The Plant Cell, Vol. 10, 1733–1745, October 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

Inefficient Reinitiation Is Responsible for Upstream Open Reading Frame–Mediated Translational Repression of the Maize *R* Gene

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Maize *R* genes encode a small family of transcriptional activators of several structural genes in the anthocyanin biosynthetic pathway. The 5′ leader region of most *R* genes contains a 38-codon upstream open reading frame (uORF) that previously was shown to be responsible for the repression of downstream gene expression in a transient transformation assay. In this study, we report that the 5′ leader also can repress translation of the downstream luciferase gene both in the rabbit reticulocyte translation system and in transgenic rice plants. The ability to visualize the uORF peptide after in vitro translation permits quantification of both products of dicistronic mRNAs. Similarly, the construction of transgenic rice plants expressing wild-type and mutant constructs permits the quantification and correlation of steady state mRNA levels and reporter gene activities. Using these assays, we demonstrate directly that translation of the uORF is required for repression, that increasing translation of the uORF peptide decreases downstream gene expression, and that repression is unaffected by either subtle or gross changes in the uORF peptide. Rather, we find that ribosomes that translate the uORF reinitiate inefficiently and that the intercistronic sequence downstream of the uORF mediates this effect.

INTRODUCTION

The initiation of translation of most eukaryotic mRNAs proceeds according to the ribosome scanning model (reviewed in Kozak, 1989b). In this model, the 40S ribosomal subunit with associated factors (the preinitiation complex) engages the mRNA at or near the 5′ cap and scans linearly in a 5′-to-3′ direction for an AUG codon. When a codon in a favorable sequence context is encountered, the 60S subunit joins the 40S complex and initiates protein synthesis. An AUG codon in an unfavorable context may be bypassed by the preinitiation complex. This process is called leaky scanning (Kozak, 1989a).

One prediction of the scanning ribosome model is that AUGs upstream of the main open reading frame (ORF) can repress downstream translation if these upstream AUGs are in a good sequence context. Although <10% of all eukaryotic mRNAs contain upstream AUGs, this frequency is much higher for regulatory genes, including those that control growth and development (Kozak, 1987a). For example, two-thirds of protooncogene mRNAs contain upstream AUG codons, and in several instances, these codons have been shown to reduce downstream translation (Arrick et al., 1991; Merrick, 1992).

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Upstream AUGs initiate ORFs that either overlap the main ORF or terminate before the downstream ORF. In the latter instance, ribosomes translating the upstream ORF (uORF) may resume scanning when translation is terminated. In these cases, the level of downstream translation is determined, in part, by the reinitiation frequency that has been shown to be influenced by factors such as uORF length (Luukkonen et al., 1995), intercistronic distance (Kozak, 1987b), and the nucleotide sequence immediately downstream of the uORF termination codon (Grant and Hinnebusch, 1994).

In a few instances, the amino acid or nucleotide sequence of the uORF is involved in repressing downstream translation. For the uORFs encoded by the mammalian *AdoMetDC* gene (Hill and Morris, 1993), *CPA1* of yeast (Werner et al., 1987), and *gpUL4* (*gp48*) of human cytomegalovirus (Schleiss et al., 1991; Cao and Geballe, 1994), missense but not synonymous mutations in the uORFs reduce or abolish the inhibitory effect on downstream translation. Detection of ribosome arrest during termination of the *gp48* uORF led to the formulation of a model whereby the nascent uORF peptide interacts with the translating ribosomes and delays translation termination, thereby slowing the flow of ribosomes to the downstream ORF (Cao and Geballe, 1996a).

It was reported previously that the 5′ leader of the maize *Lc* gene inhibits downstream translation (Damiani and Wessler, 1993). *Lc* is a member of the *R*/*B* gene family

encoding regulatory proteins required to activate transcription of the anthocyanin biosynthetic pathway. Tissue-specific expression of *Lc* appears to be controlled solely at the transcriptional level (Ludwig et al., 1989, 1990). However, because overexpression of *R* genes lacking the 5′ leader has been associated with developmental defects and even lethality in transgenic plants (Lloyd et al., 1992; Goldsbrough et al., 1996), it has been proposed that translational control of *Lc* and other *R* genes has evolved to ensure that *R* mRNA is translated inefficiently (Damiani and Wessler, 1993).

The 235-nucleotide 5′ leader of *Lc* mRNA contains three in-frame AUG codons. The first, located 60 nucleotides from the 5′ end, is in the best sequence context based on a comparison with both mammalian and plant initiation codons (Damiani and Wessler, 1993). This AUG initiates a 38-codon uORF that terminates 59 nucleotides before the *Lc* ORF. In a previous study, a transient transformation assay was used to test the effect of leader mutations on the translation of the downstream *Lc* gene. Plasmids with *Lc* genes and a variety of leader mutations were cobombarded with a reporter plasmid containing the luciferase reporter gene fused to the *Lc*-responsive *bronze1* (*bz1*) promoter. Luciferase activity thus provided an indirect measure of the efficiency of *Lc* translation. It was found that elimination of all three upstream AUGs by point mutations or deletion of the 5′ terminal 175 nucleotides increased luciferase activity by 20- to 30-fold. A few point mutations in the uORF also increased reporter gene expression and led to the suggestion that the amino acid sequence and/or the codons of the uORF may influence the magnitude of repression.

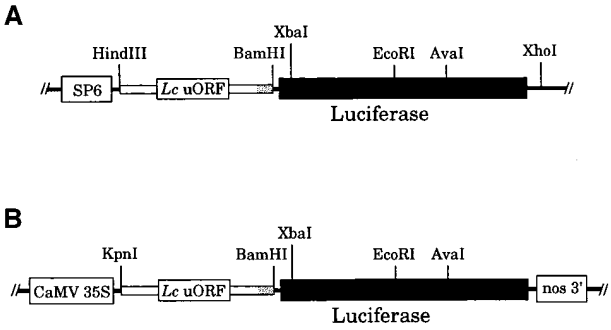


Figure 1. Expression Vectors for in Vitro and in Vivo Assays.

(A) For in vitro assays, the *Lc* 5′ leader (nucleotide +20 to +205 of the *Lc* cDNA) was placed upstream of a luciferase reporter gene. Sequences from the *Lc* 5′ leader are represented by the unfilled boxes, with the uORF indicated. The stippled region was derived from pSelect-1 (see Methods), whereas the luciferase gene and its flanking sequences are from vector pGEM–*luc* (Promega) and are indicated in black. Transcription of the uORF–*Luc* chimeric gene is under control of the SP6 promoter.

(B) For in vivo assays, the chimeric gene was transferred to the vector pJD300, which has the cauliflower mosaic virus (CaMV) 35S promoter and the 3′ nopaline synthase (nos) terminator (Luehrsen et al., 1992).

The conclusions of the previous study had to be tempered by the fact that translation repression was measured as an indirect effect of transcriptional activation and because translation of the uORF could not be verified. These limitations were overcome in this study in three ways. First, the 5′ leader was fused directly to the luciferase reporter so that translation repression could be measured directly. Second, we demonstrated that repression could be assayed in an in vitro translation system as could the synthesis of the uORF peptide, which is stable after in vitro translation. Third, wild-type and mutant constructs were transformed into rice, with both steady state mRNA levels and luciferase activities being quantified and correlated.

RESULTS

Repression of Reporter Gene Translation by the *Lc* Leader in Vitro and in Vivo

To determine whether the *Lc* leader, including the uORF, was sufficient to repress translation of a downstream reporter gene in vitro and in vivo, we fused it to the luciferase coding region (Figures 1A and 1B and Methods). For in vitro translation reactions, chimeric RNA was synthesized from an SP6-derived vector. After RNA gel blot quantitation, equal amounts of RNA were translated in the rabbit reticulocyte in vitro system. This system was chosen rather than the wheat germ translation system because of its superior ability to translate large proteins such as the 61-kD luciferase protein. For in vivo assays, the chimeric uORF–luciferase (uORF–*Luc*) gene was transferred to a plant expression vector (with the 35S cauliflower mosaic virus promoter) and bombarded into maize aleurone cells or stably transformed into rice plants (see Methods). For both in vitro and in vivo assays, luciferase activity provided an initial measure of translational efficiency.

The results presented in Figure 2A show that the *Lc* leader represses downstream luciferase translation both in vitro and in maize aleurone cells. The presence of the wild-type 5′ leader in LUCWT represses translation 2.18-fold when compared with LUCm123, in which the three upstream AUG codons have been eliminated by point mutations. A similar result also was obtained with the wheat germ in vitro translation system (data not shown). When the same constructs were tested after bombardment into maize aleurone cells, LUCm123 encoded 17.9-fold more luciferase than did LUCWT.

The most prominent feature of the 5′ leader is a 38-codon uORF. To determine whether translation of uORF was responsible for the observed repression, we tested whether increasing translation of uORF would affect downstream expression. To this end, the sequence surrounding the first AUG codon of the uORF (uAUG) was changed to match the

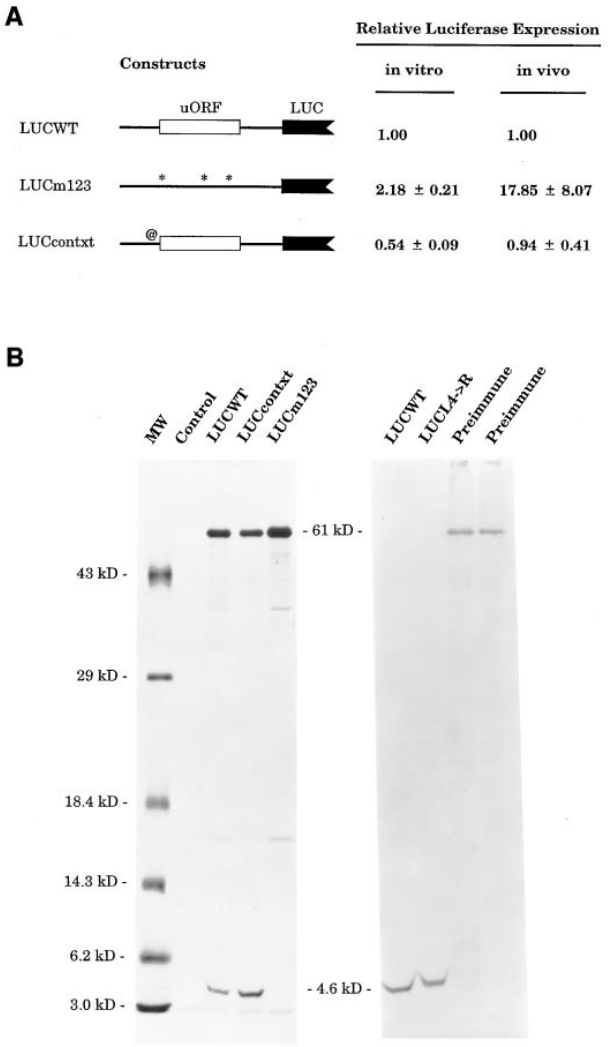


Figure 2. Translation of the uORF and Its Effect on Luciferase Expression Levels.

(A) Constructs and their relative luciferase expression in vitro and in maize aleurone cells with the relative expression level of LUCWT set at 1.00. For the in vivo data, luciferase activity was adjusted for the expression of a cobombarded *CAT* gene (see Methods). Each value (±SD) represents the average of at least four independent assays for the in vitro data and eight bombardments for the in vivo data. Asterisks represent point mutations that eliminate the three upstream AUGs of the *Lc* uORF. The mutation in LUCcontxt (indicated as @) improves the sequence context of the uORF initiation codon (5′-CGCAUGG-3′) to match the eukaryotic consensus (5′-ACCAUGG-3′) (Kozak, 1986, 1987a).

(B) Visualization of the uORF-encoded peptide. ^{35}S-methionine–labeled products were analyzed by SDS-PAGE with a 14% polyacrylamide resolving gel. The labeled reactions were analyzed directly (left) or after immunoprecipitation by using the uORF peptide–specific antisera (right). The two preimmune lanes (from left to right) are for LUCWT and LUCL-4→R, respectively. Five times more in vitro translation reactions were used for these lanes than for immunoprecipitation with the uORF peptide–specific antisera. MW stands for the protein molecular mass markers, with their sizes in kilodaltons indicated at left. The 61-kD product corresponds to the luciferase protein. The predicted size of the *Lc* uORF peptide is 4.6 kD.

eukaryotic consensus (Kozak, 1986, 1987a). This mutation (LUCWT, CGCAUGG; LUCcontxt, ACCAUGG) reduced downstream expression almost twofold in the rabbit reticulocyte in vitro system (Figure 2A, 1.0 versus 0.54) but had little effect on expression in the wheat germ system (data not shown) or after bombardment into maize kernels (Figure 2A, 1.0 versus 0.94). One explanation for this result is that the natural uORF initiation site may already be recognized efficiently in plant cells.

The *Lc* uORF Is Translated in Vitro

To confirm that the uORF peptide actually is translated and that its translation increases with LUCcontxt, we attempted to detect the products of in vitro translation. Translation products were labeled by including ^{35}S-methionine in the reactions and visualized after SDS-PAGE and autoradiography. As shown in Figure 2B, a peptide of the size predicted from the uORF sequence (4.6 kD) was visible among the translation products of LUCWT and LUCcontxt but not LUCm123. Precipitation of a peptide of this size by uORF peptide–specific antisera (raised against the synthetic uORF peptide; see Methods) indicated that this peptide is encoded by the uORF. The fact that products <4.6 kD were not observed suggests that significant levels of translation initiation do not occur from the two internal AUG codons within the uORF; this result is consistent with their poor sequence context for translation initiation.

Quantification of the 4.6-kD uORF peptide and the 61-kD luciferase protein encoded by LUCWT and LUCcontxt, respectively, confirmed that more uORF peptide and less luciferase were encoded by the latter construct (Figure 2B and Table 1). This was accomplished by measuring relative amounts of the in vitro translation products on gels by using a PhosphorImager and adjusting these values for the number of radioactive methionine residues present. For LUCWT and LUCcontxt, the relative amount of the 61-kD protein agreed well with the luciferase activity measurements (Table 1 and Figure 2A). Furthermore, the context improvement resulted in more than a twofold increase in uORF peptide with an almost twofold decrease in the 61-kD protein.

Repression Is Not Mediated by the Amino Acid Sequence of the uORF

Two opposing models can explain how the translation of the uORF could repress downstream translation. Either the uORF sequence itself mediates repression or the act of

translating the uORF, irrespective of its sequence, is repressive. In a previous study, a mutation of the fourth uORF codon from leucine to arginine (L-4→R), when incorporated into the full-length *Lc* cDNA, led to derepressed expression after bombardment into maize aleurones (Damiani and Wessler, 1993). However, we found that this same mutation did not increase downstream luciferase translation in vitro or even in vivo after bombardment into maize cells (Figure 3A). Furthermore, replacement of single uORF codons with alanine or other codons also had no effect on repression in vitro (Figure 3B and data not shown).

Because single codon mutations had no effect on downstream expression, we tested whether a dramatically different uORF sequence could still mediate repression. Twenty-nine of the 38 uORF codons were replaced without changing the length, position, or flanking uORF sequences by deletion of an A three nucleotides downstream from the uAUG and the addition of an A 15 nucleotides upstream of the uORF stop codon (Figures 3A and 3C). The fact that this construct (LUCfs) still encoded repressed levels of luciferase both in vitro and in maize aleurone cells indicates that neither the uORF codons nor the derived amino acids are responsible for the observed repression.

Translation of the Downstream ORF Is Largely Dependent on Ribosome Reinitiation in Maize Cells but Not in Vitro

The second model to explain repression postulates that the act of translating uORF reduces downstream gene expression because ribosome reinitiation is inefficient. To address this issue, we first determined which fraction of luciferase translation is due to reinitiation and which fraction is due to ribosomes that scan past the upstream AUGs in favor of the luciferase ORF (the so-called leaky scanners [Kozak, 1989b]). This was accomplished by deleting all stop codons between the two ORFs, thus eliminating the contribution of reinitiating ribosomes to luciferase expression. The constructs $LUCWT_{ovlp}$ and $LUCcontxt_{ovlp}$ are derived from LUCWT and LUCcontxt, respectively. The extended uORF overlaps the downstream luciferase ORF by 83 nucleotides, making it unlikely that reinitiation would occur after translation initiates at the uAUG (Peabody and Berg, 1986; Cao and Geballe, 1995). Thus, the residual luciferase activity encoded by the overlap constructs will be a direct measure of the contribution of ribosomes that scan past the upstream AUGs.

When translated in vitro, $LUCWT_{ovlp}$ encoded 60% of the activity of LUCWT (Figure 4A), indicating that only 40% of the luciferase encoded by LUCWT is due to ribosomes that reinitiate after translating uORF. Luciferase activity was only 8% of LUCWT in the double mutant $LUCcontxt_{ovlp}$, which contains the context change shown previously to increase translation of uORF in vitro (Figure 2). Thus, when the uAUG was efficiently recognized, the contribution of reinitiating ribosomes to total luciferase translation increased from 40 to 85% (46 of 54), whereas the contribution of leaky scanners decreased from 60 to 15% (eight of 54). Independent evidence for these data was obtained by visualizing the products of translation after SDS-PAGE and autoradiography. Due to the stability of the extended uORF peptide, it could be seen that the context change led to an increase in peptide synthesis and a dramatic decrease in the synthesis of the 61-kD (luciferase) protein (Figure 4B).

Cap-independent translation has been demonstrated in vitro (Svitkin et al., 1996). Thus, internal ribosome entry could be a source of luciferase activity that would complicate the interpretation of the data. To determine whether any of the luciferase expression was due to internal ribosome entry, scanning ribosomes were blocked by introducing an inverted

Table 1. Quantitative Analysis of in Vitro Translation Products

RNA	Relative Amount of uORF Peptide[a]	Relative Amount of Luciferase[a]	Relative Amount of Luciferase from Reinitiation[b]	Reinitiation Frequency in Vitro (%)[c]
LUCWT	133	100	40	30
LUCcontxt	212	59	51	24
LUCm123	—[d]	227	—	—
LUCint102	130	103	43	33
LUCnew1	128	169	109	85
LUCnew2	132	183	123	93
LUCnew2contxt	203	174	166	82

[a] Values have been adjusted for the number of labeled methionine residues in each product, with the relative molar amount of luciferase in LUCWT set as 100. Each value represents the average of at least four independent SDS-PAGE analyses.

[b] Relative amount of luciferase from reinitiation is calculated by subtracting the luciferase from leaky scanning from the relative amount of luciferase for each construct. The fraction of luciferase from leaky scanning is 60% of 100 (LUCWT) for wild-type uORF-containing RNA and 8% of 100 (LUCWT) for constructs containing the uORF with the context improvement (see Figure 4).

[c] Reinitiation frequency in vitro is calculated by dividing the relative amount of luciferase from reinitiation by the relative amount of uORF peptide for each construct.

[d] (—), does not apply to this RNA.

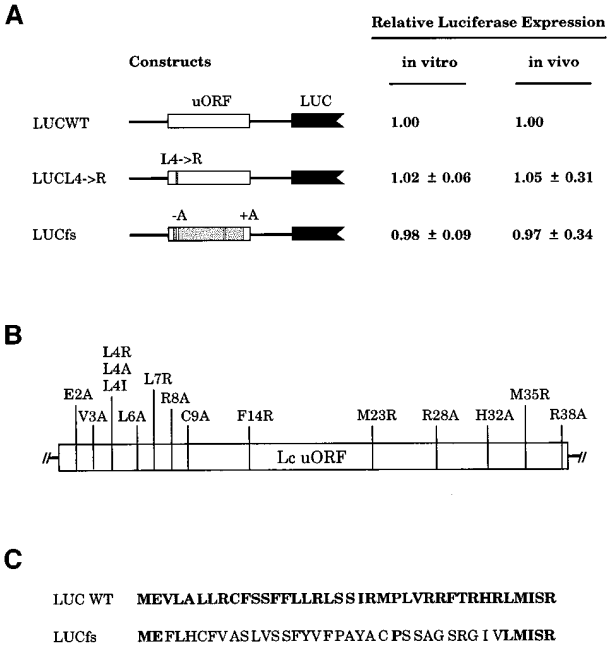


Figure 3. Mutational Analysis of the uORF Coding Region.

(A) Effect of an L-4–to–R (L4→R) and a frameshift mutation on repression. The stippled boxes within the uORF indicate the positions of changed residues. For LUCfs, −A represents the deletion of the A at nucleotide +65 of the *Lc* cDNA (Ludwig et al., 1989), and +A is the addition of an A between +158 (T) and +159 (C). Each value (±SD) represents the average of at least four independent assays for the in vitro data and eight bombardments for the in vivo data.

(B) Additional point mutations analyzed in vitro. Each construct has one of the point mutations.

(C) Amino acid sequences of the LUCWT and LUCfs uORF peptides. Residues of the wild-type peptide and the unchanged residues in the mutant peptide are in boldface.

repeat structure near the 5′ end of the RNA (Figure 4A, $LUCWT_{sl}$). The fact that this construct encoded no residual luciferase activity indicates that translation in this system is dependent on ribosomes loaded at the 5′ end of the mRNA.

When assayed after bombardment into maize cells, $LUCWT_{ovlp}$ and $LUCcontxt_{ovlp}$ encoded only 7 and 1% of the activity of LUCWT (Figure 4A). This result provides additional evidence that the uAUG of LUCWT is recognized efficiently in maize cells and indicates that almost all of the observed luciferase activity is due to reinitiation in this assay system.

Quantitative Analysis of the in Vitro Translation Products

The reinitiation frequency in vitro can be calculated based on knowledge of the proportion of luciferase protein due to ribosome reinitiation and the relative amount of uORF and luciferase proteins synthesized. As shown in Table 1, LUCWT encodes 100 molecules of luciferase for every 133 molecules of uORF peptide. Of these 100 molecules of luciferase, 60% or 60 are due to scanning ribosomes, whereas 40% or 40 are due to reinitiation. In other words, only 40 of 133 or 30% of the ribosomes that translate uORF reinitiate downstream. In contrast, ~86% of luciferase activity synthesized by LUCcontxt, or 51 of the 59 molecules, is due to reinitiating ribosomes (Table 1). Thus, only 51 of 212 or 24% of the ribosomes reinitiate; this value is very similar to that obtained for LUCWT.

Involvement of Intercistronic Sequence in Inefficient Reinitiation

The length of the intercistronic sequence has been implicated in the efficiency of ribosome reinitiation (Kozak, 1987b). Specifically, it has been hypothesized that translating ribosomes lose certain initiation factors that must be reacquired before reinitiation. To determine whether the length of the *Lc* intercistronic sequence was responsible for inefficient reinitiation, we lengthened the intercistronic sequence to 102 nucleotides in LUCint102 (Figure 5A). When this construct was tested both in vitro and in maize aleurone cells, no significant effect on luciferase expression was observed. Furthermore, labeled in vitro translation reactions revealed the presence of both translation products (Figure 5B), thus permitting a calculation of the reinitiation frequency, which was determined to be virtually identical to that of LUCWT (Table 1).

Previous studies also have demonstrated a role for a particular intercistronic sequence in the ability of ribosomes to reinitiate (Grant and Hinnebusch, 1994). To test this possibility, we replaced the *Lc* intercistronic sequence with two random sequences. LUCnew1 has a new sequence of 56 nucleotides that was constructed from LUCint102 by deleting the 46 nucleotides downstream of the uORF stop codon (Figure 5C and Methods). This construct encoded 1.85-fold more activity in vitro and 7.34-fold more activity in maize aleurone cells relative to LUCWT (Figure 5A).

Visualization and quantitation of the translation products after SDS-PAGE again permitted a determination of the reinitiation frequency. As expected, LUCnew1 encoded the same amount of uORF peptide as did LUCWT (Table 1). Furthermore, the increase in luciferase protein (100 versus 169) was almost the same as the 1.85-fold value determined from luciferase activity measurements (Figure 5A). Of these 169 molecules, 60 could be attributed to scanning ribosomes, whereas the remaining 109 were due to reinitiation. Thus, 109 of 128 or 85% of the ribosomes that translated the uORF reinitiated downstream.

LUCnew2 contains a 59-nucleotide intercistronic sequence that, like LUCWT, is AU rich downstream of the uORF stop codon (Figure 5C). When tested in vitro, LUCnew2 showed a level of luciferase activity comparable to that of LUCnew1

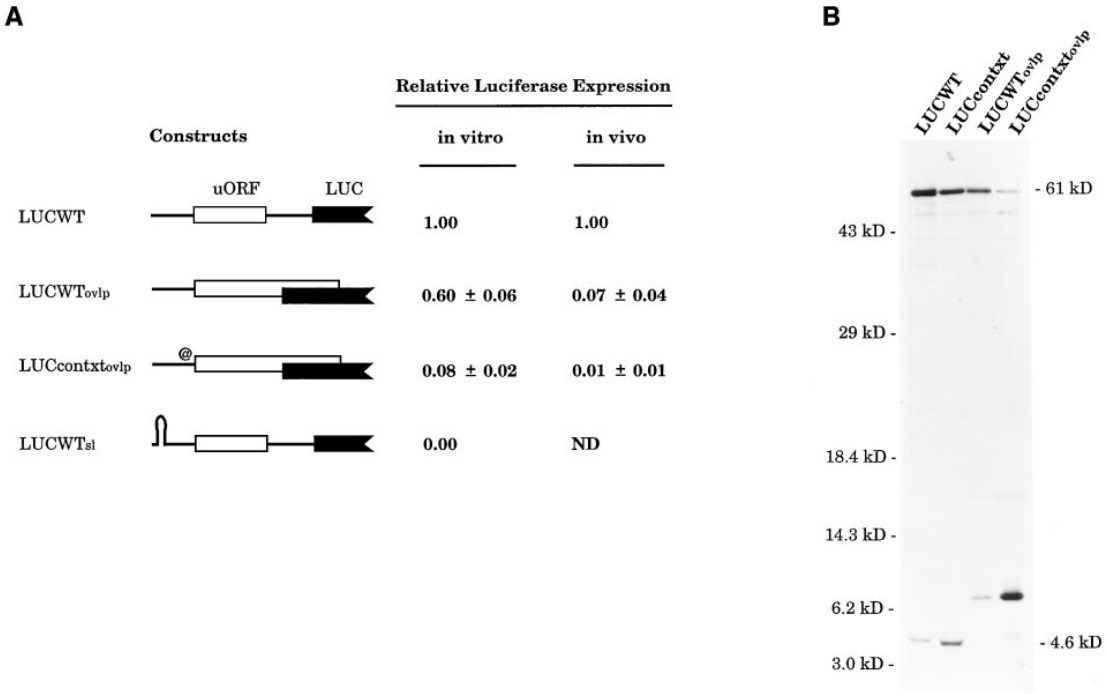


Figure 4. Determination of the Proportion of Luciferase Translation Due to Leaky Scanning.

(A) Luciferase activity measurements. In $LUCWT_{ovip}$, the four stop codons between the two ORFs of LUCWT were deleted. The extended uORF (unfilled box) overlaps with the luciferase ORF (filled box) by 83 nucleotides. In $LUCcontxt_{ovip}$, the context improvement of the uORF initiation codon is indicated as @. LUCWTsl contains a stem–loop structure seven nucleotides from the 5′ end (see Methods). Each value (±SD) represents the average of at least four independent assays for the in vitro data and eight bombardments for the in vivo data. ND, not determined.
(B) SDS-PAGE analysis of the in vitro translation products. Numbers at left indicate positions of size markers in kilodaltons. The 61-kD product corresponds to the luciferase protein. The predicted size of the *Lc* uORF peptide is 4.6 kD.

(Figure 5A). Furthermore, the reinitiation frequency of this construct was determined to be 93%, almost threefold greater than that of LUCWT (Figure 5B and Table 1).

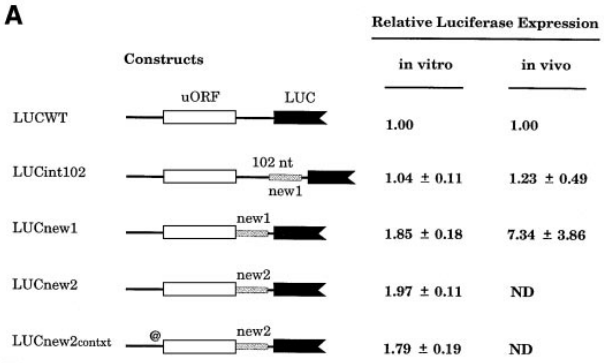
More significantly, when the sequence context of the uAUG of LUCnew2 was improved to the eukaryotic consensus, the double mutant, $LUCnew2_{contxt}$, showed increased synthesis of both the uORF peptide and luciferase protein (Figure 5B and Table 1). This result was markedly different from that of LUCcontxt, in which the same context improvement increased production of the uORF peptide but reduced luciferase translation when compared with LUCWT (Table 1). The reinitiation frequency of $LUCnew2_{contxt}$ was determined to be 82%, ~3.5-fold greater than that of LUCcontxt (Figure 5B and Table 1).

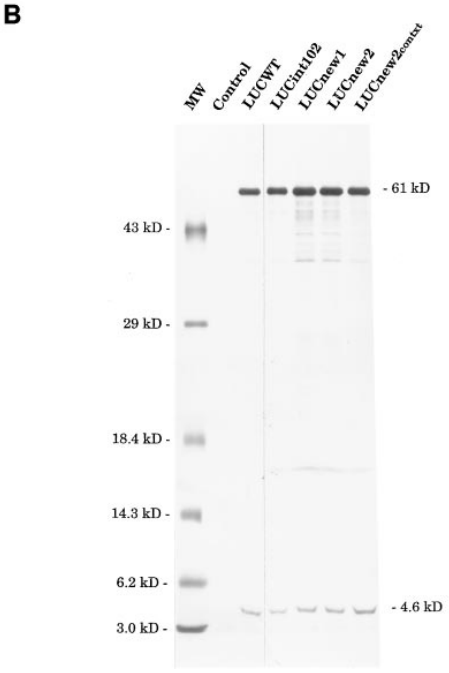
Translational Repression in Transgenic Rice Plants

Both in vitro and transient in vivo assays indicate that ribosomes that translate the uORF reinitiate inefficiently due to the intercistronic sequence downstream of the uORF. However, because construct-encoded RNA in the bombarded kernels cannot be visualized or quantified, it is a formal possibility that the introduced mutations also may influence RNA stability. To rule out this possibility, we transformed three of the constructs (LUCWT, LUCm123, and LUCnew1) into rice. Rice, like maize, is a member of the grass family; however, unlike maize, it is easily transformed.

Seven independent transgenic lines for each construct were obtained (from the International Laboratory for Tropical Agricultural Biotechnology, Scripps Institute, La Jolla, CA) and found to contain between one and six copies of the transgene after DNA gel blot analysis (data not shown). RNA gel blot analysis of all of the transgenic plants led to the detection of luciferase mRNA in only eight lines (Figure 6). For each of these plants, luciferase mRNA levels were quantified and standardized using an internal actin control. Similarly, luciferase activities were quantified and correlated with steady state mRNA levels. These results, presented in Table 2, indicate that elimination of the uAUGs increases translation efficiency by ~20-fold (LUCWT versus LUCm123). This value is very close to that obtained in the transient bombardment

assays (Figure 2A). When the wild-type intercistronic sequence was replaced, translational efficiency was also increased ~15-fold (LUCWT versus LUCnew1). These data indicate that the increased luciferase activity encoded by LUCm123 and LUCnew1 is due to more efficient translation of their mRNAs in contrast to LUCWT rather than increased mRNA stability.





C

LUCWT	5' AGA UAA **UAA** GCA AUA **UAA** UAC **UGA** UCUAGAAUCGAC
LUCnew1	5' AGA UAG UCG CGA ACU AGU AAC GGC CGCCAGUGUGCU
LUCnew2	5' AGA UAA CUA GUA UUA CAA UUC ACU GGCCGUCGUUUU

LUCWT	CUGCAGGCAACUGGAAGGAUCCAA AUG 3'
LUCnew1	GGAAUUCGAGCUCGCCGGGGAUCCAA AUG 3'
LUCnew2	ACAACGUCGUGACUGGGAAAAGGAUCCAA AUG 3'

Figure 5. Effect of Intercistronic Length and Sequence on Downstream Translation.

(A) Luciferase activity measurements. New sequences are represented as stippled boxes. The intercistronic distance in LUCint102 was lengthened to 102 nucleotides (nt) by the insertion of the new1 sequence. The context improvement of the uORF initiation site in LUCnew2$_{contxt}$ is indicated as @. Each value (±SD) represents the average of at least four independent assays for the in vitro data and eight bombardments for the in vivo data. ND, not determined.

(B) SDS-PAGE analysis of the in vitro translation products. MW stands for the protein molecular mass markers, with their sizes in kilodaltons indicated at left. The 61-kD product corresponds to the luciferase protein. The predicted size of the *Lc* uORF peptide is 4.6 kD.

(C) The intercistronic sequences in LUCWT, LUCnew1, and LUCnew2. The last codon (AGA) and stop codon (underlined) of the uORF are included. The AUG (underlined) at the 3′ end of each sequence is the luciferase initiation codon. For LUCWT, the three in-frame stop codons downstream of the uORF are in boldface.

DISCUSSION

In this study, we used three different assay systems to determine how the *Lc* uORF inhibits downstream translation. The stability of the *Lc* uORF peptide in the cell-free translation system permitted the quantitation of both uORF peptide synthesis and downstream translation. However, because cell-free translation systems do not always faithfully reproduce the in vivo condition, constructs also were assayed after bombardment into maize aleurone cells and when stably integrated into the rice genome.

A Model for uORF-Mediated Repression

Results from all three assay systems are in agreement with the model shown in Figure 7. Ribosomes that translate the uORF, whether in vivo or in vitro, are proposed to reinitiate inefficiently due to sequences downstream of the uORF termination codon. In contrast, 40S subunits that scan past the uAUG codon efficiently initiate translation at the downstream ORF. Based on the data presented, the level of repression is independent of uORF sequence but is dependent on both the fraction of ribosomes that translate the uORF and the efficiency of reinitiation.

The major difference in the results obtained from plant cells (either transiently [maize aleurone cells] or stably [rice plants] transformed or in the wheat germ translation system) and from an animal cell-free system was the magnitude of repression: ~15- to 20-fold in plants (also referred to as in vivo) versus approximately twofold in the animal in vitro system. In the model, it is proposed that this difference is due, in part, to more efficient recognition of the uAUG in plants than in animals. Support for this claim comes from the bombardment of LUCWT$_{ovlp}$, which encodes only 7% of the luciferase levels of LUCWT (Figure 4A), indicating that >90%

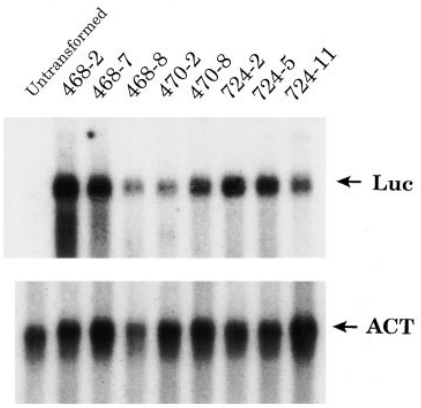


Figure 6. RNA Gel Blot Analysis of Luciferase Transcript Levels in Transgenic Rice.

mRNA isolated from the leaves of transgenic rice was probed with a 1.1-kb luciferase (Luc) fragment (top) and reprobed with a HindIII-SstI fragment of the maize actin (ACT) gene (bottom). Lines designated as 468 were transformed with LUCWT, those designated as 470 were transformed with LUCm123, and those designated as 724 were transformed with LUCnew1.

of ribosomes initiate translation at the uAUG in vivo. In contrast, $LUCWT_{ovlp}$ encodes almost 60% of LUCWT level in vitro. Improvement of the context of the uAUG in $LUCcontxt_{ovlp}$ reduces this value to 8%, making it more like the in vivo situation (Figure 4A). The stability of both the uORF peptide of LUCWT and the extended uORF peptide of $LUCWT_{ovlp}$ allowed us to confirm that the context change did, in fact, increase the synthesis of both peptides (Figure 4B and Table 1).

One prediction of this model is that increasing the translation of the uORF will decrease downstream translation. Evidence for this prediction comes from the comparison between LUCWT and LUCcontxt, in which it is shown directly that LUCcontxt encodes ~1.6-fold more uORF peptide (133 versus 212 molecules) but approximately half as much luciferase as does LUCWT (Figure 2A and Table 1). Thus, improving the uAUG context makes the in vitro system more like the in vivo condition, in which the uAUG is more efficiently recognized. The context change increases the magnitude of repression from 2.18-fold (LUCm123 versus LUCWT) to more than fourfold (LUCm123 versus LUCcontxt).

The second component of the model is that the act of translating the *Lc* uORF, but not what is translated, is repressive. Evidence for this claim is that the level of repression is unaffected by either single amino acid substitutions throughout the uORF peptide or the substitution of most of the uORF codons. Replacement of 29 of the 38 uORF codons in LUCfs (Figure 3C) has virtually no effect on repression either in vitro or in vivo (Figure 3A). For two other repressive uORFs, from the mammalian *AdoMetDC* and viral *gp48* genes, the amino acid sequence at the C terminus was required for uORF-mediated repression (Hill and Morris, 1993; Cao and Geballe, 1994). The frameshift mutations that created LUCfs left the C-terminal amino acids unchanged (Figure 3C). However, it is extremely unlikely that these amino acids contribute to repression because replacement of two of them (M-35 and R-38) had no effect on repression in vitro (Figure 3B).

The results of a previous study suggested that the uORF codons mediated repression in an in vivo bombardment assay (Damiani and Wessler, 1993). In that study, constructs contained the uORF upstream of the *Lc* (*R*) gene. The effect of uORF mutations was quantified indirectly by measuring the activity of a luciferase gene fused to the *Lc*-responsive *bz1* promoter. By using this assay system, it was found that an L-4–to–R mutation (Figure 3B) led to almost complete derepression, whereas M-23–to–R and M-35–to–R mutations relieved repression to lesser extents. In this study, we found that identical mutations had no effect on the magnitude of repression either in vivo or in vitro. At this time, we consider it likely that the previous results are due to either experimental error or an artifact of the indirect assay. This is currently under investigation. However, it is a formal possibility that the differences may reflect interactions between the peptide and the *Lc* gene product, something that cannot be assayed with the constructs tested here. If interactions between the Lc uORF peptide and the Lc protein do occur, this would add another level of regulation to those described in this report.

The third component of the proposed model is that ribosomes translating uORF reinitiate inefficiently. Again, the stability of the peptide in vitro facilitated this analysis by permitting a calculation of reinitiation frequencies once the fraction of luciferase translation due to reinitiation was known (from analysis of the overlap constructs illustrated in Figure 4). For LUCWT and LUCcontxt, we found this value to be

Table 2. Translational Efficiency in Transgenic Rice

Construct	Transgenic Rice Lines	Relative mRNA Level[a]	Relative Luciferase Activity[b]	Relative Translation Efficiency[c]
LUCWT	468-2	1.00	1.00	1.00
	468-7	0.58	0.83	1.43
	468-8	0.53	0.50	0.94
LUCm123	470-2	0.19	3.60	18.95
	470-8	0.34	7.24	21.29
LUCnew1	724-2	0.55	7.93	14.42
	724-5	0.56	9.33	16.66
	724-11	0.15	2.87	19.13

[a] Relative mRNA levels of transgenes have been normalized to rice actin mRNA level in each sample.

[b] Luciferase activity was measured as light units per microgram of total protein in each sample, with relative luciferase activity of transgenic line 468-2 set as 1.00.

[c] Relative translation efficiency is the relative luciferase activity divided by the relative mRNA level.

very similar at 30 and 24%, respectively (Table 1). The length of intercistronic sequences was shown in many systems to influence reinitiation frequencies both in vitro and in vivo (Kozak, 1987b; Grant et al., 1994). However, increasing the intercistronic length from 54 to 102 nucleotides by inserting sequences upstream of the luciferase ORF did not affect the magnitude of repression in vitro or in vivo (Figure 5). In contrast, replacement of the sequences downstream of the uORF stop codon with two random sequences (Figure 5C) increased the reinitiation frequency almost threefold in vitro (Table 1) and led to an ~15-fold increase in luciferase activity in transgenic rice plants (Table 2). The most revealing comparison is between LUCcontxt and LUCnew2$_{contxt}$ (Table 1). For both constructs, the improved context of the uAUG results in an almost twofold increase in the synthesis of the uORF peptide over their wild-type counterparts (i.e., LUCcontxt versus LUCWT; LUCnew2$_{contxt}$ versus LUCnew2). However, whereas increased uORF translation resulted in reduced luciferase synthesis in the presence of the wild-type intercistronic sequence (LUCcontxt versus LUCWT), increased uORF translation had no effect on downstream luciferase translation in constructs containing new intercistronic sequences (LUCnew2$_{contxt}$ versus LUCnew2). Thus, with the wild-type intercistronic sequence, increasing uORF translation reduced downstream expression because reinitiating but not scanning ribosomes initiate downstream inefficiently. In contrast, when the intercistronic sequence is changed, it does not matter whether ribosomes translate or scan passed uORF because reinitiation is efficient.

The efficiency of reinitiation cannot be calculated in bombarded kernels or transgenic rice plants because uORF peptide cannot be detected in these systems (data not shown). Despite this limitation, the data presented support the conclusion that inefficient reinitiation is responsible primarily for repression in maize aleurone cells. First, the use of overlapping constructs demonstrates that uAUG is recognized efficiently in vivo and that reinitiating ribosomes account for ~90% of downstream translation (Figure 3 and Table 1). Second, mutations in the uORF codons have no effect on the magnitude of repression (Figure 3). Third, like the in vitro results, increasing the intercistronic length has virtually no effect on downstream repression, but replacement of the intercistronic sequence leads to a dramatic increase in luciferase activity (Figure 5 and Table 2).

Under certain circumstances, sequence changes may alter mRNA stability and/or the secondary structure of the 5′ leader region, both of which can influence the efficiency of translation initiation. However, it is unlikely that the observed repression and derepression can be explained in terms of mRNA stability or secondary structure. First, RNAs were stable during the course of the in vitro translation reactions, as assayed by RNA gel blots for all constructs, and luciferase activity showed a linear increase during at least the first 30 min of incubation (data not shown). Second, replacement of the intercistronic sequence led to a 15-fold increase in luciferase expression in transgenic rice plants (Table 2).

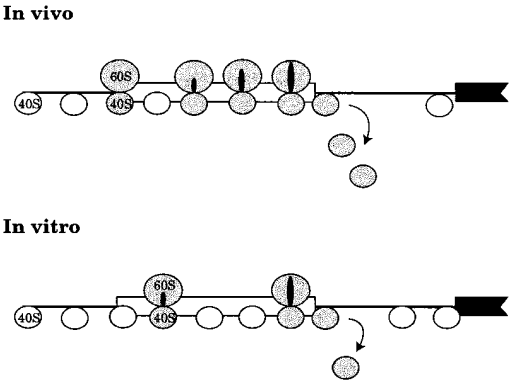


Figure 7. Model of uORF-Mediated Translational Control in Vitro and in Vivo.

The *Lc* uORF is represented as the open boxes, the downstream ORF as the black boxes, scanning ribosomes (40S) as unfilled circles, and ribosomal subunits (40S and 60S) that are translating or have translated the uORF as stippled circles. The nascent uORF peptide is represented by black ovals.

This increase resulted directly from more efficient translation of mutant mRNAs rather than differences in mRNA stability.

With regard to secondary structure, the most stable secondary structure of the *Lc* 5′ leader has predicted free energy (ΔG) of −18 kcal/mol (Damiani and Wessler, 1993). Based on previously published studies, it is unlikely that such a weak structure has a significant effect on translation initiation (Kozak, 1989c, 1994). The effect of secondary structure on translation initiation also depends on the proximity of the structure to the 5′ cap. A −30 kcal/mol structure located 52 nucleotides from the 5′ cap did not inhibit translation initiation (Kozak, 1989c). For all LUC constructs, the first 60 nucleotides from the 5′ end are identical. Similarly, the sequence replacement in LUCnew1 and LUCnew2 starts from nucleotide 174, and all of the upstream nucleotides are identical to LUCWT. Thus, any difference in secondary structure between wild-type and mutant constructs should not affect significantly the translational efficiency.

uORFs Exhibit a Variety of Repressive Mechanisms

The mechanism of uORF-mediated repression has been addressed only in a small fraction of the genes with uORFs (reviewed in Geballe, 1996). A common feature of the characterized uORFs is that they must be translated for repression of downstream translation to occur. However, aside from this feature, the mechanism of repression appears to differ between several of the well-characterized uORFs and that of *Lc*. Specifically, the amino acid sequence is a key component of repression for the 22-codon uORF2 of the human cytomegalovirus *gp48* gene (Cao and Geballe, 1994), the six-codon

uORF of the vertebrate *AdoMetDC* gene (Hill and Morris, 1993), the 25-codon uORF of the yeast *CPA1* gene (Werner et al., 1987), the 24-codon uORF of the *Neurospora crassa* homolog of *CPA1*, *arg-2* (Luo and Sachs, 1996), and the 19-codon uORF of the mammalian β_2-adrenergic receptor gene (Parola and Kobilka, 1994). For *gp48*, *AdoMetDC*, *CPA1*, and *arg-2*, missense but not synonymous replacements relieve repression, indicating that the amino acid and not the codon sequence is required for repression.

The precise manner in which a uORF peptide represses downstream translation has not been demonstrated as yet. However, for *gp48* and *AdoMetDC*, the available evidence is consistent with a model in which the peptide interacts with the translating ribosome as it encounters the termination codon and causes the ribosome to stall, thus blocking the flow of ribosomes to the downstream cistron. In both of these cases, repression is abolished if the uORF is extended by a single codon, suggesting that translation termination is a key step in the repression mechanism (Degnin et al., 1993; Hill and Morris, 1993). Direct support for this model has come recently from the analysis of ribosomes translating *gp48* in a cell-free system. Retention of the uORF2 peptide in the ribosome as a peptidyl–$tRNA^{Pro}$ complex suggests a mechanism in which the nascent uORF2 peptide blocks translation termination before hydrolysis of the last peptidyl–tRNA bond (Cao and Geballe, 1996b).

There are several other differences between our results and those of the uORFs mentioned above. For both *AdoMetDC* and *gp48*, the sequences both upstream and downstream of the uORF do not effect repression (Hill and Morris, 1993; Cao and Geballe, 1994). In addition, the uAUG of *gp48* is in a poor sequence context and is recognized in vivo by only $\sim$10% of the 40S subunits (Cao and Geballe, 1994). It is hypothesized that improving the sequence context has little effect on repression for these uORFs because stalled ribosomes block the flow of all upstream ribosomes, whether they are translating uORF or scanning downstream. In contrast, ribosome stalling is not hypothesized to occur during translation of the *Lc* uORF. In fact, all attempts to detect stalled ribosomes by using the ribosome "toeprint" technique have been unsuccessful (L. Wang and S.R. Wessler, unpublished data). Rather, we propose that ribosomes that have translated the *Lc* uORF have a $<$30% chance of reinitiating downstream because of their interaction with sequences in the intercistronic region.

Like the *Lc* uORF, the yeast *GCN4* gene is regulated in part by the frequency of reinitiation. The 5′ leader of *GCN4* has four very short uORFs. Ribosomes that translate uORF2, uORF3, or uORF4 inhibit downstream translation at *GCN4* because they reinitiate inefficiently. For the best-characterized uORF4, inefficient reinitiation is due to the high G+C content of the last uORF codon and the 10 nucleotides downstream of the uORF stop codon (Grant and Hinnebusch, 1994). The authors hypothesize that GC-rich sequences promote stable RNA–RNA interactions that may influence negatively the resumption of ribosome scanning.

Although both the *Lc* uORF and uORF4 of *GCN4* promote inefficient reinitiation, it is unlikely that the same mechanism accounts for both phenomena. The sequences immediately downstream of the *Lc* uORF stop codon are AU rich (Figure 5C), with eight of the 10 downstream nucleotides either an A or U. Furthermore, replacing the *Lc* intercistronic region with a relatively GC-rich sequence in LUCnew1 (in which five of the 10 downstream nucleotides are G or C) increases the frequency of reinitiation.

The most prominent feature of the *Lc* intercistronic sequence is the multiple stop codons downstream of the uORF (Figure 5C). Besides the uORF stop codon, three of the next six in-frame triplets are also stop codons. These stop codons may, in some manner, act to prevent newly dissociated 40S subunits from resuming a scanning mode. The process of translation termination in eukaryotes is poorly understood. It is thought that a single release factor (RF) recognizes all three termination codons and catalyzes the release of the peptide chain from the ribosome (Merrick and Hershey, 1996). In *Escherichia coli*, another protein known as ribosome release factor (RRF) has been shown to promote the dissociation of ribosomes from mRNA after the peptide is released (Ryoji et al., 1981; Janosi et al., 1994). A similar factor has yet to be identified in eukaryotes; however, the result of at least one study implies its presence (Grant and Hinnebusch, 1994). It is possible that the multiple stop codons downstream of the *Lc* uORF could facilitate the binding of a putative RRF and promote dissociation of the 40S subunit. If the multiple stop codons were found to be the feature responsible for inefficient reinitiation, analysis of the underlying mechanism might help to illuminate aspects of this ill-defined but important stage of translation.

METHODS

Plant Material

The maize inbred line W22 (*r-g*, *A1*, *A2*, *Bz1*, *Bz2*, *C1*, *C2*, *pl*, and *B-b*), provided by J. Kermicle (University of Wisconsin, Madison), was used as the recipient in all bombardment assays.

Plasmid Constructions

An EcoRI-XbaI fragment containing the *Lc* upstream open reading frame (uORF) and flanking sequences was cloned into vector pSelect-1 (Damiani and Wessler, 1993). For the in vitro assay, the *Lc* uORF region in pSelect-1 was amplified using polymerase chain reaction (PCR) with the primers 5′-GTCGAAGCTTGCGCGGAGGAGAGCTCCTCCG-3′ (HindIII site underlined) and 5′-CGGGATCCTTCCAGTTGCCTGCAGGTCGATTCTAG-3′ (BamHI site underlined). The amplified fragment was digested with HindIII and BamHI and subcloned into the corresponding sites in pGEM–*luc* (Promega), fusing 186 nucleotides of the *Lc* 5′ leader containing the uORF to the luciferase coding region. Transcripts from this chimeric gene have an intercistronic region of 54 nucleotides; of these, 29 were derived

from the *Lc* cDNA. The plasmid containing the wild-type *Lc* uORF was designated LUCWT. Mutations m123, L-4→R, L-4→I, M-23→R, and M-35→R (Damiani and Wessler, 1993) were transferred to the vector pGEM–*luc* as described above for LUCWT.

Additional mutations were created by first subcloning the HindIII-EcoRI fragment of LUCWT (Figure 1) into the pAlter-1 vector (Promega). Site-directed mutagenesis was performed using the Altered Sites II kit (Promega) with the following mutagenic oligonucleotides: 5′-TCTCTACCCTTACCATGGAAGTTC-3′ for LUCcontxt; 5′-ACCCTTCGCATGGAGTTCTTGCATTGCT-3′ and 5′-CTGATCATCAGTACGATGCCTCGTG-3′ for LUCfs; 5′-GGCGTCTTCCATTTGGATCTCTACTGATCATCAGACGATG-3′ for LUCWT$_{ovlp}$ and LUCcontxt$_{ovlp}$; 5′-GCAAGAACTGCCATGCGA-3′ for E-2→A; 5′-AATGCAAGAGCTTCCATG-3′ for V-3→A; 5′-AAGCAATGCAGCAACTTCCATG-3′ for L-4→A; 5′-GCAACGAAGCGCTGCAAGAACT-3′ for L-6→A; 5′-GCATTGCGTCGTTGCTT-3′ for L-7→R; 5′-GAGAAGCAAGCAAGCAATGC-3′ for R-8→A; 5′-AACTAGAGAAGGCACGAAGCAATG-3′ for C-9→A; 5′-ACGTAGAAGGCGGAAACTAGAG-3′ for F-14→R; 5′-CTCGTGAACGCGCGGACGAG-3′ for R-28→A; 5′-TCACGAGGGCTCGTCTGAT-3′ for H-32→A; and 5′-ATGATCAGTGCATAATAAGCA-3′ for R-38→A. After mutagenesis, DNA was cloned back into the pGEM–*luc* vector as HindIII-BamHI (or XbaI) fragments (Figure 1).

To construct LUCWT$_{ovlp}$ and LUCWTcontxt$_{ovlp}$, the sequence from the uORF stop codon to the first nucleotide of the BamHI site in the intercistronic region was deleted (Figure 1). The extended uORF is out of frame with the downstream luciferase ORF with an overlapping region of 83 nucleotides. LUCWTsl was generated by inserting a 102-bp stem–loop structure into the HindIII site of LUCWT (Figure 1). The 102-bp fragment is an inverted repeat of the pUC19 polylinker (the EcoRI-HindIII fragment; Yanisch-Perron et al., 1985). It is inverted at the EcoRI site; thus, there is a HindIII site at each end.

LUCint102 was created by inserting 48 bp into the BamHI site of LUCWT (Figure 1). The inserted sequence contains the BamHI-EcoRI fragment of the pCR2.1 polylinker (Invitrogen, Carlsbad, CA) and the BamHI-EcoRI fragment of the pUC19 polylinker with a 4-bp deletion at the KpnI site. The sequence was generated by first subcloning the XbaI-EcoRI fragment of the pUC19 polylinker into the corresponding restriction sites of pCR2.1, followed by BamHI digestion. The BamHI fragment is 52 bp, with an SpeI site near one end (from pCR2.1) and a KpnI site near the other (from pUC119). For further subcloning, the KpnI site was removed by KpnI digestion followed by treatment with the Klenow fragment of DNA polymerase I. Thus, the insert present in LUCint102 is 48 bp and is oriented such that the SpeI site lies near the uORF. LUCnew1 was derived from LUCint102 by deleting the 3′ flanking sequence of the uORF. This was done by amplifying the uORF with primers 5′-GTCGAAGCTTGCGCGGAGGAGAGCTCCTCCG-3′ (HindIII site underlined) and 5′-CGGACTAGTTCGCGACTATCTACTGATCATCAGACGATGCCTCGTG-3′ (SpeI site underlined). The PCR product was digested with HindIII and SpeI and used to replace the HindIII-SpeI fragment in LUCint102. The new intercistronic sequence of LUCnew1 transcript is 56 nucleotides, and the uORF stop codon is UAG (Figure 5C).

LUCnew2 and LUCnew2$_{contxt}$ contain a new intercistronic sequence from the LacZα coding region of pCR2.1 (bases 361 to 407) (Invitrogen). The sequence was amplified by primers 5′-CGGACTAGTATTACAATTCACTGGCCGTCG-3′ (SpeI site underlined) and 5′-GTGGATCCTTTTCCCAGTCACGACGTTGTA-3′ (BamHI site underlined). The uORF region of LUCWT and LUCcontxt was amplified using primers 5′-GTCGAAGCTTGCGCGGAGGAGAGCTCCTCCG-3′ (HindIII site underlined) and 5′-CGGACTAGTTATCTACTGATCATCAGACGATGCCTCGTG-3′ (SpeI site underlined). The HindIII-SpeI fragment was ligated with the sequence from pCR2.1 (an SpeI-BamHI fragment) by using the SpeI site and was used to replace the HindIII-BamHI fragment of pGEM–*luc* (Promega).

For expression in maize cells and in transgenic rice plants, the HindIII-AvaI fragment of LUCWT or the mutant derivatives replaced the KpnI-AvaI fragment of pJD300 (Luehrsen et al., 1992) by first amplifying the HindIII-AvaI fragment with the primers 5′-TACTATGGTACCGCGCGGAGGAGAGCTCCTCCGGTT-3′ (KpnI site underlined) and 5′-GTAACAATATCGATTCCA-3′, which converted the HindIII site to a KpnI site. The PCR product was digested with KpnI and AvaI and subcloned into the corresponding sites in pJD300, placing the *Lc* uORF–*Luc* chimeric gene under control of the cauliflower mosaic virus 35S promoter.

In Vitro Transcription and Translation

pGEM–*luc*—derived plasmids were linearized with XhoI (Figure 1), and capped RNA was synthesized using the Riboprobe Core System-SP6 kit (Promega) according to the manufacturer's instructions. RNA samples were analyzed by RNA gel blots (Sambrook et al., 1989) that were probed with a 1.1-kb EcoRI fragment of the luciferase gene in pGEM–*luc* (Promega) and were quantified by using a PhosphorImager scan (Molecular Dynamics, Sunnyvale, CA). Luciferase RNA (1.0 μg/μL; Promega) was included to standardize the RNA concentrations of the samples.

Each RNA was translated in the rabbit reticulocyte lysate in vitro translation system (Promega) at a final RNA concentration of 4 μg/μL. Reactions were performed at 30°C for 30 min and then frozen on dry ice. Luciferase activity showed a linear increase during the first 30 min of incubation (data not shown). For SDS-PAGE analysis of the in vitro translation products, ^{35}S-methionine (Amersham) was included in the translation reactions at a final concentration of 0.8 mCi/mL.

Immunoprecipitation

The ^{35}S-methionine–labeled uORF peptide was precipitated using an uORF peptide–specific rabbit antisera (raised against synthetic uORF peptide) and protein A–Sepharose CL-4B (Sigma) as described previously (Harlow and Lane, 1988).

SDS-PAGE Analysis

Samples from in vitro translation reactions or immunoprecipitation were fractionated on a 14% polyacrylamide resolving gel (Sambrook et al., 1989). Gels were treated with Entensify (Du Pont) before autoradiography. Relative amounts of luciferase and uORF peptide were determined by using the PhosphorImager (Molecular Dynamics), followed by adjustment for the number of labeled methionine residues in each product.

Transient Expression in Maize Aleurone Cells

Maize kernels used for particle bombardment were prepared as described by Damiani and Wessler (1993). Plasmid DNAs were precipitated onto 1.0-μm gold particles (60 mg/mL; Klein et al., 1989) and then delivered into maize aleurone cells with the Biolistic PDS-1000 (Du Pont). Each half kernel was cobombarded with 0.4 μg of a luciferase plasmid and 0.4 μg of pAdh1CAT (also called pAI1CN [Callis

et al., 1987]), which contains a chloramphenical acetyltransferase (*CAT*) gene under the control of the maize alcohol dehydrogenase (*Adh1*) promoter. After bombardment, the kernels were incubated on Murashige and Skoog media (Sigma) at 28°C for 48 hr before enzyme assays.

Rice Transformation

Transgenic rice plants were generated at the International Laboratory for Tropical Agricultural Biotechnology (Scripps Research Institute, La Jolla, CA), as described previously (Song et al., 1995). Transformation was confirmed by DNA gel blot analysis using the luciferase gene in pGEM–*luc* (Promega) as the probe (data not shown).

RNA Isolation and Gel Blot Analysis

Total rice RNA was isolated from 5 g of leaf tissue as described by Weisshaar et al. (1991). Poly(A)$^+$ RNA was isolated by using the Poly-A-Tract mRNA isolation system IV (Promega). RNA gel blot analysis was performed by following the standard procedure (Sambrook et al., 1989), and blots were probed with the 1.1-kb EcoRI fragment of the luciferase gene in pGEM–*luc* (Promega). The same membrane was reprobed with a 485-bp HindIII-SstI fragment of the maize actin gene as described previously (Hu et al., 1996). Probes used for the RNA gel blot analysis were labeled by using the random primers DNA labeling system (Bethesda Research Laboratories). mRNA levels were determined by scanning with the PhosphorImager.

Enzyme Assays

In vitro and in vivo expression levels were determined by luciferase activity assays as described previously (Callis et al., 1987). In vitro translation reactions were diluted 10-fold in 20 mM Tricine, pH 7.8, and 1 μL of the diluted sample was assayed with a model 3010 luminometer (Analytic Scientific Instruments, Alameda, CA). Luciferase activity was expressed as the number of light units detected in the first 10 sec of reaction at room temperature. Relative luciferase expression was calculated by dividing the luciferase activity for each construct by the activity of LUCWT.

For the bombarded maize kernels, each half kernel was ground in 350 μL of 100 mM KPO_4, pH 7.80, and 1 mM DTT at 4°C. After centrifugation, 100 and 25 μL of the supernatant were assayed for luciferase and CAT activity, respectively. CAT activity was expressed as ethyl acetate–soluble counts per minute (Sleigh, 1986). Luciferase expression levels were adjusted by CAT activity and expressed as the ratio of luciferase to CAT activities. Relative luciferase expression was calculated by dividing the average luciferase/CAT ratio for each construct by that of LUCWT.

For the transgenic rice plants, 0.5 g of leaf tissue was used for protein extraction, and luciferase expression levels were adjusted by the total protein concentration in the samples, as determined by using the Bradford (1976) method .

ACKNOWLEDGMENTS

We thank Li-Li Chen and Dr. Claude Fauquet (International Laboratory for Tropical Agricultural Biotechnology, supported by the Rockefeller Foundation and ORSTOM) for rice transformation. We also thank Dr. Ronald Damiani for helping to set up the in vitro assay system and Drs. Lane Arthur, Michael Terns, and Claiborne Glover for critical reading of the manuscript. This work was supported by a grant from the U.S. Department of Energy to S.R.W.

Received June 24, 1998; accepted August 11, 1998.

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