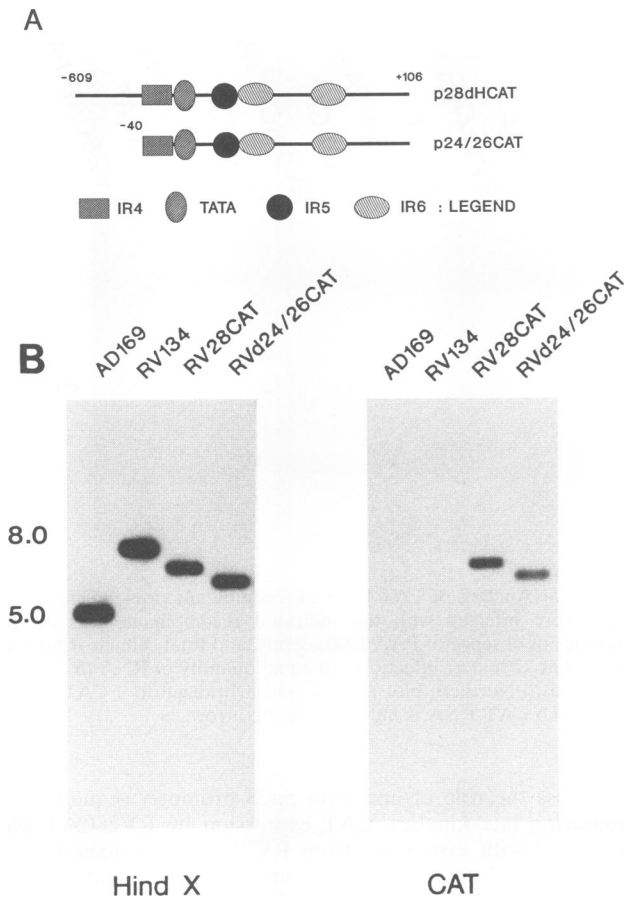


FIG. 6. (A) Structure of the full-length pp28 (p28dHCAT) promoter compared with its deleted variant (p24/26CAT). Elements which are retained in both promoters are indicated. Inverted repeats (IR) and the TATA homology are indicated. (B) Southern blot analysis of recombinant virus DNA. Total cellular DNA was isolated from cells infected with the indicated viruses and subsequently digested with *Hin*dIII. Southern blot analysis was performed as described in the legend to Fig. 3. Molecular sizes are indicated (in kilobases).

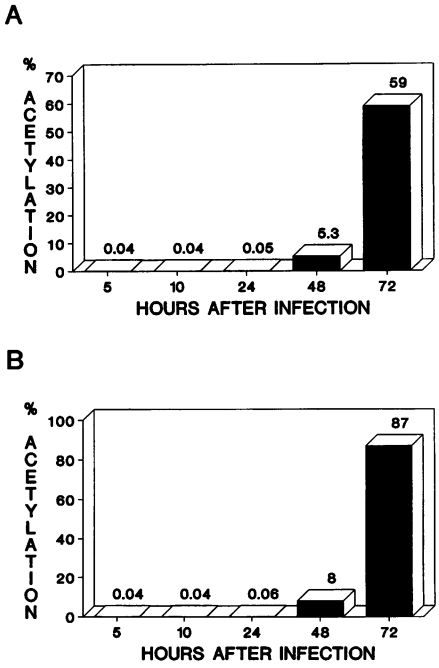


FIG. 7. Kinetic analysis of (A) RV28CAT and (B) RV24/26CAT. Cells were infected with 5 to 20 PFU per cell and harvested at the indicated times postinfection for CAT assay. Data are expressed as percent acetylation.

early-late gene, pp65 (UL83); and a true late gene, pp28 (UL99). These studies demonstrated that, compared with transient assays, promoters assessed in the context of the viral genome expressed a reporter gene with a greater fidelity which more closely mimics expression of the endogenous counterpart. In addition, we identified a region of the pp28 promoter (−40 to +106) which is sufficient to confer late kinetics. These findings demonstrate the feasibility of this approach for assessing *cis*-acting elements within a viral promoter and establishing the role of these elements in HCMV infection.

Our laboratory as well as others has invested considerable effort studying various HCMV early and late promoters (3, 6, 7, 17–20, 24, 25, 31, 35, 38, 39, 42, 50). Our previous studies on the pol, pp65, and pp28 promoters assessed the viral *trans*-acting proteins and *cis*-acting elements responsible for promoter activation in transient assays (6, 7, 17, 40, 42). While studies of this type have provided useful information on those regulatory events which control transcription, they cannot detect other viral influences that either directly or indirectly regulate viral gene expression. Clearly, these viral promoters, in particular pp65 and pp28, behave promiscuously and are expressed with inappropriate kinetics when assessed in transient assays after viral superinfection. Placement of these promoters into the viral genome returns a level of regulation which is not afforded by transient assays and which results in maintenance of the proper kinetic class.

In order to preliminarily assess the sequences that regulate kinetic class, we generated a pp28 recombinant virus variant that was deleted from −609 to −41 within the pp28 promoter. This virus (RV24/26CAT) contains a deleted version of the pp28 promoter that was previously shown to contain the minimal upstream sequences for activation by superinfecting virus in transient assays (7). Removal of the upstream sequences had no effect on the kinetics of pp28 promoter expression, as this variant remained a true late promoter. This is consistent with studies on herpes simplex virus late promoters in the context of the virus, which demonstrate that sequences in the vicinity of the TATA motif are sufficient for determining kinetic class (11). Studies to further address this possibility in HCMV are presently under way.

While expression from *d*24/26CAT was reduced approximately 50% relative to that from 28dHCAT in transient assays, RV24/26CAT consistently expressed levels (ca. one- to two-fold) of CAT RNA and protein equivalent to or slightly higher than those of RV28CAT. This result may reflect the differences between studies of promoters in transient assays and those studies that occur in the context of the viral genome. In support of this finding, we have preliminary data indicating that sequences within the pol promoter which appear to attenuate expression in transient assays are not repressive when assessed in the context of the viral genome (data not shown). These data collectively imply that observations made from transient assays may not necessarily reflect regulatory events that occur in a natural infection. This is supported by findings in the herpes simplex virus system (34).

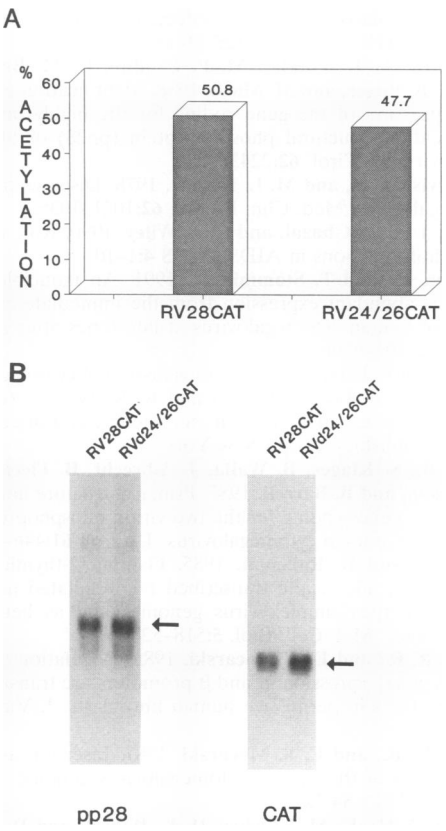


FIG. 8. Comparative expression of RV28CAT and RV24/26CAT. Cells were infected with 5 to 20 PFU of the indicated virus per cell, and at 72 h after infection, cells were harvested for CAT and total-cell RNA assays. (A) CAT activity was measured as described in the legend to Fig. 1 and is indicated as percent acetylation. (B) Analysis of pp28 and CAT RNA from RV28CAT- and RV24/26CAT-infected cells. An equal quantity of RNA (5 μg) was subjected to Northern blot analysis and hybridized to either a CAT probe or a probe for pp28 derived from a 5.3-kb *Pst*I fragment of *Xba*I C (6). The molecular sizes of these bands are indicated (in kilobases).

While genetic manipulation of the viral genome has proven to be a useful approach in studying other herpesviruses such as herpes simplex virus, manipulation of HCMV has proven difficult. Spaete and Mocarski first manipulated the HCMV genome when they inserted the *lacZ* gene under the control of the 2.7E promoter (36). This virus expressed β-galactosidase with appropriate early kinetics. However, as pointed out by the authors, this virus was genetically unstable, with approximately 10% of the progeny reverting to a white-plaque phenotype. To date, only a limited number of successful endeavors have been published (2, 14–16, 36, 47), and while several of these studies identified genes that are not required for replication in cell culture, no studies have been undertaken to specifically ascertain the function(s) of viral regulatory sequences or proteins. We have extended the studies of Jones and coworkers (14, 15) and used an existing recombinant virus (RV134) to insert promoter-CAT constructs of interest into the virus. This approach affords the advantages that (i) promoters from existing promoter-CAT variants need not be recloned adjacent to a selectable reporter gene (i.e., β-galactosidase or β-glucuronidase) and (ii) recombinant virus can subsequently be selected at low frequencies from a primary transfectant population of progeny virus. By the use of homologous flanking sequences and selecting a white-plaque phenotype from the blue-plaque phenotype parentals, it is a relatively straightforward way to identify new recombinants. These viruses appear to be genetically stable and grow to titers similar to those of the parental strain (RV134). While we have not carried out exhaustive analyses of the recombinant virus genomes, these findings imply that gross changes have not occurred during the recombination event. Consequently, this approach will be useful for assessing sequences that regulate viral gene expression in the context of the virus.

We have also attempted an alternative approach which employed the double insertion of β-glucuronidase and the promoter-CAT construct of interest followed by screening for a blue-plaque phenotype. While this approach also proved successful, it periodically yielded unstable recombinants that correlated with certain promoter constructs (data not shown). It is possible that the two bacterial genes in tandem are unstable. Regardless of the reason, this approach was less desirable and was not pursued further.

In conclusion, our studies demonstrate an approach for assessing HCMV promoter regulatory sequences in a more natural setting and that differences in regulation and kinetic class relative to transient assays can occur. We are using this approach to systematically identify those elements which are important for regulating transcription as well as temporal expression of early and late promoters. These reagents will become useful in ultimately assessing the role of HCMV *cis*-acting sequences in various cell types and tissues. Studies of this type should provide valuable information regarding the extent of host influences on virus replication.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grants AI23313 (R.M.S.) and AI34154 (R.M.S.) from the National Institutes of Health.

We thank Ann Campbell and Timothy Bos for comments and critical review of the manuscript.

REFERENCES

1. **Alford, C. A., and W. J. Britt.** 1990. Cytomegalovirus, p. 1981–2010. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
2. **Browne, H., M. Churcher, and T. Minson.** 1992. Construction and characterization of a human cytomegalovirus mutant with the UL18 (class I homolog) gene deleted. J. Virol. **66**:6784–6787.
3. **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.
4. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchinson 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**:125–170.
5. **DeMarchi, J. M.** 1981. Human cytomegalovirus DNA: restriction enzyme cleavage maps, and map locations for immediate early, early, and late RNAs. Virology **114**:23–38.
6. **Depto, A. S., and R. M. Stenberg.** 1989. Regulated expression of the human cytomegalovirus pp65 gene: octamer sequence in the promoter is required for activation by viral gene products. J. Virol. **63**:1232–1238.
7. **Depto, A. S., and R. M. Stenberg.** 1992. Functional analysis of the true late human cytomegalovirus pp28 upstream promoter: *cis*-acting elements and viral *trans*-acting proteins necessary for promoter activation. J. Virol. **66**:3241–3246.
8. **Ghazal, P., and J. A. Nelson.** 1993. Transcription factors and viral regulatory proteins as potential mediators of human cytomegalo-

virus pathogenesis, p. 360–383. *In* E.-S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag, Heidelberg, Germany.
9. **Goodart, S. A., J. F. Guzowski, M. K. Rice, and E. K. Wagner.** 1992. Effect of genomic location on expression of β-galactosidase mRNA controlled by the herpes simplex virus type 1 UL38 promoter. J. Virol. **66:**2973–2981.
10. **Heilbronn, R., G. Jahn, A. Burkle, U.-K. Freese, B. Fleckenstein, and H. zur Hausen.** 1987. Genomic localization, sequence analysis, and transcription of the putative human cytomegalovirus DNA polymerase gene. J. Virol. **61:**119–124.
11. **Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine.** 1986. Transcriptional control signals of a herpes simplex virus type 1 late (gamma 2) gene lie within bases −34 to +124 relative to the 5′ terminus of the mRNA. Mol. Cell. Biol. **6:**3652–3666.
12. **Jahn, G., E. Knust, H. Schmolla, T. Sarre, J. A. Nelson, J. K. McDougall, and B. Fleckenstein.** 1984. Predominant immediate-early transcripts of human cytomegalovirus AD169. J. Virol. **49:** 363–370.
13. **Jones, T. R., and V. P. Muzithras.** 1991. Fine mapping of transcripts expressed from the US6 gene family of human cytomegalovirus strain AD169. J. Virol. **65:**2024–2036.
14. **Jones, T. R., and V. P. Muzithras.** 1992. A cluster of dispensible genes within the human cytomegalovirus genome short component: IRS1, US1 through US5, and the US6 family. J. Virol. **66:**2541–2546.
15. **Jones, T. R., V. P. Muzithras, and Y. Gluzman.** 1991. Replacement mutagenesis of the human cytomegalovirus genome: US10 and US11 gene products are nonessential. J. Virol. **65:**5860–5872.
16. **Kaye, J., H. Browne, M. Stoffel, and T. Minson.** 1992. The UL16 gene of human cytomegalovirus encodes a glycoprotein that is dispensible for virus growth in vitro. J. Virol. **66:**6609–6615.
17. **Kerry, J. A., M. Priddy, and R. M. Stenberg.** 1994. Identification of sequence elements in the human cytomegalovirus DNA polymerase gene promoter required for activation by viral gene products. J. Virol. **68:**4167–4176.
18. **Klucher, K. M., D. K. Rabert, and D. H. Spector.** 1989. Sequences in the human cytomegalovirus 2.7-kilobase RNA promoter which mediate its regulation as an early gene. J. Virol. **63:**5334–5343.
19. **Klucher, K. M., M. Sommer, J. T. Kadonaga, and D. H. Spector.** 1993. In vivo and in vitro analysis of transcriptional activation mediated by the human cytomegalovirus major immediate-early proteins. Mol. Cell. Biol. **13:**1238–1250.
20. **Klucher, K. M., and D. H. Spector.** 1990. The human cytomegalovirus 2.7-kilobase RNA promoter contains a functional binding site for the adenovirus major late transcription factor. J. Virol. **64:**4189–4198.
21. **Kouzarides, T., A. T. Bankier, S. C. Satchwell, E. Preddy, and B. G. Barrell.** 1988. An immediate early gene of human cytomegalovirus encodes a potential membrane glycoprotein. Virology **165:**151–164.
22. **Kouzarides, T., A. T. Bankier, S. C. Satchwell, K. Weston, P. Tomlinson, and B. G. Barrell.** 1987. Sequence and transcription analysis of the human cytomegalovirus DNA polymerase gene. J. Virol. **61:**125–133.
23. **Lahijani, R. S., E. W. Otteson, J. D. Adlish, and S. C. St. Jeor.** 1991. Characterization of a human cytomegalovirus 1.6-kilobase late mRNA and identification of its putative protein product. J. Virol. **65:**373–381.
24. **Leach, F. S., and E. S. Mocarski.** 1989. Regulation of cytomegalovirus late-gene expression: differential use of three start sites in the transcriptional activation of ICP36 gene expression. J. Virol. **63:**1783–1791.
25. **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:**1498–1506.
26. **Martinez, J., R. S. Lahijani, and S. C. St. Jeor.** 1989. Analysis of a region of the human cytomegalovirus (AD169) genome coding for a 25-kilodalton virion protein. J. Virol. **63:**233–241.
27. **McDonough, S. H., and D. H. Spector.** 1983. Transcription in human fibroblasts permissively infected by human cytomegalovirus strain AD169. Virology **125:**31–46.
28. **Meyer, H., A. T. Bankier, M. P. Landini, C. M. Brown, B. G. Barrell, B. Ruger, and M. Mach.** 1988. Identification and procaryotic expression of the gene coding for the highly immunogenic 28-kilodalton structural phosphoprotein (pp28) of human cytomegalovirus. J. Virol. **62:**2243–2250.
29. **Nankervis, G. A., and M. L. Kumar.** 1978. Diseases produced by cytomegalovirus. Med. Clin. N. Am. **62:**1021–1035.
30. **Nelson, J. A., P. Ghazal, and C. A. Wiley.** 1990. Role of opportunistic viral infections in AIDS. AIDS **4:**1–10.
31. **Puchtler, E., and T. Stamminger.** 1991. An inducible promoter mediates abundant expression from the immediate-early 2 gene region of human cytomegalovirus at late times after infection. J. Virol. **65:**6301–6306.
32. **Rapp, F.** 1980. Persistence and transmission of cytomegalovirus, p. 193–232. *In* M. Frankel-Conrat and R. R. Wagner (ed.), Virus-host interactions: viral invasion, persistence, and disease, vol. 16. Plenum Publishing Corp., New York.
33. **Ruger, B., S. Klages, B. Walla, J. Albrecht, B. Fleckenstein, P. Tomlinson, and B. Barrell.** 1987. Primary structure and transcription of the genes coding for the two virion phosphoproteins pp65 and pp71 of human cytomegalovirus. J. Virol. **61:**446–453.
34. **Silver, S., and B. Roizman.** 1985. Gamma 2-thymidine kinase chimeras are identically transcribed but regulated as gamma 2 genes in herpes simplex virus genomes and as beta genes in cell genomes. Mol. Cell. Biol. **5:**518–528.
35. **Spaete, R. R., and E. S. Mocarski.** 1985. Regulation of cytomegalovirus gene expression: α and β promoters are transactivated by viral functions in permissive human fibroblasts. J. Virol. **56:**135–143.
36. **Spaete, R. R., and E. S. Mocarski.** 1987. Insertion and deletion mutagenesis of the human cytomegalovirus genome. Proc. Natl. Acad. Sci. USA **84:**7213–7217.
37. **Spector, D. H., K. M. Klucher, D. K. Rabert, and D. A. Wright.** 1990. Human cytomegalovirus early gene expression. Curr. Top. Microbiol. Immunol. **154:**21–45.
38. **Staprans, S. I., D. K. Rabert, and D. H. Spector.** 1988. Identification of sequence requirements and *trans*-acting functions necessary for regulated expression of a human cytomegalovirus early gene. J. Virol. **62:**3463–3473.
39. **Stasiak, P. C., and E. S. Mocarski.** 1992. Transactivation of the cytomegalovirus ICP36 gene promoter requires the α gene product TRS1 in addition to IE1 and IE2. J. Virol. **66:**1050–1058.
40. **Stenberg, R. M.** 1993. Immediate-early genes of human cytomegalovirus: organization and function, p. 330–359. *In* E.-S. Huang (ed.), Molecular aspects of human cytomegalovirus diseases. Springer-Verlag, Heidelberg, Germany.
41. **Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson.** 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J. Virol. **63:**2699–2708.
42. **Stenberg, R. M., J. Fortney, S. W. Barlow, B. P. Magrane, J. A. Nelson, and P. Ghazal.** 1990. Promoter-specific *trans* activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. J. Virol. **64:**1556–1565.
43. **Stenberg, R. M., and M. F. Stinski.** 1985. Autoregulation of the human cytomegalovirus major immediate-early gene. J. Virol. **56:**676–682.
44. **Stenberg, R. M., D. R. Thomsen, and M. F. Stinski.** 1984. Structural analysis of the major immediate-early gene of human cytomegalovirus. J. Virol. **49:**190–199.
45. **Stenberg, R. M., P. R. Witte, and M. F. Stinski.** 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. **56:**665–675.
46. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate-early genes of human cytomegalovirus. J. Virol. **46:**1–14.
47. **Takekoshi, M., F. Maeda-Takekoshi, S. Ihara, S. Sakuma, and Y. Watanabe.** 1991. Site specific stable insertion into the human

cytomegalovirus genome of a foreign gene under control of the SV40 promoter. Gene **101:**209–213.

48. **Tenney, D. J., and A. M. Colberg-Poley.** 1991. Expression of the human cytomegalovirus UL36-38 immediate early region during permissive infection. Virology **182:**199–210.
49. **Tenney, D. J., and A. M. Colberg-Poley.** 1991. Human cytomegalovirus UL36-38 and US3 immediate-early genes: temporally regulated expression of nuclear, cytoplasmic, and polysome-associated transcripts during infection. J. Virol. **65:**6724–6734.
50. **Wade, E. J., K. M. Klucher, and D. H. Spector.** 1992. An AP-1 binding site is the predominant *cis*-acting regulatory element in the 1.2-kilobase early RNA promoter of human cytomegalovirus. J. Virol. **66:**2407–2417.
51. **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.
52. **Weston, K.** 1988. An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. Virology **162:** 406–416.
53. **Wiley, C. A., and J. A. Nelson.** 1988. Role of the human immunodeficiency virus and cytomegalovirus in AIDS encephalitis. Am. J. Pathol. **133:**73–81.
54. **Wilkinson, G. W. G., A. Akrigg, and P. J. Greenaway.** 1984. Transcription of the immediate early genes of human cytomegalovirus strain AD169. Virus Res. **1:**101–116.