JOURNAL OF VIROLOGY, Feb. 1995, p. 1030–1036 Vol. 69, No. 2
0022-538X/95/$04.00+0

Translational Inhibition by a Human Cytomegalovirus Upstream Open Reading Frame despite Inefficient Utilization of Its AUG Codon

JIANHONG CAO[1] AND ADAM P. GEBALLE[1,2]*

Department of Molecular Medicine and Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104,[1] and Departments of Medicine and Microbiology, University of Washington, Seattle, Washington 98195[2]

Received 24 August 1994/Accepted 2 November 1994

The second of three short upstream open reading frames (uORF2) in the transcript leader of the human cytomegalovirus gp48 (gpUL4) virion glycoprotein gene inhibits downstream translation approximately 10-fold. Remarkably, this inhibition depends on the amino acid coding information of uORF2. In the current studies we demonstrate that expression of the cistron downstream from uORF2 depends on ribosomes bypassing the uORF2 AUG codon (AUG2) by a leaky scanning mechanism. Replacing the nucleotides surrounding the wild-type AUG2 codon with those optimal for translation initiation reduces downstream translation approximately 10-fold. Analyses of mutants in which uORF2 either overlaps or is in frame with the downstream reading frame reveal that the initiation frequency at the wild-type AUG2 codon is surprisingly low; rather, the majority of ribosomal subunits bypass the wild-type AUG2 codon because of its suboptimal context. We propose a model to explain this unprecedented example of a paradoxically strong inhibitory effect of an upstream ORF despite inefficient utilization of its initiation codon.

The 230-kb human cytomegalovirus (CMV) genome contains approximately 200 genes (5), whose expression is regulated sequentially during infection of cells in culture. In addition to transcription and transcript processing, translational events modulate the kinetics and abundance of CMV protein expression (reviewed in reference 17). For example, the transcripts of several CMV genes (7, 23, 27), including the virion glycoprotein gene gp48 (gpUL4) (2; unpublished data), accumulate in cells much earlier during infection than do their protein products. However, few details of the mechanisms responsible for translational regulation of CMV gene expression have been elucidated.

Previous studies aiming to identify *cis*-acting sequences that affect translation of CMV transcripts revealed that the second of three short upstream open reading frames (uORF2) in the predominant gp48 transcript (4) represses translation of a downstream cistron (21). The inhibitory effect of uORF2 depends on its amino acid coding content (6), a very unusual property reported thus far for only a few other eukaryotic genes (10, 18, 26). This amino acid sequence dependence suggests that the peptide product of uORF2 mediates the inhibitory effect, although the peptide has not been identified in infected cells.

Since proteins encoded by reading frames positioned downstream of the gp48 leader are expressed, albeit at a low level (6, 21), it is evident that the repressive effect of uORF2 is not absolute. Insights from studies of other eukaryotic genes suggest several mechanisms by which ribosomes could gain access to an AUG codon downstream from uORF2. First, according to the scanning model of eukaryotic translation (13), 40S ribosomal subunits and associated factors load onto an mRNA at the cap site and then scan in a 3′ direction until they encounter the first AUG codon, at which point the 60S subunit joins the complex and protein synthesis commences. However, if the context of nucleotides surrounding the 5′-proximal AUG codon is suboptimal (ACCaugG is optimal, with the underlined A at −3 and G at +4 being the most influential bases [11]) or if the AUG codon is very close to the cap site (14, 22), the 40S subunits may bypass the AUG codon, a process known as leaky scanning. Second, in a subset of viral and cellular mRNAs, ribosomes never encounter the upstream AUG codon because they load onto the mRNA at an internal site rather than at the cap (16). Finally, ribosomes may translate an uORF but then reinitiate at the downstream AUG codon (12).

The present studies were initiated to clarify the mechanism that accounts for translation downstream from uORF2. Our results reveal that a surprisingly large fraction of ribosomes bypass the uORF2 initiation codon, AUG2, because of the suboptimal context of surrounding nucleotides. Thus, translation of the downstream cistron results from a leaky scanning mechanism. Understanding the mechanism underlying the paradoxically potent inhibitory effect of uORF2 despite inefficient utilization of its AUG codon is relevant to studies of the regulation of other viral and cellular genes that contain uORFs (15).

MATERIALS AND METHODS

Cells and virus. Human fibroblasts (HF) and CMV (Towne) were grown in Dulbecco's modified Eagle's medium supplemented with 10% Nu serum (Collaborative Research, Inc., Bedford, Mass.).

Plasmids. The promoterless β-galactosidase (β-gal) plasmid pEQ3 and plasmids containing the CMV major immediate-early (IE) promoter/enhancer directing transcription of β-gal transcripts without (pEQ176) or with (pEQ239, pEQ325, pEQ334, and pEQ400) gp48 leader sequences have been described previously (6, 21). The plasmid pEQ276 expresses the CMV IE1 and IE2 proteins (2). To construct plasmids with AUG2 context mutations, the gp48 leader in CMV (Towne) DNA was amplified by PCR with primers 32 (5′-GAT CAAGCTTAATCAGTTGCCGGCCTT[A/C]CCATG[G/C]AGCCG-3′, where equimolar mixtures of the two bracketed bases were used at positions −3 and +4 relative to the underlined AUG2 codon) and gp48.3 (21). The PCR product was digested with *Hin*dIII and *Afl*II and ligated into pEQ239 at the *Hin*dIII and *Afl*II sites. Clones containing the sequences ACCatgG, CCCatgG, ACCatgC, and CCCatgC (named pEQ422, pEQ423, pEQ424, and pEQ425, respectively) flanking the AUG2 codon were identified by sequence analysis. Plasmid pEQ429, with the AUG2 codon context ACCatgG, was constructed by using the same strategy except that pEQ400, rather than viral genomic DNA, was used as the template for the PCR.

The 3.5-kb *Nae*I fragment from pEQ239 containing AUG1 was ligated to the 4.9-kb *Nae*I fragment from pEQ422 containing the optimal context AUG2 mu-

* Corresponding author. Mailing address: Fred Hutchinson Cancer Research Center, C2-023, 1124 Columbia St., Seattle, WA 98104. Phone: (206) 667-5122. Fax: (206) 667-6523. Electronic mail address: ageballe@fred.fhcrc.org.

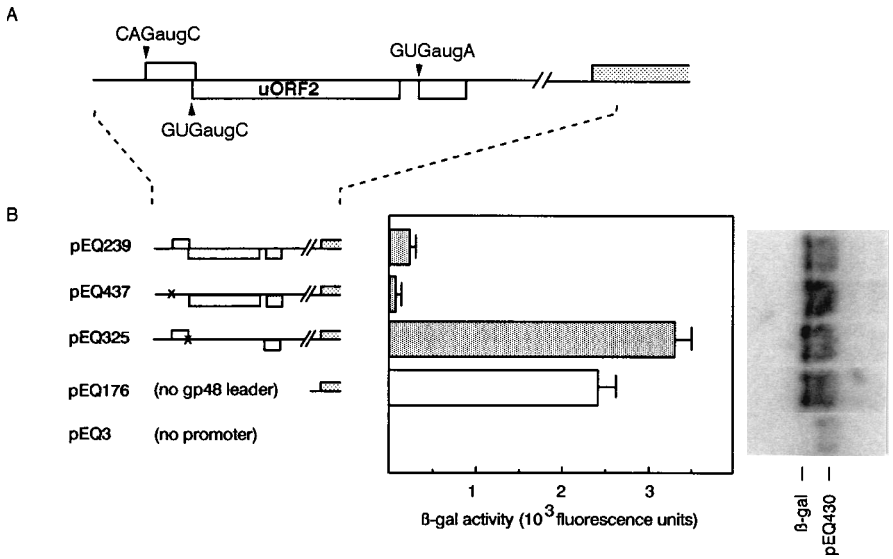

FIG. 1. Effects of mutation of AUG1. (A) Structure of the gp48 transcript leader containing three uORFs initiating at AUG codons surrounded by the indicated context of nucleotides. (B) Unlike AUG2, AUG1 is not required for translational inhibition by the gp48 transcript leader. The 5′ ends of the transcript leader expressed from plasmids with no gp48 leader (open bar, pEQ176) or containing the gp48 leader (shaded bars) with wild-type AUG1 and AUG2 (pEQ239) or mutations of AUG1 or AUG2 (X) are shown (left). The mean β-gal activity (± standard deviation) from duplicate dishes minus that expressed by the promoterless control (pEQ3) was determined after transfection and CMV infection as described in Materials and Methods. Accumulated β-gal mRNA from the test plasmids and the enzymatically inactive control plasmid pEQ430 (right) was detected by Northern blot hybridization of RNA extracted from the same cells in which β-gal activity was measured.

tation ACCatgG, resulting in pEQ436. Conversely, ligation of the 4.9-kb *Nae*I fragment from pEQ422 with the 3.5-kb *Nae*I fragment from pEQ239 resulted in pEQ437, which contains the AUG1-to-UUG mutation and a wild-type AUG2 codon.

Several plasmids were constructed in which deletions of the gp48 leader sequences resulted in overlap of uORF2 and the β-gal ORF. After pEQ334 was cut with *Xho*I, treated with DNA polymerase I Klenow fragment, and religated, sequence analysis identified one clone, pEQ346, which had lost the *Xho*I site as a result of an unexpected insertion of a single G (underlined in CTCGGAG) into the *Xho*I site. After pEQ422 was linearized with *Afl*II, blunted with mung bean nuclease, and digested with *Hin*dIII, the 91-bp gp48 leader fragment was ligated into pEQ176 which had been cut with *Bgl*II, blunted with DNA polymerase I Klenow fragment, and then cut with *Hin*dIII, resulting in pEQ426. pEQ427 was constructed in the same way except that the 91-bp fragment was isolated from pEQ425.

A series of in-frame fusions of uORF2 and β-gal were constructed by amplifying pEQ325, pEQ239, pEQ422, pEQ437, and pEQ436 with primer 27 (6) and primer 33 (5′-GATCGGTACCGGCGGGATGTATTTGC-3′). After digestion with *Hin*dIII and *Asp* 718, the amplified products were ligated into pEQ176 cut with *Hin*dIII and Asp 718, resulting in pEQ441, pEQ442, pEQ443, pEQ444, and pEQ445, respectively.

Transient-transfection assay and RNA analysis. Transfection by calcium phosphate precipitation was carried out as described previously (9). Briefly, 4×10^5 HF in a 60-mm dish were transfected with 5 μg of test plasmid and 5 μg of control plasmid (pEQ430). At 16 h after transfection, the cells were fed with fresh medium and, 8 h later, were infected with CMV (Towne) at a multiplicity of infection of 10 PFU per cell to augment transcription from the transfected plasmids. β-gal activity and β-gal RNA accumulation were measured 48 h after infection, as described previously (21). β-gal RNA was detected by Northern (RNA) hybridization of whole-cell RNA as described previously (7).

β-gal stability assay. At 2 days after cotransfection of uORF2–β-gal in-frame fusion plasmids and pEQ276 (encoding the CMV transcriptional transactivator genes IE1 and IE2) into HF, β-gal activity in cytoplasmic extracts was measured as described previously (2). The cells in the remaining dishes were fed with medium containing 100 μg of cycloheximide per ml, and β-gal activity was measured in cytoplasmic extracts prepared at various times thereafter. At each time point, all remaining cells were fed with fresh medium containing cycloheximide.

RESULTS

Inhibition by the gp48 leader is independent of uORF1. To clarify the mechanism of translation of the cistron downstream from the inhibitory uORF2, we used transfection assays of β-gal expression plasmids containing gp48 transcript leader sequences with either wild-type or mutant nucleotide sequences surrounding the AUG2 codon. A peculiar feature of the gp48 transcript leader sequence that complicated this approach was that the nucleotides flanking the AUG2 codon (GUGaugC), which are the most important determinants of the AUG2 codon translation initiation context (shown in capital letters), also contain the termination codon for uORF1 (underlined). Thus, most mutations that change the context of AUG2 by altering position −1 or −2 (where +1 is the A of the AUG codon) also eliminate the uORF1 termination codon, resulting in a carboxy-terminal extension of uORF1.

To evaluate the role of uORF1, we first constructed a mutant in which AUG1 was changed to UUG. We tested this plasmid (pEQ437; Fig. 1B) and plasmids containing no gp48 leader (pEQ176), the wild-type gp48 leader (pEQ239), or the gp48 leader with mutation of the AUG2 codon (to AAG; pEQ325) in a transfection-infected assay as described in Materials and Methods. Elimination of AUG1 slightly reduced β-gal expression compared with the wild-type sequence. In contrast, mutation of AUG2 completely alleviated the inhibitory effects of the gp48 leader, as shown previously (21).

Northern blot hybridization of whole-cell RNA from the same transfections, probed with a β-gal-specific probe, revealed similar levels of β-gal RNA, indicating that the differences in β-gal activities expressed by these plasmids were not due to differences in RNA accumulation. In this and subsequent transfections (shown in Fig. 1 to 4), we cotransfected a truncated, enzymatically inactive β-gal plasmid (pEQ430) along with each test plasmid to monitor transfection efficiency, recovery of RNA, and blotting efficiency. Consistent with previous analyses of mutants with deletions that removed AUG1 from the gp48 leader (21), these data verify that uORF1 has little impact on downstream translation.

AUG2 codon context effects in transcripts with otherwise wild-type gp48 leaders. Although the wild-type gp48 leader inhibits downstream translation approximately 90% (6, 24) (Fig. 1), the downstream cistron is still expressed to a measur-

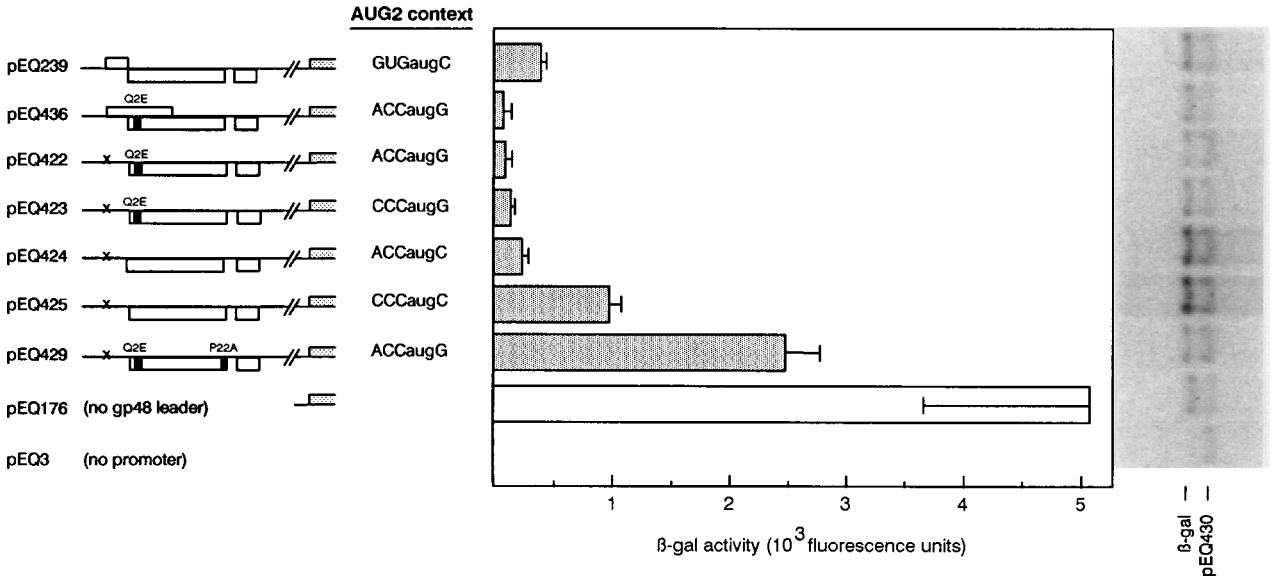

FIG. 2. Effects of AUG2 codon context mutations on downstream translation in transcripts containing the full gp48 transcript leader. Control (open) and gp48 leader (shaded) plasmids expressing transcript leaders containing the indicated mutations in AUG1 (X) and in the nucleotides surrounding AUG2 (left) were transfected into HF, and the cells were infected with CMV. As in the wild-type gp48 transcript leader, uORF2 in these plasmids is 22 codons long and terminates 141 nucleotides upstream of the β-gal AUG codon. β-gal activity (middle) and RNA levels (right) in duplicate dishes were measured as described in the legend to Fig. 1.

able level. Several mechanisms could account for this expression downstream from uORF2. A fraction of ribosomes might bypass AUG2 either because scanning ribosomes fail to recognize the AUG2 codon (for example, because the context of surrounding nucleotides is suboptimal) or because ribosomes might load onto the transcripts at an internal ribosome entry site. Alternatively, ribosomes might translate uORF2 and then reinitiate at the β-gal AUG codon. To distinguish among these mechanisms, we constructed mutants with alterations in the nucleotides adjacent to the AUG2 codon. If the nucleotides flanking the AUG2 codon are suboptimal for translation initiation and allow ribosomes to leak past AUG2, improving the AUG2 codon context should result in a further reduction in β-gal expression. In contrast, if either an internal ribosomal entry or reinitiation mechanism is responsible for β-gal expression, improving the context of AUG2 would not be expected to reduce β-gal expression.

We constructed mutations in the gp48 leader involving nucleotides −3 to −1 and +4 relative to AUG2 and analyzed expression from the mutant plasmids in transfection assays (Fig. 2). Similar to previous experiments, the wild-type gp48 leader in pEQ239 reduced β-gal expression approximately 10-fold in this experiment. Changing the AUG2 codon context to the optimal sequence, ACCaugG (pEQ436; Fig. 2), further reduced β-gal activity to a near-background level. In these experiments, the limit of detection is approximately 1 to 2% of the level expressed by the control construct that has no gp48 leader (pEQ176).

In addition to improving the context for translation initiation at the AUG2 codon, the AUG2 context mutations in pEQ436 also eliminate the uORF1 stop codon and change the second codon of uORF2 from glutamine to glutamate (Q2E). To distinguish which of these features was responsible for the reduction in β-gal expression from pEQ436 compared with the wild-type construct (pEQ239), we constructed additional mutants and assayed their β-gal expression in transfection assays. pEQ422 contains the same optimal AUG2 codon context as pEQ436 but also has a mutation eliminating the AUG1 codon (Fig. 2, pEQ422). Like pEQ436, pEQ422 expresses near-background β-gal activity, indicating that the increased inhibition in pEQ436 did not result from the extension of uORF1. The inhibitory impact of the gp48 leader in constructs containing the optimal context AUG2 codon depends on the coding information of uORF2, as does the uORF2 with a wild-type AUG2 codon (6), since mutation of the proline at codon 22 to alanine (P22A) relieved most of the inhibitory impact of the leader (compare pEQ429 with pEQ422).

Like pEQ422, mutants pEQ423, pEQ424, and pEQ425 share the mutation eliminating the AUG1 codon but have different nucleotides surrounding AUG2 (Fig. 2). Compared with the optimal context AUG2 codon (pEQ422), changing either the −3 or the +4 base to the suboptimal base cytosine (pEQ423 and pEQ424, respectively) resulted in a slight but reproducible increase in β-gal expression. A greater level of β-gal activity was expressed from a plasmid in which both the −3 and +4 bases are cytosines (pEQ425). Weakening the AUG2 codon context by changing the A to C at the −3 position increased downstream translation (compare pEQ422 with pEQ423 and pEQ424 with pEQ425). It was not possible to distinguish whether the effects of changing the +4 nucleotide from C to G were due to changes in translation initiation frequency at AUG2 or to the Q2E change in uORF2. Although the level of accumulated β-gal RNA corresponding to pEQ424 and pEQ425 appeared to be increased compared with that of RNA expressed by the other plasmids in this Northern blot, in other experiments (not shown) there was no consistent difference in accumulation of RNA expressed by the β-gal expression plasmids shown in Fig. 2. These results suggest that a measurable fraction of ribosomes bypass the wild-type AUG2 codon as a result of its suboptimal context.

AUG2 codon context effects in transcripts with overlapping uORF2 and β-gal reading frames. In earlier studies (21), we were puzzled to find that a deletion mutant of the gp48 leader that resulted in overlap of uORF2 (extended at its carboxy terminus) and the β-gal ORF expressed high levels of β-gal activity (pEQ334 in Fig. 3). Even after discovering that alter-

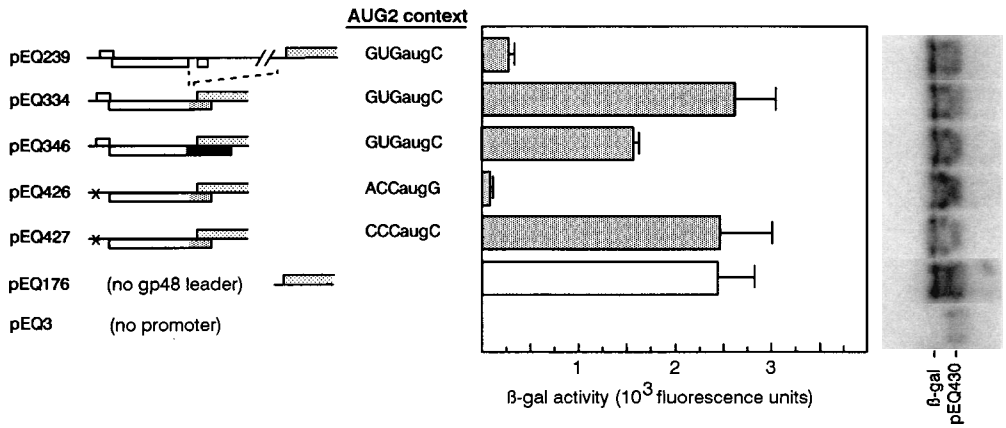

FIG. 3. AUG2 codon context effects on downstream expression in deletion constructs with overlapping ORFs. Deletion of sequences between the termination codon of uORF2 through the 3′ end of the gp48 leader (dotted line) generated mutant transcripts (left) which contain an extended uORF2 (shaded) that terminates downstream from the β-gal AUG codon. Insertion of a single nucleotide in pEQ436 generated a carboxy-terminal extension of uORF2 in an alternative reading frame compared with pEQ344, pEQ426, and pEQ427. Mutations in the AUG2 codon context are shown. β-gal enzymatic activity (middle) and mRNA accumulation (right) were assayed after transfection and CMV infection as described in the legend of Fig. 1.

ations of the amino acid coding content of uORF2 inactivated its inhibitory properties (6), we expected that such mutants would express reduced levels of β-gal since ribosomes translating the extended uORF2 would not be likely to have access to the β-gal AUG codon. Several mechanisms could account for the efficient expression of β-gal from such transcripts: (i) ribosomes might initiate at the AUG2 codon in these transcripts but undergo a frame shift into the β-gal reading frame; (ii) a large percentage of ribosomes might bypass AUG2 by a leaky scanning mechanism; or (iii) after translating, uORF2 ribosomes might scan backwards (19) and reinitiate at the β-gal AUG codon.

To distinguish among these possibilities, we constructed additional plasmids with deletions creating an extended uORF2 that overlaps with the β-gal ORF (Fig. 3). The unintentional insertion of a single extra nucleotide at the junction of the gp48 leader sequences and the polylinker adjacent to the β-gal ORF created pEQ346, containing an extended uORF2 that terminates 43 nucleotides downstream from the β-gal AUG codon. In transfection assays, pEQ346 expressed a high level of β-gal similar to pEQ334 (Fig. 3). While it is conceivable that a frameshift site was fortuitously created in constructing pEQ334 or pEQ346, it would be extremely unlikely if these two constructs, which differ by only a single base pair, both contained frameshift sites that are highly efficient yet shift the ribosome frame +1 in one case and −1 (or +2) in the other case. These data argue against frame shifting as the explanation for high-level β-gal expression from pEQ334 and pEQ346.

We next changed the AUG2 codon context in the plasmids with overlapping ORFs. If downstream expression results from leaky scanning at the AUG2 codon owing to its suboptimal context, improving the context should reduce β-gal expression. However, if either frame shifting or backward scanning followed by reinitiation is responsible, improving the AUG codon context should increase, or at least not reduce, β-gal expression. Plasmid pEQ426, with an optimal-context AUG2 codon, expressed very low levels of β-gal. In contrast, plasmid pEQ427, with a weak-context AUG2 codon, expressed high levels of β-gal, similar to expression by the wild-type AUG2 codon construct pEQ334. These data are most consistent with a leaky scanning mechanism accounting for translation downstream from the wild-type AUG2 codon.

AUG2 codon context effects in transcripts with in-frame fusions of the uORF2 and β-gal reading frames. To investigate the efficiency of translation initiation at the AUG2 codon more directly, we constructed plasmids in which uORF2 is fused in-frame to the β-gal ORF. In these plasmids, the uORF2 termination codon, the intercistronic sequences, and the β-gal AUG codon have been deleted. In previous studies we found that deletion of the uORF2 termination codon eliminated the coding sequence-dependent inhibitory effect of the uORF2. Thus, β-gal expression from these in-frame fusion constructs provides a direct measure of the initiation frequency at the AUG2 codon. Compared with the wild-type AUG2, which expressed low but detectable levels of β-gal (pEQ444 and pEQ442), the optimal-context AUG2 codon constructs expressed approximately 20- to 30-fold more β-gal (Fig. 4, compared pEQ444 with pEQ443 and pEQ442 with pEQ445). The increased expression initiating from the optimal-context AUG2 codon was independent of the AUG1 codon, again indicating that AUG1 and uORF1 do not affect translation in these assays. Northern hybridization analysis showed that differences in β-gal RNA accumulation did not account for the differences in β-gal activities expressed by the wild-type AUG2 codon constructs compared with the optimal-context construct. These data confirm that the wild-type AUG codon is used inefficiently compared with an optimal-context AUG2 codon.

Mutation of the second codon of uORF2 does not affect protein stability. Mutation of the +4 nucleotide at AUG2 codon from C to G changes the initiation context but also affects the amino acid coding information of uORF2. Thus, the effects of some our mutations involving the +4 position could be due to changes in the encoded proteins rather than to changes in translation initiation rates. Since the second amino acid in proteins is thought to be a major determinant of protein stability (24), we investigated the relative stability of proteins initiating with the wild-type AUG2 codon encoding glutamine at codon 2, compared with the optimal-context AUG2 with glutamate at codon 2. As detailed in Materials and Methods, we measured the stability of uORF2–β-gal fusion proteins in transfected cells by serially measuring β-gal activity in cells after addition of cycloheximide to prevent new protein synthesis (25). The half-lives of uORF2-β-gal fusion proteins were similar (approximately 15 h) regardless of the second codon of uORF2 (Fig. 5). Although we were unable to perform studies of the stability of the 22-codon uORF2 (because we have not

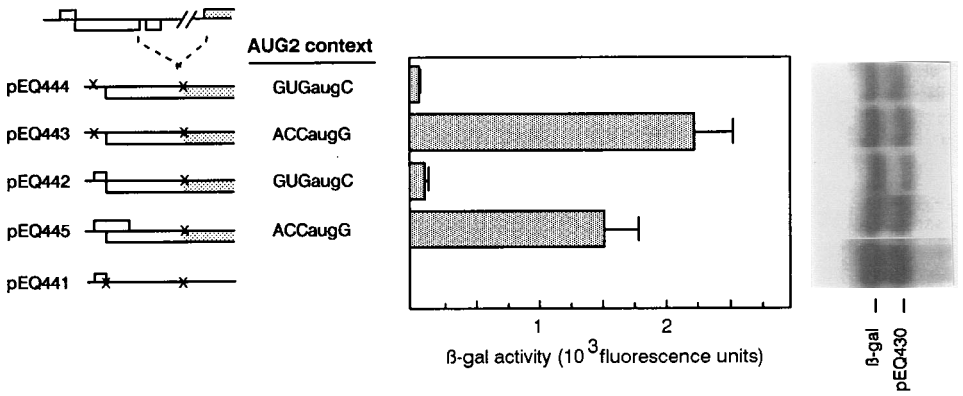

FIG. 4. Effects of the AUG2 codon context in in-frame fusion constructs. Plasmid pEQ441, containing no β-gal initiation codon, or in-frame fusion plasmids in which β-gal synthesis results from translation initiation at the gp48 AUG2 codon were transfected into HF. After CMV infection, both β-gal enzymatic activity (middle) minus the background expressed in cells transfected with pEQ441 and mRNA accumulation were assayed as described in the legend to Fig. 1.

yet been able to detect the uORF2 peptide), these data support the conclusion that the increased inhibition by the optimal-context AUG2 codon results from the increases in translation initiation at AUG2, not from changes in the uORF2 peptide.

DISCUSSION

Although uORFs are found in 5 to 10% of eukaryotic genes and in an even higher proportion of genes involved in control of cellular growth and differentiation (15), their regulatory effects have been investigated in only a few cases. The limited number of such studies is in part due to the belief that many of these uORFs are inconsequential because their initiation codons are surrounded by a suboptimal context of nucleotides. Our studies of gp48 uORF2 demonstrate that this assumption is invalid.

The magnitude of the inhibitory effect of gp48 uORF2 depends not only on its coding content but also on the efficiency of translation initiation at AUG2. When the wild-type AUG2 codon is replaced with the optimal-context mutation in plasmids with an otherwise wild-type uORF2, β-gal expression is reduced to background levels (Fig. 2). These results are consistent with reduced expression downstream from the gp48 leader, expressed from retroviral vectors, upon mutation of the nucleotides flanking the wild-type AUG2 codon to an optimal context (3). In plasmids with deletions of the gp48 leader that create an extended uORF2 overlapping the β-gal ORF, the wild-type AUG2 codon had no measurable effect on downstream translation, whereas the optimal-context AUG2 mutant inhibited downstream translation more than 10-fold (Fig. 3). Finally, studies of in-frame fusions of uORF2 to the β-gal ORF revealed a greater-than-10-fold increase in β-gal expression with the optimal-context AUG2 compared with the wild-type AUG2 (Fig. 4). Together, these three sets of data are most consistent with the interpretation that compared with the number of ribosomes that initiate at an optimal-context AUG2 codon, only approximately 10% of ribosomes initiate translation at the wild-type AUG2 codon.

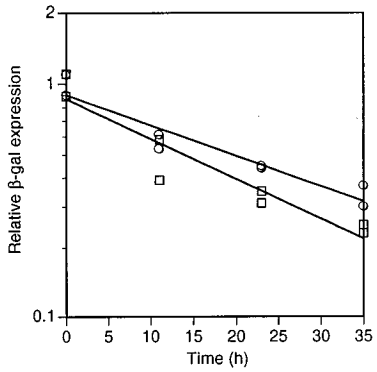

FIG. 5. Stability of uORF2–β-gal fusion proteins. uORF2–β-gal in-frame fusion plasmids, containing the either the wild-type AUG2 codon (pEQ444) (□) or the optimal-context AUG2 codon (pEQ443) (○) were cotransfected into HF with a plasmid expressing the CMV transcriptional transactivator genes IE1 and IE2. At 2 days later, β-gal activity was measured immediately and after cells were incubated in medium containing cycloheximide as described in Materials and Methods. The relative β-gal activity at each time point, compared with the activity expressed immediately prior to addition of drug ($t = 0$), is plotted on a logarithmic scale. Lines were generated by the linear least-squares method.

Although we cannot completely exclude the possibility that the effects of the optimal-context mutations are due to changes in the peptide encoded by uORF2 (Q2E) rather than to changes in the translation initiation frequency at AUG2, several lines of evidence support the latter interpretation. First, comparison of plasmids with the same nucleotide at the +4 position (and thus the same second codon of uORF2) but different nucleotides at the −3 position clearly demonstrates that at least some of the differences in β-gal expression between the wild-type and the optimal-context mutants are not due to differences in the uORF2 peptide (Fig. 2). Instead, these differences are probably due to the major impact of the nucleotide at the −3 position on translation initiation (11). Second, in previous studies (21), all mutants with carboxy-terminal extensions of uORF2 expressed high levels of β-gal, similar to those for controls lacking uORF2, suggesting that effects of uORF2 mutants with carboxy-terminal extensions are not dependent on the peptide sequence. Therefore, the reduction in β-gal expression when the AUG2 context is optimized in transcripts with overlapping ORFs (Fig. 3) most probably reflects increased translation initiation at AUG2 rather than a change in the peptide product of the extended uORF2. Finally, the Q2E mutation does not have a significant effect on protein stability, at least when expressed at the beginning of a uORF2–β-gal fusion protein (Fig. 5). Thus, our data are most consistent with the effects of AUG2 context mutations being mediated by changes in translation initiation rates at AUG2 rather than by changes in the encoded peptide.

The relative inefficiency of translation initiation at the wild-type AUG2 codon (GUGaugC) is not totally unexpected. For

example, in Kozak's studies highlighting the key role of nucleotides at the −3 and +4 positions in determining translation initiation efficiency, changing the initiation codon context from GUUaugU to AUUaugG increased translation sevenfold (11). A corollary of these observations, supported by an increasing number of examples (reviewed in reference 8), is that some or perhaps even most eukaryotic transcripts may express alternative proteins by initiating at AUG codons downstream from a 5′-proximal but suboptimal AUG codon.

Besides gp48, only a very few other eukaryotic gene transcripts have been shown to contain uORFs that inhibit downstream translation by a mechanism that depends on the amino acid coding information of the uORF (10, 18, 26). In one of these, the *S*-adenosylmethionine decarboxylase gene (Ado MetDC), leaky scanning has recently been shown to play an important role. The AdoMetDC uORF inhibits downstream translation in T-cell lines but is ignored in other cell types because of the proximity of the AUG codon to the cap site (20). These studies suggest that regulation of the leakiness at an upstream AUG codon may be a general mechanism for fine-tuning gene expression downstream from uORFs in other genes.

Although parameters such as the AUG codon context or 5′-end proximity that influence AUG codon recognition may modulate the effects of uORFs, knowledge of these parameters is insufficient for predicting the impact of uORFs. For example, even an optimal-context AUG codon may be neutral with respect to downstream translation (1) (Fig. 2, pEQ429). Conversely, the studies presented in this paper demonstrate that even an inefficiently recognized upstream AUG codon can dramatically affect downstream translation. We are not aware of any other gene transcript in which a uORF with an inefficiently utilized AUG codon has a potent inhibitory effect on downstream translation. Our results emphasize the need for further studies to define the full set of parameters that determine the regulatory effects of uORFs.

If the vast majority of ribosomes bypass AUG2, how does uORF2 exert its inhibitory effect? In Fig. 6 we present a model of translational inhibition by uORF2. Initially, 40S subunits scan past the wild-type AUG2 codon because of its poor context and translate the downstream ORF. Approximately 1 in 10 ribosomes initiates at AUG2 and translates uORF2. Some as yet uncharacterized interaction of the nascent peptide product of uORF2 with the ribosome and the mRNA prevents or delays the normal events required for termination of translation. The stalling of the ribosome during translational termination creates a roadblock that obstructs subsequent ribosomes from scanning past uORF2. This stalling phase may be prolonged such that no protein product of the downstream ORF is synthesized by the vast majority of transcripts. Thus, even though only a minority of ribosomes initiate at AUG2, they can exert a lasting inhibitory effect on downstream translation.

In mutants containing the optimal context AUG2 codon, the majority of ribosomes initiate at the AUG2 codon. If the remainder of the uORF2 is wild type, the ribosomes again stall at termination, resulting in an even greater reduction in downstream expression than occurs with the wild-type AUG codon (Fig. 2). However, if the uORF2 peptide contains a mutation such as P22A (Fig. 2, pEQ429), the ribosomes terminate efficiently at the end of uORF2 but are able to reinitiate translation at the β-gal AUG codon.

This model accounts for the strong inhibitory effect of uORF2 despite inefficient initiation at the wild-type AUG2 codon and is consistent with the observations that uORF2 acts only in *cis* (6) and that mRNAs containing uORF2 cosediment predominantly with monosomes in sucrose gradients (6, 21). However, many questions about this regulatory system remain unanswered. For example, although no viral factors are required for the inhibitory effects of uORF2 (3), we do not know whether viral factors participate in countering the inhibition during infection. Work is in progress to clarify this issue and to test additional predictions of the model.

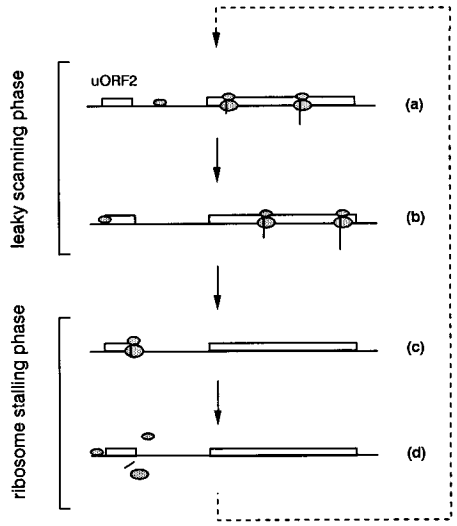

FIG. 6. Model of translational inhibition mediated by gp48 uORF2. (a) During an initial leaky scanning phase, most 40S ribosomal subunits leak past AUG2 because of its poor context and translate the downstream ORF. (b) Eventually, a 40S subunit recognizes AUG2 and translates uORF2. (c) The nascent uORF2 peptide prevents efficient termination of translation and results in the ribosomal stalling. The ribosome-peptide complex creates a roadblock that obstructs scanning of subsequent 40S past AUG2 to the downstream AUG codon. (d) Eventually, termination and release of the mRNA may occur and enable further use of the mRNA for downstream translation (dotted line).

ACKNOWLEDGMENTS

We thank Mark Schleiss for construction of plasmid pEQ346, Stephanie Child and John Alderete for critical review of the manuscript, and the Biotechnology, Biocomputing and Image Analysis Resources of the Fred Hutchinson Cancer Research for technical assistance.

This work was supported by PHS grant AI26672. J.C. was supported in part by a grant from the Bayley Construction Guild.

REFERENCES

1. **Biegalke, B. J., and A. P. Geballe.** 1990. Translational inhibition by cytomegalovirus transcript leaders. Virology **177:**657–667.
2. **Biegalke, B. J., and A. P. Geballe.** 1991. Sequence requirements for activation of the HIV-1 LTR by human cytomegalovirus. Virology **183:**381–385.
3. **Cao, J., and A. P. Geballe.** 1994. Analysis of the human cytomegalovirus gpUL4 (gp48) transcript leader translational signal by retroviral mutagenesis. Virology **205:**151–160.
4. **Chang, C.-P., D. H. Vesole, J. Nelson, M. B. A. Oldstone, and M. F. Stinski.** 1989. Identification and expression of a human cytomegalovirus early glycoprotein. J. Virol. **63:**3330–3337.
5. **Chee, M. S., A. T. Bankier, S. Beck, R. Bohni, C. M. Brown, R. Cerny, T. Horsnell, C. A. Hutchinson III, T. Kouzarides, J. A. Martignetti, E. Preddie, S. C. Satchwell, P. Tomlinson, K. M. Weston, and B. G. Barrell.** 1990. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169, p. 125–169. *In* J. K. McDougall (ed.), Cytomegaloviruses. Springer-Verlag, New York.
6. **Degnin, C. R., M. R. Schleiss, J. Cao, and A. P. Geballe.** 1993. Translational inhibition mediated by a short upstream open reading frame in the human cytomegalovirus gpUL4 (gp48) transcript. J. Virol. **67:**5514–5521.
7. **Geballe, A. P., F. S. Leach, and E. S. Mocarski.** 1986. Regulation of cytomegalovirus late gene expression: γ genes are controlled by posttranscriptional events. J. Virol. **57:**864–874.
8. **Geballe, A. P., and D. R. Morris.** 1994. Initiation codons within 5′ leaders of

mRNAs as regulators of translation. Trends Biochem. Sci. **19:**159–164.
9. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
10. **Hill, J. R., and D. R. Morris.** 1993. Cell-specific translational regulation of S-adenosylmethionine decarboxylase mRNA: dependence on translation and coding capacity of the *cis*-acting upstream open reading frame. J. Biol. Chem. **268:**726–731.
11. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44:**283–292.
12. **Kozak, M.** 1987. Effects of intercistronic length on the efficiency of reinitiation by eukaryotic ribosomes. Mol. Cell. Biol. **7:**3438–3445.
13. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108:**229–241.
14. **Kozak, M.** 1990. A short leader sequence impairs the fidelity of initiation by eukaryotic ribosomes. Gene Exp. **1:**111–115.
15. **Kozak, M.** 1991. An analysis of vertebrate mRNA sequences: intimations of translational control. J. Cell Biol. **115:**887–903.
16. **Meerovitch, K., and N. Sonenberg.** 1993. Internal initiation of picornavirus RNA translation. Semin. Virol. **4:**217–227.
17. **Mocarski, E. S.** 1991. Evidence for posttranscriptional modulation of cytomegalovirus gene expression, p. 287–299. *In* E. Wagner (ed.), Herpesvirus transcription and its regulation. CRC Press, Inc., Boca Raton, Fla.
18. **Parola, A., and B. K. Kobilka.** 1994. The peptide product of a 5′ leader cistron in the β_2 adrenergic receptor mRNA inhibits receptor synthesis. J. Biol. Chem. **269:**4497–4505.
19. **Peabody, D. S., and P. Berg.** 1986. Termination-reinitiation occurs in the translation of mammalian cell mRNAs. Mol. Cell. Biol. **6:**2695–2703.
20. **Ruan, H., J. R. Hill, S. Fatamie-Nainie, and D. R. Morris.** 1994. Cell-specific translational regulation of S-adenosylmethionine decarboxylase mRNA. J. Biol. Chem. **269:**17905–17910.
21. **Schleiss, M., C. R. Degnin, and A. P. Geballe.** 1991. Translational inhibition mediated an upstream reading frame within the cytomegalovirus gp48 gene transcript leader. J. Virol. **65:**6782–6789.
22. **Sedman, S. A., G. W. Gelembiuk, and J. E. Mertz.** 1990. Translation initiation at a downstream AUG occurs with increased efficiency when the upstream AUG is located very close to the 5′ cap. J. Virol. **64:**453–457.
23. **Spaete, R. R., R. M. Thayer, W. S. Probert, F. R. Masiarz, S. H. Chamberlain, L. Rasmussen, T. C. Merigan, and C. Pachl.** 1988. Human cytomegalovirus strain Towne glycoprotein B is processed by proteolytic cleavage. Virology **167:**207–225.
24. **Varshavsky, A.** 1992. The N-end role. Cell **69:**725–735.
25. **Wang, C.-T., J. Stegeman-Olsen, Y. Zhang, and E. Barklis.** 1994. Assembly of HIV GAG-B-galactosidase fusion proteins into virus particles. Virology **200:**524–534.
26. **Werner, M., A. Feller, F. Messenguy, and A. Pierard.** 1987. The leader peptide of yeast gene cpa1 is essential for the translational repression of its expression. Cell **49:**805–813.
27. **Wright, D. A., and D. H. Spector.** 1989. Posttranscriptional regulation of a class of human cytomegalovirus phosphoproteins encoded by an early transcription unit. J. Virol. **63:**3117–3127.