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The Positive Regulatory Function of the 5′-Proximal Open Reading Frames in *GCN4* mRNA Can Be Mimicked by Heterologous, Short Coding Sequences

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Translational control of *GCN4* expression in the yeast *Saccharomyces cerevisiae* is mediated by multiple AUG codons present in the leader of *GCN4* mRNA, each of which initiates a short open reading frame of only two or three codons. Upstream AUG codons 3 and 4 are required to repress *GCN4* expression in normal growth conditions; AUG codons 1 and 2 are needed to overcome this repression in amino acid starvation conditions. We show that the regulatory function of AUG codons 1 and 2 can be qualitatively mimicked by the AUG codons of two heterologous upstream open reading frames (URFs) containing the initiation regions of the yeast genes *PGK* and *TRP1*. These AUG codons inhibit *GCN4* expression when present singly in the mRNA leader; however, they stimulate *GCN4* expression in derepressing conditions when inserted upstream from AUG codons 3 and 4. This finding supports the idea that AUG codons 1 and 2 function in the control mechanism as translation initiation sites and further suggests that suppression of the inhibitory effects of AUG codons 3 and 4 is a general consequence of the translation of URF 1 and 2 sequences upstream. Several observations suggest that AUG codons 3 and 4 are efficient initiation sites; however, these sequences do not act as positive regulatory elements when placed upstream from URF 1. This result suggests that efficient translation is only one of the important properties of the 5′ proximal URFs in *GCN4* mRNA. We propose that a second property is the ability to permit reinitiation following termination of translation and that URF 1 is optimized for this regulatory function.

The *GCN4* gene of *Saccharomyces cerevisiae* encodes a positive regulator that stimulates transcription of amino acid biosynthetic genes in response to starvation for any amino acid. Activation of gene expression is coupled to amino acid availability by increasing the synthesis of *GCN4* protein in starvation conditions. *GCN4* expression is controlled by multiple *trans*-acting regulatory factors, both positive (*GCN2* and *GCN3*) and negative (*GCD1* and *GCD10* to *GCD13*). Genetic evidence suggests that the products of *GCN2* and *GCN3* function indirectly as positive effectors by negative regulation of *GCD* factors (reviewed in reference 7).

There are four upstream AUG codons in the *GCN4* mRNA leader, and each initiates a short open reading frame of two or three codons before an in-frame termination codon is reached (5, 25). Removal of all four leader AUG codons by point mutations leads to constitutive derepression of *GCN4* expression, independent of the *GCN* and *GCD* gene products normally required to regulate *GCN4* expression. By contrast, eliminating the AUG codons has no effect on the level of *GCN4* mRNA. These data show that the upstream AUG codons mediate translational control of *GCN4* expression by *GCN* and *GCD* regulatory factors (6, 18, 19, 25).

The various upstream AUG codons have distinct roles in translational control of *GCN4* mRNA. AUG codons 3 and 4 (counting from the 5′ end) are both necessary and sufficient for efficient repression of *GCN4* expression in nonstarvation conditions. After removal of AUG codons 1 and 2, these sequences repress *GCN4* expression in the absence of the *GCD* gene products normally required for repression of the wild-type *GCN4* gene. By contrast, AUG codons 1 and 2 are relatively weak negative elements when present alone in the mRNA leader. In fact, when AUG codons 3 and 4 are present downstream, AUG codon 1 and, to a lesser extent, AUG codon 2 act as positive elements, being required for efficient *GCN4* expression in derepressing conditions. 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 in nonstarvation conditions. These findings led to the idea that recognition of AUG codons 1 and 2 suppresses the inhibitory effects of AUG codons 3 and 4 and that this interaction is the target of *GCD* negative regulatory function (18, 19).

Just as in mammalian transcripts, upstream AUG codons occur infrequently in yeast mRNA and their insertion into the leader of a transcript generally leads to reduced translation of downstream protein-coding sequences (1, 3, 9, 11, 12, 14, 15, 20, 24). The scanning model for translation initiation accounts for this effect by postulating preferential initiation at 5′ proximal AUG codons coupled with inefficient reinitiation at downstream AUG codons (10, 12, 24). In view of these considerations, it is not surprising that AUG codons 3 and 4 efficiently block *GCN4* expression. The unique aspect of *GCN4* mRNA is that the inhibitory effects of AUG codons 3 and 4 can be overcome by a mechanism that requires additional AUG codons present further upstream. We wished to determine the flanking sequence and positional requirements of the various upstream AUG codons for their novel regulatory interactions. Towards this end, we constructed a variety of deletion mutations in the mRNA leader in an effort to define the minimal sequences surrounding particular upstream AUG codons that are needed for translational control. Our results suggest that a combination of two 30- to 40-nucleotide segments containing upstream open

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reading frames (URFs) 1 and 4, respectively, is sufficient for significant regulation of *GCN4* expression. Having shown that the functions of URF 1 and URFs 3 and 4 are relatively insensitive to changes in flanking sequences and proximity to the *GCN4* AUG codon, we proceeded to examine the effects of altering the 5′-3′ order of the URFs. In addition, we determined the effects of substituting the segment containing URF 1 with synthetic fragments containing heterologous short coding sequences flanked on the 5′ side with nucleotides found upstream from two authentic yeast initiation codons. The results of these experiments have important implications for the molecular mechanism of *GCN4* translational control.

MATERIALS AND METHODS

Construction of *GCN4-lacZ* mutations. The starting plasmid for all deletion constructions (p298) is a derivative of p180 (6), a single-copy plasmid containing a *GCN4-lacZ* translational fusion and the yeast *URA3*, *ARS1*, and *CEN4* sequences. The *lacZ* coding sequences are inserted at codon 56 of the *GCN4* protein-coding sequence (5). To construct p298 from p180, three new restriction sites in the *GCN4* mRNA leader (see Fig. 1) were generated by oligonucleotide-directed mutagenesis, all exactly as described previously (19). The *Sac*II-*Bgl*II, *Bgl*II-*Bst*EII, and *Bst*EII-*Xho*I deletions were constructed by digestion of p298 with the appropriate enzymes, treatment with Klenow fragment and deoxyribonucleotides to produce flush ends, and recircularization with DNA ligase. All other *GCN4-lacZ* alleles were constructed by replacing the *Bgl*II-*Bst*EII fragment of p298 with synthetic double-stranded oligonucleotides. For constructs *A*, *D*, *E*+*F*, *E*′+*F*, *P*, *P*′, *T*, and *T*′, the appropriate oligonucleotides were synthesized with *Bgl*II and *Bst*EII termini to allow one-step replacement of the wild-type *Bgl*II-*Bst*EII fragment with synthetic fragments. In all other cases, a second ligation step was required to insert an additional synthetic fragment or to recombine two of the above-mentioned constructs. The *Bgl*II-*Bst*EII interval of every construct was sequenced by the dideoxy-chain termination technique (21). The complete DNA sequences are shown in Fig. 1. To remove the *lacZ* coding sequences from *GCN4-lacZ* constructs and thereby reconstruct the *GCN4* coding region, plasmids were digested with *Bam*HI and recircularized at low DNA concentrations. Standard procedures were used throughout for preparation, modification, and cloning of plasmid DNA molecules (17).

Assay of GCN4 expression. Plasmids containing mutations in *GCN4-lacZ* constructs were introduced into the following three yeast strains by the transformation technique of Ito et al. (8): TD28 (*MAT*α *ura3-52 ino1*), H15 (*MAT*α *gcn2-1 ura3-52 leu2-3 leu2-112*), and F98 (*MAT*α *gcd1-101 ura3-52*). To assay β-galactosidase or steady-state fusion mRNA levels in repressing conditions (see Fig. 2 to 5), transformants were grown for 6 h from stationary phase to mid-exponential growth in SD medium (23) 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 above-described medium and growth was continued for 6 h to cause histidine starvation. Derepressing conditions for H384 transformants containing *GCN4* alleles were identical to those just described, except that the medium was supplemented with 0.3 mM histidine plus 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 previously described (16). Enzyme activity is reported as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside 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 to 4 are averages calculated from these replicate measurements and have standard errors of 30% or less. Extraction and blot hybridization analysis of total RNA were performed as described previously, by using the same radiolabeled DNA fragments to probe *PYK*, *GCN4-lacZ*, and *GCN4* mRNAs (19).

RESULTS

***GCN4* mRNA leader sequences in the vicinity of URFs 1 and 4 are sufficient for a significant degree of translational control.** The sequence requirements for translational control of *GCN4* mRNA were investigated by constructing internal deletions in the leader region of a *GCN4-lacZ* fusion construct. The effects of these mutations on expression of β-galactosidase activity were measured in nonstarved wild-type cells and in *gcn2* mutant cells for repressing conditions and in histidine-starved wild-type cells and in *gcd1* mutant cells for derepressing conditions. For selected constructs, the effects of the leader mutations on derepression of *GCN4* expression were examined by an independent assay. Following removal of *lacZ* sequences to reconstitute the wild-type *GCN4* coding region, the resulting mutant alleles were tested in vivo for complementation of a *gcn4* chromosomal deletion.

To facilitate construction of deletions, new restriction sites were introduced into the leader region by site-directed mutagenesis (Fig. 1). These point mutations caused a small reduction in the expression of fusion enzyme activity in derepressing conditions (compare wt and wt* in Fig. 2); however, because the wt* allele retained a large derepression ratio, it was selected as the starting point for additional constructions. Three deletions were generated from wt* by eliminating all sequences found between different pairs of restriction sites in the mRNA leader. Smaller deletions were constructed between the *Bgl*II and *Bst*EII sites flanking the four URFs in wt* by replacing this segment with oligonucleotides containing a subset of the sequences normally found in the interval (Fig. 1).

In agreement with earlier findings (5, 25), a deletion of all four URFs (*G*/*B*) led to high, essentially unregulated *GCN4-lacZ* enzyme expression (Fig. 2). By contrast, regulation remained largely intact following deletion of 127 base pairs (bp) located just upstream from the four URFs (*S*/*G*). Deletion of 104 bp just downstream from the four URFs (*B*/*X*) reduced the absolute amount of *GCN4-lacZ* expression by two- to threefold; however, the derepression ratio (e.g., *gcd1*/*gcn2*) was unchanged from that of the parental construct. The latter two results are in accord with our previous finding that the *Bgl*II-*Bst*EII segment is sufficient to confer translational control typical of *GCN4* mRNA upon a heterologous yeast transcript. They also confirm the conclusion that moving all four upstream AUG codons much closer to either the 5′ end of the mRNA or the GCN4 initiation codon has only a minor effect on the magnitude of *GCN4* translational control (18).

Deletions that remove URFs from the 5′ or 3′ direction have effects on *GCN4-lacZ* expression very similar to those reported previously for point mutations in the corresponding AUG codons (19). A 64-bp deletion of URFs 3 and 4 (construct *A*+*C*) led to a substantial increase in expression

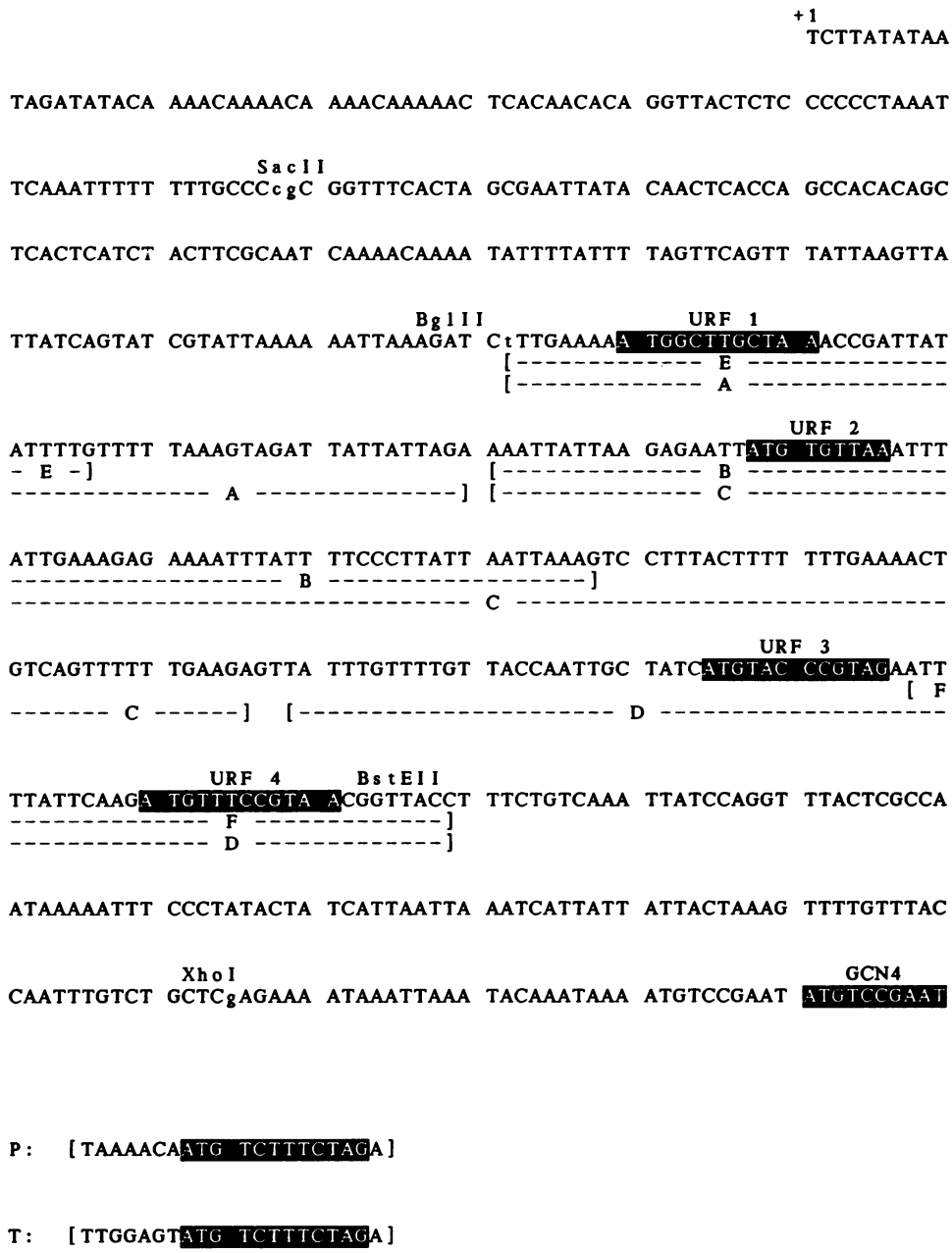
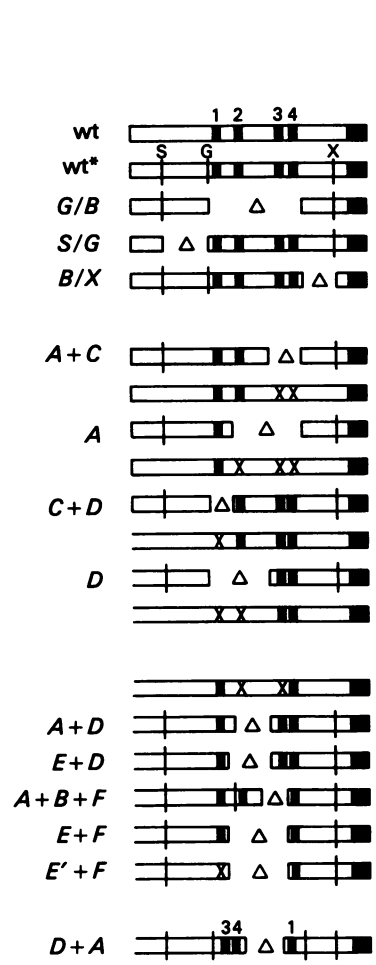


FIG. 1. Construction of *GCN4* leader mutations. The mRNA leader sequence is shown, with the four URFs and the beginning of the *GCN4* coding sequence in reverse contrast. Point mutations that produce new restriction sites are shown in lowercase. Segments joined together to create deletions between the *Bgl*II and *Bst*EII sites are shown in brackets and lettered. Brackets are placed at the last nucleotides included in each segment. For the *E* and *A* segments, the wild-type A nucleotide was restored at the first position in place of the T nucleotide residue (shown in lowercase) that was introduced into the parent plasmid to create the *Bgl*II site. The *P* and *T* segments containing heterologous URFs are shown below the *GCN4* leader sequence. The sequences at the junctions of the various segments (in italics and bracketed) in the *Bgl*II-*Bst*EII interval of each construct are as follows: *A+C*, AGATC-[*A*]-tc-[*C*]-tctGGTTACC; *A*, AGATC-[*A*]-tctGGTTACC; *C+D*, AGATC-[*C*]-tc-[*D*]; *D*, AGATC-[*D*]; *A+D*, AGATC-[*A*]-tc-[*D*]; *E+D*, AGATC-[*E*]-agatc-[*D*]; *A+B+F*, AGATC-[*A*]-tc-[*B*]-atct-[*F*]; *E+F*, AGATC-[*E*]-agatct-[*F*]; *D+A*, AGATC-[*D*]-gatc-[*A*]-GGTTACC; *P*, AGATC-[*P*]-aGGTTACC; *P+D*, AGATC-[*P*]-gatc-[*D*]; *T*, AGATC-[*T*]-aGGTTACC; *T+D*, AGATC-[*T*]-gatc-[*D*]. The letters in lowercase are non-*GCN4* nucleotides included in the synthetic oligonucleotides to generate *Bgl*II sites used for joining different oligonucleotide segments together.

under repressing conditions and reduced the *gcd1/gcn2* ratio from a value of 30 to only ~3.5 (Fig. 2). Deletion of an additional 106 bp containing URF 2 (construct *A*) resulted in even greater *GCN4-lacZ* expression and further diminished the degree of regulation compared with *A+C*. In contrast to these results, deletions of 59 or 166 nucleotides (nt) that removed URF 1 or both URFs 1 and 2 (constructs *C+D* and *D*, respectively) led to reduced *GCN4-lacZ* expression. The reductions were greater under derepressing than repressing conditions; consequently, these deletions of URFs 1 and 2 reduced the derepression ratio. This effect was particularly evident for construct *D*, which exhibited a *gcd1/gcn2* ratio of



	GCN4-lacZ Enzyme Activity						
	wt		gcn2		gcd1		gcd1/
	R	DR	R	DR	R	DR	gcn2
wt	10	80	5	6	280	340	56
wt*	10	68	5	6	170	160	30
G/B	1000	550	1000	1000	1200	1200	1.2
S/G	10	61	6	9	110	120	15
B/X	5	34	2	2	63	60	31
A+C	67	120	59	67	200	230	3.4
	140	270	180	210	400	500	2.3
A	140	200	150	140	310	260	2.0
	290	390	530	350	610	730	1.5
C+D	3	12	2	3	23	23	9.2
	3	25	2	4	36	57	16
D	4	16	4	5	10	15	2.8
	2	16	2	4	14	27	6.8
	15	130	11	21	340	430	24
A+D	17	68	13	16	180	170	12
E+D	11	45	16	21	180	200	10
A+B+F	31	77	29	46	380	210	7.9
E+F	23	64	23	32	150	130	5.1
E'+F	19	27	22	28	38	47	1.7
D+A	4	8	4	5	10	9	2.1

FIG. 2. Effects of deletion mutations in the *GCN4* mRNA leader region on regulation of *GCN4-lacZ* expression. The schematic depicts the 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. Gaps containing Δs indicate deletions. Xs indicate point mutations that remove the AUG codons of the URFs. The constructs containing only point mutations were described previously (19) and were examined in parallel with the deletion alleles generated here. Levels of β-galactosidase activity (in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein) 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. The expression measured in repressing and derepressing culture conditions in *gcd1* transformants was averaged and divided by the average of expression in the same two conditions for *gcn2* transformants. wt*, The parental construct for all of the deletion alleles, containing *Sac*II (S), *Bgl*II (G), and *Xho*I (X) restriction sites introduced into the leader by site-directed mutagenesis.

only ~3. These results support the previous conclusions that (i) URFs 3 and 4 are more effective than URFs 1 and 2 as inhibitory elements, (ii) URFs 3 and 4 are necessary and sufficient to maintain low *GCN4* expression in repressing conditions (e.g., compare *D* with *A+C*), and (iii) in the presence of URFs 3 and 4, URFs 1 and 2 act as positive elements, being required for efficient *GCN4* expression in derepressing conditions (19, 27).

We showed previously that removal of AUG codons 2 and 3 by point mutations had only a minor effect on regulation of *GCN4-lacZ* expression, leading to an approximately twofold increase in the level of expression in repressing conditions (Fig. 2). These results demonstrated that URFs 1 and 4 are sufficient for a nearly wild-type pattern of translational control (19). Deletions of 107- and 131-bp segments containing URF 2, a 78-bp segment containing URF 3, and a 171-bp segment containing URFs 2 and 3 had significant quantitative effects on regulation, reducing the derepression ratio to values that were 1/2 to 1/6 of that given by the parental construct (compare *A+D*, *E+D*, *A+B+F*, and *E+F* with wt* in Fig. 2). These reductions in the degree of regulation result primarily from elevated *GCN4-lacZ* expression under repressing conditions. The deleterious effects of the internal deletions on the efficiency of repression exceeded the effects of removing AUG codon 2 or 3 by a point mutation (Fig. 2), suggesting that non-URF sequences in the region between URFs 1 and 4 contribute to the efficiency of translational control.

Despite their considerable quantitative effects on *GCN4-lacZ* expression, none of the deletions constructed between URFs 1 and 4 completely abolished regulation. Even construct *E+F*, which lacked all but 27 bp of the 186 bp normally found between URFs 1 and 4, exhibited a degree of regulation significantly greater than that seen with alleles containing only one or no upstream URFs. To determine whether the residual derepression observed for the *E+F* construct involves the same regulatory mechanism that operates for the wild-type gene, we removed AUG codon 1

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

Construct	Complementation of Δ*gcn4*[a]	*GCN4-lacZ* expression (*gcd1*)[b]
wt*	++++	310
D	−	13
A	++++	290
A+D	++++	180
E+D	++++	190
E+F	++	140
E'+F	+	43
D+A	−	10
P+D	++	58
P'+D	+/−	10
T+D	+	36
T'+D	−	9

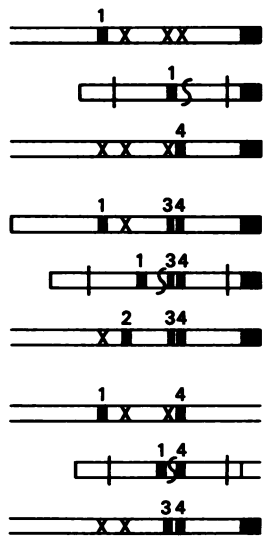
[a] To assay complementation of deletion allele *gcn4-103*, plasmids were introduced into strain H384 (*MATα gcn4-103 his1-29 ura3-52*) and the resulting transformants were replica printed to medium lacking histidine and supplemented with 30 mM 3-aminotriazole. (*his1-29* is a leaky mutation that confers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

[b] Expression levels (in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein) of the corresponding *GCN4-lacZ* fusion constructs examined in *gcd1*-carrying cells. The values are averages of expression levels in repressing and derepressing conditions taken from Fig. 2 to 4 and shown here for comparison.

from this allele by a point mutation (construct *E'+F*). This mutation led to lower *GCN4-lacZ* expression and complete loss of regulation by *GCN2* and *GCD1* (Fig. 2). Therefore, URF 1 functions in the *E+F* construct as a positive control element, the hallmark of *GCN4* translational control. The positive effect of URF 1 in the *E+F* construct was also seen by comparing the degree of complementation of a *gcn4* deletion given by the *E+F* construct versus the *E'+F* construct (Table 1).

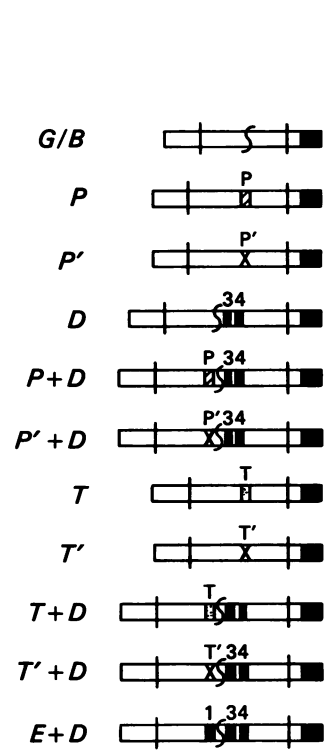
In the *A*, *A+D*, and *E+F* constructs, URF 1 was moved downstream into the approximate positions normally occupied by URF 2, 3, or 4 in the wild-type *GCN4* gene. Comparison of these deletion alleles with the corresponding AUG point mutations (Fig. 3) revealed that, despite such changes in position, alleles containing URF 1 exhibit higher *GCN4-lacZ* expression than those containing URF 2, 3, or 4 located at a similar position in the mRNA leader. This conclusion was particularly evident in the comparison between deletion construct *A* and the allele containing point mutations in AUG codons 1, 2, and 3. At similar distances from the *GCN4* AUG codon, URF 1 remained 20-fold less inhibitory to *GCN4-lacZ* expression than did URF 4. (In fact, expression from the deletion alleles shown in Fig. 3 would be even higher in the absence of the *Sac*II, *Bgl*II, and *Xho*I sites present in these constructs but absent in the corresponding point mutations with which the deletions are compared). These results suggest that the differences among the various URFs in their effects on *GCN4* expression are determined principally by their nucleotide sequences rather than by their distance upstream from the *GCN4* AUG codon.

The 5'-3' order of the URFs is important for translational control. On the basis of the results shown in Fig. 2, it appears that the *A* and *D* leader segments containing URF 1 and URFs 3 and 4, respectively, possess all of the sequences necessary for a significant degree of translational control. Sequences upstream and downstream of each segment can be deleted and at least 10-fold derepression remains intact. Relying on this finding, we inserted the *A* segment downstream from the *D* segment to determine whether URF 1 could function as a positive element when located 3' to URFs 3 and 4.



	GCN4-lacZ Enzyme Activity						
	wt		*gcn2*		*gcd1*		*gcd1/ gcn2*
	R	DR	R	DR	R	DR	
	280	390	490	380	690	730	1.6
A	140	200	150	140	310	260	2.0
	5	12	6	11	15	16	1.8
	7	61	6	15	410	510	44
A+D	17	68	13	16	180	170	12
	3	25	2	4	36	57	16
	15	130	11	21	340	430	24
E+F	23	64	23	32	150	130	5.1
	2	16	2	4	14	27	6.8

FIG. 3. Effects of altering the distance between URF 1 and the *GCN4* AUG codon on *GCN4-lacZ* expression. See the legend to Fig. 2 for a general description of the schematic; the wavy lines indicate deletion junctions. The constructs shown with Xs were described by Mueller and Hinnebusch (19) and were examined in parallel with the deletion constructs generated here. β-Galactosidase activity (in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein) was measured in wild-type (wt), *gcn2*, and *gcd1* transformants in repressing (R) and derepressing (DR) conditions. The derepression ratio *gcd1/gcn2* was calculated as described in the legend to Fig. 2.



	GCN4-lacZ Enzyme Activity						
	wt		gcn2		gcd1		gcd1/
	R	DR	R	DR	R	DR	gcn2
G/B	1000	550	1000	1000	1200	1200	1.2
P	44	88	57	53	220	230	4.1
P′	790	930	910	1900	1800	1100	1.0
D	4	16	4	5	10	15	2.8
P+D	5	29	5	7	62	54	10
P′+D	3	12	3	5	6	14	2.5
T	93	200	100	170	230	240	1.7
T′	760	920	880	1600	1700	1500	1.3
T+D	4	17	6	9	32	39	4.7
T′+D	2	13	3	4	7	10	2.4
E+D	11	45	16	21	180	200	10

FIG. 4. Replacement of URFs 1 and 2 with heterologous URFs. See Fig. 2 for a general description of the schematic; the wavy lines indicate deletion junctions. The shaded boxes P and T represent fragments containing the *P* and *T* URFs; the Xs labeled P′ and T′ represent the identical fragments containing an ACG codon in place of the ATG codon. β-Galactosidase activity (in nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein) was measured in wild-type (wt), *gcn2*, and *gcd1* transformants in repressing (R) and derepressing (DR) conditions. The derepression ratio *gcd1/gcn2* was calculated as described in the legend to Fig. 2.

As described above, when these two segments were joined in the correct order in construct *A+D*, considerable derepression was evident. By contrast, when joined in the reverse order in construct *D+A*, *GCN4-lacZ* expression was very low and showed little or no regulation (Fig. 2). In fact, the *D+A* allele exhibited *GCN4-lacZ* expression nearly indistinguishable from that of the *D* allele, which lacked URFs 1 and 2. The absence of positive function conferred by URF 1 in the *D+A* construct was also shown by a comparison of the ability of the *A+D*, *D+A*, and *D GCN4* alleles to complement a *gcn4* deletion (Table 1). These data suggest that URF 1 cannot stimulate *GCN4* expression from a position downstream of URFs 3 and 4. In addition, URFs 3 and 4 failed to function as positive elements when placed upstream from URF 1 (compare *D+A* and *A*). These findings cannot be explained by an increase in the inhibitory effect of URF 1 due to its greater proximity to the GCN4 AUG codon in construct *D+A* versus *A+D*. This statement follows from the aforementioned fact that URF 1 remained ~30-fold less inhibitory to *GCN4-lacZ* expression than URFs 3 and 4 when segment *A* was moved into the position normally occupied by segment *D* (Fig. 2). Increased proximity between URFs 4 and 1 is also an unlikely explanation for the *D+A* regulatory defect because an even closer spacing between these two URFs in construct *E+F* is compatible with significant regulation.

Replacement of URF 1 with heterologous URFs. The autonomy of segments A and D as regulatory elements enabled us to determine whether URF 1 could be functionally substituted by heterologous short coding sequences. In place of segment *A*, we inserted upstream from segment *D* two different 21-bp sequences containing three-codon open reading frames. The segment *P* URF contains the first three codons and six base pairs found immediately upstream from the initiation codon of the yeast 3-phosphoglycerate kinase (*PGK*) structural gene (2; Fig. 1). These upstream sequences match the following consensus sequence compiled from the initiator regions of highly expressed yeast genes: 5′ (A/U)-A-(A/C)-A-(A/C)-A-*AUG*-U-C-U 3′ (4). The *T* segment URF uses the same three codons as the *P* URF but contains sequences upstream from the initiation codon found at the yeast *TRP1* gene (26; Fig. 1). *TRP1* upstream sequences deviate from the above-described consensus sequence at four of six positions, including three G residues that are completely absent in the initiation regions of highly expressed yeast genes (4).

When examined as single URFs in the *GCN4-lacZ* leader, the *P* and *T* URFs each acted as negative elements, reducing *GCN4-lacZ* expression 5- to 20-fold from the level observed in the absence of all URFs (Fig. 4). The *T* URF was comparable in its inhibitory effect to authentic URF 1 when the two URFs were examined in roughly the same position in the mRNA leader (compare the *A* and *T* constructs in Fig. 3 and 4, respectively). As solitary URFs, the *P* URF is more inhibitory than URF 1 but is at least fivefold less inhibitory than URF 4 (Fig. 2 and 4). The inhibitory effects of the *P* and *T* segments depended on their ATG codons because identical oligonucleotides lacking ATG codons (*P′* and *T′*) had little or no effect on *GCN4-lacZ* expression (Fig. 4). Thus, the *P* and *T* ATG codons exerted the negative effect on *GCN4-lacZ* expression expected for an upstream ATG codon. Interestingly, the *P* URF conferred a degree of regulation on *GCN4-lacZ* expression when present alone in the mRNA leader. In this respect, the *P* construct differed form those

containing authentic *GCN4* solitary URFs; the latter exhibited derepression ratios no greater than 2.

Despite this difference in behavior as negative regulatory elements, the *P* URF resembled URF 1 in its ability to act as a positive element and stimulate *GCN4-lacZ* expression when placed upstream from the more inhibitory URFs 3 and 4. (*P+D* versus *D*, Fig. 4). This effect was most striking in a comparison between expression in *gcd1* and *gcn2* cells, in which a 10-fold difference was observed but was also evident in wild-type cells grown in derepressing versus repressing conditions. Although the *P* URF did not function as efficiently as URF 1 to stimulate *GCN4* expression when these two URFs were compared with the same flanking sequences (*P+D* versus *E+D*, Fig. 4 and Table 1), it is important to note that the stimulatory effect of the *P* URF (i) depended on its ATG codon; (ii) in wild-type cells, was restricted to derepressing conditions; (iii) required the *GCN2* gene product; and (iv) was constitutive in *gcd1* cells. The positive effect of the *P* URF on *GCN4* expression could also be seen in a comparison between the *P+D* and *P'+D* alleles for complementation of the *gcn4* deletion (Table 1). These findings strongly suggest that the *P* URF qualitatively mimics the regulatory function of URF 1.

The results with the *T* URF were less straightforward; however, it appears that the *T* URF can also mimic the regulatory function of URF 1. When placed upstream from URFs 3 and 4 in construct *T+D*, significant stimulation of *GCN4-lacZ* expression relative to the *D* construct was observed in *gcd1* cells. Importantly, this stimulation was completely dependent on the ATG codon of the *T* URF (compare the *T+D* and *T'+D* constructs). The positive function of the *T* URF was also detectable in the *gcn4* complementation assay in comparisons between the *T+D* and *T'+D* alleles (Table 1). For unknown reasons, the *T* URF failed to stimulate *GCN4-lacZ* expression in wild-type cells grown under histidine starvation conditions.

Effects of leader mutations on the size and abundance of *GCN4* mRNA. *GCN4* mRNA was examined by blot hybridization analysis for selected deletion constructs. Total RNA was isolated from transformants of the *gcn4* deletion strain after growth in starvation conditions. The results of this analysis (Fig. 5A) demonstrated that none of the mutations examined led to any significant change in the abundance of *GCN4* mRNA under conditions in which considerable changes in *GCN4* expression were evident for the same alleles (Table 1). In addition, the relative sizes of the transcripts varied in accord with the amount of DNA deleted from the leader region in each construct. These data strongly suggest that the effects of the mutations on *GCN4* expression occurred at the translational level. RNA blot hybridization analysis was also conducted for certain key *GCN4-lacZ* constructs in *gcn2* and *gcd1* transformants (Fig. 5B). Relative to *PYK* mRNA, some variations in abundance were observed among the full-length fusing transcripts expressed from these constructs; however, little difference was observed between the *E+F* versus the *E'+F* constructs or between the *P+D* versus the *P'+D* constructs in *gcd1* cells, in which important differences in fusion enzyme expression were observed between the members of each pair. The variation in transcript levels between the *A+D* and *D+A* constructs shown in Fig. 5B was not observed in independently isolated transformants; however, it remains possible that a reduction in transcript level contributed somewhat to the ~20-fold lower enzyme expression from *D+A* versus *A+D* seen in *gcd1* cells (Fig. 2). Smaller hybridizing species, presumed to be degredation products, were observed in addition to full-length fusion transcripts; however, as with the full-length mRNAs, the amounts of the smaller species cannot explain the variations in enzyme expression observed among the different constructs.

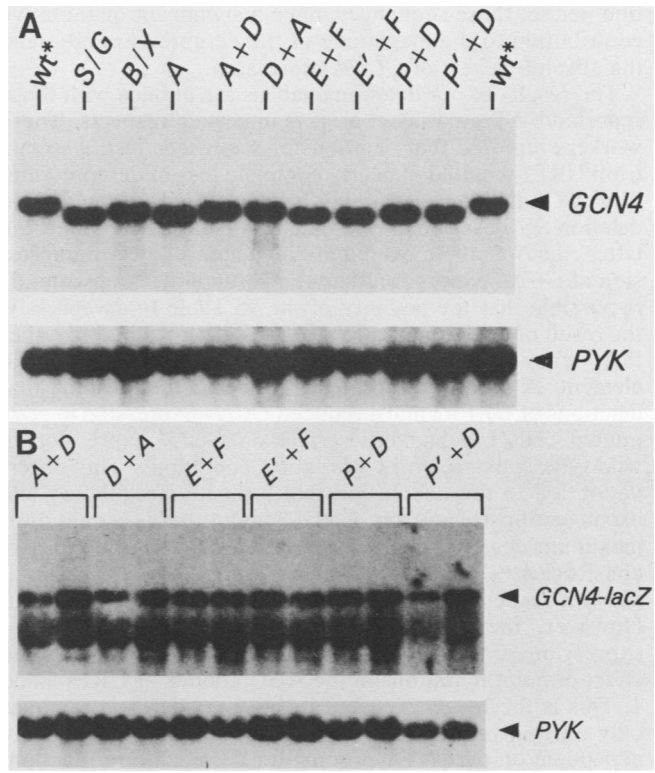


FIG. 5. Blot hybridization analysis of *GCN4* mRNAs containing URF mutations designated as in Fig. 1 and 3. (A) *GCN4* and pyruvate kinase (*PYK*) mRNAs in total RNA isolated from transformants of *gcn4* deletion strain H384 grown in tryptophan starvation conditions. (B) *GCN4-lacZ* and *PYK* mRNAs examined in total RNA isolated from *gcn2* and *gcd1* transformants grown in nonstarvation conditions. Brackets enclose RNA samples for the same construct isolated from *gcn2* and *gcd1* cells, loaded from left to right. Fusion transcripts from the parental construct wt* are not shown but were found to be expressed at levels very similar to those observed for the transcripts presented here.

DISCUSSION

Sequence requirements for translational control of *GCN4* mRNA. The upstream AUG codons are required for translational control of *GCN4* expression by the products of *GCN2*, *GCN3*, *GCD1*, and *GCD10* to *GCD13*. A ~240-nt mRNA leader segment containing the four AUG codons is sufficient to confer regulation by these factors upon a heterologous yeast transcript (18, 19). The data presented here suggest that two 30- to 40-bp segments containing URFs 1 and 4, respectively, are sufficient for a pattern of translational control typical of wild-type *GCN4* mRNA. The 3' proximal segment supplies the negative regulatory function required to maintain repression of *GCN4* expression in nonstarvation conditions. The 5' proximal segment provides the positive regulatory function needed to overcome the inhibitory effect of the 3' proximal sequences in starvation conditions. Although most of the ~500 nt in the mRNA leader located upstream, downstream, or between the segments containing URFs 1 and 4 are dispensable for regula-

tion per se, these sequences make a significant quantitative contribution to the magnitude of the derepression ratio and the absolute levels of *GCN4* expression.

The results of our deletion analysis are at odds with those reported by Tzamarias et al. (27) in certain respects. These workers reported that deletions of sequences just upstream from URF1 resulted in nearly complete loss of derepression. The *S/G* deletion described here is almost identical to deletion 9S described by Tzamarias et al., but unlike the latter, the *S/G* allele exhibited only slightly lower derepression of *GCN4* expression than the parental wt* construct. It is possible that the inability of the 9S allele to derepress is the result of nucleotides added at the deletion junction rather than removal of an important upstream positive control element. A second discrepancy concerns their finding that deletions of URFs 3 and 4 reduced *GCN4* expression in *gcd1* mutant cells but had little effect on *GCN4* expression in wild-type cells grown in starvation conditions. This observation led to the conclusion that a positive regulatory site exists just upstream from URFs 3 and 4 that is required for maximum derepression. Our deletion of URFs 3 and 4 in construct *A+C* is similar to those described by Tzamarias et al. with respect to the leader sequences that are removed. However, the *A+C* deletion had little effect on *GCN4* expression in *gcd1* mutant cells, thereby mimicking the effect of point mutations in the AUG codons of URFs 3 and 4. This is the result expected if the *A+C* deletion removes only a negative element (URFs 3 and 4) that is completely dependent on the *GCD1* product for its regulatory function. We have no explanation for this discrepancy between the two sets of results.

Analysis of point mutations in the upstream AUG codons suggested that, when present alone in the mRNA leader, URFs 1 and 2 are considerably less inhibitory to *GCN4* expression than are URFs 3 and 4 (19). Our deletion data show that URF 1 is somewhat more effective as a negative element when it is moved farther downstream towards the GCN4 initiation codon; however, it remains much less inhibitory to *GCN4* expression than URF 3 or 4 when located at their approximate positions in the mRNA leader. In addition, placing URF 1 downstream from URFs 3 and 4 is as deleterious to derepression as removal of URF 1 in the presence of URFs 3 and 4. These results indicate that the distinct regulatory properties of the various URFs are not dictated by their proximity to the GCN4 initiation codon. Rather, it appears that sequence differences among these elements exist and that the 5′-3′ order of particular URFs is critical for regulation.

Translational control with heterologous 5′ proximal URFs. A remarkable finding presented in this report is that the positive effect of URF 1 can be qualitatively mimicked by short heterologous coding sequences. Although the heterologous URFs do not function as efficiently as authentic URF1, it is noteworthy that these sequences lead to increased, rather than decreased, *GCN4* expression when inserted upstream from URFs 3 and 4. As with authentic URF 1, the regulatory functions of the *P* and *T* URFs are completely dependent on their initiation codons. These results provide strong evidence that translation of URF 1 is required for its regulatory function. We recently found that the regulatory function of URFs 3 and 4 can also be mimicked by a heterologous URF that is 43 codons long (P.P.M. and A.G.H., unpublished data). Taken together with the dispensability of most of the mRNA leader sequences for a significant degree of regulation (Fig. 2), these findings indicate that little or no strict sequence specificity is required for the basic features of the *GCN4* translational control mechanism. On the other hand, although heterologous sequences can mimic the functions of wild-type *GCN4* URFs, different URFs vary greatly in their abilities to confer the distinct regulatory properties of authentic URFs 1 and 4. The following discussion explores the possible nature of these functional differences in detail.

A comparison of the coding sequences of URF 1 with the *P* and *T* URFs (Fig. 1) suggests that neither of the two nonmethionine codons found in URF 1 is uniquely required for positive regulation. Codons 2 and 3 of the *P* and *T* URFs specify different amino acids than do the corresponding codons of wild-type URF 1. This finding is in accord with the fact that *GCN4* URFs 1 and 2 each function as positive regulatory elements but encode different peptide sequences (19). These results make it unlikely that the peptide products of URFs 1 and 2 have an essential regulatory role. It may be significant that codons rarely used in protein-coding sequences in *S. cerevisiae* are absent from URF 1, URF 2, and the *P* and *T* URFs, whereas URFs 3 and 4 each contain a rare proline codon (22). The importance of codon usage in the 5′ proximal URFs needs to be more thoroughly investigated.

One feature that URF 1 and the *P* URF have in common is a close correspondence with the consensus sequence for the initiation regions of highly expressed yeast genes. *GCN4* URF 2 shows less homology with this consensus sequence and functions less efficiently than URF 1 as a positive control element (19). Likewise, the *T* URF contains three G residues rarely found in the initiation regions of highly expressed yeast genes (4) and functions less efficiently than the *P* URF as a positive element. These comparisons suggest that efficient recognition of the initiation codon is an important property of URF 1. This conclusion is consistent with our recent finding that *lacZ* fusions to URF 1 produce high levels of β-galactosidase activity in vivo, comparable to levels expressed by the *GCN4-lacZ* construct lacking all four URFs (P.P.M. and A.G.H., unpublished data).

Efficient initiation is unlikely to be the only requirement for the positive regulatory role of URF 1. This conclusion is suggested by the fact that the *P* URF probably contains an optimum initiation region yet functions less efficiently than URF 1 to derepress *GCN4* expression (compare derepressed expression from *E+D* and *P+D*, Fig. 4). It is also suggested by our finding that URFs 3 and 4 do not act as positive elements when placed upstream from URF 1, although URFs 3 and 4 are expected to contain well-recognized initiation sites. The latter expectation is based on the following three considerations. (i) URFs 3 and 4 each contain AUG codons in a sequence context very similar to that of the initiation regions of highly expressed yeast genes (4); (ii) when fused to *lacZ* coding sequences, URFs 3 and 4 direct high levels of β-galactosidase activity in vivo, comparable to the *GCN4-lacZ* construct in the same circumstances (A. G. Hinnebusch, B. M. Jackson, and P. P. Mueller, Proc. Natl. Acad. Sci. USA, in press); and (iii) when present as a solitary URF in the mRNA leader, URF 3 or 4 each imposes a nearly complete block to *GCN4* expression and this negative function is absolutely dependent on its ATG codon (19).

If URFs 3 and 4 are efficient initiation sites, then perhaps they fail as positive regulatory elements because elongation or termination steps cannot be completed at these URFs, thus blocking the movement of ribosomes to the *GCN4* AUG codon. Alternatively, having completed translation at URFs 3 and 4, ribosomes may dissociate from the mRNA or be

unable to reassemble the factors required for subsequent reinitiation events downstream. We suggest that, by contrast, translation of URF 1 is very efficient and is also frequently followed by reinitiation at downstream AUG codons. The latter property can account for the fact that URF 1 is a much weaker negative element than URF 3 or 4 when each is examined as a solitary URF. As discussed further below, the tendency of URF 1 to allow reinitiation events downstream may underlie its ability to influence translational events at URFs 3 and 4.

One way in which URF 1 function could be regulated would be to couple the rate of URF 1 translation to amino acid availability. According to this model, *GCD* factors would act to repress URF 1 translation in nonstarvation conditions. Because it seems improbable that initiation is regulated at the heterologous *P* and *T* URFs in this specific fashion, the ability of these sequences to mimic URF 1 function suggests that regulation of URF 1 translation is not essential for its positive regulatory role. If initiation at URF 1 is unregulated, it follows that translation of URF 1 is necessary but not sufficient to regulate translational events at URFs 3 and 4. The inactivation of *GCD* proteins expected to occur in starvation conditions is additionally required for translation of URF 1 to have any effect on the 3′ proximal URFs.

Implications for the mechanism of translational control. How might translation of URF 1 sequences influence events >150 nt downstream at URFs 3 and 4? One possibility is that translation of URF 1 leads to changes in the secondary structure of the mRNA at URFs 3 and 4. Although we cannot rule out an important quantitative contribution to the regulatory mechanism, an essential role for changes in secondary structure now seems unlikely, given the dispensability of most of the leader sequences surrounding URF 1 and URFs 3 and 4 plus the lack of strict sequence specificity required for the basic operations of these regulatory elements.

We favor an alternative mechanism based on the aforementioned idea that URF 1 differs from URFs 3 and 4 primarily in its ability to permit frequent reinitiation events at downstream AUG codons following its own translation. In this view, the presence of URF 1 simply ensures that most ribosomes reaching URFs 3 and 4 have engaged in prior translation of URF 1 and must therefore execute a reinitiation event to translate these 3′ proximal URFs. The main assumption behind this model is that reinitiating ribosomes at URFs 3 and 4 can be influenced by inactivation of *GCD* gene products in derepressing conditions, whereas primary initiation events at these sites (those that occur in the absence of URF 1) are insensitive to reductions in *GCD* function. Depending on what translational event is regulated at URFs 3 and 4, reinitiating ribosomes generated by URF 1 translation in derepressing conditions would be (i) less likely to initiate at URFs 3 and 4, (ii) more likely to reinitiate again following translation of URFs 3 and 4, and (iii) less likely to stall in the course of translating URF 3 and 4 sequences. This hypothesis implies a mechanistic difference between primary and reinitiating ribosomes. It is widely accepted that certain initiation factors become associated with the initiation complex by binding to the capped 5′ end of the mRNA (N. Sonnenberg, Prog. Nucleic Acid Res. Mol. Biol., in press). If these factors dissociate following the first (primary) initiation event on a transcript, they may be unavailable for reinitiation of downstream AUG codons. Perhaps in the absence of such components of the primary initiation complex, modification of other factors (*GCD* products) in starvation conditions can alter the behavior of the translational complex at internal initiation sites.

This reinitiation model is consistent with the lack of strict sequence specificity needed for positive regulation by 5′ proximal URFs. In this scheme, all that is required for a positive element is an URF that is well translated and that permits reinitiation to occur downstream following termination of its own translation. URF 4 satisfies the first of these requirements but not the second. URF 1, the *P* URF, URF 2, the *T* URF, and URF 3 represent a set of sequences listed in descending order according to their ability to satisfy both requirements. Experiments are under way to determine the exact sequence differences among these URFs that are responsible for their differing efficiencies as positive control elements. In a previous study, all four *GCN4* URFs were substituted by two heterologous URFs, with the result that *GCN4* expression was constitutively repressed (27). Presumably, the 5′ proximal URF in this construct had properties more similar to URF 4 than to URF 1; according to the scheme just proposed, it would have a low potential for allowing reinitiation events downstream following its own translation.

It was recently reported for a mammalian transcript that the efficiency of reinitiation at a downstream AUG codon following a termination event upstream increased as the separation between the terminator and the downstream URF was increased from 2 to 79 nt. It was also reported that the strong inhibitory effect of an upstream AUG codon was partially suppressed by inserting a second AUG codon with an in-frame terminator 10 nt farther upstream (13). The stimulatory effect of the 5′ proximal URF in these experiments is reminiscent of the positive regulatory function of *GCN4* URF 1. This similarity raises the possibility that the separation between URFs 1 and 4 is a critical parameter in translational control of *GCN4* expression.

One important difference between the mammalian transcript and *GCN4* mRNA is that under normal growth conditions, *GCN4* URF 1 is unable to overcome the inhibitory effects of URFs 3 and 4; inactivation of *GCD* factors is also required for this effect. A second important consideration is that the normal separation between URFs 1 and 4 is ~200 nt. In the aforementioned study, it was found that a separation of this magnitude almost completely abolished the inhibitory effect of an upstream AUG codon on initiation at the next AUG codon downstream. If yeast and mammalian cells were similar in this respect, URF 1 would be unable to suppress initiation at URF 4 by this mechanism from its normal location 200 nt upstream from URF 4.

The internal deletion in *GCN4* construct *E+D* decreased the distance between URFs 1 and 4 to only 27 nt; the *A+B+F* construct placed URF 2 only 58 nt upstream from URF 4. These separations fall within the range defined above for the mammalian transcript in which one AUG codon can suppress initiation at a second AUG codon downstream. Interestingly, these and similar *GCN4* constructs exhibited elevated expression in repressing conditions, consistent with the occurrence of constitutive antagonism between the 5′ proximal and 3′ proximal upstream AUG codons just described for the mammalian transcript. On the other hand, inactivation of the *GCD1* product still led to considerable derepression from these deletion alleles, suggesting that a major component of *GCN4* translational control is independent of the separation between URFs 1 and 4. Analysis of the biochemical functions performed by the *GCD* factors should provide important clues about the nature of the

interaction between URFs 1 and 4 that forms the basis for translational control of *GCN4* expression.

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