JOURNAL OF VIROLOGY, Feb. 1997, p. 981–987
0022-538X/97/$04.00+0
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Vol. 71, No. 2

Translational Regulation of the Human Cytomegalovirus pp28 (UL99) Late Gene

JULIE A. KERRY,[1] MORRIS A. PRIDDY,[1] CATHERINE P. KOHLER,[1] TERRI L. STALEY,[1] DEBORAH WEBER,[1] THOMAS R. JONES,[2] AND RICHARD M. STENBERG[1]*

Department of Microbiology and Immunology, Eastern Virginia Medical School, Norfolk, Virginia 23501,[1] and Department of Molecular Biology, Infectious Diseases Section, Wyeth-Ayerst Research, Pearl River, New York 10965[2]

Received 25 July 1996/Accepted 17 October 1996

The pp28 (UL99) gene of human cytomegalovirus is expressed as a true late gene, in that DNA synthesis is absolutely required for mRNA expression. Our previous studies demonstrated that pp28 promoter sequences from position −40 to +106 are sufficient for late gene expression in the context of the viral genome (C. P. Kohler, J. A. Kerry, M. Carter, V. P. Muzithras, T. R. Jones, and R. M. Stenberg, J. Virol. 68:6589–6597, 1994). To extend these studies, we have examined the sequences in the downstream leader region of the pp28 gene for their role in late gene expression. Deletion of sequences from position −6 to +46 (ΔSS) results in a threefold increase in gene expression in transient assays. In contrast, deletion of sequences from position +46 to +88 (ΔA) has little effect on gene expression. These results indicate that the sequences from position −6 to +46 may repress gene expression. To further analyze this region, site-directed mutagenesis was performed. Mutation of residues from either position +1 to +6 (SS1) or position +12 to +17 (SS2) duplicated the effect of the ΔSS deletion mutant, indicating that sequences from position +1 to +17 were important for the inhibitory effect. To assess the biological significance of these events, a recombinant virus construct containing the ΔSS mutant promoter regulating expression of the chloramphenicol acetyltransferase (CAT) reporter gene was generated. Analysis of this virus (RVΔSSCAT) revealed that deletion of sequences from position −6 to +46 does not alter the kinetic class of this promoter. However, the ratio of CAT protein to CAT mRNA levels in RVΔSSCAT-infected cells was 8- to 12-fold higher than that observed in the parental RV24/26CAT-infected cells. These results imply that the leader sequences within the pp28 gene can regulate the translation of this late gene.

Human cytomegalovirus (HCMV) is a common infectious agent that is a significant pathogen worldwide (3). Infection of newborn infants in utero with HCMV can result in congenital defects such as mental retardation and hearing loss. In addition, HCMV is a major cause of complications in AIDS and organ transplant patients. Primary infection or reactivation of latent HCMV in immunosuppressed or immunocompromised patients results in severe disease, including retinitis, pneumonia, and encephalitis. An understanding of the mechanisms of HCMV replication and reactivation should lead to improved strategies for combating and treating HCMV disease.

Much remains to be learned regarding the mechanisms of regulation of HCMV replication. However, it is known that both viral and cellular factors can cooperate to regulate viral gene expression (15, 16, 22, 25, 26, 33, 40, 41, 43, 50). Three sequential phases of viral gene expression have been defined: immediate early (IE), early, and late. Immediate-early proteins, in association with cellular transcription factors, are responsible for the activation of viral early gene expression (22, 40–42, 45, 47). Recently, it has been demonstrated that the expression of early genes can also be stimulated by other viral regulatory proteins, including IRS1, UL36-38, UL69, and UL112-113 (4, 16, 23, 44, 53). Late gene expression is defined as that which occurs after viral DNA replication. Although early gene regulation has been extensively studied in recent years, very little is known regarding the specific mechanisms of late gene regulation in HCMV-infected cells. In part, this may be due to difficulty in studying late gene promoters, as they are promiscuously expressed in transient assays (6, 27). However, our studies on the pp28 promoter show that upstream promoter sequences appear to play little role in regulating the kinetics of this late gene (6). This is similar to what has been observed with herpes simplex virus (HSV) late genes (11, 12, 14, 38). For example, Guzowski et al. (11, 12) demonstrated that sequences required for late expression of the UL38 gene occur within the 5′ untranslated leader sequences. In this case, it was determined that the downstream element was involved in the transcriptional regulation of the UL38 gene.

The leader sequence of at least one HCMV gene has been demonstrated to translationally regulate the downstream open reading frame (ORF) (8, 39). UL4 (gp48) mRNA has a 5′ untranslated leader sequence which contains several AUG codons with short ORFs upstream of the primary ORF. These AUG sequences, as well as the coding region of the short upstream ORF, have been shown to inhibit translation of the downstream ORF. Translational regulation of other HCMV genes has also been reported (7, 54). For example, a delayed appearance of the ICP27 and ICP36 proteins relative to mRNA has been observed in HCMV-infected cells (7). However, the exact mechanism of the translational regulation for these two genes has not been reported.

The UL99 ORF is located in a complex gene region of HCMV from which a series of 3′-coterminal transcripts are expressed from several different promoters (52). The pp28 tegument protein is predicted to be expressed by two mRNAs of 1.6 and 1.3 kb (6). The 1.3-kb mRNA is of relatively low abundance in HCMV-infected cells and contains only the pp28 ORF. Our studies have focused on the promoter for the 1.6-kb mRNA. This mRNA is of high abundance and contains two

* 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. E-mail: RMS@BORG.EVMS.EDU.

potential ORFs. The AUG codon of the first ORF is located 177 bp downstream of the CAP site and corresponds to the carboxy terminus of the UL98 ORF, which encodes the viral alkaline exonuclease enzyme (1). The pp28 ORF begins 476 bp downstream of the CAP site of the 1.6-kb mRNA. Such long 5′ leader sequences can be associated with mRNAs that are regulated by translational mechanisms (13). The present study analyzes the role of downstream sequences in UL99 promoter regulation. These studies indicate that the 5′ leader region of the UL99 gene contains a repressor element. Analysis of the repressor element in the context of the viral genome reveals that this element is involved in the translational regulation of UL99 gene expression.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblast (HF) cells were grown and infected with the HCMV Towne and AD169 strains as previously described (46, 48). The AD169 recombinant virus derivatives RV134 and RV24/26CAT have been described previously (27).

Plasmids. The plasmid constructs p28USdHCAT and pd24/26CAT, containing the UL99 (pp28) 1.6-kb mRNA promoter fragment from position −609 to +106 and position −40 to +106, respectively, have been described elsewhere (6). The ΔSS deletion was generated by digesting p28USdHCAT and pd24/26CAT with *Sst*II and *Sma*I, blunting the ends by mung bean nuclease treatment, and religating. This resulted in the removal of sequences from position −6 to +46. The ΔA deletion was generated by digesting p28USdHCAT and pd24/26CAT with *Ava*I, filling in the ends with Klenow, and religating. This resulted in the removal of sequences from position +46 to +88. The regions deleted in these constructs were confirmed by DNA sequencing.

Oligonucleotide-directed mutagenesis of the UL99 promoter. The SS1, SS2, and SS4 mutations were introduced into the UL99 promoter by direct cloning of oligonucleotide fragments into the pd24/26CAT plasmid as described previously (22). Oligonucleotides were generated that spanned the unique *Sst*II and *Sma*I sites that were used to generate the ΔSS deletion mutant. These oligonucleotides contained 5- or 6-base substitutions that scanned the sequences between these two sites. The oligonucleotides used to generate the SS mutants are as follows (the mutated nucleotides are underlined): SS1, 5′ TCCCCGCGGGCAATATT GCTGGCGGCGGCGCTGATCACCACCTGCGGGGCAGCCCGGGGGA; SS2, 5′ TCCCCGCGGGCGACGGCGCTGGTTTAAACGCTGATCACCACC TGCGGGGCAGCCCGGGGGA; SS4, 5′ CCCCGCGGGCGACGGCGCTG GCGGCGGCGCTGAAATATTCCTGCGGGGCAGCCCGGGGGA; and primer, 5′ TCCCCCGGGCTGC.

Transient expression of the UL99 promoter. p28USdHCAT, pd24/26CAT, and their mutated derivatives (5 μg) were assessed by superinfection experiments using the Towne strain of HCMV (5). In brief, HF cells were transfected with the appropriate reporter plasmid by the DEAE-dextran method. At 18 h after transfection, the cells were superinfected with HCMV strain Towne (5 to 20 PFU per cell) and incubated for 2 h. At this time, the inoculum was removed and the cells were overlaid with fresh medium. Infections in the presence of phosphonoacetic acid (PAA) were performed as previously described (5). At 72 h after infection, the cells were harvested and assessed for chloramphenicol acetyltransferase (CAT) activity (5).

Generation and isolation of recombinant virus. The virus RVΔSSCAT was generated by using our modified protocol as described previously (20, 23, 27). Briefly, the *Hin*dIII-to-*Bam*HI fragment of pd24/26CAT with the ΔSS deletion was cloned into *Hin*dIII-*Bam*HI-digested pRV3. This construct was linearized at the unique *Sal*I restriction site and subsequently cotransfected with RV134 DNA (27). Purified recombinant viral isolates were then selected from the transfectant population exactly as described previously (27).

Southern blot analysis. HF cells infected with recombinant viruses were harvested for total cell DNA at 100% cytopathic effect (27). The infected-cell DNAs were digested with *Hin*dIII and subjected to electrophoresis on 0.8% agarose gels in 1× Tris-borate-EDTA buffer. The gels were transferred to positively charged nylon membranes (Biodyne B; Gibco Life Technologies, Grand Island, N.Y.) and hybridized to a probe for CAT or the *Hin*dIII X fragment of AD169 by using the Genius detection system (Boehringer-Mannheim Corporation, Indianapolis, Ind.).

Northern blot analysis. Total cell RNA was isolated from infected cells by using the RNeasy system (Qiagen, Chatsworth, Calif.). Equal quantities of RNA were subjected to Northern blot analysis and hybridized to a ^{32}P-radiolabeled probe for CAT as previously described (27). RNA levels were quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, Calif.). Multiplicity of infection was assessed by stripping the Northern blots and reprobing with a ^{32}P-radiolabeled probe to the endogenous pp28 gene (UL99). These results were confirmed by assessing the levels of the 3.0-kb UL98 transcript, which is 3′ coterminal with the pp28 mRNA (23).

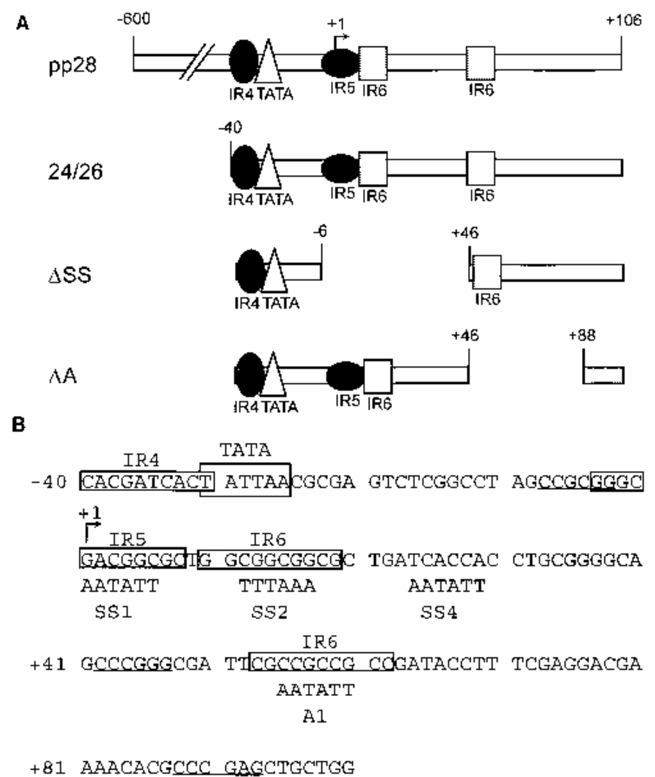

FIG. 1. (A) Schematic representation of the UL99 promoter region. The full-length (pp28) and truncated (24/26) promoter regions are shown, with the relevant inverted repeat sequences indicated. The regions deleted in the ΔSS and ΔA deletion mutants are also indicated. (B) Sequence of the truncated UL99 promoter. Relevant repeat sequences and the TATA element are boxed. Restriction sites utilized to generate the deletion mutants are underlined. Site-directed mutations introduced into the truncated promoter are indicated, with the actual sequence substitutions shown underneath.

RESULTS

Role of UL99 untranslated leader sequences in regulating gene expression. Our previous studies had demonstrated that the sequences from position −40 to +106 of the UL99 1.6-kb mRNA promoter (pd24/26CAT) were required for promoter activity in transient assays (6). Further, these sequences were sufficient to confer late gene kinetics to the CAT reporter gene in the context of the viral genome (27). As studies of late gene promoters in HSV have indicated that sequences downstream of the TATA element are important for late gene expression (11, 12, 14, 38), we examined these sequences in the UL99 promoter for their role in regulating gene expression. This region of the UL99 promoter contains three inverted repeat sequences: one copy of IR5 and two copies of IR6 in inverted orientations (6). In order to determine the potential roles of these sequences in regulating expression of UL99 in HCMV-infected cells, we generated two deletion mutations within the leader sequence of this promoter (Fig. 1A). The deletion of sequences from position −6 to +46 (pΔSSCAT) results in the removal of the IR5 repeat and one copy of the IR6 element. The second deletion, of sequences from position +46 to +88 (pΔACAT), removes the second copy of the IR6 repeat sequence. The effect of these deletions on reporter gene expression was then assessed in transient assays after viral superinfection (Fig. 2). These analyses revealed that the deletion of sequences from position −6 to +46 resulted in a threefold increase in expression of the CAT reporter gene (320% ±

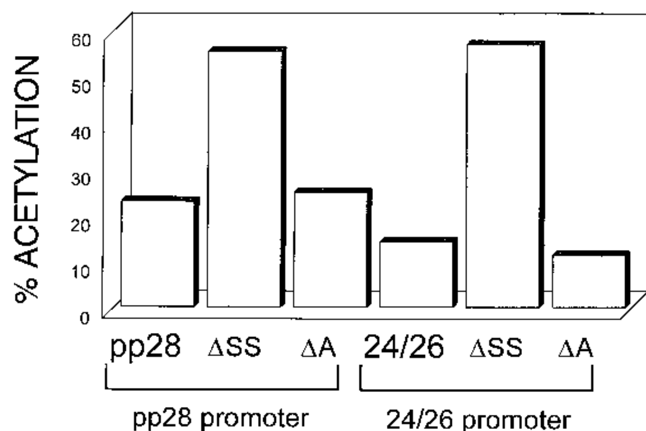

FIG. 2. Effect of UL99 promoter deletions on activation by viral proteins. The left side of the figure represents deletion mutants generated in the context of the full-length UL99 promoter (pp28). The right half shows data from the same experiment for the deletion mutants within the context of the truncated promoter (24/26). The indicated UL99 promoter-CAT plasmids were transfected into human fibroblasts and superinfected with virus. Cell extracts were prepared at 72 h after infection, diluted 1:30, and examined for CAT activity. Activity was quantitated by PhosphorImager analysis. Data from a single experiment of multiple experiments are shown.

72%; 12 duplicate experiments). However, deletion of the second copy of the IR6 element (pΔACAT) had little effect on CAT expression. These data imply that the region from position −6 to +46 contains an element capable of repressing gene expression.

Our previous studies have indicated that the truncated promoter pd24/26CAT (position −40 to +106) is less active than the full-length UL99 promoter in transient assays (6). These studies demonstrated a twofold difference in CAT expression in transient assays, although no such difference is observed in the context of the viral genome (6, 27). To confirm the role of the region from position −6 to +46 in regulating gene expression from this promoter, we generated equivalent deletion mutations in the context of the full-length pp28 promoter. Analysis of these deletions demonstrated that the repressor domain functions similarly in the context of the full-length promoter (Fig. 2). In this case, we observed a two- to threefold increase in gene expression with the ΔSS deletion as compared to the wild-type promoter. Deletion of the sequences from position +46 to +88 (ΔA) had no effect on UL99 promoter activity. These results confirm that the region from position −6 to +46 contains a repressor element that is functional in the context of the full-length UL99 promoter.

The region of the UL99 promoter from position −6 to +46 contains two repeat elements, IR5 and IR6. To eliminate the possibility that the increase in gene expression was a result of positional effects and to further define the roles of the IR5 and IR6 sequences in UL99 gene expression, substitution mutations in the repressor domain were generated (Fig. 1B). Consecutive scanning mutations of the sequences from position +1 to +29 were introduced into the UL99 promoter. Analysis of these mutants in superinfection experiments (Fig. 3) demonstrated that mutation of either the IR5 element (position +1 to +6 [SS1]) or the IR6 element (position +12 to +17 [SS2]) was individually sufficient to duplicate the effect of the ΔSS deletion mutation. In contrast, mutation of the sequences from position +24 to +29 (SS4) had minimal effects on CAT activity. These data indicate that the IR5 and IR6 elements function together to repress CAT expression from the UL99 promoter. In addition, the results suggest that the IR6 element is functional in only one orientation, as removal of this sequence in the ΔA deletion had no effect on gene expression. To directly test this, the downstream IR6 sequence was subjected to site-directed mutagenesis (Fig. 1B). Assessment of this mutation (A1) in transient assays revealed that this mutation had no effect on CAT expression (Fig. 3), confirming that the downstream IR6 element does not function to regulate gene expression in this assay.

Assessment of UL99 promoter regulation in the viral genome. These results demonstrate that in transient assays, sequences from position +1 to +17 of the UL99 promoter are involved in the repression of gene expression from this promoter. However, our previous results have shown that late gene promoters are promiscuously expressed in transient assays compared to expression in the context of the viral genome (6, 27). Generally, late gene promoters are expressed to higher levels and at earlier times after infection when assessed in transient-transfection assays. To confirm the role of the UL99 repressor element in a biologically relevant system, a recombinant virus that contained the UL99 truncated promoter (position −40 to +106) with the ΔSS region deleted was generated. This virus, RVΔSSCAT, contained the relevant promoter sequences upstream of the CAT gene inserted between the US9 and US10 ORFs by our previously described strategy (24). The integrity of the recombinant virus construct was assessed by Southern blot analysis (Fig. 4). RVΔSSCAT differs from the original UL99 promoter recombinant virus construct (RV24/26CAT) (24) by the presence of an intact *Hin*dIII site at the 5′ end of the promoter, resulting in fragments of 4.4 and 1.8 kb upon *Hin*dIII digestion (Fig. 4B, lanes 2). Only the 4.4-kb fragment is capable of hybridizing with the CAT probe. Digestion of RV24/26CAT with *Hin*dIII and *Bam*HI results in three fragments of 3.0, 2.0, and 1.2 kb, with the 3.0-kb fragment also hybridizing with the CAT probe (Fig. 4B, lanes 3). The presence of the additional *Hin*dIII site in RVΔSSCAT results in the conversion of this 3.0-kb fragment to two fragments of 1.8 and 1.2 kb, with the 1.2 kb fragment containing the CAT gene (Fig. 4B, lanes 4). These digests of RVΔSSCAT show that the appropriate promoter-CAT construct was inserted into this region.

CAT expression in RVΔSSCAT-infected cells was then compared to that in RV24/26CAT-infected cells (Fig. 5). HF cells were infected with the recombinant viruses at approximately equivalent multiplicities of infection, harvested at various times after infection, and assessed for both CAT activities and CAT RNA levels. These values were then corrected for the

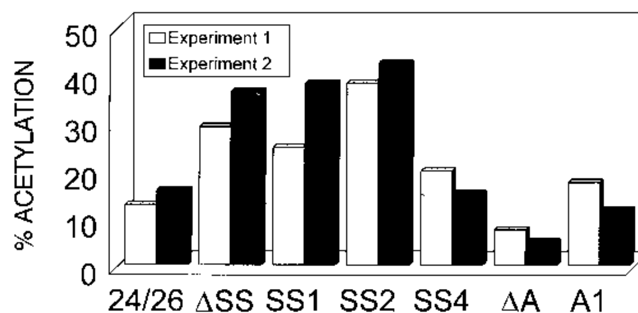

FIG. 3. Effect of UL99 promoter mutants on activation by viral proteins. Site-directed mutants generated in the truncated UL99 promoter (24/26) were assessed by transfection into human fibroblasts followed by superinfection with virus. Cell extracts were prepared at 72 h after infection, diluted 1:30, and examined for CAT activity. Activity was quantitated by PhosphorImager analysis. Data from two independent experiments are shown.

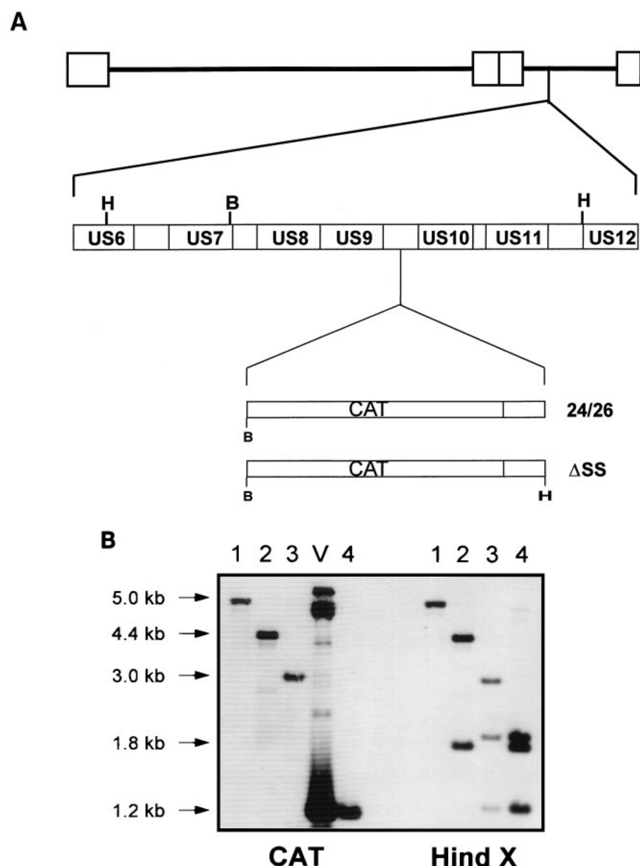

FIG. 4. (A) Schematic representation of recombinant virus constructs. The top line represents the HCMV genomic sequence, with the 5-kb *Hin*dIII X fragment shown underneath. The indicated UL99 promoter-CAT constructs were inserted into the *Apa*I site in the US9-US10 intergenic region as described in Materials and Methods. Relevant restriction sites utilized in Southern blot analysis are indicated: B, *Bam*HI; H, *Hin*dIII. (B) Southern blot hybridization analysis of recombinant viruses RV24/26CAT (lanes 1 and 3) and RVΔSSCAT (lanes 2 and 4). Total cellular DNA was isolated from cells infected with virus for 72 h, digested with *Hin*dIII (lanes 1 and 2), or *Hin*dIII and *Bam*HI (lanes 3, V, and 4), and subjected to Southern blot analysis. The blots were hybridized with a labeled probe generated from the isolated CAT gene or *Hin*dIII X fragment. The molecular sizes of the relevant bands are indicated. As shown in panel A, RVΔSSCAT differs from the original RV24/26CAT construct by the presence of an intact *Hin*dIII site at the 5′ end of the promoter, resulting in fragments of 4.4 and 1.8 kb upon *Hin*dIII digestion. Lane V, recombination vector used to generate RVΔSSCAT. The 1.2-kb *Hin*dIII-*Bam*HI UL99 promoter-CAT fragment is indicated. The image was generated with a Hewlett-Packard ScanJet II cx with Hewlett-Packard HPDeskscan II software (version 2.3.1).

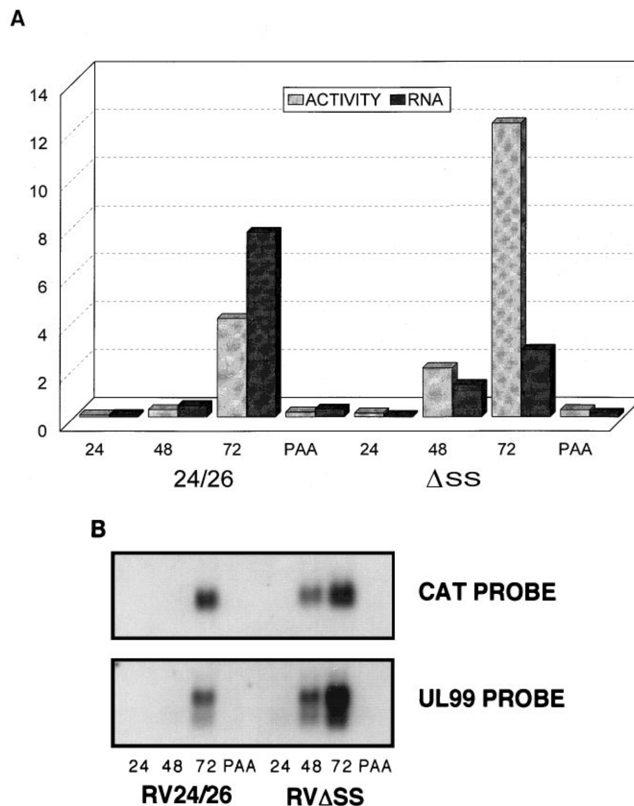

FIG. 5. Kinetics of CAT expression from recombinant viruses. HF cells were infected with 5 PFU of the indicated viruses per cell in the presence or absence of PAA (200 μg/ml). Cells were harvested at the indicated times (hours) and assessed for CAT enzyme activity (A) or CAT RNA levels (A and B) by Northern blot analysis with a ^{32}P-labeled probe to the CAT gene. Percent acetylation and RNA levels were quantitated by PhosphorImager analysis. The values shown in panel A were corrected for multiplicities of infection by stripping the blot and reprobing with a probe to the endogenous UL99 gene (B) and were confirmed by using a probe to the UL83 gene (data not shown). Values represent typical results from three replicate experiments.

multiplicity of infection by measuring endogenous UL99 mRNA levels and confirmed by assessing UL98 mRNA expression (Fig. 5B). Correction factors for CAT activity and RNA levels in RV24/26CAT-infected cells were no greater than fivefold in each experiment (Table 1). Deletion of the repressor element in the UL99 leader sequence had no effect on the kinetics of gene expression from this promoter (Fig. 5). Relative CAT expression was restricted to late times after infection. In addition, the expression of CAT was sensitive to the presence of PAA, a DNA synthesis inhibitor. These results confirm that expression of CAT from the truncated ΔSS promoter occurs with late kinetics.

Slightly reduced levels of CAT mRNA were observed in the RVΔSSCAT-infected cells compared with RV24/26CAT-infected cells when the results were corrected for the multiplicity of infection (Fig. 5). These findings indicate that the ΔSS deletion may have some influence on the activity of the UL99 promoter. In contrast, CAT activity in the RVΔSSCAT infected cells was approximately threefold higher than that observed in RV24/26CAT-infected cells at 72 h after infection (Fig. 5). This is similar to the increased level of CAT activity

TABLE 1. Ratio of CAT activity to RNA levels in RV24/26CAT- and RVΔSSCAT-infected cells at 72 h after infection in two independent experiments

Expt and virus	CAT activity[a] (% acetylation)	CAT RNA[b] (arbitrary units)	Ratio (activity/RNA)
1			
RV24/26CAT	35.7	48.9	0.73
RVΔSSCAT	201.5	24.3	8.3
2			
RV24/26CAT	4.1	7.7	0.53
RVΔSSCAT	12.25	2.79	4.4

[a] Values are corrected for multiplicity of infection as assessed by endogenous UL99 levels. The correction factor for CAT activity and RNA levels in RVΔSSCAT-infected cells in experiment 1 was fivefold, and that for RV24/26CAT-infected cells in experiment 2 was 4.4-fold.

[b] RNA levels were quantitated by PhosphorImager analysis and corrected for multiplicity of infection as assessed by endogenous UL99 levels.

observed in transient assays. To explain these results, the ratios of CAT activity to relative RNA levels were compared (Table 1). This analysis revealed that the RVΔSSCAT-infected cells had 8- to 12-fold higher CAT protein/CAT mRNA ratios than RV24/26CAT-infected cells. Similar results were observed in three independent experiments. These findings strongly suggest that the sequences from position −6 to +46 of the UL99 promoter inhibit gene expression at the level of translation.

DISCUSSION

Genes expressed during the late phase of HCMV replication are generally considered to encode primarily structural components. In addition, some late gene products which are transactivators of gene expression have been described (18, 33). Despite their importance in HCMV replication, very little is understood regarding the regulation of late gene expression in HCMV-infected cells. Such studies have been hampered by the fact that HCMV late promoters are expressed with altered kinetics in transient-transfection–superinfection systems (6, 27). In addition, the viral proteins required for late gene expression have yet to be defined. We have established a recombinant virus system to analyze the requirements for HCMV gene expression (23, 27). Our previous studies on the pp28 (UL99) 1.6-kb mRNA late promoter indicated that promoter sequences upstream of position −40 had little influence on late gene expression in the viral genome (27). This is consistent with studies of late gene promoters in HSV (11, 12, 14, 38).

Our present studies have revealed an apparent novel mechanism of gene regulation in HCMV biology. The untranslated leader of the pp28 late gene was shown to contain a repressor of gene expression. Removal of the repressor element resulted in increased levels of gene expression. Analysis in the context of the viral genome revealed that deletion of the repressor element resulted in increased protein levels. These findings demonstrate that the repressor domain acts at the level of translation. Various elements in 5′ untranslated sequences have been shown to regulate the efficiency of eukaryotic translation initiation. For example, the presence of short upstream ORFs can inhibit translation of downstream mRNAs (8, 30, 39). An HCMV early gene, UL4, is regulated in this manner (8, 39). In addition, oligopyrimidine tracts immediately downstream of the CAP site have been identified in ribosomal protein mRNAs which are selectively repressed in quiescent cells (2). However, neither upstream ORFs or oligopyrimidine tracts have been identified in the 5′ untranslated region (UTR) of the pp28 mRNA.

mRNAs with extensive secondary structure generally exhibit reduced translational efficiency (13, 21, 24, 28, 31, 32, 34, 36). For example, the long 5′ UTR of the ornithine decarboxylase mRNA can form an extremely stable secondary structure and represses the translation of this mRNA (21, 34). In some cases, it has been determined that only relatively short stem-loop structures within long 5′ UTRs are responsible for the translational inhibition. For example, a stem-loop structure from position +49 to +76 of the 840-nucleotide transforming growth factor β 5′ UTR was found to be sufficient to inhibit translation (24). The region of the UL99 leader sequence which we have defined as the repressor element is extremely GC rich. One possibility is that this region may form a short but stable structure that can inhibit translation. Computer modeling of this region has revealed that the sequences from position +1 to +25 can form a stem-loop structure (Fig. 6). A role for this structure in translational repression is consistent with our data obtained by using site-directed mutations generated in this region. For example, the SS1 and SS2 mutations,

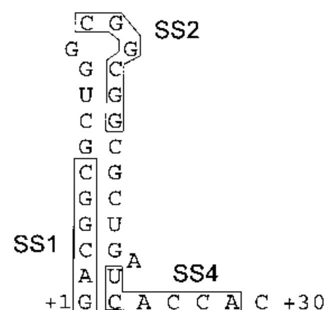

FIG. 6. Predicted stem-loop structure at the 5′ end of the pp28 mRNA. Sequences altered in the SS1, SS2, and SS4 mutants (Fig. 1B) are indicated by boxes.

which increase gene expression, either completely disrupt the stem-loop structure or result in a stem-loop with much-reduced stability. In contrast, the SS4 mutation, which has minimal effects on gene expression, maintains a stable stem-loop structure. Kozak has demonstrated that a stem-loop structure within 12 nucleotides of the CAP structure can prevent engagement of 40S ribosomal subunits by the mRNA (32). A second possibility is that the region from position +1 to +17 defines a binding site for an as-yet-unidentified protein. Therefore, it is possible that the repressor domain may bind a protein which blocks either recognition of the CAP structure or ribosomal scanning.

Perhaps a more intriguing question is why UL99 gene expression is regulated in this manner. The 1.6-kb mRNA is a very abundant mRNA that is predicted to contain two ORFs. The first begins 177 bp downstream of the CAP site and consists of the carboxy-terminal 120 amino acids of the alkaline exonuclease homolog (UL98) (1). However, no evidence of this truncated carboxy-terminal protein has been observed in either infected cells or cells transfected with a genomic construct of this region (1). The second ORF of this 1.6-kb mRNA encodes the pp28 tegument protein. The ATG of this protein occurs 476 bp downstream of the CAP site of this mRNA. It is possible that the function of the translation repressor element is to prevent translation of the truncated UL98 ORF and allow for translation of the pp28 protein from the downstream ORF, possibly by internal ribosome entry. Some mRNAs with long 5′ UTRs have been shown to promote internal ribosome binding (17, 35, 37, 49). To date little is known regarding the specific structural and sequence requirements for internal ribosome binding. Oligopyrimidine tracts upstream of picornavirus AUG sequences have been identified (17). However, such sequences are not present in cellular mRNAs which are translated in this manner (35, 49). Two cellular mRNAs known to be expressed by internal ribosome entry, those of fibroblast growth factor 2 and immunoglobulin heavy-chain-binding protein, are both extremely GC rich (35, 49). Although the 5′ region of the pp28 UTR is GC rich (87% in the first 60 bp), the remainder of the 5′ UTR is not GC rich and does not contain an oligopyrimidine tract. The ability of this region to act as an internal ribosome entry site requires further investigation.

The translational regulation of the UL99 late gene defines a novel regulatory mechanism in HCMV biology. Complex gene structures with overlapping ORFs and 3′-coterminal transcripts appear to be a common feature of the HCMV genome (10, 19, 29, 51, 52). For example, the US region of HCMV encodes multiple 3′-coterminal transcripts (10, 19). In addition, the ORFs of glycoprotein B and the viral DNA polymerase are partly overlapping, with the glycoprotein B polyadenyl-

ation site located within the polymerase ORF (29). Such overlapping transcripts are thought to be an evolutionary development to maximize usage of the viral genome. However, such complex gene arrangements can present potential regulatory problems. Our analysis of pp28 translational regulation suggests that translational control may be one mechanism utilized by the virus for regulating protein expression from such overlapping transcripts.

Several HCMV proteins are thought to be translationally regulated. In addition to pp28, translation regulation is likely to regulate levels of gp48 (UL4), the UL112-113 50-kDa protein, the major late DNA binding protein UL44, and pp65 (UL83) (7, 8, 39, 54). Several of these genes, including UL112-113, UL44, and UL83, can express mRNA with early kinetics, but protein levels do not accumulate until late times after infection (7, 54). Interestingly, pp28, UL4, and UL83 are components of the virion. Therefore, translational control rather than transcriptional mechanisms may be important for tightly regulating the level and time of appearance of proteins involved in virion structure and assembly. In fact, the virion assembly protein (UL80) is itself regulated posttranscriptionally by a proteolytic processing mechanism (9). In addition, the UL80 protein is also transcribed from a group of 3′-coterminal transcripts (51). Thus, posttranscriptional mechanisms may play a significant role in regulating the stoichiometry of virion structural components and may therefore be important in the control of virion assembly.

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