Translational Control of Cytomegalovirus Gene Expression Is Mediated by Upstream AUG Codons

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Human cytomegalovirus regulates gene expression at both the transcriptional and posttranscriptional levels. We have characterized posttranscriptional signals carried on the RNA of one β (or delayed-early) gene, signals that we show delay the translation of an actively transcribed gene. The *cis*-acting components of this signal map to two upstream AUG codons in the 5' leader of the β gene transcript that act to delay the kinetics and reduce the levels of gene expression. Both upstream AUG codons are part of short open reading frames, and the presence of either is sufficient to suppress expression of a downstream open reading frame. These upstream natural AUG codons appear to act in *cis* at the level of translation, consistent with the scanning hypothesis of eucaryotic translation. The occurrence of natural upstream AUG codons in other cytomegalovirus transcripts suggests their physiological significance in influencing the kinetics and steady-state levels of gene expression during viral growth.

Human cytomegalovirus (CMV) is a medically important herpesvirus that latently infects a majority of the adult population and causes severe disease in immunocompromised individuals (1). The virus has a large (240-kilobase-pair) double-stranded DNA genome encoding gene products expressed in a sequentially ordered cascade (7, 24, 34, 37; for a review, see E. S. Mocarski, *Transfusion Rev.*, in press). Early work showed that, as with herpes simplex virus (HSV), CMV gene expression occurs in at least three kinetic classes: immediate early or α , delayed early or β , and late or γ (35; Mocarski, in press). In contrast to the dominant role of transcriptional regulation in HSV, posttranscriptional signals moderate transcriptional activation in the regulation of CMV gene expression (9, 10, 11, 37).

The initial regulatory event following CMV infection of permissive cells is the transcriptional transactivation of α gene expression by a virion factor (32), a process that appears to be characteristic of herpesviruses and best studied in HSV (2, 3, 26, 29, 30). Following expression, one or more as yet uncharacterized α gene products transcriptionally transactivate β and some γ genes (9, 32, 37). Once transcriptional activation has occurred, there is a delay in full expression of β and γ gene products due to posttranscriptional regulatory events, a process we have characterized for one β gene (10) and two CMV γ gene products (9). In addition to our observations, changes in 3' RNA-processing events have been correlated with the temporal control of one other γ gene (11).

In a study designed to examine the *cis*-acting elements involved in posttranscriptional control of β gene expression, we identified a regulatory signal within the 142-nucleotide (nt) 5' leader of a β gene transcript (10). This transcript, encoded by a gene present in the inverted repeats flanking the L component (35) of the CMV genome, is polyadenylated, polysome associated, and extremely abundant at both early and late times in infection (12, 24, 25, 37). Although as yet no one has reported the detection of a protein product from this gene, nucleotide sequence analysis identified a 170-codon open reading frame (ORF) that may encode such

a gene product (12). This ORF is preceded by two short ORFs (7 and 35 codons in length) within the 5' leader region. Although the natural protein product has not been observed, the transcriptional and posttranscriptional regulatory signals from this β gene up to position +142 relative to the transcription start site (and including the short ORFs) have been harnessed for a high level of expression of protein from recombinant CMV (33).

Previous analyses of the regulatory signals on CMV α and β genes made use of transient assays with the *Escherichia coli lacZ* gene as an indicator of gene expression (10, 32). For detailed analysis of the β gene, *lacZ* was placed in an analogous position to the 170-codon ORF of the natural gene under posttranscriptional control of signals in the 142-nt 5' leader but under transcriptional control of α promoter-regulator signals. These chimeric constructs were used to map the posttranscriptional signal to the 3' 81 nt of the leader region by deletion analysis (10). We have now precisely defined the components of this signal, showing that transcripts carrying the 5' leader are inefficiently translated as a consequence of two upstream AUG codons that are part of short ORFs. We show that the presence of either of these two AUG codons delays expression of a downstream ORF for several hours in uninfected human fibroblast cells and that during viral infection these signals significantly reduce the levels and effectively delay the kinetics of gene expression within the regulatory cascade. The importance of translational control in CMV gene expression is supported by recent reports identifying short ORFs in the 5' leader region of other CMV transcripts (6, 14, 16, 17).

MATERIALS AND METHODS

Cells, virus, and plasmids. Human CMV (Towne) was propagated in human foreskin fibroblasts as previously described (32). The plasmids pON1, pON241, pON249, and pON261 and the bacteriophage mpON258 have been described previously (10, 32). Plasmid pRB3458 (29) was kindly provided by Bernard Roizman. pON263 was constructed by insertion of the 92-nt *NaeI-HindIII* fragment from the β gene 5' leader into the *HindIII* site of pON249 in the same orientation as in pON253 and in the viral genome. Deletion

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of the same *NaeI*-*HindIII* fragment from pON241 generated pON267, a clone directing synthesis of the same β -galactosidase (β gal) transcripts as pON261 but under the control of β rather than α transcriptional signals.

The synthetic oligonucleotides 5'-CGTTCTCCCCGTC GTCCGA-3' and 5'-GGGCTCTCCGTCTTCTCCC-3', prepared on an Applied Biosystems automated DNA synthesizer, were complementary to 19 of 20 nt flanking ATG1 and ATG2, respectively, within the 5' leader and were used to generate mutants in which the each ATG was converted to ACG. After each oligonucleotide was annealed to mpON258 phage templates and the mutant strand circle was completed with *E. coli* DNA polymerase I (Klenow fragment; New England BioLabs, Inc.), the double-stranded circles were transformed into *E. coli* TGI, and mutant plaques were identified by hybridization with a 32 P-end-labeled oligonucleotide probe (4). Dideoxy sequencing (31) confirmed that mpON290 and mpON287 had the mutations in ATG1 and ATG2, respectively. A phage with mutations in both ATG1 and ATG2, mpON295, was constructed in the same way, by annealing the first oligonucleotide on the mpON287 template. The 188-nt *SacI*-*HindIII* fragments from mpON258, mpON290, mpON287, and mpON295 were each inserted into the *XbaI*-*HindIII* sites of pON249 to generate pON286, pON290, pON287, and pON295, respectively, by using T4 polymerase to blunt the *XbaI* and *SacI* ends after the *HindIII* ends were ligated.

Transient expression assays. Transient assays were carried out by using DEAE-dextran followed by CMV infection as previously described (10, 32), except that following cycloheximide reversal (32), monolayers were washed twice with 10 mM NaPO_4 (pH 7.5)-1 mM EDTA and extracts were made (at 10 h postinfection) by the addition of 1 ml of 10 mM NaPO_4 (pH 7.5)-1 mM EDTA-1% Nonidet P-40. Lysates were centrifuged ($12,000 \times g$) for 10 min at 4°C, and the supernatants were stored at -20°C until they were assayed for β gal activity and total protein content. To measure β gal activity, 4 parts of Z buffer (26, 32) were mixed with 1 part of 0.44 mM 4-methylumbelliferyl- β -D-galactoside (MUG; Sigma Chemical Co.). One hundred milliliters of this mixture was added to 100 μ l of the cell lysate, followed by incubation at 37°C for 30 min, and fluorescence was measured by using a Microfluor microfluorimeter (Dynatech Industries, Inc.). The protein content of lysates was determined by using the fluorogenic substrate *o*-phthaldehyde (OPA; Pierce Chemical Co.). OPA (8 mg) was dissolved in 100 μ l of methanol and then added to 10 ml of 0.1% Nonidet P-40-14 mM β -mercaptoethanol-1 M borate buffer (pH 10.5; Pierce). The OPA solution (100 μ l) was added to 10 μ l of cell lysate in 90 μ l of 0.1% Nonidet P-40-14 mM β -mercaptoethanol-1% methanol-1 M borate buffer. After 5 min, the fluorescence was measured on the microfluorimeter. The specific activity was determined by dividing the β gal activity, as measured by MUG cleavage, by the protein content determined by the OPA assay.

Electroporation was carried out by suspending 4×10^6 trypsinized and washed cells in 0.8 ml of 23 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; U.S. Biochemical)-0.77 mM Na_2HPO_4 -150 mM NaCl-5.5 mM KCl-6.2 mM glucose (pH 7.05) with 30 μ g of pRB3458 and 20 μ g of pON1, pON249, or pON253. After a 200-V pulse at 540- μ F capacitance and a 5-min incubation, the cells were plated on one 100 mM tissue culture dish with 5 ml of medium. Unattached cells were decanted after 5 h, and the medium was replaced with medium containing 0.44 mM

MUG. The fluorescence in the medium was measured as described previously (10).

Analyses of RNA. Whole-cell RNA was prepared by guanidinium isothiocyanate solubilization and by pelleting through CsCl as described previously (5, 10). Immediately RNA was extracted from cells treated with cycloheximide (100 μ g/ml) from 1 h before infection until harvest at 8 h postinfection. Nuclear and cytoplasmic RNA fractions were prepared from cell monolayers rinsed with cold phosphate-buffered saline and then incubated for 5 min in 0.5 ml of 0.15 M NaCl-10 mM Tris hydrochloride (pH 7.8)-1.5 mM MgCl_2 -0.5% Nonidet P-40-4 U of RNasin (Promega) on ice. Nuclei were pelleted by centrifugation at $2,000 \times g$ for 5 min. After the nuclei were washed once with phosphate-buffered saline, nuclear RNA was harvested as described above for whole-cell RNA. The cytoplasmic fraction (1.5 ml) was added to 1 ml of guanidinium isothiocyanate solution (5), and RNA was pelleted through CsCl. To remove contaminating plasmid DNA from cytoplasmic RNA, pellets were suspended in 0.5 ml of water; then 1.5 ml of 4 M guanidinium isothiocyanate and 0.5 ml of 5 M CsCl-50 mM sodium acetate were added, and the RNA was again pelleted through cesium chloride. RNA blot analyses of nuclear and cytoplasmic RNA were performed as described previously (9), except that probes used for hybridization were synthesized by priming denatured plasmids with random hexamers (Pharmacia), followed by elongation with Klenow fragment as described elsewhere (8).

RESULTS

Transport of transcripts containing the signal to the cytoplasm. To understand how the *cis*-acting signal previously mapped within the 5' leader of the major β gene transcript exerted its effect on gene expression, we first determined the cellular location of chimeric transcripts either containing or lacking the 5' leader under conditions that allowed transcriptional activation and accumulation of α transcripts while blocking their posttranscriptional expression. Following transfection with pON249 or pON253 (Fig. 1; 10) and CMV infection in the presence of cycloheximide, RNA was isolated from nuclear and cytoplasmic fractions of cells at 8 h postinfection. RNA blot analysis revealed that α transcripts containing (pON253) or lacking (pON249) the 5' leader accumulated in the cytoplasm to similar levels (Fig. 2). As expected from previous results (10), the β promoter-regulator controlling the *lacZ* gene in pON241 was transcriptionally inactive under these conditions. Cytoplasmic localization of transcripts was confirmed by in situ hybridization following an identical transfection-infection procedure (data not shown). Thus, the presence of the 5' leader did not alter the nuclear-cytoplasmic transport or the stability of transcripts. These results were consistent with the 5' leader acting to inhibit translation of mRNA in the cytoplasm.

Mediation of translational control by upstream AUG codons carried naturally in the 5' leader. The signal within the 5' leader region was previously mapped by deletion analysis (10) to between nt 62 and 142 of the leader. Within this region (Fig. 1), there were two AUG codons, both followed by short ORFs that terminate before the AUG codon used to initiate β gal translation (Fig. 1). To determine their roles in regulating gene expression, we generated point mutations in these AUG codons, changing each initiation codon to ACG separately as well as in combination (Fig. 1). When transfected into cells that were subsequently infected with CMV under cycloheximide reversal conditions (10, 32), construct

FIG. 1. Plasmid constructs used to dissect the *cis*-acting signal in the 5' leader of the major β gene. Constructs using the CMV major α (immediate-early) gene promoter-regulator (■) or the β (delayed-early) gene promoter-regulator (▨) for transcriptional control fused to various regions from the natural or mutated leader. Plasmid constructs pON253 and pON286 differed only in polylinker sequences (see Materials and Methods). Plasmids pON253, pON286, and pON241 carried intact copies of the 142-nt leader sequence. The position of a large dyad symmetry element ($\rightarrow$) and short ORFs (□) upstream of the *lacZ* gene in these constructs are indicated on the pON286/253 diagram. Plasmid pON263 carried a deletion of 61 nt removing the 5' portion of the leader, and plasmids pON261 and pON267 carried an 81-nt deletion in the 3' portion of the leader (—). Plasmids pON290, pON287, and pON295 carried a point mutation (X) in AUG1, AUG2, and both AUGs, respectively.

pON295, which had both AUG codons altered, expressed β gal at levels similar to that of the construct (pON249) lacking the 5' leader altogether (Fig. 3). In contrast, separate mutations in either AUG had little effect on gene expression. To correct for differences in the levels of β gal transcripts expressed from these different plasmids, the data for the point mutants were corrected for β gal transcript levels as well as for cell number. The similarity of the data expressed

with either correction indicated that, as previously observed (10), transcriptional regulation did not account for the observed differences in gene expression. Our results suggested that upstream AUG codons inhibited expression of the downstream *lacZ* ORF by affecting translation initiation, a result consistent with model systems used to establish the principles of the scanning hypothesis of eucaryotic translation initiation (18, 19, 22).

Gene expression delayed independent of viral *trans*-acting functions. We had previously postulated that viral *trans*-acting factors might be responsible for reversing the inhibitory effect of the 5' leader signal at early times in infection (10). Mapping this signal to the two upstream AUG codons raised the possibility that the effect we observed was the result of a delay in translation of β gal due to the inaccessibility of this downstream ORF to the translation initiation machinery. This interpretation predicted that we would observe a delay in gene expression even in the absence of viral *trans*-acting components, that is, without viral infection. We examined the need for viral *trans*-acting factors by using two types of experiments: (i) expression of 5' leader-containing transcripts under the transcriptional control of different kinetic classes of promoters and (ii) expression of transcripts in the absence of CMV gene products altogether. Comparison of constructs containing or lacking the 5' leader signal under either α or β promoter-regulator control revealed a similar 4-h delay in high-level expression of β gal (Fig. 4). Steady-state RNA levels were not affected by the presence of the 5' leader (data not shown). Because expression was delayed regardless of when during infection transcriptional activation occurred, these results suggested that the signal could act to suppress gene expression independent of viral *trans*-acting factors.

To establish that this signal could delay gene expression independent of CMV factors, we transcriptionally transactivated plasmid constructs in the absence of any CMV gene products. On the basis of earlier work which demonstrated that the CMV α promoter-regulator could be activated by HSV (32), we used the HSV α *trans*-inducing factor (VMW65), plasmid clone pRB3458 (29), to transcriptionally activate the test constructs. We examined the kinetics of β gal expression following cotransfection of the HSV α *trans*-inducing factor clone in combination with various α

FIG. 2. RNA blot analysis to determine cellular localization of transcripts containing or lacking the 5' leader. RNA was purified from nuclear (NUC) and cytoplasmic (CYTO) fractions of cells that were mock transfected or transfected with pON249, pON253, or pON241 and then infected with CMV in the presence of cycloheximide, as described in Materials and Methods. After RNA samples (5 μ g) were separated by electrophoresis on 1% formaldehyde-agarose gels and transferred to nitrocellulose, β gal transcripts were detected by using a 32 P-labeled pON1 probe. Numbers on the left indicate size standards (in kilobases).

FIG. 3. Immediate-early expression of β gal from plasmids with mutations in the natural AUG codons within the 5' leader sequence. Extracts from mock (M)-transfected cells or from cells transfected with the indicated plasmid constructs and infected with CMV under cycloheximide reversal conditions were analyzed for expression of β gal and for the total protein content, as described in Materials and Methods. Specific activities were calculated and represent values standardized for cell number (striped bars). These specific activities were also standardized for β gal RNA levels (solid bars) obtained by densitometric scanning of a Northern blot of the RNA from transfected-infected cells.

promoter-regulator constructs containing or lacking the 5' leader signal. The presence of the leader delayed but did not eliminate β gal expression (Fig. 4), reinforcing the evidence that viral *trans*-acting factors are not essential components for the delayed expression of 5' leader-containing transcripts.

Finally, to determine whether the products of the short ORFs within the 5' leader might themselves act in *trans* to inhibit translation of the downstream *lacZ* ORF, we mixed pON295, which carried a 5' leader with mutations in both AUG codons, with pON286, which carried a wild-type 5' leader. Following transfection into cells that were subsequently infected with CMV under cycloheximide reversal conditions, the presence of pON286 did not result in a significant reduction of expression by pON295 (Fig. 5). The results of this analysis (Fig. 5) firmly established that the ability pON295 to direct expression of β gal was dominant and suggested that the short ORF products play no *trans*-acting role in the delay of β gal expression.

DISCUSSION

We have investigated the signals involved in the posttranscriptional control of a CMV gene of the β (delayed-early) kinetic class. These signals act in concert with transcriptional activation signals to confer kinetic class on the β gene. Apparently, CMV has adopted posttranscriptional control to ensure expression of the gene product at the appropriate levels and time during viral growth. Expression is thereby precisely regulated through a combination of transcriptional and translational controls. Although transcriptional control of gene expression occurs in all herpesviruses and is key to

the sequential expression of viral genes, posttranscriptional controls operating in CMV clearly have the effect of tempering transcriptional activation.

Several lines of evidence support the conclusion that translation is the posttranscriptional target through which the 5' leader signal modulates gene expression. RNA blot and in situ hybridization analyses localized transcripts containing the signal to the cytoplasm well before the time when protein is expressed at high levels, establishing that this signal did not affect mRNA transport or stability. Specific mutations that affected the natural upstream translational initiation codons destroyed the regulatory signal. We have also found that the presence of the 5' leader on β gal transcripts inhibits their translation in rabbit reticulocyte lysates and after microinjection into frog oocytes (unpublished data). Although this inhibition involves sequences within the 5' leader, the principal controlling elements do not map solely to the upstream AUG codons. Human fibroblast extracts may provide a more authentic system to investigate translation regulation.

The effects we have observed depend upon AUG codons, each of which starts a short ORF contained in the 5' leader. In the indicator gene construct, pON253, these ORFs are 7 and 25 codons long. The natural β gene transcript contains these two AUG codons and an identical first ORF (12). In our constructs, the second ORF is shortened from its natural length (35 codons) because we substituted *lacZ* sequences at a site within this ORF (10). The mechanism we presume is operating involves the AUG codons impeding the access of ribosomes to the downstream ORF according to the rules of the scanning hypothesis (18, 19, 22). It appears that CMV

FIG. 4. Effect of the 5' leader signal on gene expression under various conditions of transcriptional activation and in the absence of CMV gene products. (A) Transiently expressed β gal was measured by assaying cleaved MUG in the medium overlaying cells transfected with α promoter-regulator constructs either lacking (pON261) or containing (pON253) the 5' leader signal or with β promoter-regulator constructs either lacking (pON267) or containing (pON241) this signal and subsequently infected with CMV to activate gene expression. β gal activity was measured at 24 and 28 h postinfection with CMV and is expressed as the fluorescence level in a sample of medium from cells transfected with the indicated plasmid less that from cells mock transfected with pON1 alone. (B) Transiently expressed β gal was measured by MUG cleavage assay at various times after electroporation of cells with pRB3458 and either pON249 or pON253. β gal activity is expressed as the fluorescence level in a sample of medium from cotransfected cells less that in cells cotransfected with pRB3458 and pON1.

FIG. 5. Effect of short ORFs on gene expression in *trans*. Extracts from mock-transfected cells or cells transfected with 11 μ g of the indicated plasmid constructs (either alone or in combinations) and infected with CMV under cycloheximide reversal conditions, allowing transcription from the chimeric plasmid constructs but not from the natural copies of the β gene on the viral genome (10, 32). The levels of expression from cells transfected with 22 μ g of pON295 or pON286 were comparable to the data shown. pON1 is a plasmid clone of *lacZ* sequences lacking CMV sequences (32). The transfection, infection, and assay for β gal activity were done as described in Materials and Methods.

has taken physiological advantage of ribosome scanning to regulate its gene expression, with these upstream AUG codons acting to slow the accumulation of gene products. The scanning hypothesis suggests that the 5'-cap-proximal AUG is used preferentially, although nearby sequences (20) and secondary structure (21, 23) can influence the efficiency of initiation at a particular AUG. Our results suggest that both AUGs within the CMV β gene 5' leader are recognized. Either AUG alone is sufficient to inhibit the expression of β gal and translational control was destroyed only when both were mutagenized. We found a similar inhibitory effect of upstream AUGs on expression of the downstream ORF whether the nucleotides surrounding the upstream AUGs were in an optimal (AXXAUGG for AUG2) or relatively poor (CXXAUGG for AUG1) context (20). For the translation machinery to be impeded and still express the third ORF on the transcript, an occasional ribosome may either miss the first as well as the second AUG codons or retain the capacity to reinitiate after translating one or both short ORFs. As a result of these effects, the expression of β gal was low and the kinetics of its accumulation were delayed. Although our transient assay data indicate that the 5' leader signals can act independently of viral *trans*-acting factors, we cannot absolutely exclude a role for such factors in modulating translation of this or other genes during viral infection. Recombinant CMV carrying mutations tested here in transient assays may provide insights into viral translational controls. Regardless of the potential spectrum of

regulatory events, our work demonstrates the impact of translational control elements on CMV gene expression.

Recent work in a variety of systems has indicated that short ORFs can play a role in translation regulation. A seven-codon ORF in the 5' leader of an avian retrovirus is conserved, can be translated, and may serve a function (13). In simian virus 40, a shift in transcriptional start sites during infection results in the inclusion of an upstream ORF on the small-t-antigen message. This ORF is expressed and decreases t-antigen expression (15). The translated product of the upstream ORF in the yeast *cpa1* gene apparently acts in concert with arginine to inhibit translation of the *cpa1* gene product (38). Our genetic analyses indicated that products of the short upstream ORFs do not act in *trans* to inhibit downstream expression, suggesting that they play no role in the translational regulation of the CMV β gene product. Upstream ORFs have been identified in other CMV genes (6, 14, 16, 17) as well as in another human herpesvirus, Epstein-Barr virus (36), although the influence of these upstream ORFs on gene expression has not been assessed. The insights described here should help to unravel the *cis* functions controlling translation of other genes.

In one of the best-studied systems, four AUG codons within the mRNA upstream of the yeast GCN4 ORF have been shown to mediate the posttranscriptional control of GCN4 expression (28); *trans*-acting factors are clearly involved in this regulation. Although our results on the translational delay do not require viral *trans*-acting factors, careful studies on the translational rates in the presence or absence of viral gene products would be needed to decisively determine whether mRNA translatability increases in their presence. Our present results suggest that once transcriptional activation has occurred, the effect of the signal is to prolong the time required to accumulate the gene product.

In its natural setting, the 5' leader we have studied is upstream of the longest ORF (170 amino acids) within the most abundant CMV β transcript (12), accumulating to be approximately 20% of total viral transcripts at early times in infection (37). Even with the translation control element present, there is abundant expression of β gal from this promoter-regulator 5' leader when introduced into a recombinant viral genome (33). Because of the abundance of the transcript, it is surprising that the product of this gene remains uncharacterized. Instability of the natural protein product may account for low levels of total expression.

On the basis of gene mapping and sequencing efforts, it appears that the presence of upstream AUG codons and short ORFs is a general feature of CMV. Upstream AUG codons have been observed in the genes encoding DNA polymerase, a phosphoprotein (pp150), an envelope glycoprotein (gB), and a tegument protein (6, 14, 16, 17). These elements apparently increase the regulatory repertoire used by CMV to regulate the quantities and kinetics of gene expression. The combined use of transcriptional and translational controls enables adaptable and precise regulation of CMV gene expression. We expect that this combination of controls will regulate expression in other eucaryotic systems.

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