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5′ untranslated sequences are required for the translational control of a yeast regulatory gene

(general control of amino acid biosynthesis/*GCN4* gene/DNA sequencing/β-galactosidase fusion/initiation codon)

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ABSTRACT **In yeast, many genes encoding amino acid biosynthetic enzymes are subject to a common regulatory system called the general control of amino acid biosynthesis. The product of the regulatory gene *GCN4* is required for an increase in transcription of general control-regulated genes when yeast are grown under amino acid-starvation conditions. In this report, we show that the expression of the *GCN4* gene is regulated at the translational level: the efficiency of translation of the *GCN4* mRNA is dramatically increased during growth under amino acid-starvation conditions. The complete nucleotide sequence of the *GCN4* gene, presented here, reveals the existence of an unusually long 5′ untranslated region in the corresponding mRNA. *In vivo* analysis of the effects of a deletion in this 5′ leader has enabled us to define a region required for the translational regulation of the *GCN4* mRNA.**

Selective translation of mRNAs is one of the many mechanisms that eukaryotic cells use to modulate levels of gene expression. Among well documented examples are the translational recruitment of maternally stored mRNAs after fertilization in a variety of organisms (reviewed in ref. 1) and the translational switch that occurs upon heat shock in *Drosophila* (2). It has been suggested that sites required for such regulation reside within the 5′ untranslated region of these mRNAs (3), as has been shown to be the case in some prokaryotic systems (4). In this paper, we report the translational regulation of a yeast mRNA and show that a portion of the 5′ untranslated region is essential for modulating the levels of the corresponding translation product.

In *Saccharomyces cerevisiae*, starvation for a single amino acid stimulates transcription of genes encoding enzymes that catalyze reactions in multiple amino acid biosynthetic pathways (5–9). The coregulation of these genes has been termed the general control of amino acid biosynthesis (10). Analyses of the 5′ flanking regions of genes subject to general control have identified a consensus sequence that is required for the increase in the expression of the linked structural gene in response to starvation (8, 11). In addition to these *cis*-acting regulatory elements, the products of 5 unlinked genes, *GCN1** (formerly *NDR1* or *AAS103*), *GCN2* (*NDR2*, *AAS1*, *AAS102*), *GCN3* (*AAS2*), *GCN4* (*AAS101*, *AAS3*), and *GCN5* (*AAS104*), are required for the general control-mediated response to amino acid deprivation (7, 12, 13). The *GCN1*, *GCN3*, and *GCN4* gene products are essential for the initiation of mRNA derepression, whereas the *GCN2* gene product is required for the maintenance of increased mRNA levels during prolonged starvation (14). Of the positive regulatory gene products that function during the initiation phase, molecular and genetic analyses suggest that the *GCN4* gene product plays the most direct role in enhancing transcription (15). An attractive possibility is that the *GCN4* gene product interacts directly with the *cis*-acting elements to stimulate transcription of the linked structural genes.

We have previously reported the cloning and initial characterization of the *GCN4* gene (14). In this paper, the complete DNA sequence is presented. In conjunction with transcription mapping, these data reveal that the 5′ untranslated region of the *GCN4* mRNA is 577 bases long. We present results of a deletion analysis of the 5′ untranslated region of the *GCN4* gene and show that a portion of this sequence normally limits the translation of the corresponding message *in vivo*. mRNA from a *GCN4* derivative lacking this region is translated ≈60-fold more efficiently than mRNA that contains the intact 5′ untranslated region. Production of such high levels of the *GCN4* gene product renders cells constitutively derepressed for the expression of genes subject to general control. This result suggests that an increase in translation of the *GCN4* message might be required for general control-mediated mRNA derepression. In support of this, we find that the translational efficiency of the *GCN4* message increases upon amino acid starvation. We speculate that some of the other regulatory molecules of the general control system interact with the 5′ untranslated region to modulate the levels of the GCN4 protein.

MATERIALS AND METHODS

Strains and Media. All yeast and bacterial strains used have been described (14). Rich medium consisted of yeast minimal medium supplemented with 0.15 mM adenine/0.2 mM uracil, and all 20 amino acids at the concentrations detailed in ref. 14. Amino acid-starvation medium was minimal medium containing 10 mM 3-amino-1,2,4-triazole/0.15 mM adenine/0.2 mM uracil. 3-Amino-1,2,4-triazole elicits histidine starvation (16). The amino acid analog sensitivity of *gcn* mutants was scored by replica plating yeast onto starvation medium.

Molecular Techniques. Transformations, nucleic acid preparations, and blot analysis were as described in ref. 14.

DNA Sequence Analysis. The 1.2-kilobase (kb) *Bam*HI/*Sal* I and the 1.0-kb *Bam*HI/*Pvu* II fragments from clone C101-1 (14) were subcloned in the vector pUC-9 (17). By using these clones, a series of deletions from each end of the insert was generated with exonuclease BAL-31 (18). The deleted fragments were ligated to an appropriately restricted pUC-18 vector (19). The deletion subclones were classified by insert size, and sets of clones harboring inserts that successively differed by ≈200 base pairs (bp) were chosen for DNA sequence analysis. All four series of deletion subclones were sequenced by the dideoxy-chain termination method (20) as modified for double-stranded DNA (21). The sequence of 2089 bp from both DNA strands was thus determined.

Hybrid Selected Translations. For *in vitro* translations, the nuclease-treated reticulocyte lysate system was used (22).

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Abbreviations: kb, kilobase(s); bp, base pair(s).

*A new nomenclature for general control genes has been agreed on: *GCN* denotes general control non-derepressible.

Selection of the *GCN4* mRNA by hybridization, NaDodSO$_4$/polyacrylamide electrophoresis, and fluorography were carried out according to Griffin-Shea *et al.* (23).

Plasmid Constructions. The single-copy yeast plasmid YCp50, containing *URA3*, *ARS1*, and *CEN3*, is the vector backbone of *C101-1*. Plasmid Δ1 was constructed as follows: C101-1 was linearized with *Bst*EII (position 440) and blunt-ended. All *GCN4* sequences upstream of the *Bst*EII site were removed by secondary digestion with *Hin*dIII. The BAL-31 deletion plasmid ε7 was the source of a new 5′ end for the *GCN4* gene. This plasmid contains a 0.7-kb insert that includes nucleotides from the *Sal* I site to position 165 on the DNA sequence, cloned into *Sal* I/*Sma* I sites of pUC-18. ε7 was linearized with *Eco*RI, blunt-ended, redigested with *Hin*dIII, and ligated to the C101-1 plasmid treated as above. Thus, in plasmid Δ1, nucleotides 165–445 of the *GCN4* gene are deleted, and 14 bp from the polylinker region of pUC-18 are added.

To put expression of β-galactosidase activity under the control of sequences supplied by the *GCN4* gene, a *6.3*-kb *Bam*HI/*Sal* I fragment containing the *Escherichia coli lacZ* gene (except for the 8 amino-terminal codons of β-galactosidase), the *lacY* gene, and a portion of the *lacA* gene (24), was cloned into the *Bam*HI/*Sal* I sites of YCp50 (plasmid Z-50). Clones C101-Z and Δ1-Z were constructed by ligating the *Hin*dIII/*Bam*HI restriction fragments from C101-1 and Δ1 to a *Hin*dIII/*Bam*HI-digested Z-50 vector. The strain carrying the integrated *HIS4–LacZ* gene fusion was described by Silverman *et al.* (25).

β-Galactosidase Assays. Yeast cells were prepared as described (26). *o*-Nitrophenyl-β-D-galactoside hydrolysis was measured according to the method of Miller (27). Results were normalized to OD 550 of the culture at time of harvest.

RESULTS AND DISCUSSION

DNA Sequence of the *GCN4* Gene. The DNA sequence of the *GCN4* locus is presented in Fig. 1. The coding strand of the *GCN4* gene was identified by RNA blot analysis using end-labeled probes specific for each strand (data not shown). These experiments established that the *GCN4* gene is transcribed from the *Bgl* II site toward the *Pvu* II site. Nuclease S1 protection experiments precisely defined the endpoints of the *GCN4* transcription unit. Such an analysis established that this gene does not contain any introns and that transcription initiates and terminates at the same unique sites irrespective of growth conditions (data not shown).

A second transcript, shown to be synthesized in the opposite direction from the *GCN4* gene, was also identified in this region. Examination of the DNA sequence identified this transcript as the product of an Arg3a tRNA gene, identical in coding sequence but dissimilar in flanking regions to a previously characterized genomic Arg3a tRNA clone (30). Southern blot analysis suggests that this tRNA is repeated 8 times in the genome, whereas sequences corresponding to the *GCN4* gene are unique.

Protein-Coding Capacity of the *GCN4* mRNA. Within the transcribed region of the *GCN4* gene, there is a single long open reading frame (nucleotides 578–1324), which has the

FIG. 1. DNA sequence of the *GCN4* region and the primary structure of the GCN4 protein. The 5′ (+1) and 3′ (1520) ends of the *GCN4* transcriptional unit are indicated by arrows. The *Sal* I site referred to in the text is located at position −495. Sequences in the 5′ nontranscribed region homologous to TATA are marked by dotted lines and occur at positions −135, −122, and −10. Within the 5′ untranslated region, the positions of the four upstream ATGs and the in-frame termination codons are underlined; solid triangles indicate the end points of the internal Δ1 deletion. The presumptive yeast transcription termination signals (28, 29) are overlined in the 3′ untranslated region (positions 1406, 1423, and 1439). The Arg3a tRNA gene is indicated (positions 1674–1605).

capacity to encode a 249 amino acid protein with a calculated molecular weight of 27,304. To verify such a coding capacity, *GCN4* mRNA was selected from total RNA by hybridization to *GCN4* DNA immobilized on a nitrocellulose filter. The selected RNA was used to direct translation in the reticulocyte lysate system, and the translation products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. As shown in Fig. 2, *GCN4* mRNA directs the synthesis of a polypeptide with an apparent molecular weight of 28,000, in close agreement with the size predicted from the DNA sequence data.

The Unique Structure of the *GCN4* mRNA. In light of the fact that only 6% of the sequenced eukaryotic genes examined to date have untranslated 5′ regions >150 bases long (3), the striking feature of the *GCN4* transcript is that the 5′ untranslated region is 577 bases long. This report constitutes the first example of a yeast nuclear gene encoding such an extensive 5′ untranslated region, although certain yeast mitochondrial genes encode messages with exceptionally long leaders (31).

A second unusual feature of the untranslated region of the *GCN4* mRNA is the presence of four AUG triplets upstream from the start of translation, each of which is followed closely by an in-frame termination codon (Fig. 1). Compilations of sequenced genes have established that 95% of eukaryotic messages do not contain AUG triplets upstream from the functional initiation codon (3), nor have eukaryotic polycistronic mRNAs been reported. These observations, coupled with the results of certain genetic analyses, have supported the proposal that eukaryotic translation initiates only at the most 5′ AUG codon (32). Yet, 5% of the messages that are translated do contain upstream AUG triplets. It has been proposed that translation of these mRNAs occurs because the sequence context of the upstream AUG triplets is unfavorable for translation initiation (3). The four upstream AUG triplets in the *GCN4* mRNA, however, all closely match the proposed optimal sequence context. Thus, this model would predict that the *GCN4* message should be translated infrequently, if at all. A regulatory mechanism that bypasses the effect of the upstream AUG triplets may therefore exist to ensure adequate translation of this message. It may be of interest to note that two upstream ATG triplets, of optimal sequence context and closely followed by termination codons, are present in the yeast *PPRI* gene, which encodes a positive regulator of *URA1* and *URA3* expression (33).

Role of the 5′ Untranslated Region in the Production of the GCN4 Protein. To address the role of the 5′ untranslated region in the regulation of the expression of the *GCN4* gene product, a deletion (positions 165–445) was introduced into the *GCN4* clone C101-1. This deletion, Δ1, removes all four upstream ATG triplets, while preserving the 5′ transcription initiation site and the translation initiation codon. After transformation into yeast, the Δ1 clone retained the capacity to complement the amino acid analog-sensitive phenotype of *gcn4* mutants. In addition, the Δ1 clone gained the capacity to complement the amino acid analog-sensitive phenotype of *gcn1*, *gcn2*, and *gcn3* mutants. The single copy number clone C101-1 does not complement mutations in these genes; however, complementation of mutations in each of these three genes does occur when the *GCN4* gene is supplied in multiple copies (ref. 15; unpublished results). These results strongly suggested that the removal of the portion of the mRNA leader that includes the four upstream AUG triplets results in increased expression of the *GCN4* gene product and that increased expression permits a bypass of the requirement for the *GCN1*, *GCN2*, and *GCN3* gene products. Two explanations for such an increase in *GCN4* expression seemed plausible: deletion of this region either (*i*) increases the stability of *GCN4* mRNA, or (*ii*) enables more efficient translation of the *GCN4* message. To address the first possibility, levels of *GCN4* mRNA transcribed from Δ1 (carried on a centromere plasmid) were compared with levels transcribed from the genomic copy of the *GCN4* gene. As apparent from the RNA blot analysis presented in Fig. 3*a*, *GCN4* mRNA transcribed from Δ1 is actually less abundant than wild-type *GCN4* mRNA. These lower levels may reflect a change in mRNA stability due to the introduced structural alteration, lower rates of *GCN4* gene transcription as a consequence of its location on a plasmid, or plasmid instability. These results prompted us to consider it likely that deletion

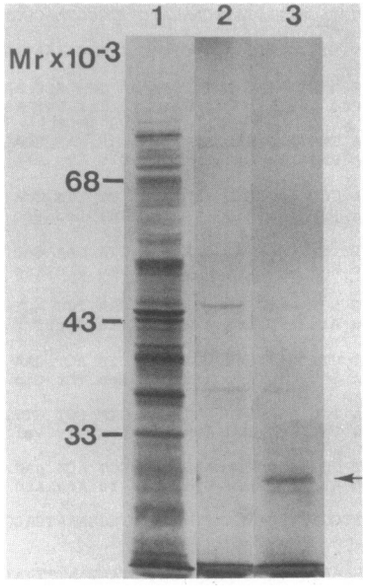

FIG. 2. Hybrid-selected translation of *GCN4* mRNA. Translation products synthesized *in vitro*, using the reticulocyte lysate system from total yeast RNA (lane 1), RNA selected by hybridization to pUC-18 (lane 2), or RNA selected by hybridization to subclone Δ17, which includes *GCN4* sequences from the *Bam*HI site to position 1471 (lane 3), were analyzed on a 10% $NaDodSO_4$ polyacrylamide gel. Arrow indicates the major *GCN4* mRNA *in vitro* translation product. A minor band of lower molecular weight is detectable and may result from initiation at a downstream AUG codon.

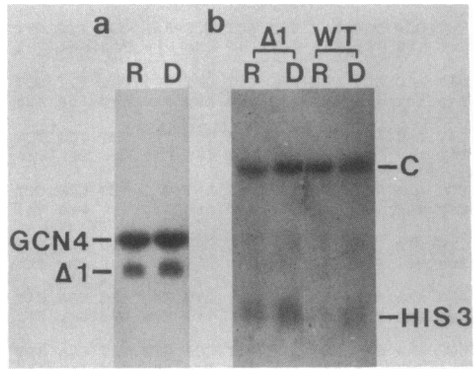

FIG. 3. (*a*) Levels of Δ1 mRNA. Total RNA extracted from yeast strains transformed with the Δ1 clone, grown in rich medium (R) or in starvation medium (D) was electrophoresed on a denaturing agarose gel, blotted onto a nitrocellulose filter, and probed with ^{32}P-labeled *GCN4* DNA. The positions of the genomic *GCN4* mRNA and the Δ1-encoded mRNA are indicated. (*b*) Effects of the Δ1 gene on *HIS3* mRNA levels. RNA was extracted from strains transformed with either the Δ1 clone or C101-1 (WT), grown in rich medium (R) or starvation medium (D). The RNA samples were electrophoresed on denaturing agarose gels, blotted onto nitrocellulose paper, and probed with a ^{32}P-labeled DNA fragment containing the *HIS3* gene and an adjacent gene (C), which is not under general control regulation (5).

of this region of the leader resulted in increased translation of the *GCN4* message.

To test this hypothesis, we fused the 5′ end of the *GCN4* gene derived from *C101-1* or *Δ1* to the *E. coli lacZ* gene, constructing plasmids C101-Z or Δ1-Z, respectively. These plasmids contain sequences which include the promoter, the untranslated mRNA region, and the coding region for the 53 amino-terminal amino acids of the *GCN4* gene fused in-frame to the *lacZ* gene. Yeast transformed with these plasmids were grown in rich medium, and levels of β-galactosidase activity were measured. As shown in Table 1, a 60-fold increase in β-galactosidase activity is observed in strains transformed with the Δ1-Z plasmid as compared to those transformed with the C101-Z plasmid. This result clearly demonstrates that the 280-nucleotide region containing the four upstream AUG triplets decreases the efficiency of translation of the *GCN4* message.

Effects of Overexpression of the GCN4 Protein. As mentioned above, overproduction of the GCN4 protein under starvation conditions is sufficient to bypass the requirement for three of the other *GCN* positive regulatory gene products, presumably by enabling the cells to derepress the amino acid biosynthetic genes subject to general control. We wished to determine whether the presence of an intracellular starvation signal was required for the activity of the GCN4 protein or whether simple overproduction of this protein was sufficient to render yeast constitutively derepressed for mRNA levels of genes regulated by the general control system. To address this question, we examined the levels of *HIS3* mRNA in strains transformed with either the C101-1 or Δ1 plasmid grown in rich medium or starvation medium. As shown in Fig. 3*b*, yeast transformed with Δ1, unlike those transformed with C101, have high levels of *HIS3* mRNA, even when grown in rich medium. *ARG4* mRNA is also constitutively expressed in the Δ1 background (data not shown). Thus, high levels of the GCN4 protein are sufficient to elicit an increase of transcription of genes subject to general control even under nonstarvation conditions.

Translational Regulation of *GCN4* Expression. We have previously shown that the *GCN4* mRNA is present at approximately the same levels in cells grown either in rich or in amino acid starvation medium (ref. 14; see also Fig. 3*a*), suggesting that the *GCN4* gene is not regulated to a large extent at the transcriptional level. The observation that a 60-fold increase in the amount of *GCN4* gene product permits bypass of the requirement for starvation conditions and for the three other positive regulatory gene products, coupled with the existence of a 5′ untranslated region that serves to limit translation of the *GCN4* message, suggest that a key feature of the general control regulatory circuit is to increase the production of GCN4 protein in response to amino acid starvation by altering the efficiency of translation of the *GCN4* mRNA.

To test this hypothesis, we assayed β-galactosidase activities in strains transformed with the C101-Z and Δ1-Z plasmids at various time points after the cells were shifted from growth in rich medium to growth in amino acid starvation medium (Table 1). The relative increase in β-galactosidase activity over a 5 hr time course, is presented in Fig. 4. There is clearly a linear increase in the expression of β-galactosidase in cells containing C101-Z. In contrast, the high levels of β-galactosidase activity in strains transformed with the *Δ1-Z* do not increase with time after a shift into starvation medium. In fact, these levels become slightly lower in such an experiment. We conclude that the translational efficiency of the *GCN4* mRNA is increased under amino acid starvation conditions and that sequences in the 5′ untranslated region are required for such translational regulation.

Two additional points support our conclusions. (*i*) As is the case for the wild-type *GCN4* mRNA (14), mRNA levels of both Δ1-Z and C101-Z remain approximately the same throughout the time course (data not shown). Thus, the dramatic change in β-galactosidase activity does not result from changes in mRNA levels. (*ii*) The two gene fusions compared code for an identical hybrid protein product. Thus, the observed increase in β-galactosidase activity cannot be the consequence of a change in protein stability. Finally, as a control, we measured the levels of β-galactosidase activity in a *HIS4–lacZ* fusion (Fig. 4). As expected for a transcriptionally regulated gene, the increase in β-galactosidase parallels the increase in *HIS4* mRNA levels that occurs under amino acid starvation conditions (14).

Concluding Remarks. The results presented in this paper necessitate revision of some ideas concerning features of the general control regulatory circuit. We have clearly shown that expression of the *GCN4* gene is subject to translational control. In addition, we have shown that an increase in the

Table 1. Effects of the Δ1 deletion on the translational efficiency of the *GCN4* mRNA

Time, min	β-Galactosidase activity, units*		Δ1-Z/C101-Z ratio
	C101-Z	Δ1-Z	
−1	0.71	42	59
0	0.71	42	59
15	1.0	42	42
30	2.1	41	20
60	4.0	39	9.8
120	5.5	38	6.9
280	7.5	38	5.1
240	9.5	38	3.9
300	11	37	3.4

Yeast cells transformed with either the C101-Z or the Δ1-Z plasmids were grown in rich medium and shifted at time 0 to starvation medium. β-Galactosidase activity was monitored in exponentially growing cells just prior to the shift (−1), just after the shift (0), and at various intervals for 5 hr. The values reported represent an average of two independent experiments.
*Units are as defined in ref. 27.

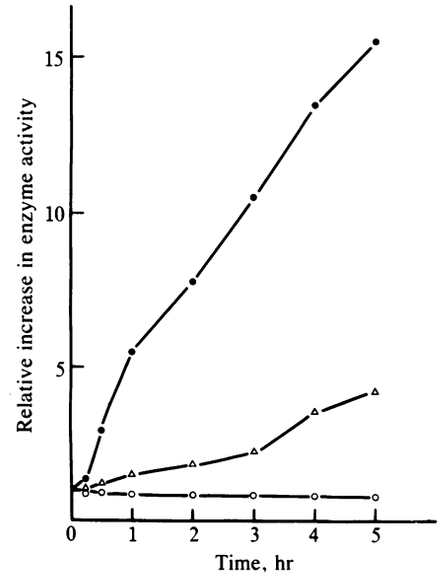

FIG. 4. Increase in translational efficiency of *GCN4* mRNA imposed by amino acid limitation conditions. The β-galactosidase activities presented in Table 1 were normalized to the activities of each strain at time 0. Enzyme activity in strains containing the C101-Z plasmid (●), the Δ1-Z plasmid (○), and of a *HIS4–lacZ* integrated fusion (△), were assayed at various times after shifting cells to starvation medium.

efficiency of translation of the *GCN4* mRNA is sufficient to activate transcription of genes subject to general control regulation. It has been previously proposed that the *GCN4* gene product exists in an inactive form, which is activated when amino acid starvation conditions are imposed (14, 15). By analogy to the regulatory circuit involved in galactose metabolism in yeast (34), the inactive state is postulated to involve a protein–protein interaction, which could be altered by the action of other *GCN* genes (15). The results presented here suggest that such a mechanism is unlikely; rather, the activity of the *GCN4* gene product simply depends on its cellular abundance. It is interesting to note that the kinetics of increase in the levels of the GCN4 protein parallels the kinetics of increase in mRNA levels of genes under general control (14). This observation supports the idea of a direct role for the GCN4 protein in the transcriptional activation of these genes.

Our results show that the translational regulation of the *GCN4* mRNA involves sequences located in the long 5′ untranslated region of this transcript, which includes the four upstream AUG triplets. It is tempting to speculate that the presence of these potential translation initiation sites decreases translational efficiency and that their effects may be circumvented by the action of other regulatory molecules of the general control system. Study of this system offers a unique opportunity to specifically define the sequences and spatial relationships required in the 5′ region of *GCN4* mRNA for efficient translation and to identify the regulatory molecules that interact with these sequences to differentially affect translation. The results of such further work in this system will be of general importance in understanding translational regulation.

Note Added in Proof. In support of our conclusions, it has been reported recently that insertions of ATGs with in-frame termination codons in the 5′ untranslated region of other eukaryotic genes also results in a decreased efficiency in translation initiation (35, 36).

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