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Regulation of Cytomegalovirus Late Gene Expression: γ Genes Are Controlled by Posttranscriptional Events

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We investigated the control of human cytomegalovirus (CMV) late (γ)-gene expression in human fibroblast cells. Transcriptional activity of two γ genes, encoding ICP27, a structural component (matrix or tegument) of virions, and ICP36, a major DNA-binding protein family, was followed by analysis of steady-state RNA levels during viral infection. Synthesis of the protein products of these genes was analyzed with specific monoclonal antibodies in conjunction with sensitive immunoblot or immunoprecipitation analysis. Although accumulation of ICP27 and ICP36 was not abundant until late times, both late genes were as transcriptionally active at early times (4 h postinfection) as at late times (48 h postinfection). Reduced amounts (<5% of late levels) of the protein products were detected at early times, demonstrating that a small proportion of the ICP27 and ICP36 RNA made at this time was translated. These observations establish that expression of at least two CMV γ genes is regulated through posttranscriptional events. The very early transcriptional activation of late genes and the relative importance of posttranscriptional regulation to late-gene expression distinguishes CMV from other well-studied herpesviruses and does not appear analogous to late-gene regulation in any other DNA animal virus.

Human cytomegalovirus (CMV) has a double-stranded DNA genome 240 kilobase pairs (kbp) in size. Viral gene expression is sequential and coordinately regulated during growth in permissive cells (10, 30, 44, 52, 53; reviewed in reference 47). As for herpes simplex virus (HSV), three classes of genes are expressed from the CMV genome, α, β, and γ (7, 8, 10, 18, 19, 50, 52). Immediate (or α) and delayed (or β) early-gene expression by HSV and CMV are controlled at the level of transcription (7, 8, 10, 12, 13, 18, 19, 24, 40, 47, 52). For both HSV (13, 22, 25–27, 39, 42, 55) and CMV (44, 48), regions upstream of the transcriptional start site contain *cis*-acting elements that increase transcription from α or β genes through interactions with viral *trans*-acting functions to confer α or β identity on a particular gene.

Herpesvirus γ genes are expressed in the greatest quantities late in infection, but their synthesis is not strictly dependent on viral DNA replication (18, 19, 41). Although studies on the mechanisms regulating herpesvirus α and β gene expression have been reported, the mechanism of regulating γ gene expression has not been resolved. In HSV, at least one β gene function appears to be involved in the control of γ gene expression (9, 18, 19, 41). The principal control of HSV late-gene expression appears to be transcriptional, although DNA replication increases the levels of gene products (8, 17, 18, 21). The *cis*-acting signals for γ gene regulation in HSV have not been identified.

Three γ gene products have been identified by analyses of proteins made during CMV infection (14, 16, 33, 46): the major DNA-binding protein family (ICP36), the matrix (or tegument) protein (ICP27), and the capsid protein (ICP4). We have mapped the ICP36 protein family to a single gene at 0.225 to 0.243 map units on the CMV (Towne) genome (33; see Fig. 1) and have shown that some members of this antigenically related protein family correspond to the previously characterized (14–16) DNA-binding protein of CMV (33). Pande et al. (37) and Nowak et al. (35) have mapped the matrix protein to 0.510 to 0.530 map units.

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In CMV the temporal class of gene expression at the transcriptional level has been defined by examining polysome-associated, rather than whole-cell, RNA (7, 11, 52). Analyses of viral mRNA associated with polysomes at late times in infection (60 to 72 h postinfection [p.i.]) have shown that late RNA is qualitatively and quantitatively different from that associated with polysomes at earlier times in infection (12 to 24 h p.i.). In contrast to polysome RNA, whole-cell viral RNA is similar at early and late times (52). It has therefore been suggested that CMV γ gene expression is influenced by posttranscriptional or translational events (7, 11, 52; W. F. Goins, T. Hermiston, and M. F. Stinski, personal communication).

In this communication we show that posttranscriptional events control the expression of CMV γ genes. The combination of available monoclonal antibody reagents, mapped genes (33, 35, 37), and identification of predominant transcripts (33, 35; this paper) enables a sensitive analysis of transcription and translation of ICP27 and ICP36 during viral infection. Our conclusions, that the principle regulatory determinants of these two CMV γ genes are posttranscriptional, place CMV in sharp contrast with the other well-studied herpesviruses, such as HSV. Although posttranscriptional events affect late-gene expression in other DNA animal viruses, the high level of transcriptional activation of late genes at early times and the importance of posttranscriptional events as primary determinants regulating gene expression have not been observed in any system. We demonstrate that transcripts similar to those detected on polysomes at late times in infection are made in abundant quantities at early times (by 4 h p.i.), when only small amounts of these gene products are being made. We present a hypothesis for the differential regulation of CMV β and γ genes which envisions viral or host-cell functions interacting with mRNA to direct its transport or translation or both.

MATERIALS AND METHODS

Virus and cell culture. Human foreskin fibroblast (HF) cells were prepared in this laboratory and grown in Dulbecco

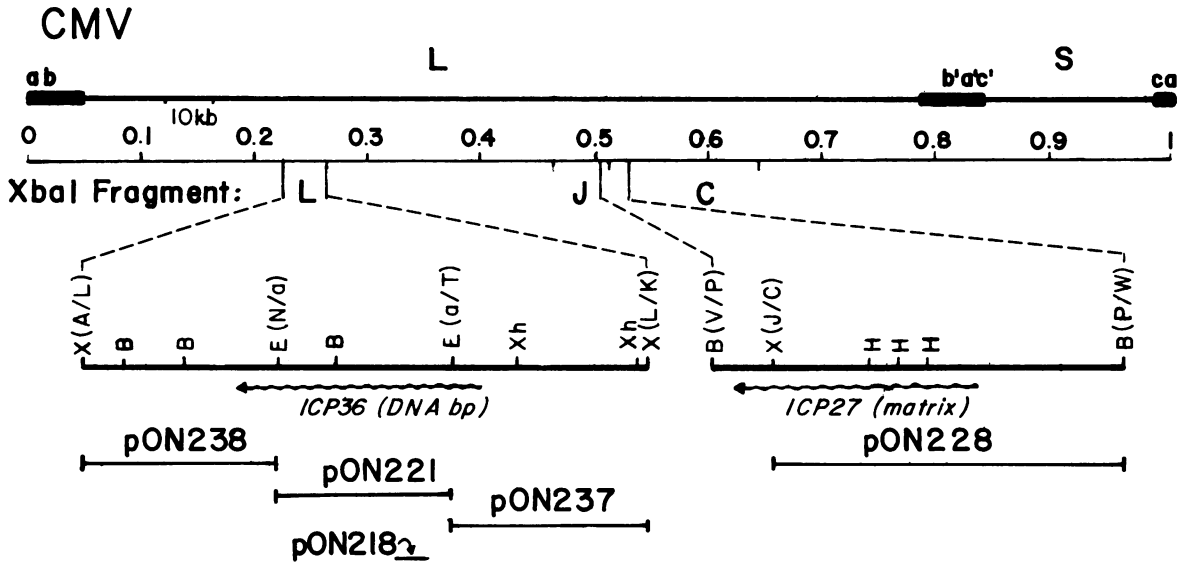


FIG. 1. Plasmid clones derived from the CMV (Towne) genome and used in transcription analyses of the ICP36 and ICP27 genes. (Top) Sequence arrangement and map positions of *Xba*I fragments L, J, and C within the long (L) component of the CMV genome (33). Also indicated are the short (S) component and the repeated elements (*a*, *b*, *c*) flanking the L and S components. (Bottom) *Bam*HI (B), *Eco*RI (E), and *Xho*I (Xh) map of *Xba*I-L; *Xba*I (X) and *Hin*dIII (H) maps of *Bam*HI-P (spanning the *Xba*I J/C junction). The letters in parentheses indicate the restriction fragments spanning relevant sites on the CMV genome. Plasmid clones pON238, pON221, pON218, pON237, and pON228 are described in the text. Arrows indicate the position and direction of the transcripts of ICP36 (this paper) in *Xba*I-L and of ICP27 (35) in *Bam*HI-P.

minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% Nu serum (Collaborative Research, Inc., Waltham, Mass.). Infection with CMV (Towne) was performed at a multiplicity of infection of 5 to 10 PFU per cell, using frozen stocks of CMV as previously described (43).

Recombinant plasmids. The procedures used for ligation, transformation of *Escherichia coli* HB101, analysis of clones, restriction enzyme analysis, and large-scale preparation of plasmid DNA have been described previously (33, 43). The plasmid pON218, which carries a small (~500-bp) insert from the coding sequences of ICP36, and pON221, which carries the *Eco*RI a fragment of the CMV (Towne) genome, were previously described (33). pON237 was prepared by subcloning the 3.2-kbp fragment located between the *Xba*I L/K and *Eco*RI a/T sites, and pON238 was prepared by subcloning the 3.1-kbp fragment located between the *Xba*I A/L and *Eco*RI N/a sites on the CMV (Towne) genome from pXba L (51) into the *E. coli* plasmid vector pMT11 (44; K. Moore, unpublished data). pON228 was prepared by digesting pXbaC (51) with *Xba*I and *Bam*HI, religating, and examining transformants for clones carrying a 5.5-kbp fragment located between the *Xba*I J/C and *Bam*HI P/W sites on the CMV (Towne) genome (see Fig. 1). This clone contains most of the ICP27 gene.

ICPs: radiolabeling and electrophoretic separation. HF cells were infected with 10 PFU per cell and radiolabeled with [^{35}S]methionine (200 μCi/ml; Amersham Corp., Arlington Heights, Ill.) in methionine-free medium (Irvine Scientific, Inc.) as previously described (33). For immediate-early (α) infected-cell proteins (ICPs), cells were treated with 50 μg of cycloheximide (CH) per ml from 1 h before infection to 7 h p.i., when CH was removed and [^{35}S]methionine was added for two more hours. Delayed-early (β) ICPs were made from cells treated with CH from 4 to 7 h p.i. and radiolabeled for 2 h (7 to 9 h p.i.) or from cells treated with 200 μg of phosphonoacetic acid (Sigma Chemical Co., St. Louis, Mo.) per ml from 1 to 20 h p.i. and radiolabeled during the final 2 hs. Other ICP samples prepared without drug blocks were either radiolabeled from 1 h p.i. to harvest (2-, 4-, and 8-h samples) or for the final 2 h before harvest (20-, 60-, 72-, and 120-h samples).

Radiolabeled cells were solubilized directly for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 9.25% gels as described previously (34). Cytoplasmic and nuclear fractions were prepared by adding 1 ml of 1% Nonidet P-40–0.5% deoxycholate–1 mM EDTA–10 mM Tris hydrochloride (pH 7.5) to a washed (10 mM Tris hydrochloride [pH 7.6], 1 mM EDTA) 25-cm^2 monolayer of radiolabeled cells, incubating on ice for 1 min, and then separating by brief centrifugation (5 s) in a microcentrifuge. The supernatant cytoplasmic fraction and pelleted nuclei were solubilized for SDS-PAGE as described previously (34). Extracts for immunoprecipitation were prepared by scraping cells into 1% Nonidet P-40–0.5% deoxycholate–140 mM NaCl–20 mM Tris hydrochloride (pH 7.5) and sonicating for 20 s with a probe-type sonicator (Ultrasonics, Inc.).

Immunoblot and immunoprecipitation. Monoclonal antibodies to ICP36 (CH16-1 and CH13-2) and to ICP27 (CH65-1 and CH69-4) were kindly provided as ascites fluids by L. Pereira (38). After electrophoresis, proteins were transferred to nitrocellulose as described previously (3). The immobilized proteins were pretreated with 3% bovine serum albumin (BSA) in 10 mM Tris hydrochloride (pH 7.5)–150 mM NaCl–0.1% Nonidet P-40 (TN) for 1 h, incubated with a 1:200 dilution of the monoclonal antibodies in TN containing 3% BSA for 1 h at 4°C, and washed three times with 200 ml of TN containing 1% BSA. After incubation for 1 h in biotinylated goat anti-mouse antibody (Tago) diluted 1:440 in TN containing 3% BSA and washing three times with 200 ml of TN with 1% BSA, bound antibody was detected with streptavidin and biotinylated alkaline phosphatase (Bethesda

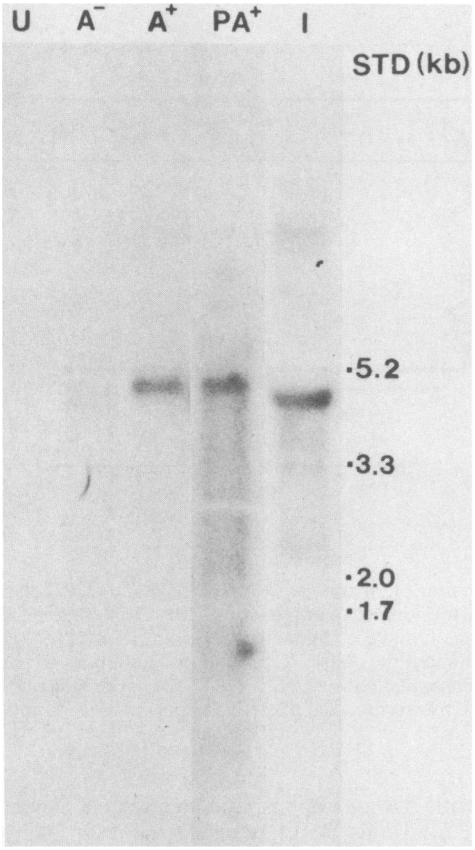


FIG. 2. RNA blot analysis of the ICP36 mRNA and its association with polysomes. Infected-cell RNA samples from 72 h p.i. were separated on 1.0% agarose–2.2 M formaldehyde gels, transferred to nitrocellulose, and then hybridized with ^{32}P-labeled pON221 as described in the text. Lanes: U, 10 μg of uninfected cell RNA; A$^-$, 10 μg of poly(A)$^-$ late CMV:infected-cell RNA; A$^+$, 3 μg of poly(A)-selected late CMV-infected-cell RNA; PA$^+$, 2 μg of late CMV-infected-cell, polysome-associated, poly(A)-selected RNA; I, 10 μg of late infected-cell RNA. Molecular weight standards (STD) are 28S rRNA (5.2 kb), 26S rRNA (3.3 kb), 18S rRNA (2.0 kb), and 16S rRNA (1.7 kb). The mobility of the ICP36 mRNA is altered in lane I because of close migration to the 5.2-kb rRNA present in the sample.

Research Laboratories, Inc., Gaithersburg, Md.) as described by the manufacturer, except that 1% BSA was added to the streptavidin incubation step. Immunoprecipitations were carried out as described previously (3, 4, 38).

RNA extraction and gel electrophoresis. Whole-cell RNA was prepared by guanidinium isothiocyanate solubilization (6). Polysomal RNA was collected by the method of Palmiter (36) as modified by Stringer et al. (50), except that the cytoplasmic fraction was not clarified after separation from nuclei.

Selection of poly(A)$^+$ RNA was by established techniques (28). Whole-cell or polysomal RNA (10 to 20 μg) or poly(A)$^+$ RNA (2 to 3 μg) was heated to 68°C for 5 min and separated by electrophoresis through 1.0% agarose gels containing 2.2 M formaldehyde (28) with recirculation of the running buffer. In some cases, the RNA gels were subjected to partial alkaline hydrolysis (28) before transfer to nitrocellulose. The immobilized RNA was then prehybridized at 58°C in 6× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate)–30% formamide–0.1% SDS–0.2% polyvinylpyrrolidone–0.2% BSA–0.2% Ficoll (hybridization buffer) for 1 h, hybridized overnight at 58°C with ^{32}P-labeled, denatured DNA probe in hybridization buffer plus 50 μg of unlabeled salmon sperm DNA, and washed as described previously (28).

Nuclease S1 analysis was performed with whole-cell or polysome-associated CMV-infected-cell RNA (isolated from infected cells at 48 or 72 h p.i.), referred to as late CMV-infected-cell RNA or as late CMV-infected-cell polysome-associated RNA, respectively (2). We followed modifications in the procedure allowing for the (high) guanine plus cytosine composition of CMV DNA (45). Late CMV-infected-cell poly(A)$^+$ RNA (8 μg) was mixed with 200 μg of carrier tRNA and ^{32}P-end-labeled DNA, heated to 85°C for 15 min, and hybridized at 60°C for 3 to 4 h. DNA fragments were end labeled with either [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham) and polynucleotide kinase (New England BioLabs, Inc., Beverly, Mass.) or [α-^{32}P]dCTP or [α-^{32}P]dATP (>3,000 Ci/mmol; Amersham) and DNA polymerase I Klenow fragment (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) as described previously (32, 43). RNA-DNA hybrids were exposed to 330 U of S1 nuclease (Bethesda Research Laboratories) for 30 min at 37°C, and after digestion the mixture was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and precipitated with isopropanol. The protected DNA was analyzed by electrophoretic separation in neutral agarose or denaturing polyacrylamide gels (28, 29), dried, and autoradiographed on Kodak XAR or RP film.

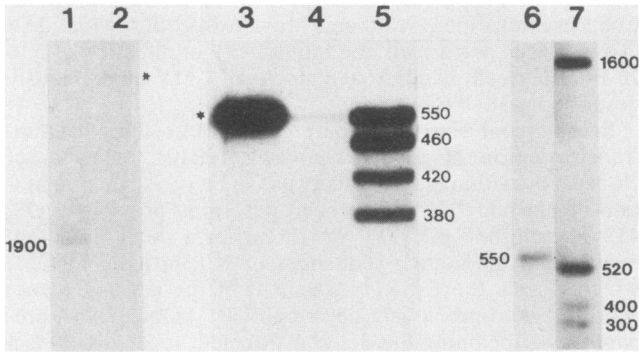


FIG. 3. Domain, direction, and start site of transcription of ICP36 mRNA. (Left lanes) S1 analysis of late infected-cell RNA to determine the direction of transcription of the ICP36 mRNA. A 4.5-kbp *Bam*HI fragment from pON221 was end labeled as described in the text, and after S1 digestion, the protected fragments were analyzed on a 1.0% agarose gel. Lanes: 1, 5′-end-labeled DNA fragment; 2, 3′-end-labeled DNA fragment. (Middle lanes) S1 analysis of late poly(A)$^+$ RNA to determine the start site of transcription. A *Bgl*II-*Kpn*I fragment from pON237 was 5′ end labeled at a unique *Bgl*II site (within the transcribed region of the ICP36 gene 88 bp 5′ to the *Eco*RI a/T site shown in Fig. 1) and digested with *Kpn*I (300 bp 3′ to the *Xho*I site shown in Fig. 1), and this 550-bp fragment was used in an S1 analysis for the start site of transcription. Protected fragments were run on a denaturing 5% polyacrylamide gel. Lanes: 3, native 550-bp *Bgl*II-*Kpn*I fragment; 4, S1 analysis with 100 μg of uninfected-cell RNA; 5, S1 analysis with 8 μg of 72-h poly(A)$^+$ RNA. (Right lanes) S1 analysis of late infected-cell RNA to detect the 3′ boundary of the ICP36 transcript with pON238 3′ end labeled at the *Eco*RI N/a site (Fig. 1). After annealing and S1 digestion, the products were analyzed on a 1.5% agarose gel. Lanes: 6, S1 analysis with pON238; 7, size markers (base pairs). All gels were dried and autoradiographed. Asterisks indicate renaturation of probes.

RESULTS

ICP36 mRNA: domain and association with polysomes. We had previously (33) determined the map position (Fig. 1) of the gene encoding the ICP36 family of proteins. Previously described characteristics, including phosphorylation of the 48-kilodalton (kDa) (ICP36b) and 49-kDa (ICP36a) species, time of synthesis, and nuclear accumulation, indicated that ICP36 corresponded to the major DNA-binding protein of CMV previously described by Gibson and co-workers (14–16). To study the temporal expression of ICP36, we first determined whether the 4.8-kb transcript previously identified (33) and mapped to 0.225 to 0.243 map units (Fig. 1) was the mRNA for ICP36 by examining its association with polysomes at late times in infection. RNA was extracted from infected cells at 72 h p.i. (multiplicity of infection of 5 PFU per cell) and processed to prepare whole-cell RNA or polysomal RNA as described in Materials and Methods. From these RNA samples, poly(A)$^+$ RNA was further fractionated. Figure 2 shows an autoradiograph of these RNA samples immobilized on nitrocellulose after electrophoretic separation and hybridized with radiolabeled pON221 probe. The 2.8-kbp *Eco*RI a fragment carried in pON221 is homologous to a region within the ICP36 transcript (Fig. 3) (33; unpublished observations). The 4.8-kb transcript is recovered in the polysome-associated poly(A)$^+$ fraction (Fig. 2). In conjunction with our previous studies (33), which showed that this transcript directed the synthesis of the predominant 48- to 49-kDa (ICP36a or -b, respectively) member of the ICP36 family when hybrid selected and translated in vitro, these data strongly suggest that the 4.8-kb transcript is the ICP36 mRNA.

The direction of transcription of the ICP36 mRNA was determined by nuclease S1 analysis of late whole-cell RNA with denatured, ^{32}P-end-labeled DNA probes. Two different probes were made from a 4.5-kbp *Bam*HI fragment which spans the larger 1.9-kbp *Bam*HI-*Eco*RI fragment of pON221 and extends through the plasmid vector sequences, by either 5′ or 3′ end labeling the *Bam*HI site. After hybridization with each of these probes separately and S1 digestion as described in Materials and Methods, a single 1.9-kb DNA species was protected with the 5′-end-labeled probe (Fig. 3). These results established that the direction of transcription

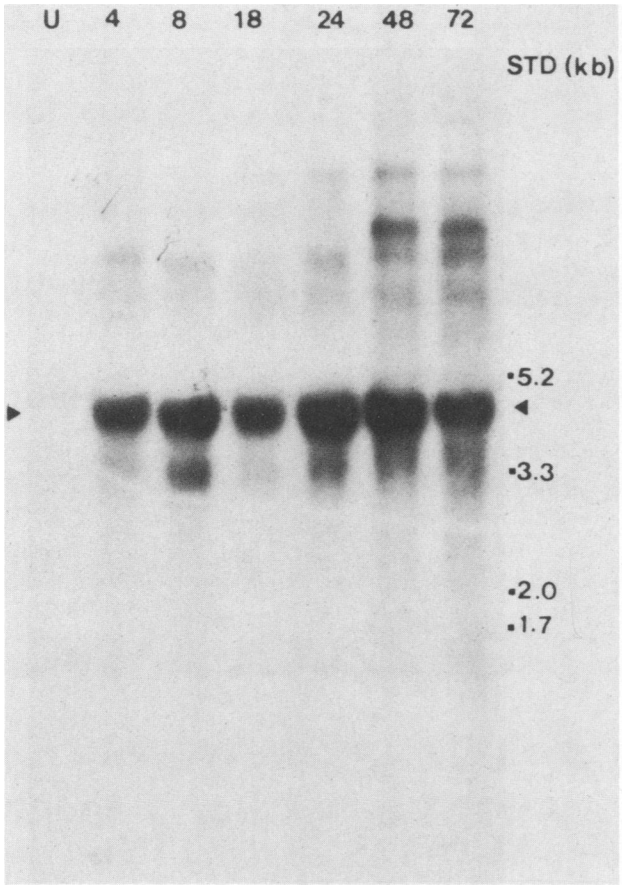


FIG. 4. RNA blot hybridization analysis of the time course of transcription of the ICP36 gene. Whole-cell RNA (10 μg) from uninfected (U) HF or at 4, 8, 18, 24, 48, or 72 h p.i. was electrophoretically separated on 1% agarose gels containing 2.2 M formaldehyde. After transfer to nitrocellulose, immobilized RNA was hybridized to ^{32}P-labeled pON218. An autoradiogram is shown with the positions of human and bacterial rRNA standards (STD) indicated on the right. This analysis showed that the 4.8-kb transcript (arrowheads) of ICP36 was found abundantly by 4 h p.i. and remained a relatively constant proportion of the total RNA through 72 h p.i. The high-molecular-weight species of RNA detectable at 48 and 72 h p.i. represent transcripts that pass trough this region of the genome (unpublished observations).

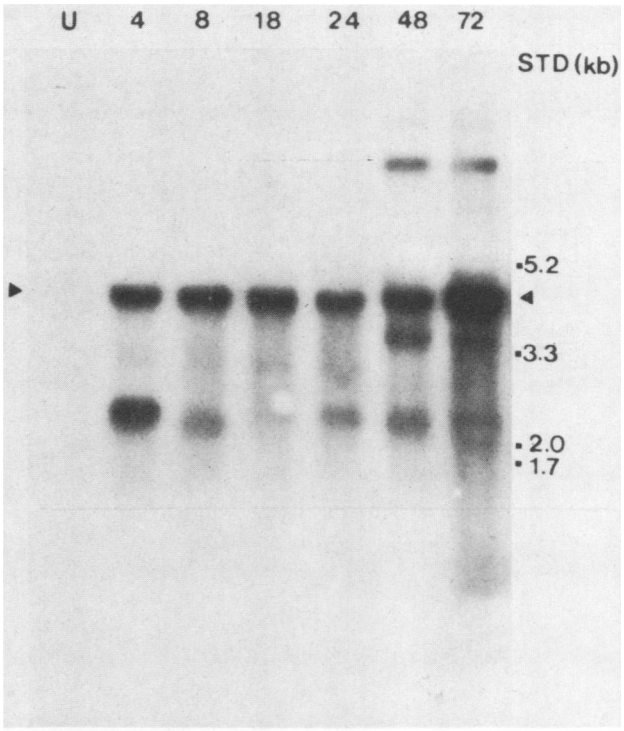


FIG. 5. RNA blot hybridization analysis of the time course of transcription of the ICP27 gene. Whole-cell RNA (10 μg) from uninfected (U) HF or at 4, 8, 18, 24, 48, or 72 h p.i. was electrophoretically separated on 1% agarose gel containing 2.2 M formaldehyde and after transfer to nitrocellulose was hybridized to ^{32}P-labeled pON228. An autoradiogram is shown with the positions of human and bacterial rRNA standards (STD) indicated on the right. These results were confirmed with a 1.5-kbp probe (*Xba*I-*Hin*dIII; Fig. 1) derived from pON228, a probe that was specific for the 4.6-kb transcript (data not shown). This analysis showed that the ICP27 transcript (arrowheads) was present by 4 h p.i. and remained at relatively constant levels through 72 h p.i.

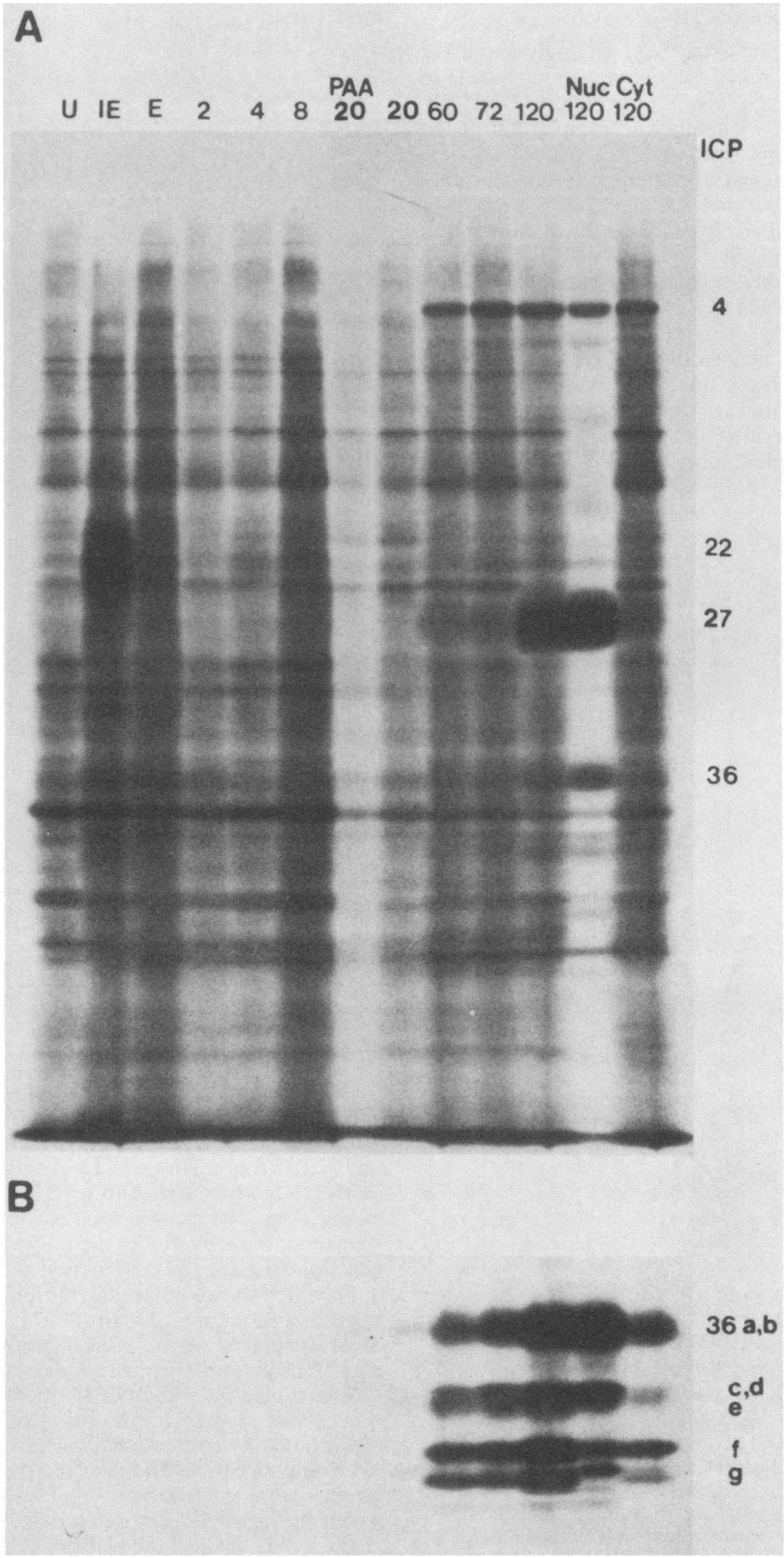


FIG. 6. SDS-PAGE and immunoblot analyses of ICP36 expression. (A) Autoradiogram of ^{35}S-labeled proteins. Whole-cell proteins were ^{35}S-labeled and prepared from uninfected (U) and infected cells at 2, 4, 8, 20, 60, 72, and 120 h p.i. Immediate-early (lane IE) proteins were made as described in the text. Early proteins were made after treatment of cells with CH from 4 to 7 h p.i. (lane E) or at 18 to 20 h p.i. while DNA synthesis was inhibited by phosphonoacetic acid (lane PAA 20). Separated nuclear (lane Nuc 120) and cytoplasmic (lane Cyt 120) proteins were prepared at 120 h p.i. as described in the text. After SDS-PAGE, proteins were transferred to nitrocellulose, and the resulting

of the ICP36 transcript was from right to left on the viral genome as depicted in Fig. 1.

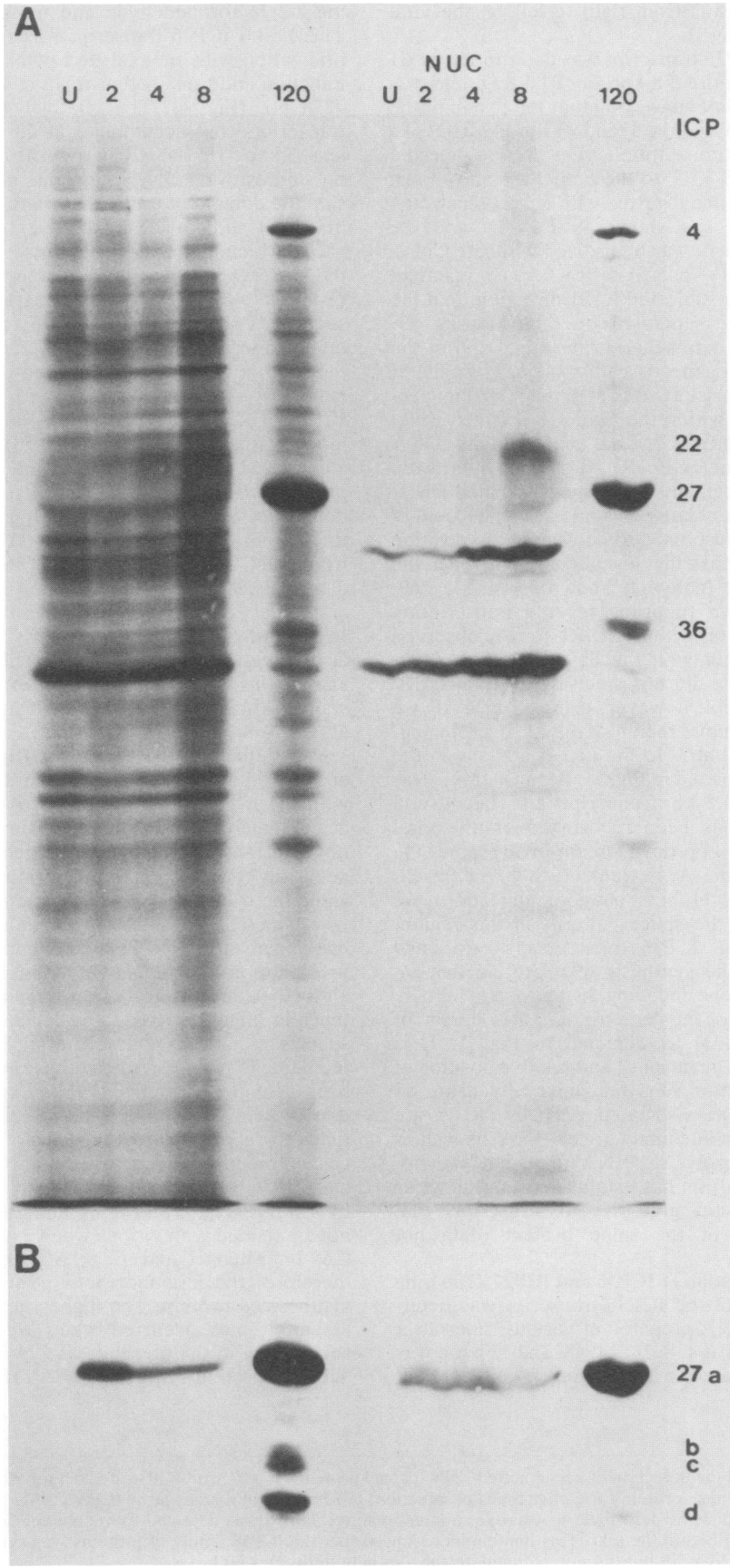
The domain of the ICP36 transcript was determined by S1 nuclease analysis. The entire 2.8-kpb *Eco*RI a fragment was protected (data not shown), suggesting that the 5′ end of the transcript was in *Eco*RI-T and the 3′ end was in *Eco*RI-N. To define the 5′ start site of transcription, pON237 was digested with *Bgl*II (which cuts 88 bp 5′ to the *Eco*RI a/T site shown in Fig. 1), 5′ end labeled, and digested with *Kpn*I (which cuts 300 bp 3′ to the *Xho*I site shown in Fig. 1), and the appropriate 550-bp fragment was hybridized with late CMV-infected-cell or late CMV poly(A)$^+$ RNA. The resultant RNA-DNA hybrids were subjected to S1 digestion, and the protected products were separated on a denaturing 5% polyacrylamide gel. Our data suggest that the 5′ end of the ICP36 transcript is heterogeneous with a major start site at 460 nucleotides and minor start sites at 420 and 380 nucleotides from the *Bgl*II site within the *Eco*RI T fragment (Fig. 3). This data was confirmed by S1 analysis with DNA fragments 5′ end labeled at the *Eco*RI a/T site and hybridized with late CMV-infected-cell and polysome-associated RNA. Primer extension analysis of late polysome-associated and of late poly(A)$^+$ RNA was also used to determine the position of the 5′ ends and to establish that no splicing occurred at the 5′ end of the transcript (data not shown). Using a 260-nucleotide DNA fragment to prime reverse transcription from the same *Bgl*II site within *Eco*RI-T, we observed cDNA products consistent with the S1 protection experiment shown in Fig. 3. We did not precisely position the 3′ end of the ICP36 transcript; however, using S1 analysis we determined that a continuous region of 3.9 kb is protected, extending to a region 550 nt 3′ to the *Eco*RI a/N site in the *Eco*RI N fragment (as depicted in Fig. 1). Because this is less than the size of the 4.8-kb transcript and because a polyadenylation signal has been recognized on the viral genome approximately 3 kbp downstream of this point (T. Kouzarides, K. M. Weston, A. T. Bankier, S. C. Satchwell, P. Tomlinson, and B. Barrel, personal communication), we are currently examining the splice patterns in this region. Thus, the 5′ end of the ICP36 transcript is positioned appropriately to direct the synthesis of a protein that includes the region of the gene encoding the epitope(s) initially used to isolate a portion of the gene from a λgt11 library of CMV DNA fragments (33) as depicted in Fig. 1. It is important to note that the occurrence and relative position of the three transcription start sites recognized by using S1 analysis on late polysome-associated poly(A)$^+$ RNA are identical to those found in late infected-cell RNA, as well as early (8 h) infected-cell poly(A)$^+$ RNA (data not shown). Nucleotide sequence analysis has established that all three transcription initiation sites give rise to transcripts that would direct synthesis of the same protein (data not shown).

Time course of transcription of ICP36 and ICP27. The time course of accumulation of the ICP36 transcript was determined with whole-cell RNA prepared at various times after infection. RNA from cells at 4, 8, 18, 24, 48, and 72 h p.i. was electrophoretically separated in 1.0% agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose. The 4.8-kb ICP36 transcript was readily detectable by hybridization with radiolabeled pON218 as early as 4 h after infection and remained at relatively constant levels through 72 h p.i. (Fig. 4). The relatively large, minor species of transcripts that accumulate at 48 and 72 h p.i. initiate at least 3 kbp 5′ to the ICP36 transcription initiation sites based on studies using DNA fragments derived from regions outside the domain of the ICP36 transcript to probe RNA blots (data not shown). The primer extention studies described above which map the ICP36 transcription initiation sites and the observation that these larger species initiate a long distance (>3 kbp) from the apparent ICP36 protein-coding sequences make it highly unlikely that these transcripts are involved in the expression of ICP36.

Pande et al. (37) demonstrated that the gene encoding a major 64-kDa structural protein with characteristics of ICP27 of CMV (Towne) is encoded near the *Hin*dIII H/N junction (map position, 0.51 to 0.53). Nowak et al. (35) found a comparable map position for the analogous 65-kDa virion protein of CMV (AD169). They also determined the size and domain of the transcript encoding ICP27. On the basis of their results, we used pON228 and DNA fragments derived from pON228 as probes to detect ICP27-specific transcripts. Our analysis (Fig. 5) of whole-cell RNA from various times after infection revealed that, like the ICP36 transcript, the ICP27 transcript appeared as early as 4 h p.i. and remained at relatively constant levels through 72 h p.i. The apparent size of the ICP27 transcript on formaldehyde-agarose gels was 4.6 kb, larger than the 4.0-kb size reported by Nowak et al. (35).

Translation of ICP27 and ICP36 during infection. The accumulation of ICP27 and ICP36 transcripts to high levels within 4 h p.i. when the respective gene products do not accumulate until nearly 2 days later (14, 46) suggests that a novel means of regulation of gene expression might be occurring through posttranscriptional activities. We investigated the translation of these gene products to determine at what time ICP36 and ICP27 accumulate in infected cells. To maximize sensitivity we used immunoblotting and immunoprecipitation techniques in conjunction with monoclonal antibodies specific for either protein. As expected, at late times in infection ICP27 and ICP36 appeared as prominent species in nuclear extracts of [^{35}S]methionine-labeled infected cells (Fig. 6), where these proteins have previously been detected as phosphorylated species (14, 16, 33). Neither protein was synthesized in sufficient amounts at early times to detect by direct analysis of [^{35}S]methionine-labeled cell extracts; however, to our initial surprise, both ICP27 and ICP36 were detected as weakly immunoreactive species by 4 h p.i. (Fig. 6 and 7) by immunoblot analysis. ICP36 is not associated with virions when examined by this technique (16; N. Michael and E. S. Mocarski, unpublished data); therefore, the immunoreactive species represented newly synthesized protein. The highest level of ICP36 expression at early times occurred when we allowed transcripts to accumulate in the presence of CH from 4 to 7 h p.i. before allowing protein synthesis for 2 h (Fig. 6B, lane E).

autoradiogram demonstrated the detection of accumulated ICP4 (capsid protein), ICP27, and ICP36 at 120 h p.i. predominantly in the nucleus and the inability to identify these proteins at earlier times of infection. (B) Immunoblot analysis of ICP36. After the proteins were prepared and transferred to nitrocellulose as described in A above, immunoreactive ICP36 was detected by incubation with monoclonal antibodies CH16-1 and CH13-2 as described in the text. This immunoblot showed that the ICP36 family of proteins was synthesized in small amounts at early times (lanes E and PAA 20) but did not accumulate substantially until after 60 h p.i.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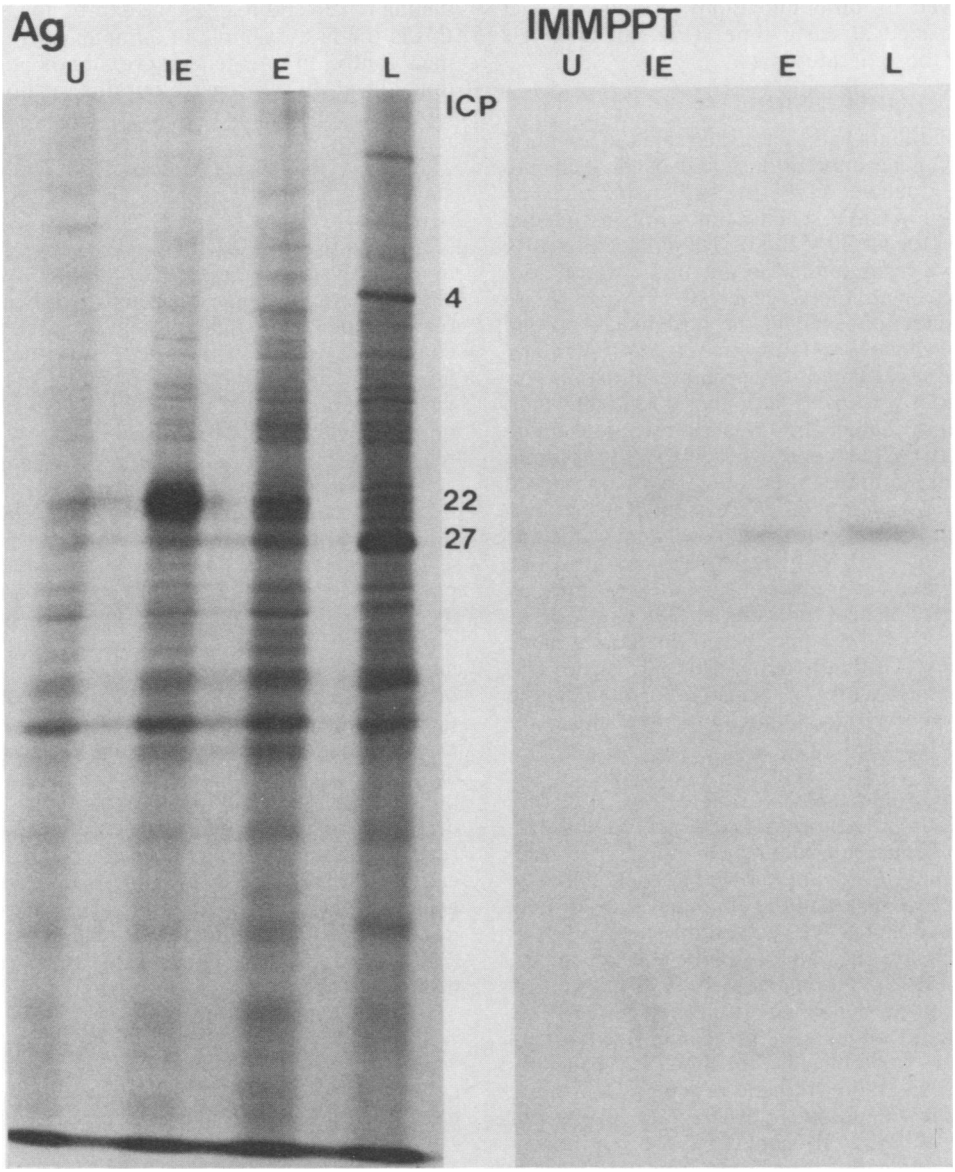

FIG. 8. Immunoprecipitation analysis of ICP27. ^{35}S-labeled proteins from uninfected (U) HF or from HF after labeling of immediate-early (IE), early (E), or late (L, 120 h p.i.) proteins as described in the legend to Fig. 6 were separated by SDS-PAGE before (Ag) or after (IMMPPT) immunoprecipitation with monoclonal antibodies CH65-1 and CH69-4. The detection of ^{35}S-labeled ICP27 in the immunoprecipitated samples indicated that ICP27 was synthesized at early as well as late times of infection. Twenty times more extract was used for the immunoprecipitation of uninfected, immediate-early, and early proteins as was used for late proteins.

Unlike ICP36, ICP27 is a structural component of virions (16, 37). Thus, it was likely that some or all of the immunoreactive protein was introduced by infecting virions. To establish whether ICP27 is synthesized at early times, we allowed transcripts to accumulate in the presence of CH from 4 to 7 h p.i. before [^{35}S]methionine pulse-labeling for 2 h and immunoprecipitating with monoclonal antibodies CH65-1 and CH69-4. Separation of immunoprecipitates by

FIG. 7. SDS-PAGE and immunoblot analyses of ICP27 expression. (A) Autoradiograph of ^{35}S-labeled proteins. ^{35}S-labeled proteins from uninfected (U) whole cells (left lanes) or nuclei (right lanes, labeled NUC) at 2, 4, 8, or 120 h p.i. were separated by SDS-PAGE, transferred to nitrocellulose, and autoradiographed. ICP4, ICP27, and ICP36 are prominent at 120 h p.i. but are not detectable at 8 h p.i. or before. (B) Immunoblot analysis of ICP27. After separation and transfer to nitrocellulose as described in A above, immunoreactive ICP27 was detected with monoclonal antibodies CH65-1 and CH69-4, streptavidin, and biotinylated alkaline phosphatase. Since ICP27 is a structural component of virions, detection of this protein at 2 h p.i. may have resulted from introduction by infecting virions and not from synthesis of ICP27 at early times of infection.

SDS-PAGE followed by autoradiography established that ICP27 was synthesized at early times (Fig. 8), at levels approximately 5% those of late times.

DISCUSSION

Previous work by others had established that the temporal regulation of CMV gene expression could be divided into immediate-early (α), delayed-early (β), and late (γ) based on differences observed in CMV-specific transcripts associated with polysomes (7, 10, 11, 30, 52, 53). The immediate-early genes of CMV have been studied in detail (20, 45, 49, 54) and, as is the case with the α genes of HSV, they are transcribed after infection even in the absence of protein synthesis. We have shown previously that, as in HSV, CMV α and β genes are regulated by promoter-proximal *cis* elements that interact with *trans*-acting viral functions (44). Here we examined, in detail, the transcriptional and translational activity of two CMV genes whose products accumulate to high levels at late times after infection. We observed posttranscriptional regulation of expression of these two late-gene products. Our results are consistent with the coordinate control of CMV γ gene expression; however, transcriptional activation of γ genes occurs at early times, by 4 h p.i., preceding full gene expression by 2 days. Furthermore, it is possible that there may be no distinction made between the transcriptional activation of β and γ genes (11, 30, 31, 44, 52). The mechanism responsible for the delay in appearance of late-gene products may be a novel means for regulating gene expression.

While investigating the time course of transcription of the ICP36 gene during productive CMV infection, we were surprised to detect, at early times, a high level of hybridization to a transcript appearing identical to the ICP36 transcript. Both ICP36 and ICP27 are known to accumulate late in infection, and yet we detected the abundant transcription of their genes at early times. We showed that the 5′ ends of the ICP36 transcript are the same at early and late times, which suggests that transcriptional activation of this gene is occurring long (2 days) before the transcript directs the synthesis of abundant amounts of protein. We confirmed that this also appears to be the case for the ICP27 gene. By assaying for the accumulation of gene products from these genes, we established that low (<5% of late levels) but detectable amounts of both ICP36 and ICP27 are synthesized by 4 h p.i. The accumulated transcripts from these genes represent a relatively constant proportion of total cellular RNA from early through late times in infection, yet the protein products are only faintly detectable at early times. At late times, however, ICP27 and ICP36 are among the most prominent of all infected-cell proteins, supporting the notion that posttranscriptional or translational controls are major factors in their regulation. As previously noted by others studying general patterns of CMV transcription, whole-cell RNA is homologous to extensive regions of the viral genome at early or late times even though polysome-associated RNA shows evidence of differential gene expression (7, 11, 52). Although extensive transcription at early times could be due to overlapping transcriptional units or differential splicing with accumulation of precursors, we showed here that the transcription units expressed appear to be the same as those destined to be fully expressed after the posttranscriptional regulatory step occurs. It has been previously suggested that nuclear transport plays an important role in determining which viral genes are expresssed in CMV-infected cells (11, 52; W. F. Goins, T. Hermiston, and M. F. Stinski, personal communication). We did not determine the mechanism regulating ICP36 and ICP27 gene expression. To our knowledge this is the first example of an animal virus whose predominant control of late-gene expression is at the posttranscriptional level. In this regard, our observations point to a clear biological distinction between HSV and CMV late-gene regulation.

The physiological significance of our observations that small amounts of late proteins are synthesized at early times is unknown. The CMV replication cycle is extremely long relative to that of HSV; however, as we showed, transcriptional acitivation of at least two γ genes occurs by 4 h p.i. In HSV infection, a matrix protein has been shown to be a regulator of α gene transcription (1, 5, 39). Similarly, a virion component of CMV plays a role in the regulation of the major immediate-early gene of CMV (44, 48). ICP27 is a virion component and is efficiently delivered to the nucleus of the infected cell. It is possible that ICP27 effects some regulatory function. To clearly relate expression of these genes to particular functions during viral infection, it will be necessary to derive viruses expressing conditionally functional gene products.

The observations we made in this study as well as our previous studies (44) lead us to propose a hypothesis on the mechanism of regulating gene expression during CMV infection. After infection, a virion component activates α gene expression, as is the case for HSV. After expression of one or several α gene products, all other transcriptional units in the CMV genome are activated; however, posttranscriptional regulatory factors encoded or induced by the virus determine the temporal order of β and γ mRNAs that are pulled from the pool of transcripts and expressed. The dramatic biological differences between CMV and HSV replication, including the species specificity of CMV replication, the fact that CMV gene expression is limited to α gene products in nonpermissive cells (47), and the observation that only differentiated cells can act as permissive hosts (23, 47), may all be related to this novel regulatory mechanism.

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