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Monitoring the Gcn4 Protein-mediated Response in the Yeast *Saccharomyces cerevisiae**

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In *Saccharomyces cerevisiae* the *GCN4* gene encodes the transcriptional activator of the "general control" system of amino acid bioynthesis, a network of at least 12 different biosynthetic pathways. We characterized the consequences of the general control response upon the signal "amino acid starvation" induced by the histidine analogue 3-aminotriazole with respect to Gcn4p levels in more detail. Therefore, we established test systems to monitor the time course of different parameters, including *GCN4* mRNA, Gcn4 protein, Gcn4p DNA binding activity, as well as Gcn4p transactivation ability. We observed a biphasic response of Gcn4p activity in the cell. At first, translation of *GCN4* mRNA is induced within 20 min after switch to starvation conditions. However, an additional increase in *GCN4* transcript steady state level was observed, leading to an additional second phase of *GCN4* expression after 3–4 h of starvation. The DNA binding activity of Gcn4p, as well as the ability to activate transcription of target genes, correlate with the amount of Gcn4 protein in the cell, suggesting that under the tested conditions there is no additional regulation of DNA binding or transactivation ability of Gcn4p, respectively.

The survival of cells in their natural environment is dependent on their ability to adapt rapidly to occurring changes. For example, yeast cells must respond to changes in the availability of amino acids to survive in nature. In yeast, complex biological mechanisms have evolved to ensure a sufficient supply of amino acids within the cell. Starvation of the yeast *Saccharomyces cerevisiae* for any of several amino acids causes a coordinated derepression of genes encoding enzymes involved in more than 10 different unlinked amino acid biosynthetic pathways, as well as of amino acid tRNA synthetases (1, 2), and enzymes for purine biosynthesis (3). This complex interplay between numerous control mechanisms is known as "general amino acid control" (4).

Under laboratory conditions, the general control response is not inducible by culturing cells on minimal medium (4). Because of the high basal expression levels of genes involved in amino acid biosynthesis, the ability of yeast to synthesize at least one of the amino acids must be impaired. A common approach to impose amino acid limitation on *S. cerevisiae* is to cultivate yeast cells in the presence of amino acid analogues, as 3-aminotriazole (3-AT)[1] or 5-methyltryptophan. The histidine analogue 3-AT is a competitive inhibitor of the imidazole glycerolphosphate dehydratase, an enzyme involved in histidine biosynthesis (5). Addition of 3-AT to exponentially growing yeast culture leads to insufficient supply of the cell with histidine which results in the activation of the general amino acid control (6).

The general control is a multifaceted regulatory mechanism that draws upon several different strategies to control gene expression. Gcn4p is the transcriptional activator of gene expression in this system. It enables the yeast cell to stimulate the expression of at least 40 different genes. Gcn4p is a member of the basic leucine zipper family (7) and binds directly as a homodimer to a conserved regulatory region of its target genes (8). This DNA sequence required for specific binding of Gcn4p is well defined and consists of the symmetric nucleotide motive TGA(C/G)TCA (9).

The rate of Gcn4p synthesis itself is regulated by the availability of amino acids. Under nonstarvation conditions the *GCN4* mRNA is poorly translated due to the negative effects of the translation of four small upstream open reading frames (uORFs) present in its 5′-untranslated region (10, 11). When cells are grown under conditions of severe amino acid limitation, an elevation in the cellular amount of Gcn4p is accomplished through an increased translation of *GCN4* mRNA. A deletion of all four uORFs (12) or point mutations in their four ATG start codons (13) results in an increased translation of *GCN4* mRNA. The model for this translational control is that the four uORFs in the leader of *GCN4* mRNA restrict the flow of scanning ribosomes from the cap site to the *GCN4* initiation codon. When amino acids are abundant, ribosomes translate the first uORF and reinitiate at one of the remaining uORFs in the leader, after which they dissociate from the mRNA. Under conditions of amino acid starvation, many ribosomes which have translated uORF1 fail to reinitiate at uORFs 2 to 4 and utilize the *GCN4* start codon instead. The failure to reinitiate at uORFs 2 to 4 in starved cells results from a reduction in the GTP-bound form of eIF-2 that delivers charged initiator $tRNA_i^{Met}$ to the ribosome. When the levels of eIF-2·GTP·Met-$tRNA_i^{Met}$ ternary complexes are low, many ribosomes will not rebind this critical initiation factor following translation of uORF1 until after scanning past uORF4, but before reaching *GCN4* (14). It has been supposed that under amino acid starvation conditions, uncharged tRNAs accumulate and stimulate Gcn2p protein kinase activity by interacting with its histidyl-tRNA synthetase-related domain (15). Gcn2p phosphorylates

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[1] The abbreviations used are: 3-AT, 3-aminotriazole; eIF-2, eukaryotic initiation factor 2; eIF-2B, guanine nucleotide exchange factor of eIF-2; GCRE, general control responsive element; $tRNA_i^{Met}$, initiator tRNA charged with methionine; UAS, upstream activation site; uORF, small upstream open reading frame; bp, base pair(s).

the α-subunit of eIF-2 (16). Phosphorylation of eIF-2 reduces the amount of active eIF-2 available for ternary complex formation by inhibiting eIF-2B, the guanine nucleotide exchange factor. The resulting low level of eIF-2·GTP and ternary complex formation diminishes the rate at which initiation complexes are reassembled.

An additional regulation mechanism for the general control system is the regulation by protein degradation of Gcn4p. Pulse-chase experiments showed that Gcn4p is highly unstable, with a half-life of about 5 min under nonstarvation conditions (17). This degradation is inhibited under conditions of starvation for amino acids. Up to now nothing is known about other regulation mechanisms of *GCN4* gene expression or Gcn4 protein itself.

The rapid turnover of Gcn4p may be the reason that Gcn4p activity could hardly be detected in yeast cells. Virtually all biochemical analyses of Gcn4p function have used Gcn4p produced by recombinant techniques, for example, analyses of *GCN4* regulation *in vivo* have used *GCN4*::*lacZ* fusion genes. Here we have characterized in detail the consequences of the general control response with respect to *GCN4* mRNA and Gcn4p levels. We have examined several parameters of the changes of Gcn4p levels when exponentially growing cells were shifted from growth in minimal medium to amino acid starvation medium. Test systems were developed in order to monitor the time course of various steps of this induction process: (*a*) *GCN4* mRNA steady state levels were determined. (*b*) Gcn4 protein was detected using a specific antibody raised against the DNA-binding domain of Gcn4p. (*c*) An assay to detect Gcn4p in a gel retardation experiment using cellular extracts was developed. (*d*) A highly sensitive *lacZ* fusion gene as efficient target of the general control was constructed carrying multiple cassettes of the Gcn4p recognition element in its promoter to monitor the trans-activation rate of the Gcn4 protein. These assays were performed with both a strain harboring the wild-type *GCN4* allele and a strain containing a *GCN4* allele which was unresponsive to the translational control due to point mutations of the four uORFs.

We observed a biphasic response of Gcn4 protein levels, Gcn4p DNA binding activity and activation ability in the cell upon the signal "amino acid starvation." The first phase started immediately after induction by 3-AT and is due to the translational control depending on the four uORFs. After 3–4 h of starvation, an additional 2-fold increase started which seems to be due to increased transcriptional initiation induced by amino acid starvation.

EXPERIMENTAL PROCEDURES

Plasmids

Construction of the Gcn4p Reporter Plasmid—pME1112 and pME1108 were constructed using the yeast integration vector pLI4 (18) containing the *CYC1*::*lacZ* reporter gene (19). pME1112 (*GCRE6*::*lacZ*) was used to integrate a Gcn4p regulated *lacZ* gene carrying six general control responsive elements (GCRE) in the *CYC1* promoter region into the genome. The same construct without GCRE was used as control and named pME1108 (Δ*UAS*::*lacZ*). Replacement of the upstream activation site (UAS) of the *CYC1*::*lacZ* with GCREs was achieved by first cutting out the *Xho*I fragment containing the UAS. Religation of the *Xho*I site resulted in pME1108. Then, two synthetic oligonucleotides (GAC1, 5′-GATCGGATGACTCATTTTTT-3′ and GAC2, 5′-GATCAAAAAATGAGTCATCC-3′) were annealed to form an optimal double-stranded GCRE (20). T4 DNA ligase was added to produce multimeric GCRE DNA probes. The products were then blunt ended with Klenow and inserted into the blunt ended *Xho*I site of pME1108. The resulting plasmid pME112 contains six GCRE sites as determined by nucleotide sequence analysis (21). Enzymatic manipulation and cloning of DNA were performed as described by Sambrook *et al.* (22).

Construction of a GCN4 Deletion Cassette—A 2.8-kilobase *Eco*RI-*Sal*I fragment containing the *GCN4* gene was isolated from vector p164 (12) and inserted between the *Eco*RI and *Sal*I sites of pBluescribe M13$^-$ to get vector pME1085. The complete *GCN4* open reading frame was deleted by removing a *Bst*EII-*Bfr*I fragment. A blunt ended *Hpa*I-*Nar*I fragment containing the *LEU2* gene isolated from vector YEp351 (23) was then inserted between the blunt ended *Bst*EII and *Bfr*I sites to obtain pME1105.

Construction of GCN4 and GCN4^m Carrying Centromeric Vectors—A 2.8-kilobase *Eco*RI-*Sal*I segment containing the *GCN4* gene was isolated from vector p164 (12) and inserted between the *Eco*RI and *Sal*I sites of vector pRS314 (24) to get vector pGCN4 (pME1092). The 640-bp *Sal*I-*Bst*EII fragment of pGCN4 carrying the four short open reading frames in the *GCN4* leader region was then replaced by a 640-bp *Sal*I-*Bst*EII fragment of vector p238 containing four mutated (nonfunctional) open reading frames in the *GCN4* leader region (13) to get vector pGCN4^m (pME1098).

GCN4::*lacZ Fusion Plasmids p180 and p227*—The plasmids were kindly provided by Alan Hinnebusch (National Institute of Child Health and Human Development, Bethesda, MD). p180 is a *URA3*,*RS1*,*CEN4* plasmid containing a *GCN4*::*lacZ* fusion with *lacZ* fused in-frame at the 55th codon of *GCN4* (12). p227 is a derivative of p180 containing substitution mutations in the AUG codons of all four uORFs in the *GCN4* mRNA leader (25).

Yeast Strains and Growth Conditions

RH1796 (*MATa ade2-101 leu2-3 leu2-112 suc2-Δ9 trp1-Δ901 ura3-52::GCRE6::lacZ Δgcn4::LEU2*) and RH1798 (*MATa ade2-101 leu2-3 leu2-112 suc2-Δ9 trp1-Δ901 ura3-52:: ΔUAS::lacZ Δgcn4::LEU2*) are derivatives of *S. cerevisiae* strain SEY6210 (*MATa ade2-101 his3-Δ200 leu2-3 leu2-112 suc2-Δ9 trp1-Δ901 ura3-52*; obtained from Scott Emr, University of California San Diego). Wild-type strain RH1415 (*Matα ura3–52 leu2*) was described earlier (26).

Strains RH1796 and RH1798 were constructed by first performing a one-step replacement of the chromosomal *GCN4* gene using the *Sca*I fragment of vector pME1105 harboring the *GCN4* deletion cassette. Then the His$^-$ phenotype was rescued by transformation with a linear *Aat*II-*Nae*I fragment containing the intact *HIS3* gene from vector pRS303 (24). Finally, the *Stu*I-linearized plasmids pME1112 and pME1108, respectively, were integrated at the *ura3-52* locus. Transformations were carried out using the lithium-acetate yeast transformation method (27). Correct integration or replacement of all manipulations was confirmed by Southern blot analysis (28).

Yeast cells were cultivated on minimal medium (29). Appropriate supplements were added in recommended amounts (30). Routinely precultures were grown overnight, diluted, and cultivated for another 12 h to an optical density of approximately 0.5 at 546 nm. Then 40 mM 3-aminotriazole was added to induce histidine starvation. After the appropriate growth period, cultures were divided and harvested for parallel measurement of β-galactosidase activity and protein or RNA isolation.

Northern Blot Analysis

Total RNA from yeast was isolated following the protocol described by Zitomer and Hall (31). 10 μg of total RNA from each sample was separated on a formaldehyde agarose gel, electroblotted onto a nylon membrane, and hybridized with ^{32}P-labeled probe prepared from a 560-bp polymerase chain reaction-generated *GCN4* fragment using the oligolabeling technique described by Feinberg and Vogelstein (32). A ^{32}P-labeled 540-bp *Cla*I fragment of *ACT1* was used as internal standard. Hybridizing signals were quantified using a BAS-1500 Phosphor-Imaging scanner (Fuji).

Western Blot Analysis and the Production of Anti-Gcn4p Antibodies

Yeast protein crude extracts for Western blot analysis were prepared as described (33). Protein contents were measured using a Bio-Rad protein assay kit according to Bradford (34). 25 μg of protein from crude cell extracts were separated on a 12% SDS-polyacrylamide gel and subsequently analyzed by Western blot analysis using polyclonal antibodies raised against the COOH terminus of Gcn4p. Pure Gcn4p (10 ng) expressed and isolated from *Escherichia coli* (9, 35) was loaded as a control. Immune complexes were visualized with an alkaline phosphatase ImmunoBlot assay kit (Bio-Rad). Gcn4p signals were quantified using the Molecular Analyst software from Bio-Rad. For calibration, a standard curve derived from measurements of defined amounts of pure Gcn4p was used.

Polyclonal anti-Gcn4p antibodies were raised in a rabbit against a synthetic peptide containing the 60 COOH-terminal amino acids of Gcn4p. The peptide was linked to keyhole limpet hemocyanin with

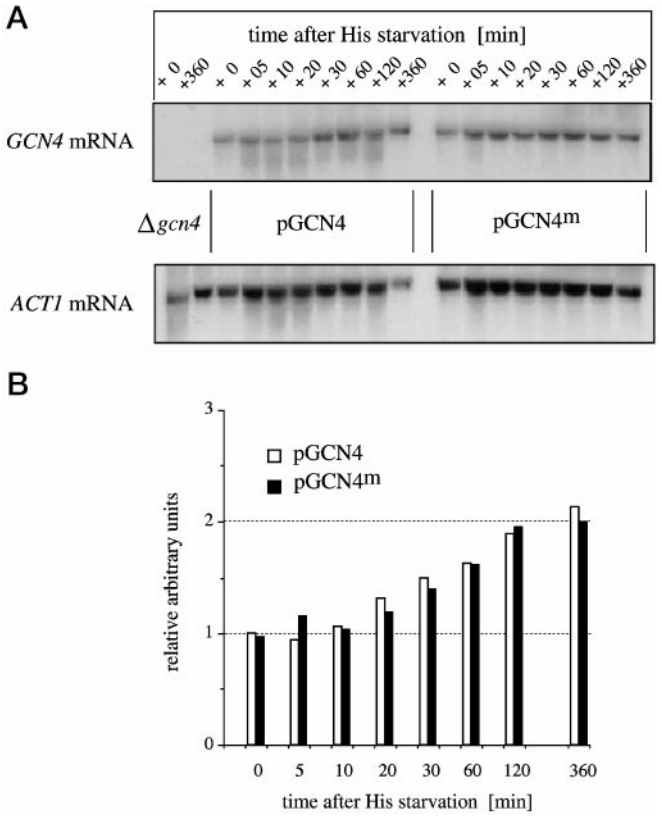

FIG. 1. **Kinetics of yeast *GCN4* steady state mRNA after shift to amino acid starvation conditions.** RNA was prepared from yeast strain RH1796 harboring either different *GCN4* alleles extrachromosomally on the centromeric vector pRS314 or containing vector pRS314 alone (Δ*gcn4*), respectively. *GCN4* alleles with intact (pGCN4) or mutated (pGCN4^m) short open reading frames in the *GCN4* leader region resulting in loss of translational control were assayed. The cultures were grown on minimal medium. Histidine (His) starvation was induced by adding 40 mM 3-AT at time 0. Samples were taken at the indicated time periods. *A,* example of a Northern blot autoradiography. 10 μg of total RNA from each sample were separated on an agarose-formaldehyde gel, blotted onto a membrane filter, then hybridized with a ^{32}P-radiolabeled *GCN4* probe and a radiolabeled *ACT1* probe as internal standard. *B,* quantifications of *GCN4* mRNA signals using a PhosphorImaging scanner. *Bars* (*white*, pGCN4; *black*, pGCN4^m) represent mean values of three independent measurements and were normalized with respect to *ACT1* levels and to the pGCN4 sample at time 0. Standard deviation was below 20%.

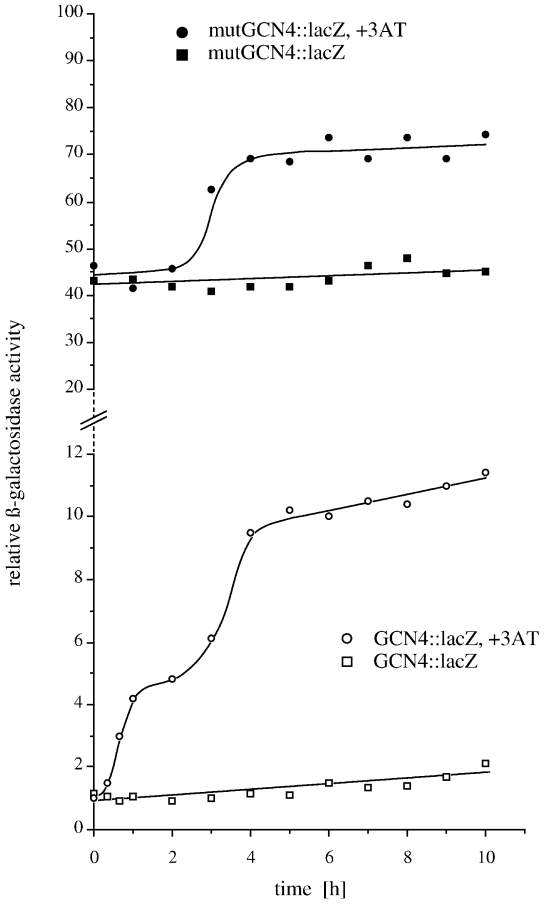

FIG. 2. **Kinetics of *GCN4::lacZ* fusions expressed in yeast after shift to 3-aminotriazole induced amino acid starvation conditions.** Wild-type (RH1415) transformants containing *GCN4::lacZ* alleles with either intact (*GCN4::lacZ*, *white*) or mutated four uORFs (*mutGCN4::lacZ*, *black*) were assayed for specific β-galactosidase activity. Transformants were cultivated in minimal medium (*quadrangle*) and minimal medium with 3-AT (*circle*). Values represent the average of at least three independent measurements and were normalized with respect to *GCN4::lacZ* at time 0 (before 3-AT addition). Standard errors were below 25%.

glutaraldehyde and injected subcutaneously after being mixed with an equal volume of incomplete Freud's adjuvant. After 5 weeks, additional injections (six times, once per week) served as boosts.

Gel Retardation Assay

The gel retardation assay using Gcn4 protein expressed in *E. coli* was described earlier (36). The DNA probe was a ^{32}P-end-labeled synthetic 49-bp *TRP4*-UAS1 DNA fragment (37). The *TRP4*-UAS1 fragment was obtained by annealing of two synthetic oligonucleotides identical to the *TRP4* promoter region −273 to −225 containing one GCRE site. 15 μg of protein extracts were incubated with 10 fmol of ^{32}P-radiolabeled probe, separated on a native 6% polyacylamide gel, and visualized by autoradiography. UAS1-Gcn4p signals were quantified using a BAS-1500 PhosphorImaging scanner (Fuji).

β-Galactosidase Assay

β-Galactosidase activities of permeabilized yeast cells were determined by using 4-methylumbelliferyl-β-D-galactoside as a fluorogenic substrate (38). Yeast cells were cultivated in minimal medium. After addition of 3-AT, specific β-galactosidase activities were assayed. Concentration of product formation during the reaction was determined based on a standard curve with commercial products (4-methylumbelliferone; Fluka, Buchs, Switzerland). Product concentrations were normalized to the reaction time and the optical density of the culture. One unit of β-galactosidase activity is defined as 1 nmol of 4-methylumbelliferone h^{-1} ml^{-1} OD_{546}^{-1}. The given values are means from measurements of at least five independent cultures. The standard errors of the means were below 25%.

RESULTS

Histidine Starvation by 3-Aminotriazole Leads to 2-Fold Increased GCN4 mRNA Transcript Levels—We performed a kinetic analysis of the steady state amount of *GCN4* mRNA after induction of the general control system by 3-AT. A yeast strain carrying a deletion of the chromosomal *GCN4*-locus (RH1796, Δ*gcn4* strain) was transformed with either the wild-type *GCN4* allele (pGCN4) or a mutated *GCN4* allele (pGCN4^m) on a centromer-based plasmid. The translational control of mRNA expression of the *GCN4* allele of pGCN4^m is abolished by point mutations in the start codons of the four uORFs (13). Histidine starvation was induced by adding 3-AT to exponentially growing cultures. Samples were taken at different time points and *GCN4* mRNA levels were subsequently analyzed by Northern blot analysis and quantified. *GCN4* expression from both alleles, wild-type *GCN4* as well as *GCN4*^{m}, showed identical kinetics after induction by 3-AT (Fig. 1). A 2-fold increase in *GCN4* mRNA steady state levels was reached after approximately 2 h and remained constant for at least 6 h.

Expression of *GCN4::lacZ* fusions were measured to distinguish between initiation of *GCN4* transcription and the stabil-

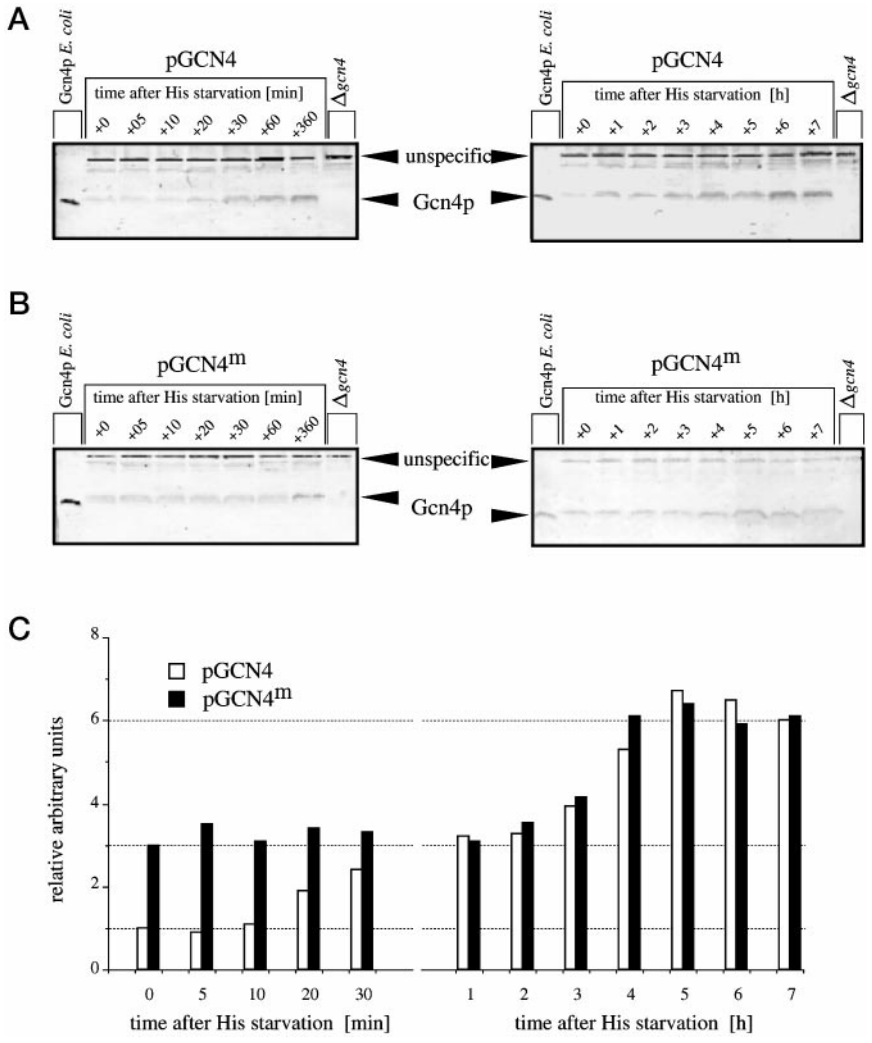

FIG. 3. **Kinetics of yeast Gcn4 protein level after shift to amino acid starvation conditions caused by 3-aminotriazole.** Yeast protein crude extracts were prepared from strain RH1796 containing pGCN4 (*A*), pGCN4^m (*B*), or the empty vector pRS314 (Δgcn4), respectively. Samples were taken before (+0) and after histidine (His) starvation induced with 40 mM 3-AT at the indicated periods of time. 25 μg of protein from each sample of yeast protein crude extracts were separated on a 12% SDS-polyacrylamide gel and subsequently analyzed by Western blot analysis using anti-Gcn4p-antiserum. Homogeneous Gcn4 protein purified from *E. coli* was loaded as control. Various amounts of this protein were used to calibrate the quantification of the immunoblots signals in the described experiments. Gcn4 protein migrates at an apparent molecular mass of 45 kDa and is indicated by a *black arrow*. The unknown protein marked as "unspecific" was used as an internal standard. *C*, Gcn4p levels were quantified using the Molecular Analyst software from Bio-Rad. *Bars* (*white*, pGCN4; *black*, pGCN4^m) represent mean values of three independent measurements and were normalized with respect to the pGCN4 sample at time 0. Standard deviation did not exceed 25%.

ity of *GCN4* mRNA. A wild-type strain (RH1415) containing a *GCN4*::*lacZ* allele with point mutations in the AUG codons of the four uORFs (plasmid p227 (25)) was grown in the absence and presence of 3-AT. Kinetic analysis of the specific β-galactosidase enzyme activity showed an approximately 2-fold increase after 3 h of histidine starvation (Fig. 2). These results suggest that amino acid starvation induced by 3-AT results in an increased *GCN4* mRNA steady state level due to initiation of transcription and not due to increased *GCN4* mRNA stability.

Gcn4 Protein Levels Increase in Two Steps after Histidine Starvation—The changes of Gcn4 protein levels in yeast cells after the switch to histidine starvation conditions were analyzed in Western blot hybridization experiments. Crude protein extracts were prepared from cells cultivated for different time periods after addition of 3-AT to the medium. Gcn4 protein levels were assayed using a polyclonal antiserum raised against the DNA-binding domain of Gcn4p. In a strain harboring the wild-type *GCN4* allele, a biphasic increase in Gcn4p after addition of 3-AT was observed (Fig. 3, *A* and *C*). Elevation of Gcn4 protein levels started after 20 min and increased by a factor of 3 during the first hour. Afterward, Gcn4 protein levels remained constant for 2 h. Three to four hours after induction an additional 2-fold increase was observed leading to a 6-fold total increase in the amount of Gcn4p after the initial induction by 3-AT.

Expression of a *GCN4*::*lacZ* fusion construct (plasmid p180 (12)) after histidine starvation corroborated the biphasic increase in Gcn4p. A biphasic increase in the specific β-galactosidase activity was induced by 3-AT, suggesting that one step correlated to translational derepression and a second step comprises increased transcriptional initiation (Fig. 2). However, the obtained biphasic increase in the specific β-galactosidase activity of the *GCN4*::*lacZ* fusion correlate in time but not in value with the results obtained by Western blot analysis of Gcn4p shown before. One reason might be an increased stability of the chimeric Gcn4-β-galactosidase fusion protein in comparison to the native Gcn4 protein.

To discriminate between transcriptional and translational control, we performed kinetic measurements of Gcn4 protein levels in a strain containing the *GCN4*^{m} allele that is no longer translationally regulated. The basal level of Gcn4 protein in these cells corresponds to the expression after the first elevation phase of the wild-type allele induced with 3-AT (Fig. 3, *B* and *C*). The Gcn4 protein level was as high as in the wild-type after 1 h of starvation. However, mutant cells exhibited only one elevation step in Gcn4 protein levels after 3–4 h under starvation conditions which was similar in time and value to the second increase in Gcn4 protein levels in wild-type cells, indicating that the second elevation phase is independent of the translational control by the four uORFs.

In summary, our data suggest that amino acid starvation by 3-AT results in a first quick response due to translational control of already synthesized *GCN4* mRNA, but there is a second slow response which might be due to increased transcriptional initiation at the *GCN4* promoter.

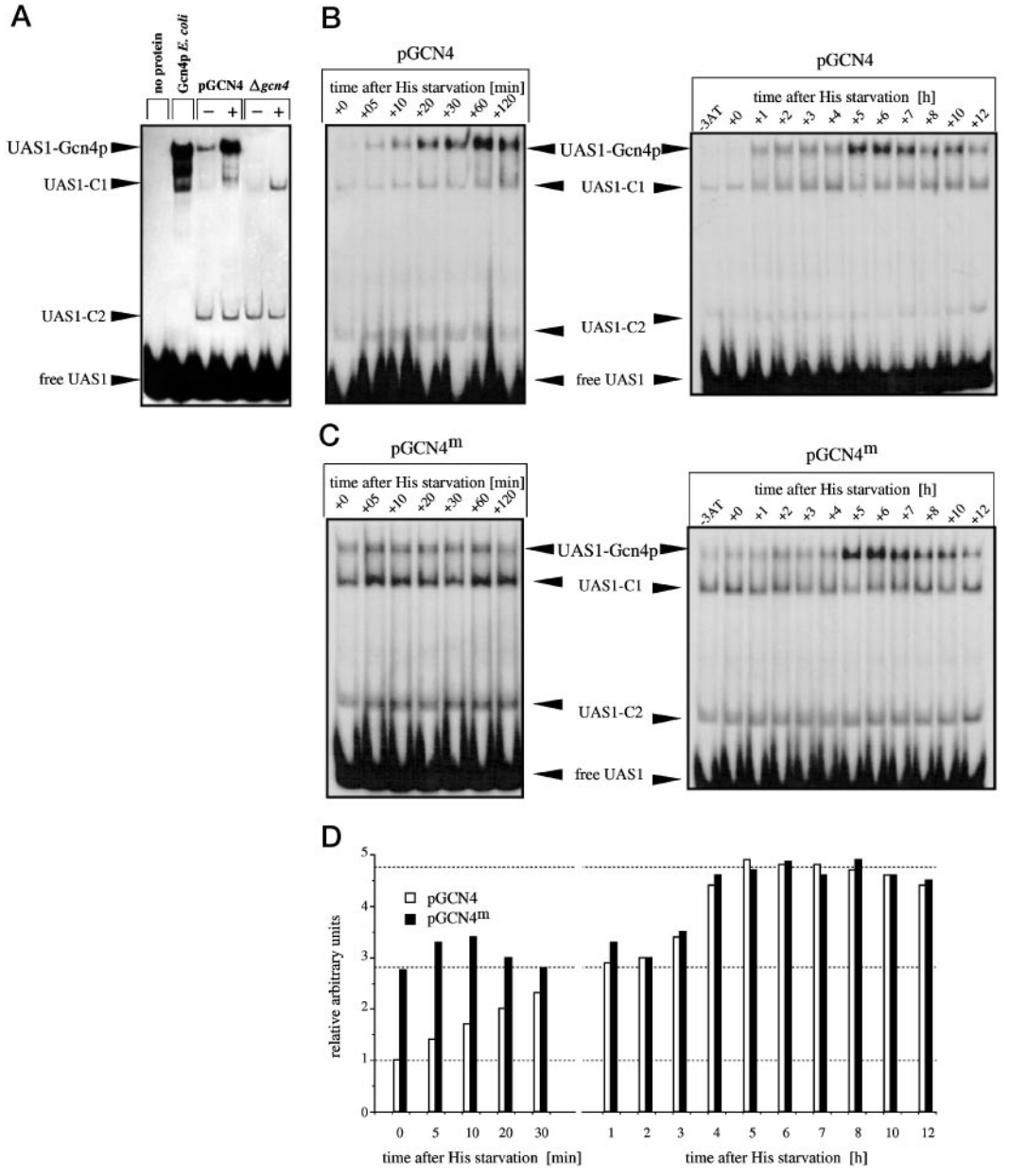

FIG. 4. **Kinetics of yeast Gcn4p DNA binding activity after shift to 3-AT induced amino acid starvation conditions.** *A*, strain RH1796 transformed with pGCN4 or the empty vector pRS314 (Δgcn4), respectively, was cultivated in minimal medium (−) and 8 h in minimal medium with 40 mM 3-AT (+). Yeast crude protein extracts were incubated with a ^{32}P-radiolabeled synthetic 49-bp *TRP4*-UAS1 DNA fragment, separated on a native 6% polyacrylamide gel and the signals were visualized by autoradiography. No added protein and partially purified Gcn4p heterologously expressed in *E. coli* were used as control. Specific Gcn4 protein-DNA complexes are indicated as UAS1-Gcn4p, and unbound DNA as free UAS1. Two unknown protein-UAS1 complexes are marked as UAS1-C1 and UAS1-C2. *B*, an example of an autoradiography of the wild-type *GCN4* allele (pGCN4) after histidine (His) starvation. The protein samples were taken after induction with 40 mM 3-AT at the indicated time. *C*, an example of an autoradiography of the *GCN4* allele lacking the translational control (pGCN4^m) after histidine (His) starvation. The protein samples were taken after induction with 40 mM 3-AT at the indicated time. *D*, quantifications of Gcn4p DNA binding activity using a PhosphorImaging scanner. *Bars* (*white*, pGCN4; *black*, pGCN4^m) represent mean values of at least five independent measurements and were normalized with respect to the pGCN4 sample at time 0. The *dotted line* at 2.9 represent the average value of the samples of pGCN4^m from 0 to 120 min. The average of all samples from 5 to 8 h is represented by a *dotted line* at 4.8. Standard deviation did not exceed 25%.

Gcn4p DNA Binding Activity Correlates with Changes of Gcn4p Levels—The increased amount of Gcn4 protein upon amino acid starvation prompted us to analyze whether increased protein levels also correlate with increased functionality. As a first function of the transcriptional activator Gcn4p we analyzed its ability to bind to its specific DNA-binding site. Therefore, we established a gel retardation assay using crude protein extracts. Yeast cells were grown 8 h on minimal medium in the presence or absence of 3-AT. Crude extracts including Gcn4p expressed from pGCN4 were tested for specific binding to a synthetic DNA fragment that contains a Gcn4p responsive element originally derived from the *TRP4* promoter (37). Induction of the general control system by 3-AT resulted in an increased intensity of the retarded band, suggesting an increased DNA binding activity in yeast crude cell extracts. The increased DNA binding activity correlated with the increased amount of Gcn4 protein in the cell as described above, suggesting that the affinity of the protein to its DNA was unaffected and only the amount of DNA-binding protein increased (Fig. 4*A*).

The time course of the elevation of Gcn4p DNA binding ability was analyzed in the same crude protein extracts as

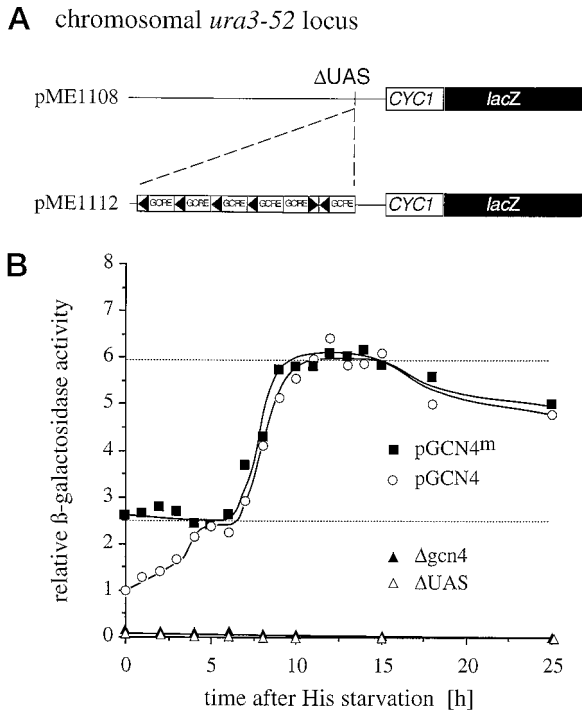

FIG. 5. **Monitoring of Gcn4 protein-dependent trans-activation rate.** *A, CYC1::lacZ* gene fusions for reporting Gcn4p trans-activation rate. pME1112 contains six synthetic Gcn4p responsive elements in tandem orientation (indicated by *GCRE* and *black arrows*) replacing the UAS element of the *CYC1* gene. pME1108 is the parental construction lacking the UAS element (indicated by Δ*UAS*). Reporter genes were stably integrated at the chromosomal *ura3–52* locus in the desired strains RH1796 (*GCRE6::lacZ*) and RH1798 (Δ*UAS::lacZ*), respectively. *B,* measurement of specific Gcn4 protein-dependent β-galactosidase activity of the synthetic GCRE::*lacZ* fusion gene. Transformants of yeast strain RH1796 containing pGCN4 (*white circle*), pGCN4^m (*black triangle*), or vector pRS314 alone (Δgcn4, *gray quadrangle*), respectively, were assayed after addition of 40 mM 3-AT. As a further control RH1798 containing pGCN4 (*white triangle*) was also assayed. Values represent the average of at least five independent measurements and were normalized with respect to pGCN4 at time 0 (before 3-AT addition). Standard errors were below 20%.

examined by Western blot analysis. The increase in gel retardation activity also proceeded in two steps when Gcn4 protein was expressed from pGCN4 (Fig. 4, *B* and *D*). Within the first hour Gcn4 protein able to bind DNA increased by a factor of 3 and remained constant up to 3 h. After 4 h of starvation a second maximum of 5-fold total increase was reached. This biphasic increase correlated both in time and in value with the elevation of protein levels analyzed by Western blot experiments, suggesting that the ability of Gcn4p to bind to DNA correlates to the amount of protein available in the cell and is not further regulated under these conditions.

Gel retardation analysis of crude protein extracts prepared from a yeast strain lacking the translational control of *GCN4* gene expression showed only one elevation phase of Gcn4 protein bound to DNA in the assay. The level of Gcn4 protein able to bind DNA *in vitro* was approximately constant during the first 3 h after addition of 3-AT (Fig. 4*C*). Nevertheless, the Gcn4p DNA binding level was as high as in the case of pGCN4 after 1 h of starvation (Fig. 4*D*). An elevation could only be observed after 4 h of histidine starvation. This increase corresponds to the second increase as observed for pGCN4. Both elevation steps occurred 4 h after addition of 3-AT by the same value. This suggests the existence of an additional regulation mechanism of *GCN4* gene expression beyond the translational control dependent on the four uORFs in the *GCN4* leader region.

A General Control Regulated Reporter Gene Responds in Two Equivalent Phases to the Amino Acid Starvation Signal—We wanted to analyze whether the two-step increase of the Gcn4 protein after 3-AT induced histidine starvation is also reflected in the induction of a general control regulated target gene. To make sure that even small changes in the activation of the Gcn4p regulated gene can be detected, we constructed an artificial general control reporter gene based on a *CYC1*::*lacZ* fusion construct (19). The promotor region of the *CYC1*::*lacZ* gene was replaced by six Gcn4p responsive elements resulting in a highly sensitive general control regulated target gene. This construct was stably integrated into yeast strain RH1796 at its *ura3–52* locus. The consequences of the observed biphasic increase of the Gcn4p levels were monitored by the transactivation ability of Gcn4 protein using this Gcn4p regulated *CYC1*::*lacZ*-reporter gene (Fig. 5*A*).

Quantification of the Gcn4p dependent β-galactosidase activity in a kinetic induction experiment showed that the response to histidine starvation also occurs in two steps (Fig. 5*B*). In a yeast strain containing the wild-type *GCN4* allele, a first phase of increased *CYC1*::*lacZ* expression immediately started after addition of 3-AT. Induction by a factor of 2.5 was reached after 4 h and did not change up for to 6 h. After 6 h of histidine starvation, however, an additional 6-fold increase with respect to the basal level, that lasted until 8 h after induction, was measured.

The basal level of the *lacZ*-reporter gene in uninduced yeast cells carrying pGCN4^m was as high as in a strain harboring wild-type *GCN4* after 4 h of starvation. This is in agreement with a constant derepression of the translational control of *GCN4*. Accordingly, the specific β-galactosidase activity was constant during 4 h after addition of 3-AT and only a single elevation step of the *lacZ*-reporter gene was observed. This increase of β-galactosidase activity started 6 h after starvation, reaching a maximal level of induction after 8 h.

DISCUSSION

We examined the time course of the Gcn4 protein elevation of yeast cells after switch to amino acid starvation cultivation medium induced by 3-aminotriazole. *GCN4* mRNA is already present in the cell under nonstarvation conditions, but only under starvation conditions does efficient translation occur (39). In this report, we find that amino acid starvation induced by 3-AT results in a biphasic response of the cell. A first step of increased *GCN4* expression is caused by translational control and depends on the four uORFs of the *GCN4* leader region. As a consequence, more Gcn4 protein is produced within 20 min after histidine starvation. In addition, *GCN4* mRNA levels increase and subsequently a second elevation phase of Gcn4 protein levels can be observed 4–6 h after induction. Accordingly, expression of a Gcn4p-dependent *CYC1*::*lacZ* reporter gene also responds in two phases. Cells with a nonfunctional translational control of *GCN4* show only the second phase of response under the same conditions.

The elevation of *GCN4* transcript levels after induction of the general control system raises the question of whether this increase is due to changes in stability of the *GCN4* mRNA or whether it is due to an increased transcription of *GCN4*. The *GCN4*::*lacZ* fusion constructs suggest that histidine starvation rather affects transcription initiation of *GCN4* than the stability of the mRNA. It has been shown that the gene expression of the Gcn4p homologues cpcAp of *Aspergillus niger* and CPC1 protein of *Neurospora crassa* are regulated at a transcriptional level (40, 41). However, transcriptional regulators of *GCN4* are not known yet. In *S. cerevisiae*, premature translation termination promotes rapid degradation of numerous mRNAs (42). It was speculated that the four uORFs preceding the *GCN4* cod-

ing region at which translational initiation and reinitiation events occur would function in a manner analogous to nonsense codons, promoting rapid degradation of the mRNA (43). However, previous studies demonstrated that the *GCN4* transcript is not degraded by the nonsense-mediated mRNA decay pathway and reported a half-life of 16–18 min of *GCN4* mRNA (44). Whereas it cannot be excluded, that there is regulated *GCN4* mRNA degradation in yeast, we do not think that this is important under the conditions we have tested here.

The DNA binding activity and the ability to activate transcription of target genes both correlate with the amount of Gcn4 protein in the cell. However, there are quantitative differences between the ratio of Gcn4p levels and of β-galactosidase activities of corresponding *GCN4*::*lacZ* fusion constructs during the amino acid starvation kinetic experiments. This is presumably due to differences in protein stability compared with the native Gcn4 protein and the chimeric protein encoded by the *GCN4*::*lacZ* fusion constructs. The half-life of β-galactosidase expressed in *S. cerevisiae* is over 20 h (45). It is also known that the half-lives of chimeric β-galactosidase fusion proteins can possibly range between 3 min and over 20 h, depending on the nature of the amino acids at the amino terminus of the protein (45). The used *GCN4*::*lacZ* fusion constructs include only 55 amino acids of the amino terminus of Gcn4p excluding the reported instability region of Gcn4p (17). Thus, it is likely that the chimeric protein encoded by the *GCN4*::*lacZ* fusion lacking the translational control accumulates also under nonstarvation conditions. In contrast, Gcn4 protein under nonstarvation conditions is highly unstable with a reported half-life of 5 min (17). Therefore, stabilization of Gcn4 protein plays an additional role under certain circumstances (17).

The observed results also raise the question of how a 6-fold increase in Gcn4p levels in the cell is sufficient to elevate expression of at least 40 genes 2–5-fold. One possibility would be an improved DNA binding ability of Gcn4p under starvation conditions as found for the human oncoprotein c-Jun that displays a regulated DNA binding activity (46). It was shown that the DNA-binding domains of c-Jun and Gcn4p are functionally homologous (47). Phosphorylation of distinct sites near the DNA-binding domain inhibits DNA-binding and dephosphorylation correlates with increased transactivation activity of c-Jun (46). In contrast, Gcn4 protein levels and its ability to bind DNA *in vitro* correlate in time and value, suggesting that there is no such post-translational regulation of Gcn4p. Therefore, we conclude that the DNA binding ability is not affected under the tested conditions. Another possibility could be a regulated nuclear localization of Gcn4p. Such a regulation is shown for the yAP-1 protein (48). The corresponding *YAP1* gene encodes another member of the leucine zipper family and recognizes a Gcn4p DNA-binding sequence motif (49). Upon imposition of oxidative stress, a small increase in the DNA binding capacity of yAP-1 protein occurs (48). However, the major change is at the level of nuclear localization; upon induction the yAP-1 protein relocalizes from the cytoplasm to the nucleus (48). It remains to be elucidated whether a similar transport regulation mechanism will be found for Gcn4p.

In summary, our results suggest the existence of an additional mechanism to control Gcn4p levels upon amino acid limitation in yeast. So far, translational control via the uORFs in *GCN4* has been found to be the major mechanism to regulate *GCN4* expression. This report shows that upon prolonged amino acid starvation Gcn4p levels can be increased by a mechanism independent of the uORFs. Whether this mechanism includes elements of the known general control system or consists of as yet novel factors remains to be elucidated in the future.

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REFERENCES

1. Meussdoerffer, F., and Fink, G. R. (1983) *J. Biol. Chem.* **258,** 6293–6299
2. Mirande, M., and Waller, J.-P. (1988) *J. Biol. Chem.* **263,** 18443–18451
3. Mösch, H.-U., Scheier, B., Lahti, R., Mäntsälä, P., and Braus, G. H. (1991) *J. Biol. Chem.* **266,** 20453–20456
4. Hinnebusch, A. G. (1992) in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression* (Broach, J. R., Jones, E. W., and Pringle, J. R., eds) pp. 319–414, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
5. Klopotowski, T., and Wiater, A. (1965) *Arch. Biochem. Biophys.* **112,** 562–566
6. Wolfner, M., Yep, D., Messenguy, F., and Fink, G. R. (1975) *J. Mol. Biol.* **96,** 273–290
7. Ellenberger, T. E., Brandl, C. J., Struhl, K., and Harrison, S. C. (1992) *Cell* **71,** 1223–1237
8. Hope, I. A., and Struhl, K. (1986) *Cell* **46,** 885–894
9. Oliphant, A. R., Brandl, C. J., and Struhl, K. (1989) *Mol. Cell. Biol.* **9,** 2944–2949
10. Hinnebusch, A. G. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6442–6446
11. Thireos, G., Driscoll Penn, M., and Greer, H. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 5096–5100
12. Hinnebusch, A. G. (1985) *Mol. Cell. Biol.* **5,** 2349–2360
13. Mueller, P. P., Harashima, S., and Hinnebusch, A. G. (1986) *Cell* **45,** 201–207
14. Hinnebusch, A. G. (1997) *J. Biol. Chem.* **272,** 21661–21664
15. Lanker, S., Bushman, J. L., Hinnebusch, A. G., Trachsel, H., and Mueller, P. P. (1992) *Cell* **70,** 647–657
16. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992) *Cell* **68,** 585–596
17. Kornitzer, D., Raboy, B., Kulka, R. G., and Fink, G. R. (1994) *EMBO J.* **13,** 6021–6030
18. Sengstag, C., and Hinnen, A. (1988) *Gene* (*Amst.*) **67,** 223–228
19. Guarente, L., Yocum, R. R., and Gifford, P. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 7410–7414
20. Hope, I. A., and Struhl, K. (1985) *Cell* **43,** 177–188
21. Tabor, S., and Richardson, C. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 4767–4771
22. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
23. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) *Yeast* **2,** 163–167
24. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
25. Mueller, P. P., and Hinnebusch, A. G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2863–2867
26. Berben, G., Legrain, M., and Hilger, F. (1988) *Gene* **66,** 307–312
27. Ito, H., Jukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **134,** 48–59
28. Southern, E. M. (1975) *J. Mol. Biol.* **98,** 503–517
29. Miozzari, G., Niederberger, P., and Hütter, R. (1978) *J. Bacteriol.* **134,** 48–59
30. Rose, M. D., Winston, F., and Hieter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Zitomer, R. S., and Hall, B. D. (1976) *J. Biol. Chem.* **251,** 6320–6326
32. Feinberg, A. P., and Vogelstein, B. (1984) *Anal. Biochem.* **137,** 266–267
33. Arndt, K., Styles, C. A., and Fink, G. R. (1987) *Science* **237,** 874–880
34. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
35. Arndt, K., and Fink, G. R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 8516–8520
36. Braus, G., Mösch, H.-U., Vogel, K., Hinnen, A., and Hütter, R. (1989) *EMBO J.* **8,** 939–945
37. Mösch, H. U., Graf, R., Schmidheini, T., and Braus, G. (1990) *EMBO J.* **9,** 2951–2957
38. Künzler, M., Balmelli, T., Egli, C. M., Paravicini, G., and Braus, G. H. (1993) *J. Bacteriol.* **175,** 5548–5558
39. Abastado, J.-P., Miller, P. F., Jackson, B. M., and Hinnebusch, A. G. (1991) *Mol. Cell. Biol.* **11,** 486–496
40. Wanke, C., Eckert, S., Albrecht, G., van Hartingsveldt, W., Punt, P. J., van den Hondel, C. A. M. J. J., and Braus, G. H. (1997) *Mol. Microbiol.* **23,** 23–33
41. Paluh, J. L., Orbach, M. J., Legerton, T. L., and Yanofsky, C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3728–3732
42. Jacobson, A., and Peltz, S. W. (1996) *Annu. Rev. Biochem.* **65,** 693–739
43. Peltz, S. W., He, F., Welch, E., and Jacobson, A. (1994) *Prog. Nucleic Acid Res. Mol. Biol.* **47,** 271–298
44. Ruiz-Echevarria, M. J., and Peltz, S. W. (1996) *EMBO J.* **15,** 2810–2819
45. Bachmair, A., Filey, D., and Varshavsky, A. (1986) *Science* **234,** 179–186
46. Lin, A., Frost, J., Deng, T., Smeal, T., Al-Alawi, N., Kikkawa, U., Hunter, T., Brenner, D., and Karin, M. (1992) *Cell* **70,** 777–789
47. Struhl, K. (1987) *Cell* **50,** 841–846
48. Kuge, S., Jones, N., and Nomoto, A. (1997) *EMBO J.* **16,** 1710–1720
49. Moye-Rowley, W. S., Harshman, K. D., and Parker, C. S. (1989) *Genes Dev.* **3,** 283–292