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Requirements for Intercistronic Distance and Level of Eukaryotic Initiation Factor 2 Activity in Reinitiation on *GCN4* mRNA Vary with the Downstream Cistron

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Translational control of the *GCN4* gene in response to amino acid availability is mediated by four short open reading frames in the *GCN4* mRNA leader (uORFs) and by phosphorylation of eukaryotic initiation factor 2 (eIF-2). We have proposed that reducing eIF-2 activity by phosphorylation of its α subunit or by a mutation in the eIF-2 recycling factor eIF-2B allows ribosomes which have translated the 5′-proximal uORF1 to bypass uORF2 to uORF4 and reinitiate at *GCN4* instead. In this report, we present two lines of evidence that all ribosomes which synthesize *GCN4* have previously translated uORF1, resumed scanning, and reinitiated at the *GCN4* start site. First, *GCN4* expression was abolished when uORF1 was elongated to make it overlap the beginning of the *GCN4* coding region. Second, *GCN4* expression was reduced as uORF1 was moved progressively closer to *GCN4*, decreasing to only 5% of the level seen in the absence of all uORFs when only 32 nucleotides separated uORF1 from *GCN4*. We additionally found that inserting small synthetic uORFs between uORF4 and *GCN4* inhibited *GCN4* expression under derepressing conditions, confirming the idea that reinitiation at *GCN4* under conditions of diminished eIF-2 activity is proportional to the distance of the reinitiation site downstream from uORF1. While uORF4 and *GCN4* appear to be equally effective at capturing ribosomes scanning downstream from the 5′ cap of mRNA, these two ORFs differ greatly in their ability to capture reinitiating ribosomes scanning from uORF1. When the active form of eIF-2 is present at high levels, reinitiation appears to be much more efficient at uORF4 than at *GCN4* when each is located very close to uORF1. Under conditions of reduced recycling of eIF-2, reinitiation at uORF4 is substantially suppressed, which allows ribosomes to reach the *GCN4* start site; in contrast, reinitiation at *GCN4* in constructs lacking uORF4 is unaffected by decreasing the level of eIF-2 activity. This last finding raises the possibility that time-dependent binding to ribosomes of a second factor besides the eIF-2–GTP–Met-tRNA$_i^{Met}$ ternary complex is rate limiting for reinitiation at *GCN4*. Moreover, our results show that the efficiency of translational reinitiation can be strongly influenced by the nature of the downstream cistron as well as the intercistronic distance.

Translation of most eukaryotic mRNAs occurs by a scanning mechanism whereby the 40S ribosomal subunit binds at or near the 5′ cap and scans along the mRNA leader until reaching the first AUG codon. The 60S ribosomal subunit then joins, forming an 80S ribosome, and synthesis of the polypeptide begins (for a review, see reference 25). The efficiency with which an AUG triplet is selected as the start codon is affected by both the surrounding sequence context and its distance from the 5′ cap (5, 21). In most cases, these requirements are satisfied by the first AUG codon encountered by the ribosome while scanning from the cap. Both viral and cellular mRNAs which contain one or more open reading frames upstream of the coding region (uORFs) have been identified (8, 9, 12, 19, 23, 32). The AUG codons of these uORFs have been shown to be recognized as translational start sites in several of these mRNAs, where they inhibit translation of the downstream coding regions (6, 13, 36). The inhibitory effect of uORFs on downstream translation reflects the fact that reinitiation at internal start sites occurs inefficiently with eukaryotic ribosomes (18, 20, 26, 34). The best-studied example of a cellular gene in which uORFs control the expression of the downstream coding region occurs with *GCN4* mRNA of the yeast *Saccharomyces cerevisiae*, which has served as a model system for the study of translational reinitiation in this simple eukaryote (15, 16).

The GCN4 protein is a transcriptional activator of more than 30 genes involved in multiple amino acid biosynthetic pathways. Under normal growth conditions, *GCN4* is expressed at a low basal level but is derepressed in response to starvation for any single amino acid or a defective aminoacyl tRNA synthetase. This control mechanism operates at the level of translation initiation and is mediated by four short uORFs in the *GCN4* mRNA leader. uORF4 (counting from the 5′ end) is a strong translational barrier that is sufficient to prevent *GCN4* expression in the absence of the other uORFs. In contrast, uORF1 is a weak translational barrier and is required to overcome the inhibitory effect of uORF4 under conditions of amino acid starvation (15). We have proposed (2) that under both repressing and derepressing conditions, the majority of ribosomes that bind at the 5′ end of *GCN4* mRNA will translate uORF1 and that a substantial fraction of these ribosomes will resume scanning following translation at uORF1. Under conditions of amino acid sufficiency, ribosomes will reinitiate at one of the downstream uORFs (uORF2, uORF3, or uORF4) but then fail to reinitiate again at *GCN4* following termination at these uORFs. Under starvation conditions, by contrast, many ribosomes will bypass the AUG codons at uORF2, uORF3, and uORF4 and reinitiate at

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GCN4 instead. Amino acid starvation activates the protein kinase GCN2 that phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2α) (7). By analogy with mammalian systems, this modification is expected to reduce the concentration of the ternary complex consisting of eIF-2, GTP, and Met-tRNA$_i^{Met}$ that binds initiator tRNA to the small ribosomal subunit. Following translation of uORF1 and the resumption of scanning, ribosomes must rebind the ternary complex in order to reinitiate translation downstream. As a consequence of the lower levels of ternary complex expected under starvation conditions, it is believed that many ribosomes scanning downstream from uORF1 will fail to rebind ternary complex and thus be incompetent to reinitiate translation until after scanning past uORF4. These ribosomes will acquire ternary complexes in the uORF4-*GCN4* interval and reinitiate translation at *GCN4* instead (2, 7).

In support of this model, it has been shown that *GCN4* translational control is strongly dependent on the spacing between the uORFs and *GCN4* (2). Increasing the distance between uORF1 and uORF4 leads to a reduction in *GCN4* expression specifically under derepressing conditions, whereas an increase in the uORF4-*GCN4* spacing has little or no effect on expression. To explain these findings, we suggested that expansion of the uORF1-uORF4 interval increased the time required to scan from uORF1 to uORF4 and thus increased the probability that ribosomes would rebind ternary complex and reinitiate at uORF4 under conditions of reduced eIF-2 function. Moreover, if the uORF1-*GCN4* interval were already sufficiently large for efficient reinitiation at *GCN4* under starvation conditions, increasing the time it takes to reach *GCN4* after bypassing uORF4 would have no effect on reinitiation at *GCN4*.

Our model is also in accord with the fact that mutations in the three subunits of eIF-2 (encoded by *SUI2*, *SUI3*, and *GCD11*) that partially impair eIF-2 function lead to constitutive derepression of *GCN4* translation in the absence of the GCN2 protein kinase, mimicking the inhibitory effect of eIF-2α phosphorylation on the level on the level of ternary complexes (11, 37). The same explanation applies to mutations in five different genes (*GCD1*, *GCD2*, *GCD6*, *GCD7*, and *GCN3*) which encode the subunits of eIF-2B in *S. cerevisiae*, the guanine nucleotide exchange factor for eIF-2 (3, 4). Phosphorylation of eIF-2α in mammalian cells reduces the level of active eIF-2 by impairing the activity of eIF-2B (28, 33, 35). Thus, mutations in eIF-2B subunits in *S. cerevisiae* should simulate the effects of eIF-2α phosphorylation and decrease ternary complex formation. As expected from our model, the derepressing effect of a mutation in the *GCD1* subunit of eIF-2B on *GCN4* expression was diminished by increasing the distance between uORF1 and uORF4 (2).

A critical feature of our model is that ribosomes must translate uORF1 and then engage in a reinitiation process in order to bypass the start sites at uORF2 through uORF4 under starvation conditions when ternary complex formation is reduced by eIF-2α phosphorylation. In this report, we present two lines of genetic evidence supporting the notion that uORF1 is translated by the majority of ribosomes that ultimately reach *GCN4*. We also present additional evidence that the frequency of reinitiation under conditions of limiting eIF-2 activity increases with the distance of the reinitiation site downstream from uORF1. The results of two other experiments, however, indicated that our understanding of reinitiation on *GCN4* mRNA is incomplete. We found that efficient reinitiation at the *GCN4* AUG codon required a relatively large scanning distance between uORF1 and *GCN4* even when levels of eIF-2 activity were high and that this intercistronic length dependence was unaffected by a reduction in eIF-2 activity. These findings may indicate that another factor besides the eIF-2–GTP–Met-tRNA$_i^{Met}$ complex must rebind to ribosomes scanning from uORF1 for reinitiation to occur at *GCN4*. Interestingly, efficient reinitiation at uORF4 under conditions of high eIF-2 activity appeared to require very little separation between uORF1 and uORF4, suggesting that the mechanisms of ribosomal reinitiation at uORF4 and *GCN4* are different in some respect. In addition to refining our molecular model for *GCN4* translational control, these results provide new insights into the requirements for translational reinitiation by eukaryotic ribosomes.

MATERIALS AND METHODS

Construction of mutant *GCN4* alleles. Plasmid constructions were generated by standard procedures (27) or by PCR with oligonucleotide primers specific for *GCN4* sequences. All constructs are derivatives of plasmid-borne *GCN4* alleles contained on the *Escherichia coli*-yeast shuttle vector YCp50, which contains the yeast *URA3*, *ARS1*, and *CEN4* sequences for selection and single-copy maintenance in *S. cerevisiae*, as described previously (2, 30, 31).

Plasmid pM199 was constructed by combining the 457-nucleotide (nt) *Sal*I-*Bgl*II *GCN4* leader fragment from plasmid pM37 (30), the 8.8-kb *Hin*dIII-*Sal*I backbone fragment of pA44 (2), and a *Bgl*II-*Hin*dIII linker oligonucleotide so as to move uORF1 into the exact position normally occupied by uORF4 with respect to the *GCN4* AUG codon. Deletion derivatives of pM199 (pM230 and pM231) were constructed by replacing the *Bgl*II-*Bam*HI fragment from pM199 with similar PCR-generated fragments of 234 (pM230) or 184 (pM231) nt which were truncated at their 5′ ends. The PCR primers used in both cases were a 3′ primer containing the *Bam*HI site within the *GCN4* coding region and a 5′ primer containing a *Bgl*II site beginning either 75 (pM230) or 25 (pM231) nt upstream from the *GCN4* AUG codon. pG40, generated by a PCR, is identical to pM231 except for a T-to-A substitution which changes the ATG codon of uORF1 to AAG. The uORF1-*GCN4* overlap construct pM226 was derived from construct pM29, in which the uORF1 TAA stop codon was mutated by insertion of a T residue between the two A residues in plasmid pM23. This insertion elongated uORF1 to a position 304 nt upstream of *GCN4*, beyond the downstream *Bgl*II site, which is situated 324 nt upstream of *GCN4*. A PCR fragment was generated from the position of the *Bgl*II site in plasmid pM199 to the *Bam*HI site in *GCN4* by using the uORF4-*GCN4* overlap construct pA59 as a template (2). The 457-nt *Sal*I-*Bgl*II fragment from pM29 and the 274-nt *Bgl*II-*Bam*HI PCR fragment just described were then ligated with the 8.6-kb *Sal*I-*Bam*HI backbone of pA44 (see below) to create pM226. pG30 is identical to pM226 except for a T-to-A substitution which changes the ATG codon of uORF1 to AAG, made by PCR.

Plasmid pA44 (2) contains a *GCN4* allele with wild-type uORF1 and uORF4 and with uORF2 and uORF3 removed by point mutations in their ATG codons. In addition, C-to-T and G-to-C substitutions have been made to create a *Sna*BI site 23 nt upstream of uORF4. Deletion derivatives of pA44 (pG26 and pG29) were constructed by replacing the 605-nt *Sal*I-*Sna*BI fragment from pA44 with similar PCR-generated fragments of 459 (pG29) and 437 (pG26) nt which were truncated at their 3′ ends. The PCR primers used in both cases were a 5′ primer containing a *Sal*I site located at the 5′ end of the *GCN4* leader and a 3′ primer containing a *Sna*BI site beginning either 31 (pG29) or 9 (pG26) nt downstream from uORF1. Plasmid

pG4 was derived from pG26 by inserting two copies of a previously described oligonucleotide, designated S1 (2), containing sequences normally present downstream from uORF4 into the *Hin*dIII site situated 93 nt upstream of *GCN4*. This insertion increases the uORF4-*GCN4* interval by 144 nt. Plasmid pG67 was derived from pG26 by means of a PCR-directed deletion which moves the *GCN4* AUG codon into exactly the same position as that of the uORF4 AUG codon. Thus, plasmid pG67 has 32 nt normally present between uORF1 and uORF4 in pG26, separating uORF1 and *GCN4*. pG142 is identical to pG67 except for a T-to-A substitution which changes the ATG codon of uORF1 to AAG, made by PCR. pG82, generated by a PCR, is identical to pG26 except for a T-to-A substitution which changes the ATG codon of uORF4 to AAG. pG143 is identical to pG82 except for a T-to-A substitution which changes the ATG codon of uORF1 to AAG, made by PCR. The uORF4-*GCN4* overlap construct pG83 was made by replacing the *Sna*BI-*Bam*HI fragment of pG26 with a PCR-generated fragment of 324 nt by using construct pA59 as the template (2). The PCR primers used were a 3′ primer containing the *Bam*HI site within the *GCN4* coding region and a 5′ primer which introduces a *Sna*BI site 24 nt upstream of uORF4.

Plasmid pG7 was derived from pA44 by making T-to-A and T-to-G substitutions by PCR, creating an ATG codon 50 nt upstream of *GCN4*. The uORF created in pG7, called uORF6, is 12 codons long and terminates 11 nt upstream of the *GCN4* AUG codon at a naturally occurring TAA stop codon. Plasmid pG9 was generated by making the same nucleotide substitutions in p238 (31), creating uORF6 in a *GCN4* leader lacking any other uORF. Plasmid pG56 is identical to pA44 except for a PCR-mediated substitution which replaces 21 nt of sequence normally present between uORF4 and *GCN4*, starting 50 nt upstream of *GCN4*, with 21 nt of sequence normally present at the very beginning of the *GCN4* coding region. In addition, the nucleotides CTC normally present at 30 nt upstream of *GCN4* were replaced with TAA, creating the small (7-codon) uORF8 which terminates 30 nt upstream of the *GCN4* AUG codon.

Plasmid pG17 was derived from p292 (38) by means of a PCR-mediated insertion which introduces the first 33 nt from the beginning of the *GCN4* coding region, followed by a TAA stop codon and 11 nt normally present immediately upstream of *GCN4*, at the normal position of the *GCN4* AUG codon. This insertion creates a small (11-codon) ORF, called uORF7, upstream of the *GCN4* coding region. pG24 is identical to pG17 except for a T-to-A substitution made in the ATG codon of uORF7 by PCR. pG34 is identical to pG17 except that it contains uORF8 from plasmid pG56 in place of uORF7. Plasmid pG37 is identical to p292 except for a PCR-mediated insertion which introduces uORF6, from plasmid pG7, starting at the *GCN4* ATG codon followed by 11 nt normally found immediately upstream of *GCN4*. Plasmid pG55 is identical to pG37 except for a T-to-A substitution made in the ATG codon of uORF6 by PCR.

GCN4-lacZ fusion derivatives of all of the above plasmids were made by inserting a 3.2-kb *Bam*HI fragment containing codons 9 through 1023 of *lacZ* at the *GCN4 Bam*HI site (14).

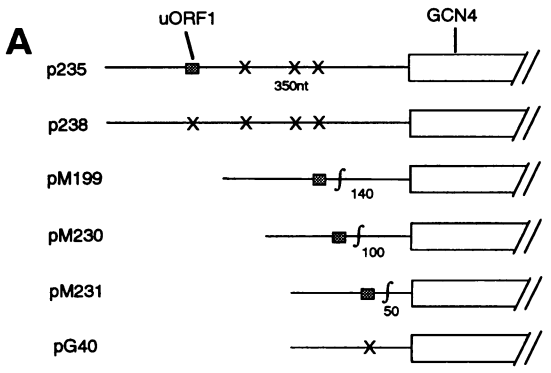
Assays of GCN4 expression. Methods for the assay of β-galactosidase activity from *GCN4-lacZ* fusions and for complementation of a *gcn4* deletion for sensitivity to 3-aminotriazole (3-AT) have been described previously (31). Briefly, plasmid-borne *GCN4* alleles were introduced by transformation (17) into strain H384 (*MATα his1-29 gcn4-103 ura3-52*), and transformants were replica plated to minimal medium lacking histidine or containing excess (40 mM) leucine and 30 mM 3-AT. Plasmids containing the corresponding *GCN4-lacZ* fusions were introduced into strains H15 (*MATα gcn2-1 leu2-3 leu2-112 ura3-52*) and F98 (*MATα gcd1-101 ura3-52*), and β-galactosidase activity was assayed. β-Galactosidase activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute per microgram of total protein (U).

RESULTS

Evidence that ribosomes which translate *GCN4* have previously translated uORF1 and resumed scanning. Our model for the translational control of *GCN4* expression makes the following two predictions about uORF1: (i) uORF1 is translated by most ribosomes scanning from the 5′ end of *GCN4* mRNA, and (ii) translation of uORF1 is frequently followed by a resumption of scanning, with the possibility of reinitiation downstream. A relatively high frequency of reinitiation at *GCN4* following uORF1 translation can explain the fact that the presence of uORF1 alone in the mRNA leader reduces *GCN4* expression only by a factor of 2.5 (Fig. 1, compare p235 and p238), whereas wild-type uORF4 reduces *GCN4* expression by a factor of ca. 50 (31). We believe that translation of uORF1, resumption of scanning, and reinitiation at *GCN4* occur at roughly equal rates under repressing and derepressing conditions. This conclusion follows from the fact that *GCN4* expression from a construct containing uORF1 alone is very similar in wild-type cells grown under nonstarvation versus starvation conditions (31) and in mutants that are constitutively repressed (*gcn2*) or derepressed (*gcd1*) for *GCN4* expression (Fig. 1, p235). According to our model, ribosomes which resume scanning after translation of uORF1 in *gcn2* cells reform an initiation complex very rapidly and reinitiate at uORF2, uORF3, or uORF4; after translating these uORFs, they dissociate from the mRNA and fail to reach the *GCN4* start site. In *gcd1* cells, many of the ribosomes scanning from uORF1 do not reform an initiation complex until after scanning past uORF2 to uORF4 and, consequently, reinitiate at *GCN4* instead.

In our model, prior translation of uORF1 is absolutely required for ribosomes to skip over the start sites at uORF2 to uORF4 and initiate translation at the *GCN4* AUG codon. The reason for this requirement is that only reinitiating ribosomes that must bind ternary complex while scanning downstream from uORF1 will be able to leaky scan past uORF2 to uORF4 when the levels of ternary complexes are reduced under derepressing conditions. This is because small ribosomal subunits normally bind ternary complex before interacting with the mRNA 5′ cap. Thus, a small reduction in ternary complexes is not expected to allow leaky scanning at uORF2 to uORF4 by ribosomes which have not previously translated uORF1 and consumed the ternary complexes they acquired prior to mRNA binding.

We tested our predictions about the behavior of ribosomes at uORF1 by analyzing the effects of two different types of mutations in the *GCN4* mRNA leader. We began by examining the effect of decreasing the intercistronic distance between uORF1 and the *GCN4* start site on the efficiency of *GCN4* expression. If ribosomes that translate *GCN4* have previously translated uORF1, then *GCN4* expression should be reduced by shortening the uORF1-*GCN4* interval because of the decreased scanning time available to reassemble a preinitiation complex before reaching *GCN4* (2, 24). To test this prediction, several deletions were made that bring uORF1 progressively closer to the beginning of *GCN4* in constructs containing uORF1 alone in the leader (Fig. 1). The three deletions made in constructs pM199, pM230, and pM231 have a common 5′



B

Construct	uORF1-*GCN4* (nt)	*GCN4-lacZ* activity (U) *gcn2*	*gcd1*	Complementation of *gcn4Δ*
p235	350	570	680	+++++
p238	n.a	1500	1600	+++++
pM199	140	340	500	++++
pM230	100	380	500	++++
pM231	50	160	190	+
pG40	n.a	1200	1700	+++++

FIG. 1. Reinitiation at *GCN4* is dependent on the intercistronic distance from uORF1. (A) Schematic showing the *GCN4* mRNA leader in a series of constructs containing deletions between uORF1 and *GCN4*. uORF1 is shown as a stippled box, and the *GCN4* coding sequence is shown as an open rectangle. X's indicate point mutations in the ATG codons of uORF2, uORF3, and uORF4 in construct p235 and of uORF1 in construct pG40. Constructs are drawn approximately to scale. The three deletions in pM199, pM230, and pM231 have a common 5′ junction 25 nt downstream of the uORF1 stop codon and different 3′ junctions which move uORF1 to positions 140, 100, and 50 nt upstream of the *GCN4* start site, respectively (indicated by ∫). pG40 is identical to pM231 except for a point mutation removing the uORF1 AUG codon. (B) The plasmids listed carry the *GCN4* alleles with the leader sequences shown in panel A. *GCN4* expression from these constructs was quantified in two different ways. First, *GCN4* constructs were tested for complementation of a chromosomal *gcn4* deletion by measuring the growth rate of transformants after replica plating to medium supplemented with 3-AT. Growth was scored after 2 or 3 days at 30°C. Second, β-galactosidase activities expressed from the corresponding *GCN4-lacZ* fusions were measured in several independent transformants of the nonderepressible *gcn2-1* strain H15 and the constitutively derepressed *gcd1-101* strain F98. The individual measurements differed from the mean value by less than 27%. n.a., not applicable.

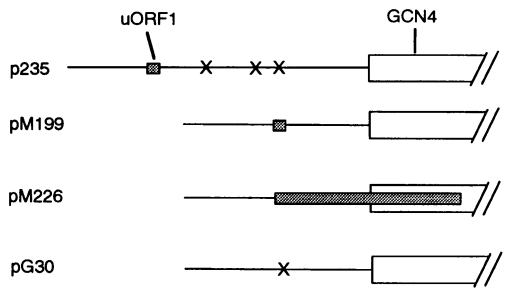
junction located 25 nt downstream from the uORF1 stop codon and different 3′ junctions located 115, 75, or 25 nt upstream from the *GCN4* start site, respectively. These and other mutations described below were analyzed in two different ways. First, *GCN4* expression was quantified by measuring β-galactosidase activity from *GCN4-lacZ* fusions in *gcd1* and *gcn2* mutant strains. Second, constructs containing the intact *GCN4* coding region were introduced into a *gcn4* deletion strain and tested for the ability to restore growth in the presence of 3-AT. 3-AT inhibits the activity of the *HIS3*-encoded enzyme in the histidine biosynthetic pathway, and derepression of *HIS3* transcription by GCN4 is required for growth on medium containing 3-AT. As shown below, the level of 3-AT resistance conferred by the plasmid-borne *GCN4* alleles in the *gcn4* deletion strain correlated well with expression of the corresponding *GCN4-lacZ* fusions in the derepressed *gcd1* mutant.

The results shown in Fig. 1 indicate that decreasing the uORF1-*GCN4* interval led to substantial reductions in *GCN4* expression, reducing it by a factor of 3 to 4 relative to that of p235 when the spacing was decreased to only 50 nt in pM231. The reduction in *GCN4* expression seen for pM231 did not arise from an inhibitory sequence introduced at the deletion junction, since a point mutation that removed the uORF1 ATG codon from pM231 restored *GCN4* expression to the high levels characteristic of *GCN4* leaders lacking all four uORFs (Fig. 1, compare pG40 with pM231 and p238). Additional constructs that document the inhibitory effect of moving *GCN4* closer to uORF1 will be presented below. These results would not be expected if *GCN4* was being translated by a population of ribosomes which have scanned past uORF1 without initiating translation; however, they are in accord with the intercistronic length dependence of reinitiation noted previously for preproinsulin mRNAs containing an uORF (24).

In a second experiment, the deletion construct pM199 was modified to lengthen uORF1 and cause it to overlap the beginning of the *GCN4* coding region (Fig. 2, pM226). This procedure involved eliminating the uORF1 stop codon by insertion of 1 bp, thereby extending uORF1 in a different translational reading frame that terminates 130 nt downstream from the *GCN4* AUG codon. Previously, no effect on *GCN4* expression was seen when uORF4 was made to overlap *GCN4* to the same extent and in the same translational reading frame used by the elongated version of uORF1 in pM226 (2). This latter result was taken as a strong indication that ribosomes which reinitiate at *GCN4* under starvation conditions have scanned past uORF4 without initiating translation. In contrast with these previous findings, making uORF1 overlap the beginning of *GCN4* in construct pM226 was found to drastically reduce *GCN4* expression under both repressing and derepressing conditions (Fig. 2). This result provides additional evidence that *GCN4* is not being translated by ribosomes which have scanned past uORF1 without initiating translation, because such ribosomes should be unaffected by the location of the uORF1 stop codon. In view of our previous findings that *GCN4* expression was unaffected by making uORF4 overlap *GCN4* and that many ribosomes continue to translate uORF4 under derepressing conditions (2), it seems unlikely that the low expression from construct pM226 results from a population of ribosomes which are translating elongated uORF1 and obscuring the *GCN4* start site from other ribosomes which have leaky scanned uORF1. Finally, removing the ATG codon of elongated uORF1 by a point mutation restored *GCN4* expression to constitutively high levels (Fig. 2, pG30), demonstrating that the reduced *GCN4* expression from pM226 did not arise from an inhibitory sequence introduced in making this construct. Together, the results in Fig. 1 and 2 provide strong support for the idea that ribosomes must first initiate translation at uORF1, terminate, and resume scanning in order to reach the *GCN4* start codon.

Reinitiation occurs efficiently at uORF4 under repressing conditions even with a greatly reduced uORF1-uORF4 inter-

A



B

Construct	*GCN4-lacZ* activity (U)		Complementation of *gcn4Δ*
	gcn2	*gcd1*	
p235	570	680	+++++
pM199	340	500	++++
pM226	12	23	-/+
pG30	1300	1600	+++++

FIG. 2. Elongating uORF1 to overlap the beginning of the *GCN4* coding region abolishes *GCN4* expression. (A) Schematic of constructs p235, which contains uORF1 as the only uORF upstream of *GCN4*, and pM199, in which the sequences between uORF1 and uORF4 have been deleted to move uORF1 to the exact position normally occupied by uORF4 upstream of *GCN4*. pM226 has a 1-bp insertion in the stop codon of uORF1, as well as point mutations in two downstream in-frame termination codons, which together lengthen uORF1 and cause it to overlap the beginning of the *GCN4* coding region by 130 nt. Construct pG30 is identical to pM226 except for a point mutation removing the uORF1 AUG codon. The constructs were drawn as described in the legend to Fig. 1. (B) Analysis of *GCN4* expression from the constructs shown in panel A, conducted exactly as described in the legend to Fig. 1. The individual β-galactosidase measurements differed from the mean value by less than 25%.

val. We showed previously that increasing the distance between uORF1 and uORF4 by 146 nt led to a substantial reduction in *GCN4* expression under starvation conditions or in *gcd1* mutants (2). This result is in agreement with our model, since the proportion of ribosomes competent to reinitiate translation at uORF4 when eIF-2–GTP–Met-tRNA$_i^{Met}$ ternary complexes are at low levels should increase as uORF4 is moved progressively further away from uORF1. The converse of this experiment would be to bring uORF4 closer to uORF1. This alteration would be expected to produce higher *GCN4* expression under repressing conditions if many ribosomes failed to reassemble a preinitiation complex in the reduced time it takes to scan the shortened uORF1-uORF4 interval, causing them to bypass the uORF4 start site and reinitiate at *GCN4* instead. In contrast, *GCN4* expression should decrease under derepressing conditions, since fewer ribosomes would rebind the ternary complex while scanning the shortened uORF1-*GCN4* interval.

We tested these predictions of our model by deleting sequences present between uORF1 and uORF4 in construct pA44, which contains the wild-type sequences between these two uORFs, except for the ATG codons at uORF2 and uORF3 (Fig. 3). The deletions in constructs pG26 and pG29 have a common 3′ junction located 23 nt upstream of the uORF4 start codon and 5′ junctions 9 and 31 nt downstream of the uORF1 stop codon, respectively. In accord with our predictions, when the distance between uORF1 and uORF4 was reduced from 200 nt to either 54 or 32 nt, *GCN4* expression increased under repressing conditions but decreased under derepressing conditions, yielding a substantially reduced derepression ratio (Fig. 3B, *gcd1/gcn2*). We then increased the distance between uORF4 and *GCN4* in construct pG26 from 139 to 283 nt by inserting two copies of a sequence normally found between uORF4 and *GCN4* at a site 43 nt downstream from uORF4. This insertion had the effect of returning the uORF1-*GCN4* spacing to approximately the wild-type spacing (Fig. 3, pG4). The insertion in pG4 increased *GCN4-lacZ* expression relative to the parental construct pG26 under both repressing and derepressing conditions (Fig. 3), suggesting that more ribosomes could reinitiate at *GCN4* with the increased scanning distance between uORF1 and *GCN4* provided by the insertion. The results from pG4 suggested that the lowered *GCN4* expression seen under derepressing conditions for construct pG26 was attributable to the decreased uORF1-*GCN4* scanning distance, rather than an inhibitory sequence introduced in the construction of pG26. These results support the idea that the magnitude of *GCN4* translational control is strongly dependent on the relative sizes of the uORF1-uORF4 and uORF1-*GCN4* sequence intervals.

Although *GCN4* expression increased 2.5-fold under repressing conditions when the distance between uORF1 and uORF4 was reduced to 32 nt in construct pG26, it remained significantly lower than that given by an otherwise identical construct lacking uORF4 (Fig. 4, compare pG26 with pG82 in *gcn2* cells). Thus, following translation of uORF1, it appears that most ribosomes continue to reinitiate at uORF4 and subsequently dissociate from the mRNA even when only 32 nt separates uORF1 and uORF4. Using the β-galactosidase expression levels measured for pG26 and pG82 in *gcn2* cells, we deduced that 76% of the ribosomes which reinitiate at *GCN4* when uORF4 is absent in construct pG82 reinitiate at uORF4 instead in construct pG26 [(160 U − 39 U)/160 U = 0.76]. In contrast, the results shown in Fig. 1 for pM199, pM230, and pM231 suggested that a distance much greater than 50 nt was required for efficient reinitiation at *GCN4* following translation of uORF1 in constructs lacking uORF4. For example, when the uORF1-*GCN4* interval was reduced to 50 nt in pM231, *GCN4* expression under repressing conditions was only 28% of that given by construct p235 with 350 nt separating uORF1 from *GCN4* (160 U/570 U). These comparisons suggested that some aspect of uORF4 or the sequences present in the 32 nt remaining between uORF1 and uORF4 in pG26 can promote very efficient reinitiation at uORF4 under repressing conditions. In the absence of these sequences, an extended intercistronic interval seems to be required between uORF1 and *GCN4* to achieve efficient reinitiation at *GCN4*.

One possible explanation for the high level of reinitiation at uORF4 exhibited by construct pG26 is that sequences remaining in its truncated uORF1-uORF4 interval form a structure that retards the scanning process. The presence of this structure would increase the time needed to scan from uORF1 to uORF4 by an amount equivalent to the time it takes to traverse a relatively unstructured segment of RNA several hundred bases long. One argument against this possibility is that construct pG29, containing only 54 nt between uORF1 and uORF4, also exhibits efficient reinitiation at uORF4 and

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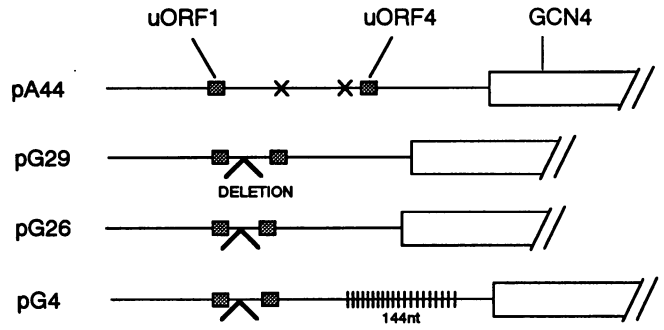

B

Construct	1 - 4 (nt)	*GCN4-lacZ* activity (U) *gcn2*	*gcd1*	*gcd1/ gcn2*	Complementation of *gcn4Δ*
pA44 (wt)	200	16	190	12	++++
pG29	54	37	130	3.5	++
pG26	32	39	140	3.6	++
pG4	32	84	200	2.4	+++

FIG. 3. Decreasing the spacing between uORF1 and uORF4 impairs *GCN4* translational control. (A) Schematic of constructs pG26 and pG29, in which 168 and 146 nt, respectively, normally present between uORF1 and uORF4 have been deleted. Also depicted is construct pG4, identical to pG26 except for an insertion of 144 nt between uORF4 and *GCN4* that restores the separation between uORF1 and *GCN4* to approximately the wild-type distance. The constructs were drawn as described in the legend to Fig. 1. (B) Analysis of the constructs shown in panel A for *GCN4* expression was conducted exactly as described in the legend to Fig. 1. The individual β-galactosidase measurements differed from the mean value by less than 30%. The spacing between uORF1 and uORF4 is listed for each construct in the column labeled "1 -4 (nt)". The column labeled *gcd1/gcn2* gives the ratio of *GCN4-lacZ* expression in the *gcd1* and *gcn2* strains. wt, wild type.

relatively low *GCN4* expression under repressing conditions (Fig. 3). A similar result was obtained previously for a deletion construct containing only 27 nt between uORF1 and uORF4 (38). The fact that three different deletion constructs with drastically shortened uORF1-uORF4 intervals retain high-level reinitiation at uORF4 suggests that this phenomenon is not an artifact of a secondary structure introduced between the two uORFs.

In an attempt to address directly whether an inhibitory structure had been introduced between uORF1 and uORF4 in pG26, we removed the uORF1 ATG codon from construct pG82 by a single-base substitution and compared expression of the resulting uORF-less construct (pG143 [Fig. 4]) with that of other uORF-less constructs, such as p238 (Fig. 1) and pG30 (Fig. 2). Expression from pG143 was about 50% lower than that given by the other two uORF-less constructs, suggesting that the sequences present between uORF1 and uORF4 in the pG26-pG82-pG143 series of constructs may exert a modest inhibitory effect on scanning compared with the wild-type leader between uORF1 and *GCN4*. To determine whether this inhibitory effect is sufficient to explain the high frequency of reinitiation at uORF4 seen in construct pG26, we deleted the uORF4 coding region and all the sequences present between uORF4 and *GCN4* from pG26, placing the *GCN4* coding region at the position of uORF4 in construct pG67 (Fig. 4). If the 32 nt between uORF1 and uORF4 is responsible for the high-level reinitiation at uORF4 seen under repressing conditions with construct pG26, then we should observe much higher *GCN4* expression from pG67 than from pM231 (Fig. 1), in which *GCN4* is 50 nt downstream from uORF1. At odds with this prediction, very low *GCN4* expression from pG67 under repressing conditions was observed (Fig. 4). By comparing expression from pG67 with the corresponding control construct pG142 lacking the uORF1 ATG codon, we deduced that only 4% of the ribosomes could reinitiate at *GCN4* in construct pG67 (55 U/1,200 U). The fact that the majority of ribosomes skip over *GCN4* in the pG67 construct, whereas most ribosomes reinitiate at uORF4 after scanning the identical intercistronic interval in pG26, suggests that the 32 nt between uORF1 and uORF4 in pG26 is not sufficient to account for the high rate of reinitiation at uORF4 versus *GCN4*.

To determine whether the high level of reinitiation seen at uORF4 is a function of its small size rather than of particular

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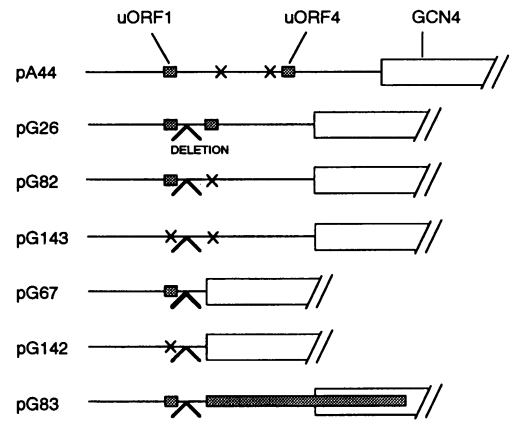


B

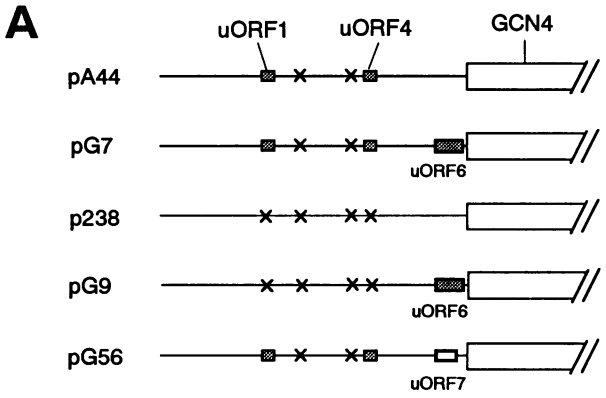
Construct	1 - 4 (nt)	*GCN4-lacZ* activity (U) *gcn2*	*gcd1*	*gcd1/ gcn2*	Complementation of *gcn4Δ*
pA44 (wt)	200	16	190	12	++++
pG26	32	39	140	3.6	++
pG82	n.a	160	180	n.a	++
pG143	n.a	730	850	n.a	+++++
pG67	n.a	55	90	n.a	+
pG142	n.a	1200	2000	n.a	+++++
pG83	32	32	122	3.8	++

FIG. 4. Reinitiation is more efficient at the uORF4 start site than at *GCN4* under repressing conditions. (A) Schematic of constructs pA44 and pG26, already depicted in Fig. 3, and pG82 in which the ATG codon of uORF4 in pG26 has been mutated to AAG. pG143 is identical to pG82 except for a T-to-A substitution that changes the ATG codon of uORF1 to AAG. Construct pG67 was derived from pG26 by means of a deletion that moves the *GCN4* coding region to the exact position normally occupied by uORF4 in pG26. pG142 is identical to pG67 except for a T-to-A substitution that changes the ATG codon of uORF1 to AAG. Construct pG83 was derived from pG26 and contains the same mutations in the stop codon of uORF4 and near the beginning of *GCN4* described above for pM226 that lengthen uORF4 and cause it to overlap the beginning of the *GCN4* coding region by 130 nt. The constructs were drawn as described in the legend to Fig. 1. (B) Analysis of the constructs shown in panel A for *GCN4* expression was conducted exactly as described in the legend to Fig. 1. The individual β-galactosidase measurements differed from the mean value by less than 23%. The spacing between uORF1 and uORF4 is listed for each construct in the column labeled "1 - 4 (nt)". The column labeled *gcd1/gcn2* gives the ratio of *GCN4-lacZ* expression in the *gcd1* and *gcn2* strains. wt, wild type; n.a., not applicable.

sequences, we expanded uORF4 into a large ORF. By inserting 1 bp, we eliminated the uORF4 stop codon in pG26 and extended uORF4 in a different reading frame that terminates 130 nt downstream from the *GCN4* start codon (pG83 [Fig. 4]). If efficient reinitiation at uORF4 in construct pG26 requires that uORF4 be only three codons in length or that its stop codon be present in a particular sequence context, then elongating uORF4 in pG83 should reduce reinitiation at uORF4 and lead to increased *GCN4* expression compared with that in pG26. At odds with this prediction, pG83 gave very low *GCN4* expression under repressing conditions in the *gcn2* mutant, essentially identical to that seen for pG26. Note that elongating uORF4 did not significantly reduce *GCN4* expression under derepressing conditions in the *gcd1* mutant relative to that given by construct pG26 (122 U versus 140 U [Fig. 4]). Thus, the elongated version of uORF4 in pG83 is being skipped under derepressing conditions to the same extent that occurs with wild-type uORF4 in pG26. These results indicate that the high efficiency of reinitiation at uORF4 is not dependent on the short length of its coding region or the sequence context of its stop codon that is believed to promote ribosome dissociation as a result of a slow step in the termination process at uORF4 (10). Although the sequences immediately upstream from uORF4 are not sufficient to confer efficient reinitiation at *GCN4*, as shown by our results with construct pG67, they may act in conjunction with other sequences 3′ to the uORF4 AUG codon to promote reinitiation at uORF4.

Insertion of uORFs between uORF4 and *GCN4* impairs derepression of *GCN4* expression. Our model proposes that under derepressing conditions in a *gcd1* mutant, many ribosomes ignore the uORF4 start site and reinitiate at *GCN4* instead because they fail to rebind the ternary complex until after scanning past uORF4. The results just described strongly suggest that under repressing conditions, reinitiation is substantially more efficient at uORF4 than at *GCN4* when both start sites are equidistant from uORF1. In view of these unexpected results, we sought to determine whether the enhanced ability of ribosomes to reinitiate at *GCN4* versus uORF4 under derepressing conditions depends on some special feature of the *GCN4* coding region or whether, as our model predicts, the enhanced ability could be attributed to the greater scanning distance between uORF1 and *GCN4* versus uORF1 and uORF4. If ribosomes gradually regain the ability to reinitiate translation under derepressing conditions as they scan from uORF4 to *GCN4*, we would expect to find that insertion of a heterologous uORF between uORF4 and *GCN4* would reduce but not abolish *GCN4* expression, whereas insertion of an uORF at the normal start site of *GCN4* would completely eliminate reinitiation further downstream at *GCN4*.

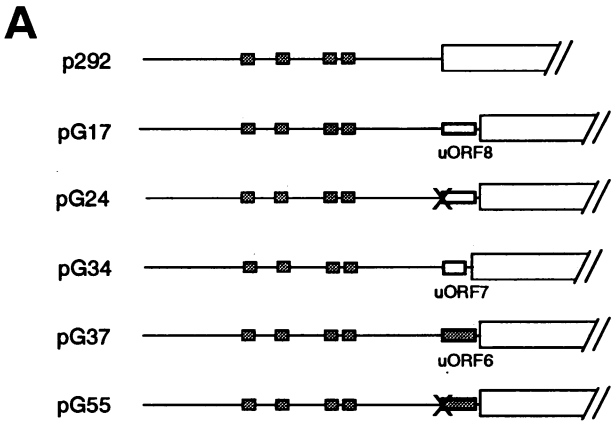
To test this prediction, two 1-nt substitutions were made that introduced an ATG codon 50 nt upstream of the *GCN4* ATG codon, creating a 12-codon ORF (uORF6) that terminates 11 nt upstream of *GCN4*. This new ORF is predicted to be in a favorable sequence context for translation initiation (5), and this expectation was verified by making the same two substitutions in a *GCN4* leader from which all of the uORFs had been removed by point mutations. The presence of uORF6 in pG9 decreased *GCN4-lacZ* expression by a factor of ca. 25 relative to that of the parental uORF-less construct (Fig. 5, compare pG9 and p238). This result suggested that uORF6 is recognized and translated by >95% of the ribosomes scanning from the cap and that reinitiation at *GCN4* following translation of uORF6 is very inefficient. In contrast with the results from pG9, when uORF6 was introduced into a *GCN4* leader containing uORF1 and uORF4 in their normal locations, *GCN4* expression under derepressing conditions was reduced only by a factor of 2 relative to that of the parental construct pA44, and a 13-fold derepression ratio (*gcd1/gcn2*) was still observed (Fig. 5, pG7). Thus, under derepressing conditions, it appeared that ca. 50% of the ribosomes which translated uORF1, resumed scanning, and then bypassed the start site at



B

Construct	*GCN4-lacZ* activity (U)			
	gcn2	*gcd1*	*gcd1/ gcn2*	Complementation of *gcn4Δ*
pA44(wt)	16	190	12	++++
pG7	7	93	13	+++
p238	1500	1600	n.a	+++++
pG9	32	59	n.a	+
pG56	12	100	8.3	+++

FIG. 5. Insertions of uORFs between uORF4 and *GCN4* lower *GCN4* expression. (A) Schematic of constructs pG7 and pG9 in which an ATG codon has been introduced by site-directed mutagenesis 50 nt upstream of the *GCN4* start site, creating a 12-codon uORF (uORF6). In construct pG56, the first 7 codons of *GCN4* and a UAA stop codon have been introduced starting at the same position as uORF6, creating uORF7. The constructs were drawn as described in the legend to Fig. 1. (B) Analysis of the constructs shown in panel A for *GCN4* expression was conducted exactly as described in the legend to Fig. 1. The individual β-galactosidase measurements differed from the mean value by less than 20%.



B

Construct	*GCN4-lacZ* activity (U)			
	gcn2	*gcd1*	*gcd1/ gcn2*	Complementation of *gcn4Δ*
p292(wt)	5	170	34	+++
pG17	3	6	2	-
pG24	14	120	8.6	++
pG34	2	9	4.5	-
pG37	3	18	6	-
pG55	11	150	14	+++

FIG. 6. Insertions of uORFs at the *GCN4* start site abolish *GCN4* expression. Schematic of construct pG17, which has a stop codon introduced at codon position 12 of the *GCN4* coding region (creating uORF8), followed by the 11 nt normally found upstream of the *GCN4* ATG codon and the intact *GCN4* coding region. Also shown are constructs pG34 and pG37, which have uORF7 and uORF6, respectively, inserted at the position of the *GCN4* ATG codon, followed by the 11 nt normally found upstream of the *GCN4* coding region and the intact *GCN4* coding sequence. The constructs were drawn as described in the legend to Fig. 1. (B) Analysis of the constructs shown in panel A was conducted exactly as described in the legend to Fig. 1. The individual β-galactosidase measurements differed from the mean value by less than 22%. wt, wild type.

uORF4 also failed to recognize the start codon at uORF6 and reinitiated 50 nt further downstream at *GCN4*. A very similar reduction in *GCN4* expression was observed with the analogous construct pG56 (Fig. 5), in which the first seven codons of *GCN4* were inserted into pA44 (creating uORF7) at exactly the same position as uORF6 in pG7, 50 nt upstream from the *GCN4* start codon.

We next wished to determine whether the residual *GCN4* expression observed in the presence of heterologous uORF6 and uORF7 in constructs pG7 and pG56, respectively, would be eliminated if the entire 150-nt sequence normally found between uORF4 and *GCN4* were present between uORF4 and these heterologous uORFs. According to our model, including these extra 50 nt would provide the additional scanning time needed to ensure that all ribosomes which have bypassed uORF4 will become competent to reinitiate at the heterologous uORF and be excluded from the *GCN4* start site. To address this possibility, we converted the beginning of the *GCN4* coding region into a small uORF by introducing a stop codon after *GCN4* codon 11 and inserting the 11 nt normally found immediately upstream of the *GCN4* start codon between the resulting small uORF (uORF8) and the authentic *GCN4* coding region (pG17 [Fig. 6]). Similar constructs were generated (pG34 and pG37) containing the uORF7 sequence of pG56 or the uORF6 sequence in pG7, discussed above, instead of uORF8. (To facilitate construction of the alleles in Fig. 6, we had to begin with constructs containing all four *GCN4* uORFs instead of only uORF1 and uORF4; however, the same regulatory mechanism operates in both situations [31].)

When uORF8 was introduced into the wild-type leader at the normal location of the *GCN4* start site, *GCN4* expression under derepressing conditions was virtually abolished (Fig. 6, compare p292 and pG17). Removing the start codon of uORF8 by a point mutation from ATG to AAG (construct pG24) nearly restored wild-type levels of *GCN4* expression, demonstrating that *GCN4* expression from pG17 was very low because ribosomes translate uORF8 and then fail to reinitiate again at *GCN4* (Fig. 6, compare pG17 and pG24). The insertion of uORF7 (in pG34) or uORF6 (in pG37) at the normal location of *GCN4* led to similar drastic reductions in *GCN4* expression, and removal of the ATG codon from uORF6 (pG55) nearly restored wild-type expression.

The various heterologous uORFs studied in Fig. 5 and 6 had essentially identical effects on *GCN4* expression under derepressing conditions, reducing it by ca. 50% when inserted 50 nt upstream from *GCN4* and by >90% when introduced at the normal location of the *GCN4* start site. These results support the idea that the ability of ribosomes to reinitiate at *GCN4* under conditions of reduced eIF-2 function increases with the distance scanned from uORF1. The fact that the heterologous uORFs essentially abolished *GCN4* expression when inserted at the normal position of the *GCN4* start site suggests that virtually all ribosomes scanning downstream from uORF1 are competent to reinitiate translation by the time they reach the *GCN4* AUG codon. The ability of uORF6, uORF7, and uORF8 to function indistinguishably in this manner would not be expected if sequences at the beginning of the *GCN4* coding region were required for efficient reinitiation by the ribosomes which have bypassed uORF4 under derepressing conditions. However, because these *GCN4* sequences remain a short distance downstream from uORF6, uORF7, and uORF8 in the constructs shown in Fig. 6, we cannot completely rule out the possibility that the *GCN4* coding region contains a sequence or structure that facilitates rebinding of initiation factors to ribosomes located just upstream from *GCN4* and thereby stimulates reinitiation at the heterologous uORFs.

DISCUSSION

Evidence that ribosomes translate uORF1 en route to the *GCN4* start codon. The experiments presented here confirm and extend our understanding of the translational control mechanism underlying general amino acid control in *S. cerevisiae*. Previous work has shown that uORF1 and uORF4 play different roles in controlling the flow of scanning ribosomes to the *GCN4* AUG codon. uORF4 functions as a strong barrier to *GCN4* translation and by itself is sufficient to repress *GCN4* expression to low levels. In contrast, uORF1 is a weak translational barrier and is required upstream from uORF4 to derepress *GCN4* translation when cells are starved for an amino acid (31). The strong inhibitory effect of uORF4 has been attributed to the inability of ribosomes to resume scanning and reinitiate at *GCN4* after completing translation of uORF4 (30). The weak inhibitory effect of uORF1 is thought to derive from the ability of ribosomes to resume scanning and reinitiate downstream following translation termination. Because the ability of uORF1 to function as a weak translational barrier has been correlated with its capacity to stimulate *GCN4* translation, we have proposed that translation of uORF1 under derepressing conditions allows ribosomes which resume scanning to ignore the uORF4 start codon and reinitiate at *GCN4* instead. Under nonstarvation conditions, essentially all of these ribosomes reinitiate at uORF4 and are excluded from the *GCN4* start site.

Alternative explanations could be proposed to explain the stimulatory effect of uORF1 on *GCN4* translation. For example, it could be suggested that uORF1 is a weak translational barrier because many ribosomes fail to initiate at uORF1 (leaky scanning) and continue scanning downstream. This pool of ribosomes might also ignore the uORF4 start site under derepressing conditions in response to a conformational change in the mRNA induced by a second group of ribosomes which translate uORF1 and then dissociate from the mRNA. Another possibility would be that low-level translation of uORF1 perturbs mRNA structure in a way that facilitates direct binding to the *GCN4* start codon by ribosomes which completely circumvent the scanning process (internal initiation).

These alternative hypotheses are inconsistent with the results of the experiments presented in Fig. 1, 2, and 4. Decreasing the distance between uORF1 and *GCN4* in constructs containing uORF1 alone led to a stepwise reduction in *GCN4* expression (Fig. 1 and 4). This relationship between the uORF1-*GCN4* intercistronic distance and *GCN4* expression would not be expected if ribosomes which translate *GCN4* have previously scanned past uORF1 without initiating translation. It is also not obvious why the frequency of internal initiation would exhibit a continuous decline with decreasing separation between uORF1 and *GCN4*. However, this trend is in accord with the idea that ribosomes must translate uORF1 en route to *GCN4* and that the probability of rebinding one or more factors required for reinitiation increases with the distance scanned following uORF1 translation.

Our observation that elongating uORF1 to make it overlap the beginning of *GCN4* completely abolishes *GCN4* expression is also consistent with the notion that all ribosomes must translate uORF1 to reach the *GCN4* start site. In the region of overlap between the elongated uORF1 and *GCN4* in construct pM226, there are four AUG codons; thus, after translating the elongated uORF1, ribosomes would have to scan for 130 nt in a 3′-to-5′ direction and ignore the four AUG codons in the overlap region in order to reinitiate at the authentic *GCN4* start codon. The extremely low-level *GCN4* expression given by construct pM226 indicates that this improbable scenario does not occur. Elongating uORF4 and causing it to overlap *GCN4* in exactly the same way had no detectable effect on *GCN4* expression, as expected if ribosomes do not translate uORF4 en route to *GCN4* (2). Thus, the results from construct pM226 provide independent evidence against any models in which ribosomes reach *GCN4* by leaky scanning at uORF1. Although translation across the *GCN4* start site might still be expected to interfere with an internal initiation mechanism, our previous finding that increased local secondary structure at uORF4 abolishes *GCN4* expression provides a third argument against internal initiation at *GCN4* (2).

***GCN4* translational control is strongly dependent on the relative distances between uORF1 and uORF4 and between uORF4 and *GCN4*.** According to our model, a substantial fraction of ribosomes fail to reinitiate at uORF4 under derepressing conditions because the time it takes to scan from uORF1 to uORF4 is insufficient to rebind the ternary complex to all ribosomes which have translated uORF1 and resumed scanning. We proposed that the additional time it takes to reach the *GCN4* start site after bypassing uORF4 would allow rebinding of ternary complexes to nearly all of the remaining ribosomal subunits, ensuring efficient reinitiation at *GCN4* (2). One piece of evidence supporting this hypothesis was that increasing the spacing between uORF1 and uORF4 to roughly the wild-type distance between uORF1 and *GCN4* almost completely abolished derepression of *GCN4* expression. We reasoned that the additional scanning time provided by in-

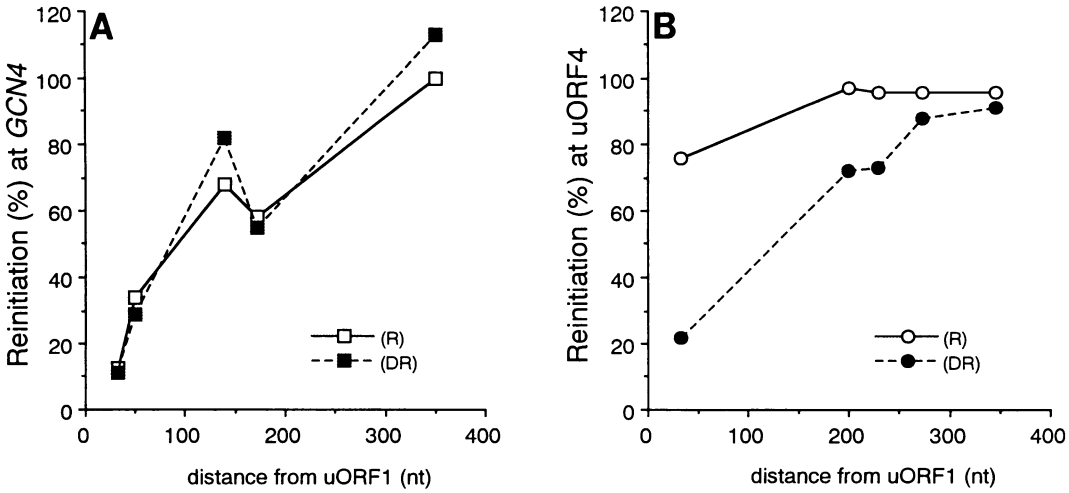


FIG. 7. Relationship between the percentage of ribosomes competent to reinitiate translation after translating uORF1 and the distance scanned downstream of uORF1. The percentage of ribosomes that are able to reinitiate at uORF4 or *GCN4* after scanning various distances from uORF1 was calculated from the data presented in Fig. 1 to 4. (A) For reinitiation at *GCN4*, constructs pG67 (32 nt), pM231 (50 nt), pM199 (140 nt), pG82 (172 nt), and p235 (350 nt) were compared with the following control constructs lacking uORF4: pG142, pG40, pG30, pG143, and pG238, respectively. The reinitiation frequencies at *GCN4* in p235 are 38% (570 U/1,500 U) under repressing conditions (R) and 43% (680 U/1,600 U) under derepressing conditions (DR). We set the reinitiation frequency for p235 in *gcn2* cells to 100% and normalized all of the other calculated frequencies to this value. (B) For reinitiation at uORF4, constructs pG26 (32 nt) and pA44 (200 nt), along with constructs pA56, pA60, and pA61 described previously (2) in which the spacing between uORF1 and uORF4 was increased by 30, 73, or 146 nt, respectively, were compared with the following control constructs lacking uORF4: pG82, p235, pA75, pA76, and pA77, respectively. (The last three control constructs were also described previously [2].) For example, the reinitiation frequencies at uORF4 in construct pG26 are 76% [(160 U − 39 U)/160 U] under repressing conditions (R) and 22% [(180 U − 140 U)/180 U] under derepressing conditions (DR).

creasing the separation between uORF1 and uORF4 enabled the majority of ribosomes to rebind ternary complexes before reaching uORF4 and to reinitiate there instead of further downstream at *GCN4*. It was also found that a large deletion between uORF4 and *GCN4* led to a significant reduction in *GCN4* expression (38), as expected if many ribosomes had failed to rebind ternary complexes before reaching *GCN4* and bypassed this start site as well as the uORF4 AUG codon.

The inhibitory effects on *GCN4* expression of inserting small heterologous uORFs between uORF4 and *GCN4* shown in Fig. 5 and 6 are in complete accord with the idea that reinitiation at *GCN4* under derepressing conditions is dictated primarily by the distance scanned from uORF1. The presence of wild-type uORF4 at its normal location 200 nt downstream from uORF1 reduces the number of ribosomes that reach *GCN4* under derepressing conditions by ca. 70% (compare p235 [Fig. 1] with pA44 [Fig. 3], 1 − [190 U/680 U]). We interpret this finding to indicate that only ca. 30% of the ribosomes scanning downstream from uORF1 under conditions of reduced eIF-2 recycling in the *gcd1* mutant will ignore the uORF4 start codon and continue scanning to *GCN4*; the remaining 70% will reinitiate at uORF4 and subsequently dissociate from the mRNA. Insertion of heterologous uORF6, uORF7, or uORF8 in the uORF4-*GCN4* interval at a position 50 nt upstream from the *GCN4* start codon lowered *GCN4* expression under derepressing conditions by a factor of 2. This result implies that after scanning 90 nt downstream from uORF4, half of the ribosomes which bypassed uORF4 have become competent to reinitiate and will recognize the heterologous uORFs we inserted 50 nt upstream from *GCN4*, and the remaining half will continue scanning and reinitiate at *GCN4*. When the heterologous uORFs were inserted at the exact location of the *GCN4* start site, they completely eliminated reinitiation downstream at the authentic *GCN4* coding sequences, implying that essentially all ribosomes had rebound the ternary complex by the time they scanned the complete 350-nt interval separating uORF1 from the *GCN4* start site. In accord with this interpretation, increasing the distance between uORF4 and *GCN4* in an otherwise wild-type construct produced only a small increase in *GCN4* expression (2), whereas insertion of 144 nt between uORF4 and *GCN4* offset the reductions in *GCN4* expression associated with a deletion of ca. 170 nt between uORF1 and uORF4 (constructs pG26 and pG4 in Fig. 3). These results provide strong confirmation of one of the central tenets of our model, that the probability of rebinding the ternary complex under derepressing conditions increases with the time elapsed in scanning downstream from uORF1. Of course, we would expect to find that reinitiation is stimulated more effectively by segments of RNA with structure-forming potential than by unstructured segments of the same length. This may explain why the 50 nt immediately 5′ of *GCN4* appeared to promote reinitiation at the heterologous uORFs to the same extent as did the 100-nt segment immediately 3′ of uORF4 (Fig. 5 and 6).

Analysis of differential requirements for reinitiation at uORF4 versus *GCN4*. Two observations presented in this report suggest that intercistronic distances and the level of eIF-2 activity are not the sole determinants of reinitiation frequency on *GCN4* mRNA. The *gcd1* mutation is believed to decrease the efficiency of eIF-2 recycling by eIF-2B (4), whereas the *gcn2* mutant lacks the protein kinase activity required to reduce eIF-2 recycling by phosphorylation of eIF-2α (7). Thus, the *gcd1* and *gcn2* mutants used in our study should represent opposite extremes in the levels of active eIF-2. In Fig. 7A, the calculated percentage of ribosomes that reinitiate at *GCN4* following translation of uORF1 has been plotted versus the distance between uORF1 and *GCN4* for five different constructs containing uORF1 alone that we described

above (pG67, pM231, pM199, pG82, and p235). To calculate the reinitiation frequency for each construct, we first divided the *GCN4-lacZ* expression determined for that construct by the value measured for the matching construct containing a point mutation in the uORF1 ATG codon. For the p235 construct, in which uORF1 is present at the wild-type position 350 nt upstream from *GCN4*, this calculated reinitiation frequency in *gcn2* cells is 0.38 (570U/1,500U [Fig. 1]) and 0.425 in *gcd1* cells (680 U/1,600U [Fig. 1]). We set the reinitiation frequency for this construct to 100% to take into account our previous finding (2) that reinitiation at *GCN4* increases very little when the distance between uORF1 and *GCN4* is increased beyond the wild-type spacing. This indicates that the wild-type spacing between uORF1 and *GCN4* is sufficient to allow nearly all ribosomes to reinitiate at *GCN4*. Accordingly, the upper limit on reinitiation observed with construct p35 should represent the fraction of ribosomes which remain attached to the mRNA and resume scanning after translating uORF1 (2). The plot shown in Fig. 7A reveals that the frequency of reinitiation at *GCN4* decreases dramatically as the distance between uORF1 and *GCN4* is reduced in the interval from 350 to 32 nt, in both *gcn2* (open squares) and *gcd1* cells (filled squares). It is noteworthy that the two plots in Fig. 7A obtained from the *gcd1* and *gcn2* strains are nearly superimposable. In Fig. 7B, we plotted the calculated frequencies of reinitiation at uORF4 following translation of uORF1 for the following constructs: (i) the wild-type construct pA44, in which uORF4 is 200 nt downstream from uORF1, (ii) for pG26, where only 32 nt separates the two uORFs, and (iii) for constructs pA56, pA60, and pA61 described previously (2), in which the spacing between uORF1 and uORF4 was increased by 30 to 146 nt over the wild-type spacing. We calculated the reinitiation frequency at uORF4, as already illustrated in the Results section, by comparing the amount of *GCN4* expression measured for each construct with the value determined for the matching construct lacking uORF4. The reduction in *GCN4* expression associated with the presence of uORF4 was attributed to reinitiation at uORF4. While this method of measuring reinitiation at uORF4 is indirect, it was validated previously by determining the rate of synthesis of an uORF4-LacZ fusion protein under repressing and derepressing conditions (1, 2).

Comparison of the resulting plots in Fig. 7B with those discussed above in Fig. 7A illustrates two important differences between uORF4 and *GCN4* regarding the dependence of reinitiation on the distance scanned from uORF1 and the amount of active eIF-2 present in the cell. Figure 7B shows that for intercistronic spacings between 32 and 200 nt, the efficiency of reinitiation at uORF4 is substantially reduced in the *gcd1* mutant versus the *gcn2* strain. In sharp contrast, Fig. 7A shows that reinitiation at *GCN4* in constructs lacking uORF4 is virtually indistinguishable in the two mutants over the entire range of intercistronic distances we analyzed. The reduction in reinitiation at uORF4 in the *gcd1* mutant illustrated in Fig. 7B is predicted by our model to be the result of decreased binding of ternary complexes to ribosomes scanning between uORF1 and uORF4. Those ribosomes which fail to reinitiate at uORF4 proceed to reinitiate at *GCN4* instead, accounting for the derepression of *GCN4* expression that occurs in *gcd1* cells. The suppression of reinitiation at uORF4 seen in the *gcd1* mutant is greatest when the uORF1-uORF4 spacing is only 32 nt but is barely detectable when the spacing is increased to 344 nt. In our model, this length dependence reflects the fact that more scanning distance (time) is needed to rebind ternary complexes to ribosomes scanning downstream from uORF1 to allow reinitiation at uORF4 when the levels of ternary complex are diminished by the *gcd1* mutation. Because ternary complex levels are constitutively high in the *gcn2* mutant, there is only a small reduction in the efficiency of reinitiation at uORF4 as uORF1 is moved progressively closer to uORF4 in this strain (Fig. 7B). Thus, in accord with previous formulations of our model, rebinding of ternary complexes to ribosomes scanning downstream from uORF1 appears to be the principal rate-limiting event for reinitiation at uORF4. Consequently, reinitiation at uORF4 is suppressed only when the level of active eIF-2 is reduced under derepressing conditions.

In contrast to what occurs at uORF4, reinitiation at *GCN4* in constructs lacking uORF4 is relatively inefficient for short intercistronic distances in the *gcn2* mutant, where levels of active eIF-2 are high, and is essentially unaffected by reducing the level of active eIF-2 by the *gcd1* mutation (Fig. 7A). We suggest that the decrease in *GCN4* expression that occurs when uORF1 is brought very close to *GCN4* reflects the failure to rebind one or more factors besides the eIF-2–GTP–Met-tRNA$_i^{Met}$ ternary complex that are needed for reinitiation at *GCN4* (Fig. 8). These might be unidentified factors or a known initiation factor like eIF-5 with an established role in subunit joining (for a review, see reference 29). To explain why reducing the level of ternary complexes does not lead to a further decrease in reinitiation at *GCN4* in the constructs lacking uORF4, it could be proposed that the hypothetical factor binds more slowly than the ternary complex to scanning ribosomes, even when the level of ternary complexes is diminished by a *gcd1* mutation. Alternatively, there could be a high-affinity binding site for the ternary complex in the vicinity of the *GCN4* start codon, allowing ribosomes to bind this factor upon entering the *GCN4* initiation region instead of during the scanning process.

The second important conclusion arising from Fig. 7 is that reinitiation is much more efficient at uORF4 than at *GCN4* for intercistronic distances of less than 200 nt in the *gcn2* mutant when eIF-2–GTP–Met-tRNA$_i^{Met}$ ternary complexes are abundant. With only 32 nt separating the two uORFs, 76% of the ribosomes reinitiate at uORF4 following translation of uORF1 in the *gcn2* strain (Fig. 7B), whereas only 12% of the ribosomes reinitiate at *GCN4* after scanning the same 32-nt interval in a construct lacking uORF4 (Fig. 7A). The observation that reinitiation occurs at uORF4 with little or no requirement for prior scanning from uORF1 can be explained in several different ways. One possibility would be that the uORF4 initiation region (consisting of sequences both 5′ and 3′ of the start codon) either does not require or contains a high affinity-binding site for the hypothetical factor postulated to be limiting for reinitiation at *GCN4* (Fig. 8). Either possibility would obviate the need for prior scanning from uORF1 to reassemble an initiation complex at uORF4, provided that ternary complex levels are high. An alternative model is that ribosomes are forced to pause in the uORF4 initiation region, allowing all the necessary factors to rebind in the absence of an extended period of scanning from uORF1. A stable secondary structure immediately 3′ to uORF4 could be responsible for this pause. In fact, a similar mechanism has been proposed to explain how a stable stem-loop structure in the beginning of a coding region can improve the utilization of a start codon present in an unfavorable sequence context for AUG recognition in mammalian cells (22). Alternatively, a late step in the initiation pathway or one of the elongation steps at uORF4 might occur slowly and lead to queuing of ribosomes upstream of uORF4. The idea that a stable RNA structure is responsible for ribosomal pausing at uORF4 leads to some difficulty in explaining how ribosomes can scan past uORF4 without rebinding ternary complex under derepressing conditions. This

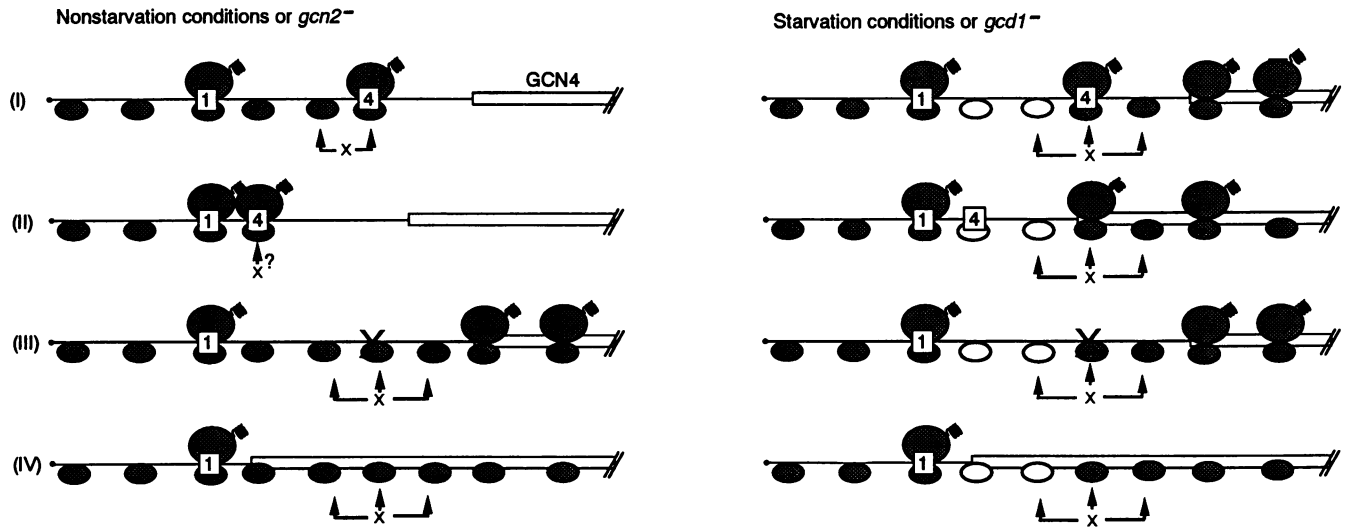


FIG. 8. Model for *GCN4* translational control. The *GCN4* mRNA leader is shown with uORF1 and uORF4 and *GCN4* represented by boxes. Scanning 40S subunits containing eIF-2 in a ternary complex with GTP and Met-tRNA$_i^{Met}$ are shaded, as are 80S ribosomes; subunits lacking the ternary complex are unshaded. 40S subunits which have translated uORF1 and resumed scanning must rebind this ternary complex in a time-dependent fashion while scanning from uORF1 in order to reinitiate either at uORF4 or at *GCN4*; a second factor (X) must also rebind to scanning 40S subunits for reinitiation to occur at *GCN4*. This hypothetical factor is either dispensable for reinitiation at uORF4 or can be acquired by ribosomes in the uORF4 initiation region without extensive prior scanning from uORF1, at least under repressing conditions (nonstarved or *gcn2* cells) when eIF-2–GTP–Met-tRNA$_i^{Met}$ ternary complexes are abundant. Ribosomes could acquire factor X at uORF4 if there is a binding site for the factor present at this site or if ribosomes are delayed just upstream of uORF4 by an RNA structure or by another ribosome stalled in the translation of uORF4 (see text for additional details). For the wild-type *GCN4* leader (construct I) under nonstarvation conditions, the levels of ternary complex in the cell are high so that essentially all ribosomes will reinitiate at uORF4 and subsequently dissociate from the mRNA, preventing *GCN4* translation. Under starvation conditions, the reduction in levels of ternary complex allows about 30% of the ribosomes scanning from uORF1 to bypass the uORF4 start site and reinitiate at *GCN4* instead. Decreasing the distance between uORF1 and uORF4 (construct II) does not lead to a large increase in *GCN4* expression under nonstarvation conditions, because levels of ternary complex are high and because factor X either binds efficiently at uORF4 or is dispensable for reinitiation at uORF4. Under starvation conditions, reduced levels of ternary complex cause skipping of uORF4 and hence *GCN4* expression. Removing uORF4 by a point mutation (construct III) leads to high-level *GCN4* expression under both starvation and nonstarvation conditions because the distance between uORF1 and *GCN4* is sufficient to rebind both the ternary complex and factor X, even when ternary complex levels are low. In the absence of uORF4, factor X must rebind to 40S subunits as they scan from uORF1 to *GCN4*. Thus, decreasing the distance between uORF1 and *GCN4* (construct IV) reduces the time available to rebind factor X, causing skipping of the *GCN4* AUG codon and reduced *GCN4* translation, regardless of ternary complex levels.

difficulty is not encountered, however, if the pause is imposed by a slow step in uORF4 translation, because the duration of the pause would be proportional to the number of ribosomes that initiate at uORF4. As initiation at uORF4 drops with a reduction in ternary complex levels, the length of the pause would decrease simultaneously, reducing the rate of reinitiation at uORF4 even further. Eventually, ribosome queuing upstream of uORF4 would be eliminated, and those ribosomes which did not rebind ternary complex while scanning between uORF1 and uORF4 would scan past the uORF4 start site and proceed to *GCN4* (Fig. 8).

Although at present we can only speculate about the molecular basis for the different scanning time requirements for reinitiation at uORF4 versus *GCN4*, these differences may be important for the efficiency of *GCN4* translational control. The high efficiency of reinitiation at uORF4 under repressing conditions that occurs without the need for prior scanning over long distances would ensure that all ribosomes will reinitiate at uORF4 even if they have translated uORF2 or uORF3 in addition to uORF1. In this way, ribosomes would fail to reinitiate at uORF4 only when the availability of ternary complexes is limited by phosphorylation of eIF-2 or by a mutation in eIF-2B. The much longer time required for ribosomes to reach the *GCN4* start site following termination at uORF1, uORF2, or uORF3 would ensure that ribosomes bind all the factors needed for reinitiation while scanning to *GCN4*, even when ternary complex levels are low. In future studies, we hope to identify the hypothetical factor which limits reinitiation at *GCN4* and also to determine the sequences at uORF4 that permit efficient reinitiation at this site after very short periods of scanning from uORF1. Our results may have important implications for the translation of other eukaryotic mRNAs containing uORFs by showing that the efficiency of reinitiation depends not only on the sequence of the first uORF and the scanning distance to the next start site but also on the sequence of the downstream ORF.

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