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The First and Fourth Upstream Open Reading Frames in *GCN4* mRNA Have Similar Initiation Efficiencies but Respond Differently in Translational Control to Changes in Length and Sequence

PETER P. MUELLER, BELINDA M. JACKSON, PAUL F. MILLER, AND ALAN G. HINNEBUSCH*

Unit on Molecular Genetics of Lower Eukaryotes, Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland 20892

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The third and fourth AUG codons in *GCN4* mRNA efficiently repress translation of the *GCN4*-coding sequences under normal growth conditions. The first AUG codon is ~30-fold less inhibitory and is required under amino acid starvation conditions to override the repressing effects of AUG codons 3 and 4. *lacZ* fusions constructed to functional, elongated versions of the first and fourth upstream open reading frames (URFs) were used to show that AUG codons 1 and 4 function similarly as efficient translational start sites in vivo, raising the possibility that steps following initiation distinguish the regulatory properties of URFs 1 and 4. In accord with this idea, we observed different consequences of changing the length and termination site of URF1 versus changing those of URFs 3 and 4. The latter were lengthened considerably, with little or no effect on regulation. In fact, the function of URFs 3 and 4 was partially reconstituted with a completely heterologous URF. By contrast, certain mutations that lengthen URF1 impaired its positive regulatory function nearly as much as removing its AUG codon did. The same mutations also made URF1 a much more inhibitory element when it was present alone in the mRNA leader. These results strongly suggest that URFs 1 and 4 both function in regulation as translated coding sequences. To account for the phenotypes of the URF1 mutations, we suggest that most ribosomes normally translate URF1 and that the mutations reduce the number of ribosomes that are able to complete URF1 translation and resume scanning downstream. This effect would impair URF1 positive regulatory function if ribosomes must first translate URF1 in order to overcome the strong translational block at the 3′-proximal URFs. Because URF1-*lacZ* fusions were translated at the same rate under repressing and derepressing conditions, it appears that modulating initiation at URF1 is not the means that is used to restrict the regulatory consequences of URF1 translation to starvation conditions.

The *GCN4* protein of *Saccharomyces cerevisiae* stimulates the transcription of genes encoding amino acid biosynthetic enzymes in response to starvation for any amino acid (for a review, see reference 7). Activation of gene expression occurs as the result of increased synthesis of *GCN4* under starvation conditions. Regulation of *GCN4* expression in response to amino acid availability is mediated by positive (*GCN*) and negative (*GCD*) *trans*-acting factors (4, 6, 8). Each of four AUG codons present in the leader of *GCN4* mRNA initiates a short open reading frame of only two or three codons before an in-frame termination codon is reached (5, 27). Removal of all four upstream AUG codons by deletion or point mutations leads to constitutive derepression of *GCN4*, independent of the *GCN* and *GCD* gene products that normally regulate its expression. The AUG mutations have no effect on the steady-state level of *GCN4* mRNA. These results indicate that the upstream AUG codons mediate translational control of *GCN4* expression by *GCN* and *GCD* regulatory factors (20, 21, 27).

The various upstream AUG codons play different roles in translational control. Either the third or the fourth AUG codon (from the 5′ end) is both necessary and sufficient for efficient repression of *GCN4* expression under nonstarvation conditions (21, 28). The strong inhibitory effect of these sequences is expected, given that insertion of an AUG codon into the leader of a eucaryotic transcript generally leads to a large reduction in translation of the protein-coding sequences (10, 12, 17, 23, 26). This effect has been explained as the result of preferential initiation at the 5′-proximal AUG codon coupled with inefficient reinitiation at downstream AUG codons (11, 12, 17, 23, 26). By comparison with AUG codons 3 and 4, AUG codon 1 is ~30-fold less inhibitory to *GCN4* expression when it is present alone in the mRNA leader. In addition, when AUG codons 3 and 4 are present downstream, the first AUG codon functions as a positive control element, as it is required for efficient derepression of *GCN4* expression under starvation conditions. The second AUG codon also acts as a positive element in this situation, but functions less efficiently than the first. Only when AUG codon 1 or 2 is present upstream from AUG codon 3 or 4 is there a strong requirement for *GCD* gene products to maintain repression of *GCN4* expression under nonstarvation conditions (20, 21, 28). These findings led us to suggest that the first and second AUG codons are required to overcome the inhibitory effects of the third and fourth AUG codons and that this antagonistic interaction between AUG codons is blocked under nonstarvation conditions by the *GCD* factors (21).

Although it was shown that the upstream AUG codons are required for translational control of *GCN4* mRNA, it has not been determined whether each of these AUG codons can actually function as an initiation site for protein synthesis. The 5′-proximal and 3′-proximal AUG codons could have drastically different initiation efficiencies that are responsible for the large differences in their inhibitory effects as solitary upstream open reading frames (URFs) and for their opposing roles in *GCN4* translational control. In addition, it is possible that the positive regulatory function of URF1 is restricted to

* Corresponding author.

starvation conditions by blocking initiation at this site under normal growth conditions. To address these questions, we fused *lacZ*-coding sequences to the first, third, and fourth URFs (URFs 1, 3, and 4, respectively), to provide a means of measuring URF translation rates in vivo. In order to construct the URF-*lacZ* fusions, we used site-directed mutagenesis to remove the termination codons of URFs 1, 3, and 4. These mutations lengthened the URFs and allowed the fusion of *lacZ* sequences at a considerable distance downstream from the URF AUG codons. In this way, we hoped to avoid changing the initiation properties of the URFs. Measurements of protein synthesis from the resulting *lacZ* fusions suggested that translation initiation can occur at URFs 1 and 4 with an efficiency comparable to that of the *GCN4* AUG codon itself. This observation raises the possibility that translational steps following initiation distinguish the regulatory properties of URFs 1 and 4.

In the case of URFs 3 or 4, elongating the URF by removing the termination codon was found to have little or no effect on regulatory function. By contrast, certain mutations that lengthened URF1 were found to impair its regulatory function almost as severely as removal of the URF1 initiation codon, suggesting that at URF1, elongation steps, termination steps, or both are very important for the positive role that this sequence plays in *GCN4* translational control. To account for the deleterious effects of altering URF1-coding sequences, we propose that ribosomes must efficiently translate URF1 and continue scanning downstream in order to pass beyond URFs 3 and 4 under derepressing conditions and subsequently initiate translation at the *GCN4* AUG codon.

MATERIALS AND METHODS

Construction of *GCN4-lacZ* mutations. The starting plasmids for all constructions were related to p180 (6), an *Escherichia coli*-yeast shuttle vector containing a *GCN4-lacZ* translational fusion and the *S. cerevisiae URA3*, *ARS1*, and *CEN4* sequences. The *lacZ* sequences are inserted at codon 56 of the *GCN4* protein-coding sequence (5). In many cases, we used derivatives of p180 containing substitution mutations in one or more of the upstream AUG codons described previously (21). A ~0.9-kilobase-pair *Sal*I-*Bam*HI fragment was isolated from each starting plasmid and inserted into the polylinker of M13 mp10 (22) or pBSM13(−). The latter was furnished by Stratagene Vector Cloning Systems. Point mutations in these fragments were constructed by the two-primer method of Zoller and Smith (31) for oligonucleotide-directed mutagenesis, as described previously (21). The entire nucleotide sequence of the *GCN4* fragment was determined by the dideoxy chain-termination technique of Sanger et al. (24) before it was used to replace the corresponding wild-type fragment of p180. *GCN4* protein-coding sequences were reconstructed by digestion of the appropriate plasmids with *Bam*HI followed by ligation at low DNA concentrations. *lacZ* fusions to the elongated URFs were constructed from the appropriate *GCN4* alleles by using the same ~3-kilobase *Bam*HI *lacZ* fragment present in the *GCN4-lacZ* fusion. Standard procedures were used throughout for the preparation, modification, and cloning of plasmid DNA molecules (19).

Assay of *GCN4* expression. Plasmids containing the URF mutations of interest in *GCN4-lacZ* constructs were introduced into the following three yeast strains by the transformation technique of Ito et al. (9): TD28 (*MATα ura3-52 ino1*), H15 (*MATα gcn2-1 ura3-52 leu2-3 leu2-112*), and F98 (*MATα gcd1-101 ura3-52*). For all assays of *lacZ* fusion expression under repressing conditions, transformants were grown for 6 h from the stationary phase to the mid-exponential growth phase in SD medium (25) supplemented with 2 mM leucine, 0.5 mM isoleucine, 0.5 mM valine, 0.25 mM arginine, and 0.2 mM inositol. For derepressing conditions, 3-aminotriazole was added to 10 mM after 2 h in the medium described above, and growth was continued for an additional 6 h to produce histidine starvation. Derepressing conditions for the H384 transformants containing *GCN4* alleles were identical to those just described except that the medium was supplemented with 0.3 mM histidine–0.25 mM arginine, and after 2 h of growth, 5-methyltryptophan was added to 0.5 mM to cause tryptophan starvation. Cells were harvested and extracted for β-galactosidase assays as described previously (18). Enzyme activity is reported as nanomoles of *o*-nitrophenyl-β-D-galactoside hydrolyzed per minute per milligram of protein. Expression of each construct was examined in at least three independent transformants of each strain. The values reported in Fig. 2 through 5 are the averages calculated from these replicate determinations and have standard errors of 30% or less. Extraction and blot hybridization analysis of total RNA were performed as described previously (6) by using the same radiolabeled DNA probes used by Mueller and Hinnebusch (21) to probe *PYK*, *GCN4-lacZ*, and *GCN4* mRNAs.

In protein radiolabeling experiments, 5 ml of cells was pulsed with 150 μCi of [^{35}S]methionine at 800 Ci/mmol for the last 45 min of growth, after which unlabeled methionine was added to 2 mM for 10 min (or 90 min when a chase was done). Cycloheximide was added to 50 μg/ml, and cultures were chilled on ice. Cells were harvested and total protein was extracted as described above for the β-galactosidase assays. The amount of labeled methionine incorporated into trichloroacetic acid-precipitable material was determined for 5 μl of each extract. Samples containing 5×10^6 acid-insoluble cpm in 300 μl of IP buffer (25 mM Tris hydrochloride [pH 7.0], 0.225 mM NaCl, 1.5% Triton X-100, 0.15% sodium dodecyl sulfate [SDS], 7.5 mM EDTA, 1.5 mM phenylmethylsulfonyl fluoride) were reacted twice with 35 μl of 20% (vol/vol) protein A-Sepharose (CL-4B; Pharmacia Fine Chemicals, Piscataway, N.J.), which was suspended in IP buffer, for 60 min at 4°C with rotary agitation, followed by centrifugation to remove material that bound nonspecifically to protein A-Sepharose. The resulting supernatants were reacted with 1.1 μg of monoclonal β-galactosidase antibody (Promega) for 12 h at 4°C. Immunoprecipitates were collected by incubating supernatants with 70 μl of 20% protein A-Sepharose for 1 h at 4°C with agitation, followed by centrifugation. Immunoprecipitates were washed twice with 1 ml of TBS (100 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 5 mM EDTA) containing 0.5% Nonidet P-40 and twice with 1 ml of TBS containing 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. Immune complexes were eluted by boiling the immunoprecipitates for 10 min in 45 μl of electrophoresis sample buffer (60 mM Tris hydrochloride [pH 7.4], 2% SDS, 2% β-mercaptoethanol, 10% glycerol). Insoluble material was removed by centrifugation, and 20 μl of each supernatant was electrophoresed for 1,300 V-h on a 12% SDS-polyacrylamide slab gel by using the discontinuous gel system described by Laemmli (15). After electrophoresis, gels were treated for fluorography with En^{3}Hance (Dupont, New Research Products, Boston, Mass.) by the instructions of the vendor, dried under vacuum, and subjected to autoradiography.

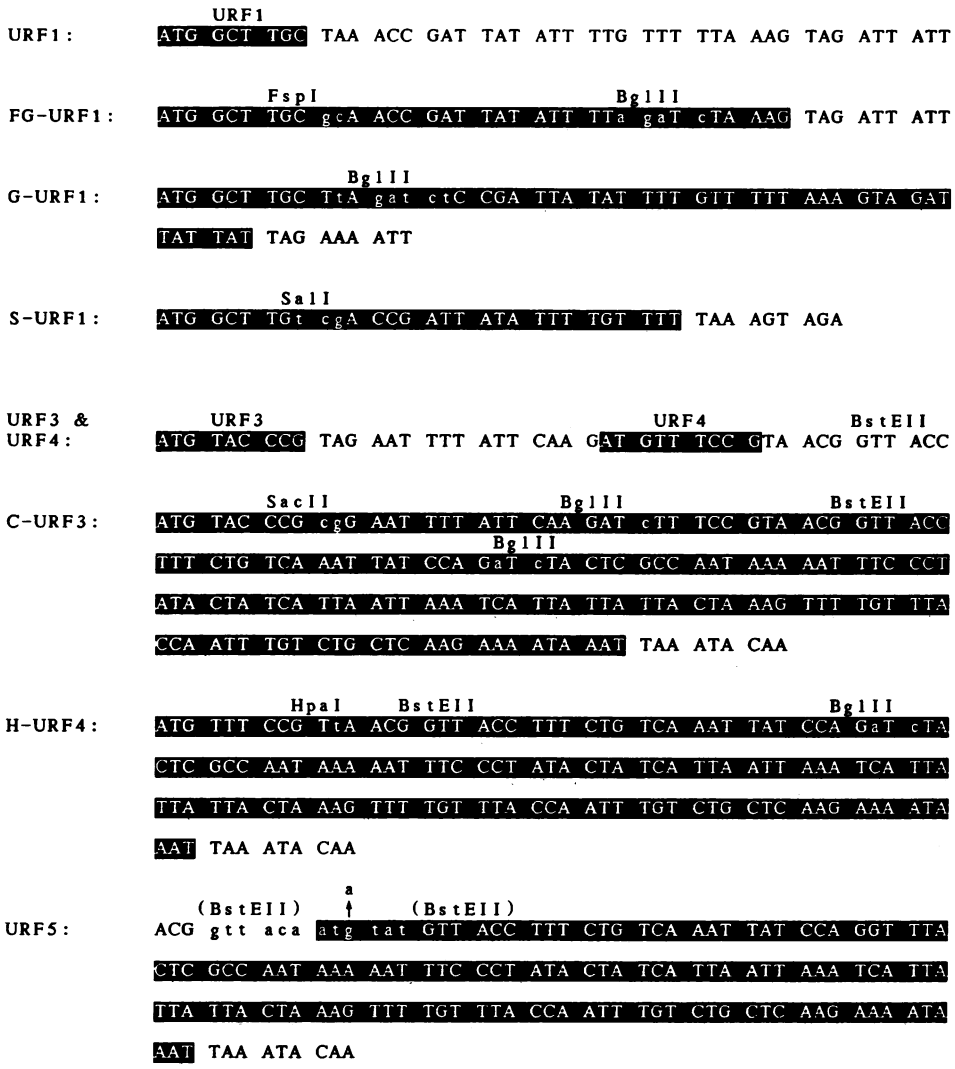
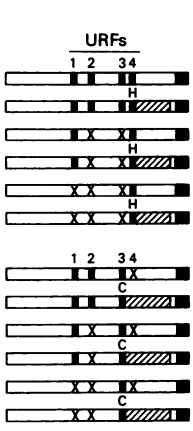


FIG. 1. Nucleotide sequences of altered URFs in the *GCN4* mRNA leader. The coding sequences of various URFs are in reverse contrast; insertion or substitution mutations that remove termination codons are given as lowercase letters. Also shown for FG-URF1, C-URF3, and H-URF4 are substitution mutations introduced secondarily to create *Bgl*II sites for insertion of *lacZ*-coding sequences. For C-URF3, these secondary mutations created the 3'-proximal *Bgl*II site shown; the 5'-proximal *Bgl*II site, which was present in the starting plasmid, was generated in the course of removing the ATG codon of URF4 (21). The data shown in Fig. 2 were collected for alleles containing URFs without the *Bgl*II sites used for *lacZ* insertion. URF5 was constructed by inserting the 12-nucleotide linker indicated by lowercase letters at the *Bst*EII site located downstream from URF4. A control linker contained the ATG to ATA substitution shown by an arrow.

RESULTS

The length, sequence, and termination sites of URFs 3 and 4 can be altered with little effect on *GCN4* translational control. As a prelude to the construction of *lacZ* fusions to the various URFs, we wished to determine the effect on *GCN4* translational control of lengthening the URFs by changing their termination sites. The stop codons of URFs 3 and 4 were removed by a 2-base-pair (bp) substitution or a 1-bp insertion, respectively (Fig. 1). The URF3 mutation created a *Sac*II site and lengthened URF3 from 3 to 52 codons (C-URF3). The *Sac*II mutation was generated in *GCN4-lacZ* alleles from which the ATG codon of URF4 was removed. The URF4 mutation created a *Hpa*I restriction site and extended URF4 from 3 to 43 codons (H-URF4). The URF4 mutation also shifted the reading frame into that used by C-URF3, such that both elongated URFs terminated at the same naturally occurring stop codon located 14 nucleotides upstream from the *GCN4* initiation codon. The effects of these mutations on expression of *GCN4-lacZ* enzyme activity were measured in nonstarved wild-type and *gcn2* mutant cells under repressing conditions and in histidine-starved wild-type and *gcd1* mutant cells under derepressing conditions.

As shown previously (21) and illustrated in Fig. 2, the second and third AUG codons could be removed from the *GCN4* mRNA leader without serious effects on translational control. *GCN4* expression from this allele is elevated somewhat under fully repressing conditions (*gcn2* cells); however, the *GCN2* requirement for derepression under starvation conditions and the *GCD1* requirement to maintain repressed expression under normal growth conditions both remained in effect. Alleles containing only the first and third AUG codons also exhibited significant regulation; however, expression under repressing conditions was further elevated



URFs	wt R	wt DR	gcn2 R	gcn2 DR	gcd1 R	gcd1 DR	gcd1/gcn2
1 2 3 4	12	89	5	9	340	380	51
H	12	120	5	8	230	220	32
	15	130	11	21	340	430	24
H	13	83	9	27	350	240	16
	5	16	8	14	22	20	2
H	5	16	6	11	22	17	2
1 2 3 4	20	100	20	30	250	360	12
C	12	98	15	31	280	280	12
	29	110	29	41	450	470	13
C	21	120	18	41	460	380	14
	18	33	56	59	65	100	1
C	7	23	11	27	49	34	2

GCN4-lacZ ENZYME ACTIVITY

FIG. 2. Effects of lengthening URFs 3 and 4 on the regulation of *GCN4-lacZ* expression. The diagram depicts the *GCN4* mRNA leader sequences in the various alleles drawn approximately to scale. The small solid rectangles symbolize the URFs. The larger solid region designates the beginning of the *GCN4*-coding sequences. The Xs indicate the point mutations that were used to remove the AUG codons of the URFs described by Mueller and Hinnebusch (21). The *Hpa*I (H) and *Sac*II (C) mutations in the termination codons of URFs 3 and 4, respectively, elongated these URFs to the extent shown by hatched shading. Levels of β-galactosidase activity were measured in wild-type (wt), *gcn2*, and *gcd1* transformants under repressing (R) and derepressing (DR) conditions. The derepression ratio *gcd1/gcn2* was calculated as follows. Expression measured under starvation and nonstarvation conditions in *gcd1* transformants was averaged and divided by the average of expression under the same two conditions for *gcn2* transformants.

because of the lesser inhibitory effect of AUG 3 compared with that of AUG 4 (21). To simplify our analysis of URF function in the experiments described below, we often chose to examine alleles containing only two URFs, a 3′-proximal URF required for efficient repression and a 5′-proximal URF needed for derepression of *GCN4* expression.

Removal of the termination codons of URF 3 or 4 has, at most, a small quantitative effect on *GCN4-lacZ* expression under the circumstances we examined (Fig. 2). For those alleles containing no other upstream ATG codons, elongated URFs 3 and 4 repressed *GCN4-lacZ* expression efficiently, and their inhibitory effects were expressed constitutively, independent of the *GCD1* gene product. For alleles containing the first and second ATG codons or only the first ATG codon upstream from the elongated URFs, *GCN4-lacZ* expression increased in wild-type cells in response to starvation. As in the case of the wild-type *GCN4-lacZ* construct, this derepression was dependent on the *GCN2* gene product and occurred constitutively in *gcd1* cells. The derepression ratios (*gcd1/gcn2*) for alleles containing elongated URFs were within 50% of the values observed for the corresponding alleles containing only wild-type URFs. We conclude that, as in the case of wild-type URFs 3 and 4, the strong inhibitory effects of elongated URFs 3 and 4 on *GCN4* expression are efficiently suppressed by URF1 under derepressing conditions.

The effects of these mutations were measured independently by using an in vivo assay for expression of authentic *GCN4* protein. The *lacZ*-coding sequences were removed from selected constructs to reconstitute the *GCN4* protein-coding sequences. The ability of the resulting *GCN4* alleles to complement a chromosomal *gcn4* mutation was then

TABLE 1. Complementation of a *gcn4* deletion by plasmid-borne *GCN4* alleles containing URF mutations

URF present in leader[a]	Complementation of Δ*gcn4*[b]
URF4	±
H-URF4	±
URF1–URF4	++++
URF1–H-URF4	+++
URF3	++
C-URF3	++
URF1–URF3	+++
URF1–C-URF3	+++
URF5	++
URF1–URF5	++++
F-URF1–URF4	++
FG-URF1–URF4	+
G-URF1–URF4	±
S-URF1–URF4	±

[a] H-, F-, FG-, G-, S-, and C-URFs stand for mutated URFs, as designated in Fig. 2 and 4. If an URF is not listed, its AUG codon is missing in that allele.

[b] To assay complementation of deletion allele *gcn4-103*, plasmids were introduced into strain H384 (*MATα gcn4-103 his1-29 ura3-52*) and the resulting transformants were replica plated onto solid medium lacking histidine and supplemented with 30 mM 3-aminotriazole. (*his1-29* is a leaky mutation that confers histidine auxotrophy in the presence of a *gcn4* mutation.) The complementation response shown is a qualitative summary of the growth rates.

tested in several independent transformants of a *gcn4* deletion strain (Table 1). The results of this analysis are in good agreement with the data obtained from *lacZ* fusions and support the conclusion that the mutations that lengthen URFs 3 and 4 have relatively minor effects on *GCN4* expression.

The regulatory function of URFs 3 and 4 can be partially reconstituted by a heterologous URF. Having found that URFs 3 and 4 could be substantially lengthened without disturbing their regulatory function, we decided to examine the effects of changing the initiation site of these elongated URFs. This was done by inserting a 12-bp linker containing an ATG codon into a *Bst*EII site located 2 bp downstream from the termination codon of wild-type URF4 (Fig. 1). This insertion was constructed in alleles lacking the third and fourth ATG codons. The resulting URF (URF5) began with an ATG codon located 20 bp downstream from the ATG codon of URF4 and was 43 codons in length, of which the carboxyl-terminal 41 codons also occurred at the 3′ ends of C-URF3 and H-URF4. Control constructs were made by inserting the same linker containing an ATA codon instead of an ATG codon.

As expected from previous studies on the inhibitory effects of upstream AUG codons on the translation of downstream coding sequences (10, 12, 17, 23, 26), the construct containing URF5 and no other upstream ATG codons exhibited relatively low constitutive *GCN4-lacZ* expression. Figure 3 shows that the inhibitory effect of URF5 was comparable to that seen for an allele containing only URF3 but was greater than that exhibited by URF1 when it was present alone in the mRNA leader. When the weakly inhibitory URF1 sequence was placed upstream from URF5, it acted as a positive element and partially suppressed the inhibitory effect of URF5, leading to increased *GCN4-lacZ* expression compared with that of the construct containing URF5 alone. The degree of regulation exhibited by the URF1-URF5 allele was significantly less

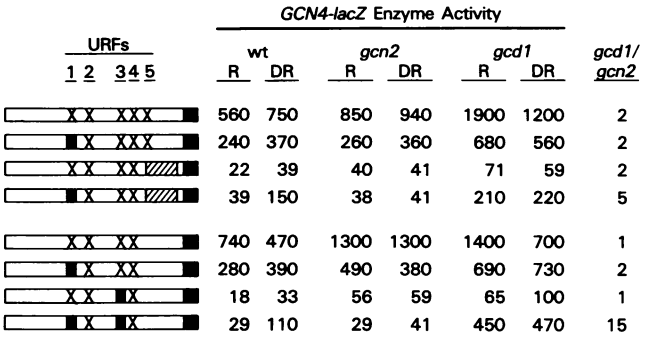


FIG. 3. Substitution of URFs 3 and 4 with heterologous URF5 (see Fig. 2 legend for a general description of the diagram). URF5 is designated by the hatched shading. The construct with five Xs contained the same linker insertion used to create URF5, except that it lacked an ATG codon. β-Galactosidase activity was measured in wild-type (wt), *gcn2*, and *gcd1* transformants under repressing (R) and derepressing (DR) conditions. The *gcd1/gcn2* ratio was calculated as described in the legend to Fig. 2.

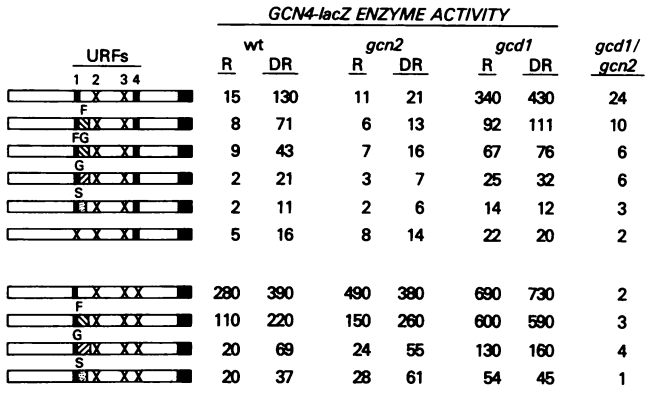


FIG. 4. Effects of lengthening URF1 on *GCN4-lacZ* expression (see Fig. 2 legend for a general description of the diagram). The *Fsp*I (F), *Fsp*I-*Bgl*II (FG), *Bgl*II (G), and *Sal*I (S) mutations removed the termination codon of URF1 and lengthened it, as shown by the shaded areas. The different types of shading designate different translational reading frames. β-Galactosidase activity was measured in wild-type (wt), *gcn2*, and *gcd1* transformants under repressing (R) and derepressing (DR) conditions. The *gcd1/gcn2* ratio was calculated as described in the legend to Fig. 2.

than that shown by the URF1-URF3 and URF1-URF4 constructs; however, the URF1-URF5 allele displayed all of the following important characteristics of *GCN4* translational control: (i) in wild-type cells, URF1 stimulated expression only under starvation conditions; (ii) this derepression was completely dependent on the *GCN2* gene product; and (iii) derepression was constitutive in *gcd1* cells. The stimulatory effect of URF1 on *GCN4* expression when URF5 was present downstream was also evident in the *gcn4* complementation analysis (Table 1). The addition of URF1 upstream from URF5 reduced the inhibitory effect of URF5 on *GCN4* expression.

Allele-specific effects of altering the length and sequence of URF1. The results described above indicate that URFs 3 and 4 can be lengthened considerably without significant effects on their negative regulatory functions. We wished to determine the effects of lengthening URF1 on its positive regulatory role in the control mechanism. In addition, we hoped to generate a functional elongated version of URF1 to which we could fuse *lacZ* sequences at a considerable distance downstream from the URF1 initiation site.

The termination codon of URF1 was removed in three different ways (Fig. 1). The simplest change was a 2-bp substitution in the termination codon that created an *Fsp*I restriction site and lengthened URF1 from 3 to 12 codons in the same reading frame (F-URF1). A derivative of this allele containing a 4-bp substitution that created a *Bgl*II site at the 3′ end of F-URF1 was constructed (FG-URF1) for the purpose of making an URF1-*lacZ* fusion (see below). The second mutation in URF1 was a 3-bp substitution and a 1-bp deletion that created a *Sal*I site, changed the third codon, and added seven codons in the +1 reading frame (S-URF1). The third mutation was a 2-bp deletion and a 6-bp insertion that created a *Bgl*II site and added 14 codons in the −1 reading frame (G-URF1).

The *Fsp*I mutation had the least effect of all three mutations. Derepression of *GCN4-lacZ* expression from this allele was reduced by a factor of 2 to 4. However, the derepression that remained was *GCN2* dependent, and *GCD1* function was still required for efficient repression under nonstarvation conditions (Fig. 4). The S-URF1 and G-URF1 mutations had significantly greater effects. When these two extended URFs were situated upstream from wild-type URF4, *GCN4-lacZ* expression under derepressing conditions was ~1/10 the level seen for the corresponding allele containing wild-type URF1. Expression under repressing conditions was also reduced by these mutations; consequently, a pattern of regulation similar to that of the wild-type gene was observed for these alleles, albeit at substantially lower absolute levels. S-URF1 and G-URF1 were very inefficient positive regulatory elements by comparison with authentic URF1. Correlated with their reduced positive function when situated upstream from URF4, S-URF1 and G-URF1 were also much more inhibitory than wild-type URF1 when they were present in the leader as solitary URFs (Fig. 4). This correlation has been observed for many additional point mutations in URF1 that both reduce its positive regulatory function and increase its inhibitory effect as a solitary URF (P. F. Miller and A. G. Hinnebusch, unpublished data). Interestingly, expression from the construct containing G-URF1 as the sole URF exhibited a significant derepression response. This result suggests that regulation by *GCN2* and *GCD1* may occur for certain solitary heterologous URFs, whose distinguishing sequence characteristics remain to be defined. By contrast, in the case of the wild-type *GCN4* URFs, URF5 and S-URF1, multiple URFs must be present in the leader for a derepression ratio in excess of 2 to be observed. The deleterious effects of the URF1 mutations on *GCN4-lacZ* expression under derepressing conditions are in good agreement with the degree of *gcn4* complementation observed for the corresponding *GCN4* alleles (Table 1).

Upstream AUG codons 1, 3, and 4 are efficient translation initiation sites. To determine whether translation initiation can occur efficiently at the upstream URFs, we fused them in-frame with an NH_2-terminally deleted *lacZ*-coding sequence. A 2-bp substitution that created a *Bgl*II site was made in C-URF3 and H-URF4 at a position ~40 nucleotides downstream from the wild-type fourth ATG codon and ~60 nucleotides downstream from the third ATG codon, respectively (Fig. 1). These mutations had no detectable effect on the function of either elongated URF (data not shown). *lacZ*-coding sequences were inserted at the new *Bgl*II sites in-frame with the ATG codons of C-URF3 or H-URF4. In the case of URF1, *lacZ*-coding sequences were inserted at

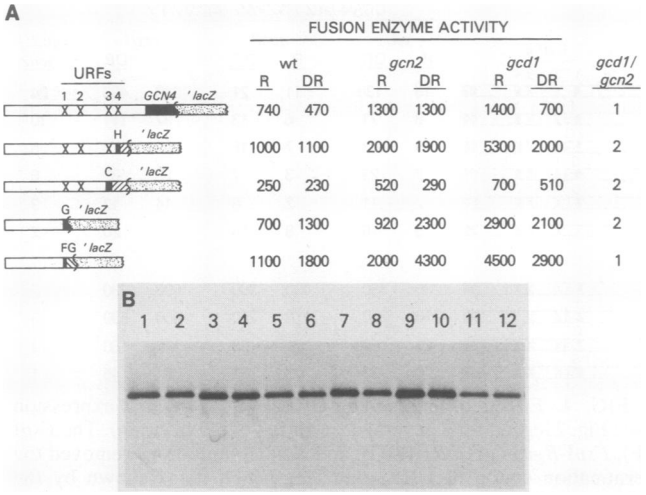


FIG. 5. Expression of *lacZ* fusions to upstream URFs. (A) The top construct in the diagram designates the *GCN4-lacZ* fusion used in all previous experiments, in this case with all four upstream AUG codons removed. The remaining constructs contained exactly the same *lacZ* sequences as the former, but they were fused at the *Bgl*II restriction sites introduced into the elongated URFs shown in Fig. 1. H, C, G, and FG designate H-URF4, C-URF3, G-URF1, and FG-URF1, respectively. β-Galactosidase expression was measured in wild-type (wt), *gcn2*, and *gcd1* transformants under repressing (R) and derepressing (DR) conditions. The *gcd1*/*gcn2* ratio was calculated as described in the legend to Fig. 2. (B) Immunoprecipitations of radiolabeled fusion proteins from the *GCN4-lacZ* (lanes 1, 2, 7, and 8) H-URF4–*lacZ* (lanes 3, 4, 9, and 10), and FG-URF1–*lacZ* fusions (lanes 5, 6, 11, and 12). Samples prepared from *gcn2* and *gcd1* transformants are shown side by side for each fusion with *gcn2* samples in lanes 1, 3, 5, 7, 9, and 11 and *gcd1* samples in lanes 2, 4, 6, 8, 10, and 12.

the *Bgl*II sites located at the termination codon of G-URF1 or 26 bp downstream from the ATG codon in FG-URF1 (Fig. 1). The *Bgl*II site in the latter reduced its positive regulatory function compared with that in F-URF1; however, FG-URF1 still conferred significant stimulation of *GCN4* expression under derepressing conditions (Fig. 4 and Table 1). In all of the URF-*lacZ* constructs, no ATG codons were present upstream from the URF-*lacZ* coding sequences and no *lacZ* sequences were present in the *GCN4*-coding region downstream.

lacZ fusions to URFs 1, 3, or 4 all give rise to high levels of β-galactosidase activity in vivo (Fig. 5). As in the case of the *GCN4-lacZ* fusion, there was little regulation of enzyme expression for any of the URF-*lacZ* fusions when no other upstream ATG codons were present. The URF3-*lacZ* fusion produced a steady-state level of enzyme activity that was two- to threefold lower than that of the *GCN4-lacZ* fusion, whereas expression levels for the URF4-*lacZ* and URF1-*lacZ* fusions were somewhat higher than that observed for *GCN4-lacZ*. The reasons behind the lower expression observed for the URF3-*lacZ* fusion remain to be determined. Because URFs 1 and 4 are sufficient for nearly wild-type regulation, expression from these two fusions was characterized further.

A precise comparison of the levels of different *lacZ* fusion proteins cannot be made from enzymatic assays without knowing the specific activity and stability of each protein. Therefore, we decided to measure the synthesis rates of the fusion proteins more directly. *gcn2* and *gcd1* transformants containing the *GCN4-lacZ*, URF4-*lacZ*, or FG-URF1–*lacZ* fusions were pulse-labeled with [^{35}S]methionine for 45 min under nonstarvation conditions. Total proteins were extracted and fusion proteins were immunoprecipitated with β-galactosidase antibody from samples containing equal amounts of acid-insoluble radioactivity. Immunoprecipitated proteins were fractionated by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. The same procedure was repeated for identical cultures except that a 90-min chase with unlabeled methionine followed completion of the pulse. The results shown in Fig. 5B indicate that little or no degradation of the fusion proteins occurred in the chase period for the *GCN4-lacZ* and URF4-*lacZ* fusions (compare lanes 1 to 4 with lanes 7 to 10). Thus, the intensity of labeling achieved during the pulse time for these two fusions should be indicative of their synthetic rates. In the case of the URF1-*lacZ* fusion, about one-half of the labeled protein was generally found to decay during the chase period (lanes 5 and 6 versus lanes 11 and 12); therefore, the synthetic rate for this fusion is slightly underestimated in the pulse-labeling experiment (by less than 30%) because of degradation during the pulse. The results in lanes 1 to 6 of Fig. 5B are in general accord with the enzyme activities shown in Fig. 5A in suggesting that the first and fourth upstream AUG codons are recognized as initiation sites with an efficiency comparable to that of the *GCN4* AUG codon itself.

The URF mutations have little effect on the size or steady-state amount of *GCN4* mRNA. *GCN4-lacZ* fusion mRNAs were examined for key URF mutant constructs in total RNA isolated from *gcn2* and *gcd1* cells grown under nonstarvation conditions. Only slight variations were detected between the steady-state amounts of these mRNAs and the corresponding fusion transcripts containing only wild-type URFs (Fig. 6A); these variations were not observed consistently in independently isolated transformants. The steady-state amounts of the three URF-*lacZ* transcripts were also similar to that of the corresponding *GCN4-lacZ* transcript (Fig. 6B). Authentic *GCN4* mRNAs were examined for several URF mutant alleles generated by removal of *lacZ* sequences. Again, only small differences in the relative amounts of these mRNAs were observed compared with those of *GCN4* transcripts containing only wild-type URFs (Fig. 6C). We showed previously that a deletion of all four URFs has no effect on the 5′ end of *GCN4* mRNA (6). If any of the mutations lead to the generation of new mRNA 5′ ends in the vicinity of the URFs, an altered transcript size would be evident in the analysis shown in Fig. 6C. Changes in transcript size of ~5% are detectable by this technique (31). Only in the case of the URF5-containing constructs were novel mRNA species observed. These transcripts were smaller and less abundant than the normal-size transcripts produced by these two alleles. Since the smaller transcripts were made in equal amounts by both URF5 alleles, they appear to be unrelated to the increased *GCN4* expression conferred by URF1 when it is inserted upstream from URF5.

DISCUSSION

The upstream AUG codons function in regulation as sites for translation initiation. Utilization of internal AUG codons as translation initiation sites appears to be very inefficient in *S. cerevisiae* (1, 26). This fact is consistent with the idea that a preinitiation complex is assembled at the 5′ end of the mRNA, scans in the 3′ direction, and selects the first AUG codon suitable for initiation. Following translation of a 5′-proximal URF, reinitiation at downstream AUG codons

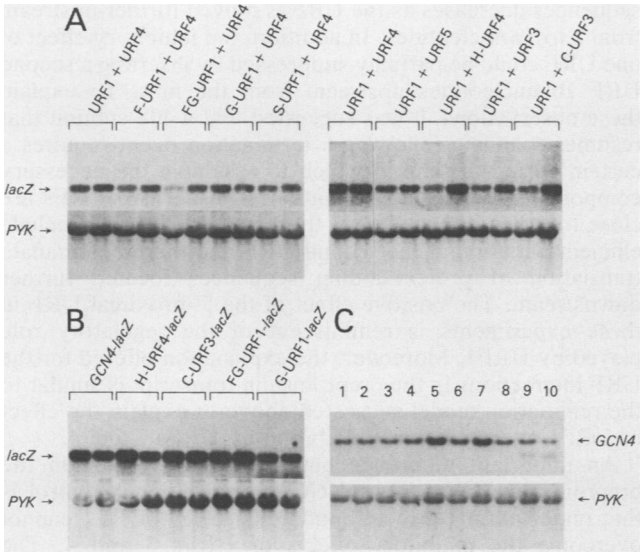


FIG. 6. Blot hybridization analysis of *GCN4* mRNAs containing URF mutations. (A) *GCN4-lacZ* and pyruvate kinase (*PYK*) mRNAs examined in total RNA isolated from *gcn2* and *gcd1* transformants grown under nonstarvation conditions, containing different fusion constructs designated as in Table 1. Brackets enclose RNA samples for the same construct isolated from *gcn2* and *gcd1* cells, from left to right. Because specific activities of the probes used in the two adjacent panels were not identical, levels of different mRNAs should be compared relative to the URF1-URF4 transcript included in each panel. (B) *GCN4-lacZ* and URF-*lacZ* fusion transcripts in total RNA from *gcn2* and *gcd1* transformants (consecutive lanes) grown under nonstarvation conditions. Fusion constructs are designated as described in the legend to Fig. 5. The specific activity of the probes used in panel B varied from those used in panel A, however, the abundance of the *GCN4-lacZ* transcript in panel B was shown previously (21) to be indistinguishable from that observed for the URF1-URF4 transcript in panel A. (C) *GCN4* mRNAs in total RNA isolated from a *gcn4* deletion strain grown under starvation conditions, containing the following constructs designated as in Table 1: F-URF1–URF4 (lane 1), FG-URF1–URF4 (lane 2), G-URF1–URF4 (lane 3), S-URF1–URF4 (lane 4), URF1–URF4 (lane 5), URF1–H-URF4 (lane 6), URF1–URF3 (lane 7), URF1–C-URF3 (lane 8), URF1–URF5 (lane 9), and URF5 (lane 10).

tends to occur very inefficiently (11, 12, 26). Accordingly, if the upstream AUG codons in *GCN4* mRNA are well-recognized initiation sites, then translation of *GCN4* protein-coding sequences under derepressing conditions must involve one of the following novel mechanisms for translation initiation: direct binding to an internal AUG codon, efficient translational reinitiation, or selective interference with the upstream AUG codons as start sites.

Several lines of evidence suggest that the *GCN4* upstream AUG codons are efficient translation initiation sites. The nucleotides surrounding the first and fourth AUG codons match those most commonly found at the initiation sites of highly expressed yeast genes (2). In particular, URFs 1 and 4 contain an A nucleotide located three residues upstream from the AUG codon. This is the most highly conserved nucleotide at known yeast initiation sites, and substitution mutations at this position lead to about twofold lower rates of translation at *CYC1* (16) and *HIS4* (1); changes at other positions have little or no effect on the expression of these two yeast genes. Therefore, based on their surrounding nucleotide sequences, the first and fourth AUG codons are each expected to be efficient initiation sites. This expectation is supported by our finding that URF1-*lacZ* and URF4-*lacZ* fusions are translated with an efficiency comparable to that of the *GCN4-lacZ* coding sequence. Furthermore, translation of the FG-URF1–*lacZ* fusion is constitutive, suggesting that regulation of initiation at URF1 is not a major factor in limiting URF1 regulatory function to starvation conditions.

In addition to showing that the first and fourth AUG codons are efficient initiation codons, our results suggest that these sequences function as start sites for protein synthesis in carrying out their respective roles in translational control. In the case of URF1, we showed that certain mutations that lengthen the coding sequence of URF1 impair its positive regulatory function nearly as much as removing its initiation codon does. By contrast, deletions of large leader segments beginning ~15 nucleotides upstream or downstream from URF1 have much less of an effect on derepression of *GCN4* expression than either the *Sal*I or *Bgl*II mutations in the URF1 termination codon described here do (20, 30); therefore, not all sequence alterations in the vicinity of the first AUG codon impair its positive regulatory role to the extent observed for these URF1 point mutations. Our data suggest that URF1 is normally well translated and that certain constraints exist on its length, codon usage, or termination site for its efficient function as a positive control element.

The conclusion that the *GCN4* URFs function in regulation as translated coding sequences is also suggested by the fact that heterologous URFs can qualitatively mimic the regulatory properties of the authentic URFs. This result was presented here for the 3′-proximal URFs by showing that, under starvation conditions, URF1 can reduce by a factor of ~4 the inhibitory effect on *GCN4* expression of heterologous URF5. In addition, we recently succeeded in showing that URF1-positive function can be mimicked by a heterologous URF containing the first three codons and upstream sequences normally found at the highly expressed yeast gene *PGK*. The *PGK*-related URF produces an ~10-fold reduction in the inhibitory effects of URFs 3 and 4 under derepressing conditions (30). Wild-type URF1 functions much more efficiently as a positive control element than the *PGK* URF does. In addition, URFs 3 and 4 do not act as positive elements when they are inserted upstream from URF1, and URF1 remains at least 10-fold less inhibitory to *GCN4* expression as a solitary URF when moved into the position normally occupied by URF4 (30). Therefore, although the 5′- and 3′-proximal URFs all function in regulation as translated coding sequences, there are important differences among these elements that determine their distinctive regulatory properties.

Reinitiation following URF1 translation may be important for its regulatory function. When present alone in the *GCN4* mRNA leader, the first and fourth URFs differ in their inhibitory effects on *GCN4* expression by ~30-fold, and when inserted upstream from URF4, URF1 acts as a positive element and leads to increased rather than decreased *GCN4* expression. The fact that the first and fourth AUG codons function with similar efficiencies as translation start sites raises the possibility that translational events following initiation distinguish the regulatory properties of URFs 1 and 4. Mutations that remove the termination codon and that lengthen URF1 cause it to more closely resemble URF4 in effectively blocking *GCN4* expression as a solitary URF. This phenotype can be explained if we assume that most scanning ribosomes normally initiate at URF1 and a significant fraction can resume scanning and reinitiate downstream. By changing the length, sequence, or termination

site at URF1, we reduced the number of ribosomes that were able to complete URF1 translation and subsequently reinitiate. The fact that these same mutations impaired the ability of URF1 to suppress the inhibitory effects of URFs 3 and 4 suggests that this regulatory interaction also depends on efficient reinitiation following URF1 translation. Accordingly, we propose that only those ribosomes that translate URF1 and that resume scanning downstream can overcome the blockade effect of the 3'-proximal URFs and initiate translation at the *GCN4* AUG codon. In this view, prior translation of URF1 makes ribosomes either more likely to bypass AUG codons 3 and 4 or more likely to reinitiate again following translation of URFs 3 and 4. By contrast, primary initiation events at URFs 3 and 4 (those that occur in the absence of URF1) are never expected to be followed by reinitiation at the *GCN4* AUG codon.

It is not obvious why moving the termination site of URF1 downstream would reduce the efficiency of reinitiation following URF1 translation, nor why the different URF1 mutations we constructed would differ so markedly in this respect. Perhaps certain codons or secondary structures present in the more inhibitory elongated URF1 elements impede elongation steps in URF1 and thereby attenuate the number of ribosomes available for reinitiation events downstream. The deleterious effect on URF1 function associated with the *Bgl*II mutation in the coding sequence of F-URF1 (the FG-URF1 construct) is consistent with an elongation effect. It is also possible that particular sequences surrounding the different stop codons of the various elongated URF1 elements could affect the efficiency with which scanning resumes following translation termination. Recent experiments demonstrate that particular sequences both within the coding region and downstream from the termination codon are required for the novel regulatory function of URF1 (Miller and Hinnebusch, unpublished data).

Although we favor the idea that the termination codon mutations affect the efficiency of reinitiation following URF1 translation, we cannot rule out the possibility that these mutations increase the efficiency of initiation at the first AUG codon. If reinitiation following URF1 translation is normally very inefficient, then by increasing the fraction of ribosomes that stop and initiate at URF1, we would reduce the number that can move beyond this site and initiate at *GCN4*. According to this alternative model, translation of URF1 by one pool of ribosomes would have an indirect effect on the behavior of a second pool that skips over the first AUG codon and initiates at URFs 3 and 4. For example, translation at URF1 could specifically alter the secondary structure at URFs 3 and 4 and thereby influence initiation or reinitiation events at these downstream sites. The ability of heterologous URFs to partially mimic the functions of the wild-type sequences seems incompatible with models of this kind that require strict sequence specificity to explain the interactions between the 5'- and 3'-proximal URFs. Of course, given the low derepression ratio observed for the URF1-URF5 construct, it is possible that changes in secondary structure make an important quantitative contribution to the regulation.

The results of a recent study by Kozak on the translational effects of upstream AUG codons in a mammalian transcript (14) are consistent with the idea that interactions between URFs affecting downstream gene expression can occur without highly specialized nucleotide sequences or secondary structures present in the mRNA. It was reported for a preproinsulin transcript that the inhibitory effect of inserting a single URF on translation of downstream protein-coding sequences decreases as the URF is moved further upstream from 2 to 79 nucleotides. In addition, the inhibitory effect of one URF could be partially suppressed by inserting a second URF 10 nucleotides upstream from the first. To explain these observations, it was suggested that a 40S subunit that resumes scanning following a termination event requires a certain period of time in which to assemble the necessary components for the next initiation. Thus, if two URFs are close together, translation of the first URF would preclude efficient initiation at the second URF and thereby stimulate translation of protein-coding sequences located further downstream. The positive effect of the 5'-proximal URF in these experiments is reminiscent of the regulatory role played by URF1. Moreover, the explanation offered for the URF interactions in the preproinsulin transcript is similar to the reinitiation model suggested above to explain the effect of URF1 on translational inhibition by URFs 3 and 4.

An important difference between these results on the preproinsulin transcript and *GCN4* translational control is that under normal growth conditions, *GCN4* URF1 cannot overcome the inhibitory effects of URFs 3 and 4. The inactivation of *GCD* factors that occurs under starvation conditions is also required for regulation. This complication could be resolved easily if *GCD* factors act to block translation of URF1 under nonstarvation conditions; however, our results with the URF1-*lacZ* fusions suggest that translation initiation at URF1 is unregulated. A second difference is that the separation between URF1 and URFs 3 and 4 is ~200 nucleotides. In the experiments performed by Kozak (14), a separation of this magnitude almost completely abolished the inhibitory effect of the first URF on initiation at a second URF downstream. If yeast and mammalian cells are similar in this respect, URF1 should not be able to suppress initiation at URFs 3 and 4 by this mechanism.

If proximity is not the key to the ability of URF1 to suppress translational inhibition by URFs 3 and 4, then perhaps reinitiating ribosomes generated by URF1 can be specifically modified under starvation conditions to alter their behavior at URFs 3 and 4. Since the postulated modification would have no effect on primary initiation events at URFs 3 and 4, this explanation implies that primary and reinitiation events differ mechanistically. It is widely accepted that certain initiation factors become associated with the initiation complex by binding to the 5' end of the mRNA (for a review, see Sonenberg [N. Sonenberg, Prog. Nucleic Acid Res. Mol. Biol., in press]). If these factors dissociate following the first initiation event on a transcript, they may be unavailable for reinitiation at internal AUG codons. Perhaps, in the absence of certain components of the primary initiation complex, it is possible to modify or replace other factors and thereby change the properties of the translational apparatus. Our previous genetic analyses suggest that the *GCD* gene products are good candidates for translational factors that are targeted for alteration or replacement in amino acid-starved cells and that the products of *GCN1*, *GCN2*, and *GCN3* are responsible for promoting such modifications (3, 4).

It was recently shown that the *CPA1* transcript in *S. cerevisiae* is also subject to translational control (29). In this case, a single URF mediates regulation of *CPA1* expression, as opposed to the combination of multiple URFs with different translational properties required for efficient regulation of *GCN4* expression. However, our finding that G-URF1 alone confers a modest regulatory response over *GCN4* expression raises the possibility that transcripts like *CPA1* with single URFs may be targets for the same *GCN*

and *GCD* factors that regulate *GCN4* expression. Multiple URFs may be necessary only to widen the range of the regulatory response. More experiments should be done to examine this interesting possibility.

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LITERATURE CITED

1. **Donahue, T. F., A. M. Cigan, B. A. de Castilho, and H. Yoon.** 1988. Translation initiation in yeast: a genetic and mutational analysis, p. 361–372. *In* M. F. Tuite, M. Picard, and M. Bolotin-Fukuhara (ed.), Genetics of translation. New approaches. Springer-Verlag, Berlin.
2. **Hamilton, R., C. K. Watanabe, and H. A. de Boer.** 1987. Compilation and comparison of the sequence context around the AUG start codons in *Saccharomyces cerevisiae*. Nucleic Acids Res. **15**:3581–3593.
3. **Harashima, S., E. M. Hannig, and A. G. Hinnebusch.** 1987. Interactions between positive and negative regulators of *GCN4* controlling gene expression and entry into the yeast cell cycle. Genetics **117**:409–419.
4. **Harashima, S., and A. G. Hinnebusch.** 1986. Multiple *GCD* genes required for repression of *GCN4*, a transcriptional activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:3990–3998.
5. **Hinnebusch, A. G.** 1984. Evidence for translational regulation of the activator of general amino acid control in yeast. Proc. Natl. Acad. Sci. USA **81**:6442–6446.
6. **Hinnebusch, A. G.** 1985. A hierarchy of *trans*-acting factors modulate translation of an activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **5**:2349–2360.
7. **Hinnebusch, A. G.** 1988. Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **52**:248–273.
8. **Hinnebusch, A. G., and G. R. Fink.** 1983. Positive regulation in the general amino acid control of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **80**:5374–5378.
9. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163–168.
10. **Johansen, H., D. Schumperli, and M. Rosenberg.** 1984. Affecting gene expression by altering the length and sequence of the 5′ leader. Proc. Natl. Acad. Sci. USA **81**:7698–7702.
11. **Kozak, M.** 1978. How do eukaryotic ribosomes select initiation regions in messenger RNA? Cell **15**:1109–1123.
12. **Kozak, M.** 1984. Selection of initiation sites by eucaryotic ribosomes: effect of inserting AUG triplets upstream from the coding sequence for preproinsulin. Nucleic Acids Res. **12**:3873–3893.
13. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:282–292.
14. **Kozak, M.** 1987. Effects of intercistronic length on the efficiency of reinitiation by eukaryotic ribosomes. Mol. Cell. Biol. **7**:3438–3445.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
16. **Laz, T., J. Clements, and F. Sherman.** 1987. The role of mRNA sequences and structure in eukaryotic translation, p. 413–427. *In* J. Ilan (ed.), Translational regulation of gene expression. Plenum Publishing Corp., New York.
17. **Liu, C., C. C. Simonsen, and A. D. Levinson.** 1984. Initiation of translation at internal AUG codons in mammalian cells. Nature (London) **309**:82–85.
18. **Lucchini, G., A. G. Hinnebusch, C. Chen, and G. R. Fink.** 1984. Positive regulatory interactions of the *HIS4* gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:1326–1333.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Mueller, P. P., S. Harashima, and A. G. Hinnebusch.** 1987. A segment of *GCN4* mRNA containing the upstream AUG codons confers translational control upon a heterologous yeast transcript. Proc. Natl. Acad. Sci. USA **84**:2863–2867.
21. **Mueller, P. P., and A. G. Hinnebusch.** 1986. Multiple upstream AUG codons mediate translational control of *GCN4*. Cell **45**:201–207.
22. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26**:101–106.
23. **Peabody, D. S., and P. Berg.** 1986. Termination-reinitiation occurs in the translation of mammalian cell mRNAs. Mol. Cell. Biol. **6**:2695–2703.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
25. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics, p. 164. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Sherman, F., and J. W. Stewart.** 1982. Mutations altering initiation of translation of yeast iso-1-cytochrome *c*; contrasts between the eukaryotic and prokaryotic initiation process, p. 301–304. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast Saccharomyces: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Thireos, G., M. Driscoll Penn, and H. Greer.** 1984. 5′ Untranslated sequences are required for the translational control of a yeast regulatory gene. Proc. Natl. Acad. Sci. USA **81**:5096–5100.
28. **Tzamarias, P., D. Alexandraki, and G. Thireos.** 1986. Multiple cis-acting elements modulate the translational efficiency of GCN4 mRNA in yeast. Proc. Natl. Acad. Sci. USA **83**:4849–4853.
29. **Werner, M., A. Feller, F. Messenguy, and A. Pierard.** 1987. The leader peptide of yeast gene *CPA1* is essential for the translational repression of its expression. Cell **49**:805–813.
30. **Williams, N. P., P. P. Mueller, and A. G. Hinnebusch.** 1988. The positive regulatory function of the 5′-proximal open reading frames in *GCN4* mRNA can be mimicked by heterologous, short coding sequences. Mol. Cell. Biol. **8**:3827–3836.
31. **Zoller, M. J., and M. Smith.** 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. **10**:6487–6500.