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Transcriptional-Translational Regulatory Circuit in *Saccharomyces cerevisiae* Which Involves the *GCN4* Transcriptional Activator and the GCN2 Protein Kinase

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GCN4 protein mediates the transcriptional activation of amino acid biosynthetic genes in *Saccharomyces cerevisiae* by specifically binding to DNA sequences in their 5′-regulatory regions. *GCN4* expression is regulated at the level of translation, with translational derepression occurring under conditions of amino acid starvation. The product of the *GCN2* gene is essential for translational derepression of *GCN4*. Sequence analysis of the *GCN2* gene reveals that the GCN2 protein has a domain highly homologous to the catalytic domain of all known protein kinases. Furthermore, *gcn2* strains are deficient in a protein kinase activity corresponding to a protein with the calculated molecular weight deduced from the *GCN2* open reading frame. Therefore it is likely that *GCN2* encodes a protein kinase, which may be directly involved in translational regulation of the *GCN4* mRNA. Transcription of the *GCN2* gene is increased when cells are cultured in amino acid starvation medium. This transcriptional activation is mediated by the GCN4 protein, which binds to the promoter region of the *GCN2* gene. Thus, this system is modulated by a transcriptional-translational regulatory circuit, which is activated by amino acid starvation. Activation is not the result of a simple quantitative increase of either one of the identified components of the circuit.

In *Saccharomyces cerevisiae*, amino acid biosynthetic genes are transcriptionally coregulated in response to amino acid availability. This regulatory pathway, which coordinates expression of at least two dozen genes (13), constitutes the general control of amino acid biosynthesis (26). The removal of amino acids from the extracellular milieu results in the rapid transcriptional derepression, which is sustained when the severity of amino acid deprivation is maintained by metabolic inhibitors (2). The key regulatory molecule involved in the sustained derepression is the GCN4 protein, which activates transcription by binding to the common sequence 5′ TGA(C/G)TCA 3′ located in the 5′-flanking region of genes under general control (9, 10). When cells are cultured under conditions of amino acid starvation, the level of GCN4 protein is increased via a translational control mechanism, resulting in the enhanced transcription of the coregulated genes. It is well established that a quantitative increase in the GCN4 protein in vivo is sufficient to activate transcription even in nonstarving cells (7, 20, 24).

The translational derepression of *GCN4* mRNA depends on four small open reading frames (ORFs) located in the 5′-untranslated region of the message (20, 25) and on the functions of the *GCN1*, *GCN2*, and *GCN3* gene products (8). Strains carrying mutations in any one of these genes do not exhibit translational derepression when starved for amino acids. Furthermore, mutations in the *GCN2* gene result in a fivefold reduction in basal level expression of the *GCN4* translation product, suggesting that the *GCN2* gene product plays a more direct role in regulating translation of the *GCN4* mRNA. Analysis of *cis*-acting mutations in the 5′ leader of the *GCN4* message suggests that the *GCN2* gene product functions in conjunction with the two most 5′-proximal ORFs (20, 25). Finally, translation of the *GCN4* mRNA is subject to negative regulation by a number of genes designated *GCD*, for general control derepressed (5, 8). The cascade of events that is initiated after amino acid deprivation, which ultimately results in the translational derepression of *GCN4* mRNA, is largely unknown. It is expected that at least a subset of the *GCN* and *GCD* genes is directly involved in this pathway. Elucidation of the steps involved necessitates a detailed analysis of the function and regulation of their respective gene products. As an initial attempt toward this goal, we have chosen to focus on the *GCN2* gene, since, as mentioned above, it appears to play a direct role in translational control of *GCN4*.

The *GCN2* gene has been previously cloned by complementation and shown to encode a 4.5-kilobase (kb) mRNA (2). The level of the *GCN2* message is increased after amino acid starvation only when the *GCN4* product is functional, suggesting that transcription of this gene might be regulated directly by the GCN4 protein. To further investigate the transcriptional regulation of the *GCN2* gene and to identify its product, we have determined the complete nucleotide sequence. This analysis reveals the presence of a single long ORF with the capacity to encode a protein of 1,020 amino acids. The predicted amino acid sequence contains a domain which is highly homologous to the conserved consensus domain of all metazoan protein kinases as well as yeast protein kinases. This finding is in agreement with our results that *gcn2* strains are deficient in a protein kinase activity, which is similar in size to that predicted for the *GCN2* ORF. In addition, the 5′-promoter region of the *GCN2* gene contains a *GCN4* recognition site, which binds the GCN4 protein in vitro. Our results demonstrate that the *GCN2* gene, which is transcriptionally regulated by the GCN4 protein, most likely encodes a protein kinase which may be directly involved in the translational regulation of the *GCN4* message.

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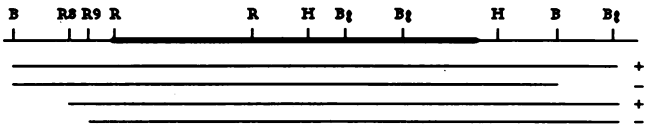

FIG. 1. Restriction map of the *GCN2* locus and complementation properties of subcloned derivatives. The cleavage sites of *Bam*HI (B), *Eco*RI (R), *Hin*dIII (H), and *Bgl*II (Bg) are indicated. The *Eco*RI-*Eco*RI DNA fragment is 1.2 kb long. The thick line depicts the *GCN2* ORF. R8 and R9 are deletion endpoints constructed in vitro. The lines beneath the map delineate regions of the locus that either complement (+) or fail to complement (−) *gcn2* mutant strains.

MATERIALS AND METHODS

Strains and media. The wild-type strain *S. cerevisiae* S288C was used in this study, and all mutant strains were derivatives of this strain. Mutations included *ura3-52*, *ura3-52 leu2-2*, *gcn2-15*, *gcn2-15 ura3-52*, and *gcd1-1 ura3-52*. The rich medium consisted of minimal dextrose supplemented with all 20 amino acids; amino acid starvation medium was minimal dextrose medium containing 15 mM 3-amino-1,2,4-triazole, which leads to histidine starvation (2).

DNA sequence and data analysis. Overlapping subclones of the *GCN2* locus from the leftmost *Bam*HI site to the rightmost *Bgl*II site (Fig. 1) were constructed in M13 vectors and sequenced from both strands by the dideoxynucleotide method. The homology search FASTP algorithm of Lipmann and Pearson (15) was used to search the Protein Identification Resource protein sequence database containing 4098 entries.

Protein kinase assay. A modification of the autophosphorylation assay of Celenza and Carlson (1) was used. Briefly, total yeast proteins were prepared from exponentially grown wild-type and *gcn2* strains by breakage in the presence of glass beads in sodium dodecyl sulfate-electrophoresis sample buffer. Then 50 μg of total protein from each strain was fractionated on a 7.5% sodium dodecyl sulfate–polyacrylamide gel and electroblotted onto a nitrocellulose filter. The filter was blocked either with 1% bovine serum albumin (BSA) or 1% total histones in 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5). Proteins bound to the filter were denatured with 7 M guanidine hydrochloride in 50 mM Tris hydrochloride (pH 8.3)–50 mM dithiothreitol–2 mM EDTA–0.05% BSA for 1 h and then allowed to renature in 50 mM Tris hydrochloride (pH 7.5)–100 mM NaCl–2 mM dithiothreitol–2 mM EDTA–0.1% Nonidet P-40–0.05% BSA for 20 h at 4°C. The filter was treated with 0.05% BSA in 30 mM HEPES (pH 7.5), and the phosphorylation assay was performed in the same buffer supplemented with 2 mM $MnCl_2$–10 mM $MgCl_2$–0.03 μM [γ-^{32}P]ATP (3,000 Ci/mmol) at 25°C for 30 min. The filter was washed extensively with 30 mM HEPES (pH 7.5)–1 mM ATP, dried, and visualized by autoradiography.

Genomic disruption. The 1.5-kb *Hin*dIII fragment of the *GCN2* gene was substituted for a 1.1-kb fragment containing the yeast *URA3* gene. The resultant 4.1-kb *Bam*HI fragment was used to transform a *ura3-52* strain to uracil prototrophy. The transformants were analyzed by Southern analysis to verify the genomic structure of the substituted *GCN2* locus. Disrupted strains of the opposite mating type were used to assay for mating efficiency, sporulation, and germination.

Transcription initiation mapping. The 5′ end of the *GCN2* mRNA was determined by primer extension with a synthetic 19-mer (5′ GCAATTGACTATCCATTAC 3′) which is complementary to the *GCN2* coding strand from positions 83 to 102. Labeling of the 5′ end was carried out with polynucleotide kinase and [γ-^{32}P]ATP. The primer (5 ng) was annealed with 1 to 5 μg of poly(A)$^+$ RNA extracted from wild-type yeast strains cultured in amino acid starvation medium. Annealing was accomplished by incubating the mixture at 100°C for 2 min, followed by quick chilling on ice in a buffer containing 100 mM Tris hydrochloride (pH 8.3) and 100 mM KCl. Extension of the annealed primer with reverse transcriptase was performed as described by Reeder et al. (22).

DNA-protein-binding assays. The DNAs employed in the binding assays included a 790-base-pair (bp) *Bam*HI-*Eco*RI fragment which contained the *GCN2* promoter and deletions extending from the *Bam*HI site toward the promoter region. Deletions were generated by the exonuclease III method of Guo and Wu (4) and subcloned into the vector pUC18. The *HIS3* DNA employed corresponds to the promoter region described by Hope and Struhl (9). Before use the DNA fragments were excised with appropriate restriction enzymes. The GCN4 protein was produced by in vitro translation of an Sp6 polymerase-synthesized *GCN4* RNA in reticulocyte lysates in the presence of [^{35}S]methionine (800 Ci/mmol). GCN4-DNA-binding reactions and electrophoresis were performed as described previously (9).

Increased expression of *GCN2*. Overexpression of *GCN2* was accomplished by subcloning the locus onto a yeast episomal vector carrying *ARS1* and *LEU2* as a selectable marker. This clone was used along with an single-copy vector containing an in-frame fusion of the *GCN4* gene with the *Escherichia coli lacZ* gene and *URA3* as a selectable marker (24) to transform a *ura3-52 leu2-2* strain. Alternatively, increased expression was accomplished by cotransforming the same strain with a clone containing the *GCN4* gene deleted for the upstream ORFs (24). β-Galactosidase assays were performed as described previously (24).

Other methods. 5′ Deletions of the *GCN2* promoter region which were generated for the GCN4-binding assays were used to substitute the *Bam*HI-*Eco*RI fragment in the original *c102-2* clone (2). These clones were used to assay for complementation of *gcn2* strains. Finally time course analyses of RNA levels were performed as described by Driscoll-Penn et al. (2).

RESULTS

Sequence analysis of the *GCN2* gene. Plasmid c102-2 was isolated as a genomic clone which complements *gcn2* mutations when harbored on the yeast centromere vector Ycp50 (2). A map of the *GCN2* clone is shown in Fig. 1. By deletion analysis we have established that the minimum fragment required for in vivo function is included between the leftmost *Bam*HI site and the rightmost *Bgl*II site (Fig. 1). This DNA fragment was sequenced in an effort to identify the *GCN2* gene product. The sequence of 4,978 nucleotides is presented in Fig. 2. The coding strand of the *GCN2* gene was established by Northern blot and S1 protection analysis with single-strand probes specific for both orientations (data not shown). Within the transcribed region there is a single long ORF with the capacity to encode a protein of 1,020 amino acids with a calculated molecular weight of 118,160. The authenticity of the ORF was confirmed by constructing in-frame fusions with the *E. coli trpE* gene by using the contiguous regions of the putative GCN2 protein included within the 1.2-kb *Eco*RI fragment, the 0.5-kb *Eco*RI-*Hin*dIII fragment, and the 1.5-kb *Hin*dIII-*Hin*dIII fragment. Each of these fusion genes was expressed in *E. coli*, and in all cases a fusion protein of the predicted size was produced.

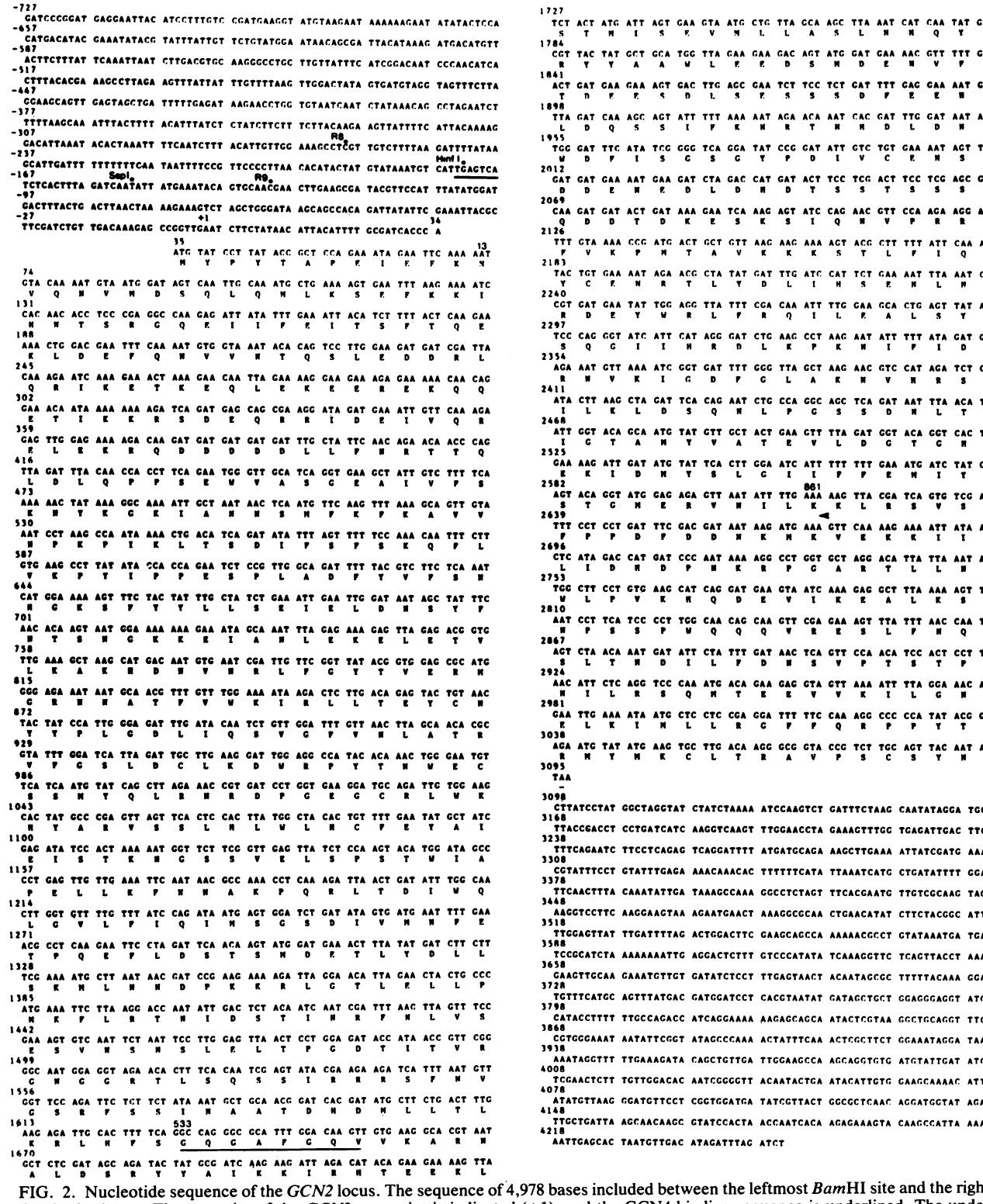

FIG. 2. Nucleotide sequence of the *GCN2* locus. The sequence of 4,978 bases included between the leftmost *Bam*HI site and the rightmost *Bgl*II site is shown. The start site of the *GCN2* transcript is indicated (+1), and the GCN4-binding sequence is underlined. The underlined portion of the translated region corresponds to an ATP-binding consensus sequence. The region of the protein sequence which is homologous to the protein kinase domain is delineated by the arrowheads.

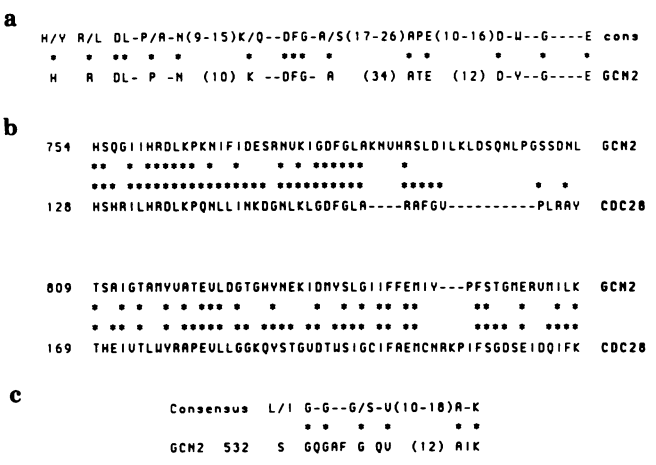

FIG. 3. Homology of the GCN2 protein with protein kinases. (a) Homology of the GCN2 protein from amino acid 760, with the conserved consensus sequence of the catalytic domain of protein kinases. (b) Homology of the same region with the catalytic domain of the yeast CDC28 protein kinase, displayed as the output of the alignment produced by the Lipman-Pearson algorithm. The double asterisks indicate homology, and a single asterisk represents a conservative change. (c) Homology of the GCN2 protein domain beginning at amino acid 532 with the conserved consensus ATP-binding domain.

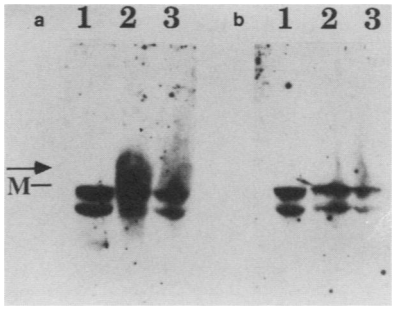

FIG. 4. *gcn2* strains lack a protein kinase activity. Total protein from a *gcn2* strain grown in amino acid starvation medium (lane 1), from a wild-type strain grown in the same medium (lane 2), and from a wild-type strain grown in rich medium (lane 3) was size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto nitrocellulose filters, denatured, renatured, and incubated in the presence of [γ-^{32}P]ATP as described in Materials and Methods. The filter in the left panel was coated with total histones, whereas the filter on the right was coated with BSA. The position of a marker protein, phosphorylase *b*, which has a molecular weight of 97,400, is indicated (M). The position of the putative *GCN2* kinase activity is indicated by the arrow. The two additional bands in the figure correspond to proteins which are autophosphorylated under these reaction conditions. In addition, yeast cells contain several other proteins which are phosphorylated in this reaction but migrate faster than the putative *GCN2* kinase activity and are therefore not included.

Homology of the GCN2 protein with protein kinases. A computer homology search with the Lipman-Pearson algorithm (15) and the PIR database revealed that the region of the GCN2 protein starting at amino acid 754 and extending for 100 amino acids toward the carboxy-terminus was highly homologous to the catalytic domain (12) of all known metazoan protein kinases (Fig. 3a). A comparison of this region with that of the yeast threonine-serine protein kinase encoded by the *CDC28* gene (16, 21) is presented in Fig. 3b. In addition, starting at amino acid position 522, the GCN2 protein contains the conserved consensus sequence for the ATP-binding domain required for kinase activity (14) (Fig. 3c).

***gcn2* strains lack a protein kinase activity.** To assay for the presence of a *GCN2*-dependent protein kinase activity, total yeast protein was isolated from a wild-type and a *gcn2* strain grown in either rich or amino acid starvation medium. The proteins were size fractionated by sodium dodecyl sulfate-polyacrylamide gel elèctrophoresis and electroblotted onto nitrocellulose paper. The filter-bound proteins were renatured and used in an autophosphorylation reaction by incubation with [γ-^{32}P]ATP in the appropriate buffer (1). A duplicate filter was blocked with 1% total histone rather than BSA (see above) to allow detection of protein kinases that are not autophosphorylated under the assay conditions but are able to phosphorylate a general substrate. A protein kinase activity capable of phosphorylating the histone coating was detected in association with a protein species migrating with an apparent molecular weight of >100,000 (Fig. 4a). This activity was readily detected in extracts from amino acid-starved cells (lane 2), whereas only very low levels were observed in cells cultured in rich medium (lane 3). Furthermore, the kinase activity was completely absent in extracts made from *gcn2* strains (lane 1) irrespective of the growth conditions. Autophosphorylation of the same species was not observed (Fig. 4b). This result demonstrates that the *gcn2* background is characterized by the absence of a protein kinase activity, which is increased when wild-type yeast cells are grown under amino acid starvation conditions. The size of the protein is in good agreement with the calculated size of the GCN2 protein.

This experiment was repeated three times; in each case results identical to those presented in Fig. 4 were observed. The reason for the diffuse nature of the band corresponding to the putative *GCN2* kinase activity is not entirely clear. The most likely explanation for the fuzziness resides in the nature of the experiment. The general substrate for the phosphorylation reaction (histone) is simply coated onto the filter and therefore may be free to diffuse to a limited extent. In contrast, the two discrete bands corresponding to autophosphorylated species are filter bound.

Dispensable GCN2 protein. It is likely that the previously described alleles of *gcn2* (2) correspond to point mutations, based on their relatively high frequency of spontaneous reversion. This raises the formal possibility that these mutations are leaky and do not reflect the true null phenotype. To address this issue, we disrupted the endogenous *GCN2* gene by replacing the 1.5-kb *Hin*dIII fragment encompassing the protein kinase domain with the yeast *URA3* gene. The genomic structure of the disruption was verified by Southern blot analysis (data not shown). The resultant strain exhibited the same phenotype as the strains with UV-induced mutations: normal growth in both rich and minimal media, no growth in minimal medium supplemented with amino acid analogs (*gcn* phenotype), normal diploidization, and sporulation.

5′-Upstream region of *GCN2* containing the consensus GCN4-binding DNA sequence. The steady-state levels of the *GCN2* mRNA increased with time when wild-type yeast cells were cultured under amino acid starvation conditions (Fig. 5). We have previously shown that this derepression depends on the presence of a functional *GCN4* gene product (2). Therefore, we searched for a potential GCN4-binding sequence in the 5′ promoter region of the *GCN2* gene. The 5′

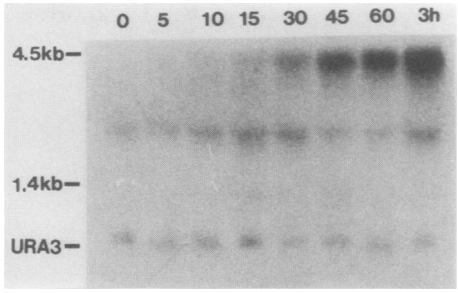

FIG. 5. Time course analysis of the *GCN2* RNA levels in cells cultured under amino acid starvation conditions. Wild-type yeast cells were precultured in rich medium, harvested by filtration, and immediately transferred to amino acid starvation medium. At the indicated times samples were removed for RNA extractions; 20 μg of total RNA from each time point was denatured with glyoxal-dimethyl sulfoxide, size fractionated on an agarose gel, and blotted onto a nylon membrane. The blot was hybridized simultaneously with the 4.1-kb *Bam*HI *GCN2* probe and a yeast *URA3* probe. URA3 mRNA serves as an internal control to quantitate the amount of RNA in each lane.

end of the *GCN2* gene was established by primer extension with a synthetic 19-mer which is complementary to the *GCN2* coding region from position +83 to position +102. This experiment demonstrates that transcription of *GCN2* initiates at three positions, each differing by 1 bp (Fig. 6). The distal most site is depicted as +1 in Fig. 2, located 34 bp 5′ to the AUG initiation codon. Upstream of the start site at position −174, there is a sequence 5′ TGAGTCA 3′ which constitutes the perfect target for GCN4 binding (6, 11).

GCN4 protein binds to the *GCN2* 5′-regulatory region. To investigate whether the GCN4 protein binds to the 5′-regulatory region of the *GCN2* gene, a DNA-binding experiment was performed employing the strategy of Hope and Struhl (9). Briefly, GCN4 protein was synthesized in vitro by translating an SP6-derived *GCN4* RNA in a reticulocyte system containing [^{35}S]methionine. The radiochemically pure protein was incubated with the appropriate DNA fragments, and the protein-DNA complexes were resolved on nondenaturing polyacrylamide gels and detected by fluorography. The *GCN2*-specific DNA fragment employed was the 790-bp *Bam*HI-*Eco*RI segment which includes the entire 5′-flanking region required for in vivo expression of the gene (Fig. 1 and 2). The GCN4 protein bound this fragment with an affinity similar to that of the *HIS3* regulatory region (Fig. 7a, lane 4), which is known to bind GCN4 protein (lane 7). With DNA fragments generated by *Exo*III deletion it was further established that the GCN4-binding site resides to the right of the R8 and to the left of the R9 deletion endpoints (Fig. 7a, lanes 2, 3, 5, and 6).

Moreover, a 100-bp DNA fragment delineated by the *Ssp*I site at position −150 (Fig. 2) and the R8 deletion endpoint bound the GCN4 protein (Fig. 7b, lane 4). Finally, binding of the GCN4 protein was completely abolished when the R9 *Eco*RI fragment was cleaved with *Hin*fI, which cleaves within the consensus sequence at position −172. Binding was unaffected when the R9 *Eco*RI fragment was cleaved with *Alu*I (Fig. 7b, lanes 2 and 3, respectively). The binding studies are consistent with our results that the DNA sequences between the R9 and R8 endpoints are essential for the expression of *GCN2* in vivo, since *GCN2* mutants with deletions of the sequences upstream from the R9 endpoint failed to complement *gcn2* strains, whereas sequences upstream of the R8 endpoint were dispensable for in vivo expression of the *GCN2* gene (Fig. 1).

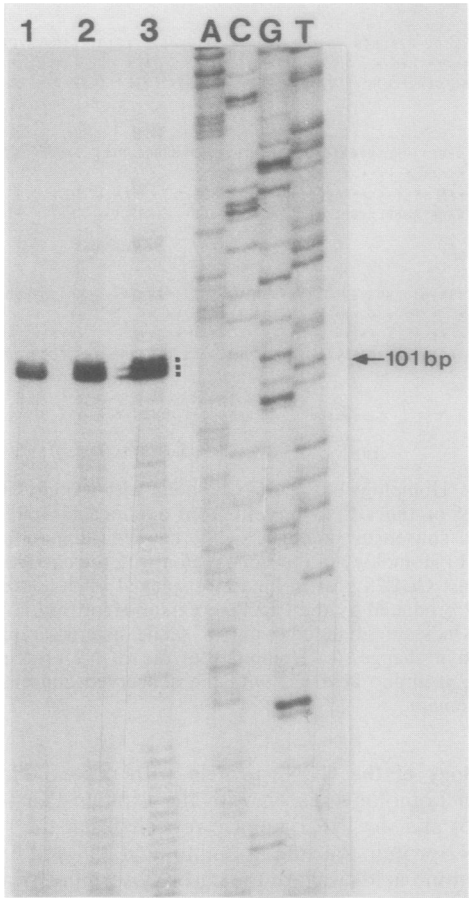

FIG. 6. Mapping the start site of the *GCN2* transcript. A 5-ng sample of a 19-mer primer complementary to the *GCN2* coding region starting at position 102 was end labeled and annealed with 1, 2, or 4 μg of poly(A)$^+$ RNA extracted from cells cultured in amino acid limitation medium (lane 1, 2, or 3, respectively). The annealed primer was extended with reverse transcriptase in the presence of deoxynucleoside triphosphates. The size of the extended product was determined by gel electrophoresis under denaturing conditions.

Effects of increased expression of the *GCN2* gene in rich medium. The transcription of the *GCN2* gene was regulated by the GCN4 protein as a function of amino acid availability (Fig. 5). To investigate the mechanism of activation of this transcriptional-translational regulatory circuit, we asked whether increased expression of the *GCN2* gene affects the translation of the *GCN4* mRNA in rich medium. An increase in expression was accomplished by either introducing the *GCN2* gene into yeast strains on a multicopy yeast vector or by transforming yeast cells with a derivative of the *GCN4* gene with a deletion of the upstream ORFs that is translated with high efficiency (24). In the latter case, overproduction of *GCN2* was accomplished indirectly by overproducing the GCN4 protein. Strains containing multiple copies of the *GCN2* gene displayed a 5-fold increase in basal level expression of *GCN2* mRNA, whereas strains overproducing the GCN4 protein resulted in a 10-fold increase in these levels (data not shown). In both strains the levels of *GCN4* mRNA were unaffected. These strains were also transformed with a *GCN4-lacZ* fusion plasmid routinely employed to monitor

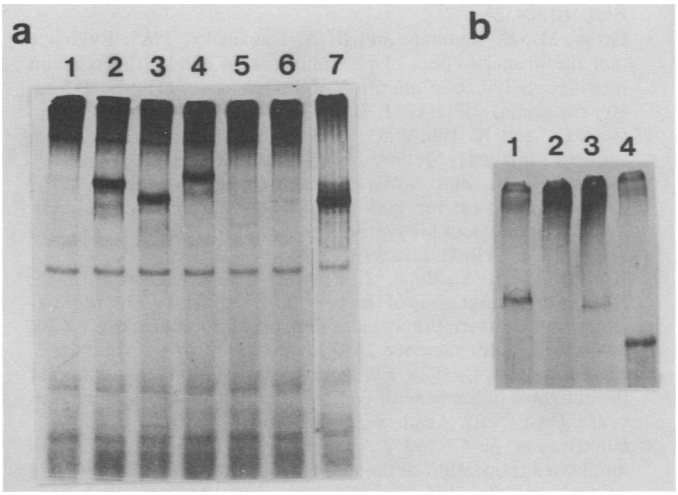

FIG. 7. Binding of the GCN4 protein to the *GCN2* 5'-regulatory region. (a) ^{35}S-labeled, in vitro-synthesized GCN4 protein was used in binding reactions with the following DNAs; Puc18 DNA (lane 1), the *Bam*HI-*Eco*RI fragment (lane 4), the R7-*Eco*RI deletion with an endpoint at base −305 (lane 2), the R8-*Eco*RI deletion fragment (lane 3), the R9-*Eco*RI deletion fragment (lane 5), the R10-*Eco*RI deletion fragment with an endpoint at base −90 (lane 6), and a DNA fragment containing the *HIS3* regulatory region (lane 7). The protein-DNA complexes were resolved on native polyacrylamide gels and detected by fluorography. (b) Binding assays with an intact R8-*Eco*RI DNA fragment (lane 1), the R8-*Eco*RI fragment digested with *Hin*fI (lane 2), the R8-*Eco*RI fragment digested with *Alu*I (lane 3), and the R8-*Ssp*I DNA fragment.

the translational efficiency of *GCN4* mRNA (24). In both cases a twofold increase in the efficiency of *GCN4* mRNA translation was observed in the absence of a starvation signal (Table 1). An additional increase occurred when cells were subjected to amino acid starvation. These results demonstrate that the translational efficiency of the *GCN4* mRNA is influenced by the level of expression of the *GCN2* gene, even in rich medium, whereas full activation of the circuit requires a starvation signal.

DISCUSSION

The *GCN2* gene is essential for the translational derepression of *GCN4* mRNA, which in turn is required for the transcriptional regulation of amino acid biosynthetic genes. Sequence analysis of the *GCN2* gene reveals two new elements of the general control regulatory pathway. The putative GCN2 protein contains a domain highly homologous to protein kinases, and the gene is itself transcriptionally regulated by the GCN4 protein. The homology of the GCN2 protein with all known metazoan protein kinases and with protein kinases of budding and fission yeasts is striking. This homology includes all known members of both the serine-threonine and the tyrosine families of protein kinases. All of the invariant amino acids common to protein kinases are conserved in *GCN2* (Fig. 3a). In addition, *GCN2* has a perfect ATP-binding site containing the highly conserved consensus Gly-X-Gly-X-Phe-Gly-X-Val, starting at amino acid 522, and the Ala-X-Lys tripeptide located 12 residues downstream (14). The sequence comparison shows that the GCN2 protein exhibits the highest homology with the *raf* protein, a serine-threonine kinase (19). The possibility that the *GCN2*-encoded protein kinase belongs to the serine-threonine family is further suggested by the presence of the sequence Lys-Pro-Lys-Asn at position 765, which is highly conserved among members of this family (12). The possibility that the GCN2 protein has an intrinsic protein kinase activity is strengthened by our in vitro kinase assay, which demonstrates that a kinase activity is present in wild-type cells cultured under conditions of amino acid starvation but absent in *gcn2* strains. This kinase activity is associated with a protein of the predicted size of the GCN2 protein. This evidence strongly suggests that the *GCN2* gene encodes a protein kinase; however, direct validation will require purification of the GCN2 protein.

TABLE 1. Levels of β-galactosidase activity produced by a *GCN4-lacZ* fusion gene in strains with increased expression of either *GCN2* or *GCN4* genes[a]

Overproduced protein	Strain	β-Galactosidase activity (U)	
		Rich medium	Starvation medium
GCN2	YEp21	4.8	22.7
	YEp21-GCN2	10.3	27.5
GCN4	GCN4	3.8	14.8
	δ1-GCN4	8.3	17.9

[a] The results are given in units of β-galactosidase produced from a *GCN4-lacZ* fusion in strains which overproduce the *GCN2* protein or the *GCN4* protein. Increased expression was accomplished as described in Materials and Methods. The effect of increased expression on the levels of β-galactosidase activity compared with the levels produced in strains harboring either the multicopy vector (YEp21) or the wild-type *GCN4* gene. β-Galactosidase activity was assayed in strains grown in rich medium (repressing conditions) or in amino acid starvation medium (derepressing conditions).

The amino acid sequence comparisons have not revealed any significant homologies between other regions of the GCN2 protein and proteins in the database. The domain that includes the first 500 amino acids is rich in charged residues. Application of various secondary structure algorithms suggests that this area is highly structured with alternating α-helices and β-turns (M. Kokinidis, personal communication). Both of these facts are consistent with the possibility that this domain could interact directly with nucleic acids, such as the 5' region of the *GCN4* mRNA, rRNA, or tRNAs. Such interaction may account for the specificity of the GCN2 protein in modulating translation of the *GCN4* mRNA.

The *GCN2* disruption strain exhibits only the *gcn2* phenotypes, namely, no growth under conditions of severe amino acid starvation and reduced basal levels of *GCN4* mRNA translation. No other apparent growth or developmental defect has been observed. Southern blot analysis of genomic DNA coupled with hybridization at low stringency does not reveal additional sequences homologous to those of *GCN2* which could potentially substitute for the disrupted gene. These data suggest that the GCN2 protein functions specifically in the general control system.

We have previously shown that accumulation of the *GCN2* mRNA is increased when cells are starved for amino acids and that the increase depends on the presence of a functional *GCN4* gene (2). The sequence analysis of the *GCN2* gene combined with transcript mapping reveals the presence of a perfect consensus sequence for *GCN4* binding (6, 11) residing 174 bp upstream of the transcription initiation site. We have shown that the GCN4 protein binds in vitro to fragments harboring this sequence. Moreover, this region is essential for the in vivo expression of the *GCN2* gene. The binding assays argue that the *GCN4*-dependent accumulation of the *GCN2* mRNA is the direct result of the GCN4

protein binding to the 5'-regulatory region of the *GCN2* gene.

A positive regulatory circuit operates at the final stages of the general control response: the *GCN2* product, which is more than likely a protein kinase, regulates the expression of its transcriptional activator. The end result is that transcriptional derepression of the *GCN2* gene parallels the increase in the translation of the *GCN4* mRNA (24) when yeast cells are grown in amino acid starvation medium. This suggests a quantitative contribution of the *GCN2* gene product in modulating the translational efficiency of the *GCN4* mRNA. Such a contribution is evident also when cells are grown in rich medium: the absence of the *GCN2* gene product results in a fivefold reduction in basal level translation of the *GCN4* mRNA (25), whereas increased expression of *GCN2* elevates the basal level (this paper). The latter increase is not proportional to the extent of *GCN2* expression, demonstrating the necessity for activation of the circuit by amino acid starvation. Such an activation could be mediated directly by the *GCN2* gene product or, alternatively, inactivation of negative regulators of *GCN4* translation, such as members of the *GCD* genes (5, 8). Given the likelihood that *GCN2* encodes a protein kinase, it is tempting to speculate that protein phosphorylation plays an important role in mediating translational derepression of the *GCN4* message.

How might a protein kinase specifically increase the translation of *GCN4* mRNA? Protein phosphorylations are known to regulate translational efficiencies in a general manner affecting the translation of most cellular mRNAs. Examples include the phosphorylation of subunits of the eIF2 complex (3, 18) and phosphorylation of the S6 ribosomal protein (17). Recent experiments suggest that in the absence of a functional *GCN2* gene 80S ribosomes are stalled in the upstream ORFs of the *GCN4* mRNA and *GCN2* is required for a destalling process (D. Tzamarias and G. Thireos, manuscript in preparation). The putative *GCN2* kinase activity could modify the agent responsible for ribosome stalling (possibly a product of one of the *GCD* genes) so as to allow the migration of 40S subunits to the AUG initiation codon of the *GCN4* mRNA. Identification of the substrate for the putative *GCN2*-encoded kinase will reveal information on the mechanism of translational regulation of the *GCN4* mRNA. In addition, identification of the factor(s) that activates the transcriptional-translational regulatory circuit will elucidate steps involved in the signaling pathway for amino acid starvation.

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