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Repression of *GCN4* mRNA Translation by Nitrogen Starvation in *Saccharomyces cerevisiae**

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Saccharomyces cerevisiae **activates a regulatory network called "general control" that provides the cell with sufficient amounts of protein precursors during amino acid starvation. We investigated how starvation for nitrogen affects the general control regulatory system, because amino acid biosynthesis is part of nitrogen metabolism. Amino acid limitation results in the synthesis of the central transcription factor Gcn4p, which binds to specific DNA-binding motif sequences called Gcn4-protein-responsive elements (GCREs) that are present in the promoter regions of its target genes. Nitrogen starvation increases *GCN4* transcription but efficiently represses expression of both a synthetic *GCRE6::lacZ* reporter gene and the natural amino acid biosynthetic gene *ARO4*. Repression of Gcn4p-regulated transcription by nitrogen starvation is independent of the ammonium sensing systems that include Mep2p and Gpa2p or Ure2p and Gln3p but depends on the four upstream open reading frames in the *GCN4* mRNA leader sequence. Efficient translation of *GCN4* mRNA is completely blocked by nitrogen starvation, even when cells are simultaneously starved for amino acids and eukaryotic initiation factor-2α is fully phosphorylated by Gcn2p. Our data suggest that nitrogen starvation regulates translation of *GCN4* by a novel mechanism that involves the four upstream open reading frames but that still acts independently of eukaryotic initiation factor-2α phosphorylation by Gcn2p.**

In bakers' yeast, *Saccharomyces cerevisiae,* starvation for a single amino acid induces the transcription of more than 50 genes encoding enzymes involved in several amino acid biosynthetic pathways, amino acid tRNA synthetases (1, 2), or enzymes of purine biosynthesis (3). This genetic system is called "general control of amino acid biosynthesis" (4). Amino acid limitation results in the synthesis of the transcription factor Gcn4p (5), which binds to specific Gcn4-protein-responsive elements (GCREs)[1] present in the promoter regions of its target genes. Gcn4p stimulates transcription of its target genes by a factor of 2–10 (6). Whereas some target genes are regulated via several GCREs in their promoter regions like *HIS3* or *TRP4*, for example (7–10), others contain only a single GCRE site that is both essential and sufficient for regulation by Gcn4p as found for the *ARO4* gene (11).

The best understood regulatory mechanism of Gcn4p expression is translational control of its mRNA, which requires four small upstream open reading frames (uORFs) present in the *GCN4* 5′-untranslated region (12, 13). When cells are growing under non-starvation conditions, ribosomes translate the first uORF, reinitiate at uORFs 2–4, and are unable to recognize the *GCN4* start codon (14). In cells starved for amino acids, ribosomes that have translated the first uORF reinitiate at the *GCN4* translational start site leading to high expression of Gcn4p. Inactivation of the four uORFs in the GCN4 leader by either deletion or mutation of the four ATG start codons uncouples translational control of *GCN4* from the general control system, leading to high expression of Gcn4p already under non-starvation conditions (15). Translational regulation of *GCN4* depends on the sensor kinase Gcn2p, which includes an N-terminal protein kinase domain and a C-terminal aminoacyl tRNA synthetase-like domain (16, 17). Under amino acid starvation conditions, Gcn2p detects uncharged tRNAs and in response phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF-2). Phosphorylated eIF-2 inhibits translation of most mRNAs mediated by the inhibition of the guanine nucleotide exchange activity of eIF-2B. This allows ribosomes to scan past the remaining uORFs in the *GCN4* 5′-untranslated region and translate the mRNA of the *GCN4* gene (18, 19).

Amino acid biosynthesis is part of the general nitrogen metabolism of yeast cells. Ammonium is among the inorganic nitrogen sources in nature that support optimal growth of yeast cells (20). Three ammonium permeases, Mep1p, Mep2p, and Mep3p, are known that control uptake of ammonium into the cytoplasm (21). Deletion of all three *MEP* genes renders yeast cells inviable on media containing less than 5 mM ammonium sulfate as the sole nitrogen source. In addition to its functions as ammonium permease, Mep2p is thought to act as an ammonium sensor protein in a signaling system that controls pseudohyphal development of diploid Σ*1278b* strains, because diploid *mep2*Δ mutant strains are unable to form pseudohyphae in response to nitrogen starvation. Pseudohyphal development of *mep2*Δ mutants can be restored by expression of dominant activated forms of Gpa2p or Ras2p, GTP-binding proteins that both regulate intracellular cAMP levels (22, 23). These studies have led to the model that Mep2p is a central ammonium sensor protein that activates Gpa2p and cAMP-dependent protein kinase in response to nitrogen starvation. The proteins Ure2p and Gln3p are two additional important regulators in the ammonium utilization pathway and are required under all conditions (24).

As amino acid biosynthesis is part of the general nitrogen metabolism in yeast, we investigated how starvation for nitrogen affects the general control system of amino acids. We found

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[1] The abbreviations used are: GCREs, Gcn4-protein-responsive elements; uORFs, upstream open reading frames; eIF-2, eukaryotic initiation factor 2; 3AT, 3-aminotriazole.

TABLE I
Yeast strains used

Strain	Genotype	Source
RH2396	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG*	This study
RH2397	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 leu2::hisG/leu2::hisG his3::hisG/HIS3 trp1::hisG/trp1::hisG gcn2Δ::LEU2/gcn2Δ::LEU2*	This study
RH2398	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 leu2::hisG/leu2::hisG his3::hisG/HIS3 trp1::hisG/trp1::hisG gcn4Δ::LEU2/gcn4Δ::LEU2*	This study
RH2399	*MAT***a**/α *ura3–52/ura3–52 trp1::hisG/trp1::hisG*	This study
RH2400	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 leu2::hisG/LEU2 his3::hisG/his3::hisG trp1::hisG/trp1::hisG ras2Δ::ura3::HIS3/ras2Δ::URA3*	This study
RH2401	*MAT***a**/α *ura3–52/ura3–52 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG gcn2Δ::LEU2/gcn2Δ::LEU2*	This study
RH2402	*MAT***a**/α *ura3–52/ura3–52 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG gcn4Δ::LEU2/gcn4Δ::LEU2*	This study
RH2403	*MAT***a**/α *ura3–52/ura3–52 leu2::hisG/leu2::hisG trp1::hisG/trp1::hisG gcn4Δ::LEU2/gcn4Δ::LEU2 gcn2Δ::kanR/gcn2Δ::kanR*	This study
RH2404	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG gpa2Δ::kanR/gpa2Δ::kanR*	This study
RH2405	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 his3::hisG/his3::hisG leu2::hisG/leu2::hisG mep1Δ::HIS3/mep1Δ::LEU2*	This study
RH2406	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 his3::hisG/his3::hisG leu2::hisG/leu2::hisG mep2Δ::HIS3/mep2Δ::LEU2*	This study
RH2407	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3* (*S288c*-background)	This study
RH2445	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 his3::hisG/his3::hisG leu2::hisG/leu2::hisG mep1Δ::HIS3/mep1Δ::HIS3 mep2Δ::LEU2/mep2Δ::LEU2*	This study
RH2446	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 ADE2/ade2–101 TRP1/trp1-Δ901 SUC2/suc2-Δ9 LEU2/leu2–3 leu2–112 gcn4Δ/gcn4–101* (*S288c*-background)	This study
RH2485	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG aro3Δ::kanR/aro3Δ::kanR*	This study
RH2486	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG aro3Δ::kanR/aro3Δ::kanR ARO4m/ARO4m*	This study
RH2489	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG ure2Δ::kanR/ure2Δ::kanR*	This study
RH2490	*MAT***a**/α *ura3–52/ura3–52::GCRE6::lacZ::URA3 his3::hisG/HIS3 trp1::hisG/trp1::hisG gln3Δ::kanR/gln3Δ::kanR*	This study
GP3153	*MAT***a** *ura3–52 leu2–3 leu2–112 trp1-Δ63 gcn3Δ::LEU2* (*S288c*-background)	26
RH2520	*MAT***a** *ura3–52* (*S288c*-background)	This study

that deprivation of a suitable nitrogen source efficiently counteracts the activation of Gcn4p-controlled gene expression. Expression of a synthetic *GCRE6*::*lacZ* reporter gene and natural amino acid biosynthetic genes such as *ARO4* is suppressed when cells are starved for nitrogen, whereas starvation for glucose has only minor effects on Gcn4p-controlled gene expression. We find *GCRE*-mediated repression although the mRNA level of *GCN4* strongly increases, suggesting that nitrogen starvation specifically suppresses translation of *GCN4*. The four intact uORFs in the *GCN4* mRNA leader sequence are necessary components for the repression mechanism, whereas eIF-2α phosphorylation, the ammonium sensing system that includes Mep2p or Gpa2p, and the proteins Ure2p or Gln3p are not required.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—All strains used in this study are derivatives of the *S. cerevisiae* strain background Σ*1278b* with exception of GP3153, RH2407, RH2446, and RH2520, which derive from the *S288c* background (Table I). Deletion mutants for *GCN2* (*gcn2*Δ) were obtained by using the *gcn2*Δ deletion plasmids pME1658 or pME1659, respectively (Table II). Plasmids pME1105 and pME1660 were used for constructing *gcn4*Δ and *gpa2*Δ mutant strains. *Aro3*Δ and *aro4*Δ mutants were obtained by using plasmids pME1756 and pME1757. *Aro4*Δ strains were transformed with a linear fragment carrying the *ARO4m* mutant gene that was excised from plasmid pME643 to construct *ARO4m* strains. *Mep1*Δ and the *mep2*Δ mutant strains were gifts from G. R. Fink (Whitehead Institute, Cambridge, MA). The plasmids pME1911 and pME1912 were used to delete the genes *URE2* and *GLN3*, respectively. *Ras2*Δ mutants were constructed using plasmid pras2Δ::HIS3 (25). A *gcn3*Δ strain, GP3153 (26), was kindly provided by A. G. Hinnebusch (NICHD, National Institutes of Health, Bethesda). The *GCRE6*::*lacZ*::*URA3* reporter gene cassette was introduced by transformation with the integrative *GCRE6*::*lacZ* reporter construct pME1112. Transformations were carried out using the lithium-acetate yeast transformation method (27). All gene deletions, integrations, or replacements were confirmed by Southern blot analysis (28). For log phase measurements (non-starvation conditions), strains were cultivated in liquid synthetic minimal medium (SD) overnight at 30 °C, diluted, and cultivated for 8 h before assaying enzymatic activities or isolation of total RNA. For amino acid starvation, 3-aminotriazole (3AT) was added to cultures grown to mid-log phase to a final concentration of 10 mM, and cells were further incubated for 6–8 h before all assays. For nitrogen starvation, cells grown to mid-log phase were washed with 2% glucose and incubated for 24 h in liquid SD medium containing only 50 μM ammonium sulfate (instead of 50 mM) as the sole nitrogen source. Carbon starvation was measured by incubation in medium with only 0.05% glucose for 8 h.

Plasmids—All plasmids used in this study are listed in Table II. The kanamycin-resistant cassette (*kan*^{r}) was amplified by polymerase chain reaction from plasmid pUG6 (29) using the two primers UG6-1 (5′-CGCGGATCCGAACGCGGCCGCCAGCTGAAGC-3′) and UG6-2 (5′-CGCGGATCCCGCATAGGCCACTAGTGGATCTG-3′) and subsequent insertion of the polymerase chain reaction product into plasmid pBluescriptKS$^+$ (Stratagene) to obtain plasmid pME1765. Deletion cassettes for *GCN2* were created by replacement of *GCN2* coding sequences by either *LEU2* (pME1658) or *kan*^{r} (pME1659) as selectable markers. To obtain plasmids pME1660, pME1756, pME1911, or pME1912, *GPA2*, *ARO3*, *URE2,* or *GLN3* open reading frames were replaced by the *kan*^{r} gene as selectable markers. Plasmid pME2157 was constructed by homologous recombination in yeast using p180 and a linear *ura3*::*TRP1 URA3*-disruption cassette (provided by Yona Kassir, Technion, Haifa, Israel).

Northern Hybridization Analysis—Total RNA from yeast was isolated following the protocol described by Cross and Tinkelenberg (30). RNA was separated on 1.4% agarose gel containing 3% formaldehyde and transferred onto nylon membranes by electroblotting. Gene-specific probes were ^{32}P-radiolabeled with the MBI Fermentas HexaLable™ DNA Labeling Kit. Hybridizing signals were quantified using a BAS-1500 PhosphorImaging scanner (Fuji).

β-Galactosidase Assay—Assays were performed with extracts of cultures grown on liquid media. Specific β-galactosidase activity was normalized to the total protein (31) in each extract and equalized (A_{415} × 1.7)/(0.0045 × protein concentration × extract volume × time) (32). Assays were performed for at least three independent transformants,

TABLE II
Plasmids used

Plasmid	Description	Source
pME1105	Cassette for full deletion of *GCN4*-open reading frame (*LEU2*)	34
pME1658	Cassette for full deletion of *GCN2*-open reading frame (*LEU2*)	This study
pME1659	Cassette for full deletion of *GCN2*-open reading frame (*kan^r*)	This study
pME1660	Cassette for full deletion of *GPA2*-open reading frame (*kan^r*)	This study
pME1756	Cassette for full deletion of *ARO3*-open reading frame (*kan^r*)	This study
pME1757	Cassette for full deletion of *ARO4*-open reading frame (*LEU2*)	G. H. Braus
pME1911	Cassette for full deletion of *URE2*-open reading frame (*kan^r*)	This study
pME1912	Cassette for full deletion of *GLN3*-open reading frame (*kan^r*)	This study
pras2Δ::URA3	Cassette for full deletion of *RAS2*-open reading frame (*URA3*)	25
pME1765	pBluescriptKS$^+$ containing *kan^r* cassette	This study
pME643	Integration cassette for *ARO4m*	11
pRS314	*TRP1*-marked centromere vector	48
pME1092	2.8-kb[a] fragment containing *GCN4* in pRS314	34
pME1098	2.8-kb fragment containing *GCN4m* in pRS314	34
pME1108	Integrative Δ*UAS*::*lacZ* reporter construct	34
pME1112	Integrative *GCRE6*::*lacZ* reporter construct	34
p180	*GCN4*::*lacZ* reporter construct on centromere vector (*URA3*)	42
pME2157	*GCN4*::*lacZ* reporter construct on centromere vector (*TRP1*)	This study
p227	*GCN4m*::*lacZ* reporter construct on centromere vector	15
B2389	*ura3*::*TRP1* marker exchange construct	Yona Kassir
Ep69	*GCN3* on centromere vector	49
p2304	*GCN3* on 2-μm vector	50

[a] kb, kilobase pair.

and the mean value is presented. The standard errors of the means were below 15%.

Western Hybridization Analysis—Strains were cultivated as described above. Crude protein extracts were prepared in the presence of a protease inhibitory mix (33). Routinely, 10 μg of crude protein extracts were separated on a 12% polyacrylamide gel, and proteins were transferred onto a nitrocellulose membrane by electroblotting. Gcn4p was visualized using ECL technology (Amersham Pharmacia Biotech) after incubation of membranes with a polyclonal rabbit anti-Gcn4p antibody (34, 35) and a peroxidase-coupled goat anti-rabbit IgG secondary antibody. Expression of Aro7p was used as internal standard in all measurements, and Aro7p was visualized using a specific anti-Aro7p antiserum (35, 36). Gcn4p and Aro7p signals were quantified using the Molecular Analyst software (Bio-Rad) as described previously (1). The Gcn2p, eIF-2α, and eIF-2α~P Western analyses were carried out using polyclonal antibodies that specifically recognize Gcn2p, eIF-2α (both kindly provided by Alan Hinnebusch (37)) or the phosphorylated form of eIF-2α, eIF-2α~P (BIOSOURCE International, Camarillo, CA). The eIF-2α and eIF-2α~P Western were quantified by using a Image Station 440CF (Eastman Kodak Co.) for detecting luminescence of ECL technology and Kodak 1D Image Analysis Software (Kodak) for quantification of the obtained signals.

Gel Retardation Assay—Gel retardation assays using crude yeast extracts or Gcn4p purified from *Escherichia coli* were performed as described (7). As DNA probe, a ^{32}P-labeled synthetic GCRE fragment was used that was obtained by annealing two synthetic oligonucleotides GCRE-1A (5′-GATCTGCTCGAGATGACTCATTTTTTGATCAATT-3′) and GCRE-1B (5′-TTGATCAAAAAATGAGTCATCTCGAGCAGATCT-T-3′). Essentially, 10 μg of crude protein extract were mixed with 10 fmol of ^{32}P-radiolabeled probe, separated on a native 6% polyacrylamide gel, and visualized by autoradiography. Protein-DNA complexes were quantified using a BAS-1500 PhosphorImaging scanner (Fuji).

RESULTS

Gcn4p-dependent Reporter Gene Expression Is Repressed by Nitrogen Starvation—We examined the effects of nitrogen starvation on expression of a Gcn4p-specific reporter gene *GCRE6*::*lacZ* that contains six GCRE-binding sites for Gcn4p in front of a *CYC1*::*lacZ* minimal promoter. Efficient expression of *GCRE6*::*lacZ* requires the presence of Gcn4p and accurately reflects the Gcn4p-transcriptional activity in the cell (1). Two different genetic backgrounds of *S. cerevisiae*, *S288c* and Σ*1278b*, were chosen for these measurements. The Σ*1278b* background is ideal for measuring the effects of nitrogen starvation, because Σ*1278b* strains are highly responsive to changes in the ammonium concentrations in the environment. However, most studies addressing Gcn4p activity and regulation by the general control system in the past were performed using the *S288c* background. Thus, the *GCRE6*::*lacZ* gene was integrated into the *URA3* locus of wild-type control and *gcn4*Δ mutant strains of both genetic backgrounds. β-Galactosidase activities of resulting strains were determined under three different growth conditions as follows: mid-log phase (no starvation), starvation for amino acids by addition of the histidine analogue 3AT, and starvation for nitrogen by 1000-fold reduction of the ammonium sulfate concentration (Fig. 1). We found that under non-starvation conditions (mid-log phase) expression of the *GCRE6*::*lacZ* reporter was ~4-fold higher in the Σ*1278b* than in the *S288c* strain. However, in the absence of Gcn4p (in a *gcn4*Δ mutant) or under conditions of amino acid starvation when the general control system is fully activated, expression of the *GCRE6*::*lacZ* gene was almost identical in both genetic backgrounds. Thus, the maxima and minima of the measured values representing the maximal Gcn4p-dependent gene expression and the Gcn4p-independent basal expression are identical. However, the basal activity of Gcn4p under non-starvation conditions is at a significantly higher level in the Σ*1278b* background. This is in agreement with a recent study reporting a higher basal Gcn4p activity in a Σ*1278b* strain when compared with a strain that carries the SP1 background (38). When cells were starved for nitrogen, however, GCRE-driven gene expression was drastically reduced in strains with both genetic backgrounds. Expression of the *GCRE6*::*lacZ* gene was decreased 11-fold in Σ*1278b* and 3.3-fold in the *S288c* strains, respectively. The absolute amounts of β-galactosidase activities measured under these conditions were similar with 25 units in the Σ*1278b* or 22 units in the *S288c* strain. In the absence of *GCN4*, expression of *GCRE6*::*lacZ* was not further reduced by nitrogen starvation.

Results in this section show that Gcn4p-dependent gene expression is repressed by nitrogen starvation in different genetic backgrounds.

Nitrogen Starvation Represses Gcn4p-dependent Transcription of the Amino Acid Biosynthetic ARO4 Gene—We next examined the effects of nitrogen starvation on expression of the general control regulated amino acid biosynthetic gene *ARO4* and of *GCN4* itself. For this purpose, steady-state mRNA levels of *ARO4* and *GCN4* were measured under different nutritional conditions and compared with expression of two control genes *DUR1,2* and *PDA1*. Whereas *ARO4* and *GCN4* are genes known to be regulated by amino acid starvation, *DUR1,2* was

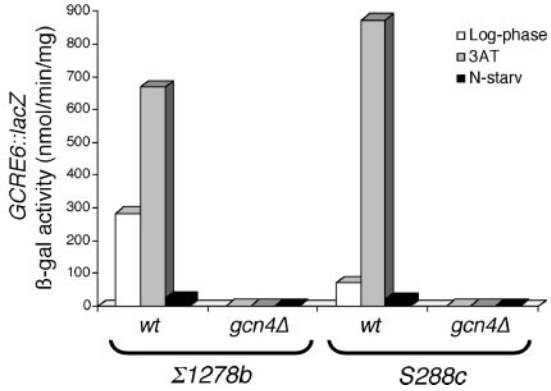
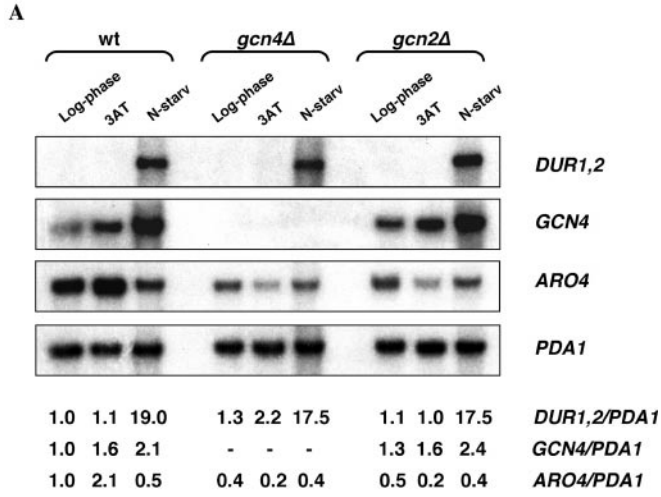


FIG. 1. **Gcn4p-dependent gene expression is repressed by nitrogen starvation.** Expression of the *GCRE6*::*lacZ* reporter gene was measured in Σ*1278b* yeast strains RH2396 (wild type, *wt*) and RH2398 (*gcn4*Δ) and in *S288c* strains RH2407 (*wt*) and RH2446 (*gcn4*Δ) under different nutritional conditions. Cultures grown to mid-log phase were used for assaying non-starvation conditions. Amino acid starvation was induced by addition of 3-amino-triazole (3*AT*) to 10 mM and nitrogen starvation (*N-starv*) by growth on minimal medium containing 50 μM ammonium sulfate as the sole nitrogen source. Strains RH2396 and RH2398 were carrying the *TRP1* centromeric plasmid (pRS314) to obtain Trp+ prototrophy. β-Galactosidase activities are given in nmol/min/mg. *Bars* depict means of at least three independent measurements with a standard deviation not exceeding 15%.



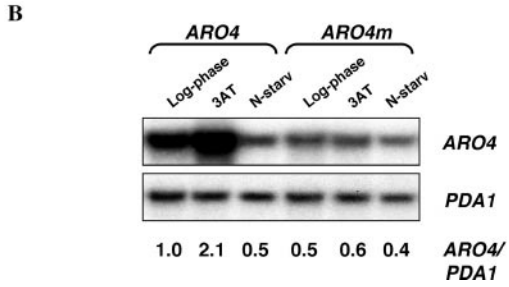


FIG. 2. **Gcn4p-dependent but not basal expression of *ARO4* is repressed by nitrogen starvation.** *A,* expression of *DUR1,2*, *GCN4*, *ARO4,* and *PDA1* under different nutritional conditions. Total RNA was prepared from yeast strains RH2396 (*wt*), RH2397 (*gcn2*Δ), and RH2398 (*gcn4*Δ) all carrying the *TRP1* centromeric vector pRS314 and grown to mid-log phase or starved for either amino acids (*3AT*) or for nitrogen (*N-starv*). For measurements of *DUR1,2*, *GCN4*, *ARO4,* and *PDA1* transcript levels, 10 μg of total RNA from each sample was subjected to a Northern hybridization analysis. Signals were quantified using a BAS-1500 PhosphorImaging scanner. Numbers given indicate relative expression levels of *DUR1,2*, *GCN4,* and *ARO4* when compared with *PDA1* as internal standard and with a value for mid-log phase expression set to 1. *B,* nitrogen starvation-induced repression of *ARO4* transcription is mediated by Gcn4p and a single GCRE site in the *ARO4* promoter. Total RNA was prepared from yeast strains RH2485 (*ARO4*) and RH2486 (*ARO4m*) grown to mid-log phase or starved for either amino acids (*3AT*) or for nitrogen (*N-starv*). *ARO4* and *PDA1* transcript levels were analyzed as described above. Numbers given indicate relative expression levels of *ARO4* when compared with *PDA1* as internal standard and with a value for mid-log phase expression set to 1.

used as a control gene that is strongly up-regulated by nitrogen starvation (39). *PDA1* was chosen as a control gene that is unaffected by starvation conditions (40). Total RNAs of a control strain or of *gcn2*Δ and *gcn4*Δ mutant strains that had either been grown to mid-log phase (no starvation) or had been starved for amino acids or for nitrogen were isolated, and expression of *DUR1,2*, *GCN4*, *ARO4,* and *PDA1* mRNA was monitored by quantitative Northern hybridization analysis (Fig. 2*A*). Amino acid starvation induced by addition of 3AT increased mRNA levels of *ARO4* by a factor of 2.1 when compared with *PDA1*. No induction of *ARO4* by 3AT was observed in the two general control mutant strains *gcn2*Δ or *gcn4*Δ, respectively. As observed previously, a 1.6-fold increase in the *GCN4* transcript levels was detected under these conditions (34). In contrast, transcription of the amino acid catabolic gene *DUR1,2* was not affected by the addition of 3AT. Thus, starvation for amino acids stimulates transcription of genes required for amino acid biosynthesis, whereas amino acid catabolic genes do not appear to be affected.

When cells were starved for nitrogen, transcription of *DUR1,2* was induced 19-fold independently of either *GCN2* or *GCN4*. Similarly, steady-state mRNA levels of *GCN4* were enhanced 2-fold under nitrogen starvation conditions, even in the absence of *GCN2*. This suggests that *GCN4* is transcriptionally regulated by nitrogen via factors that also control expression of *DUR1,2*. However, when cells were starved for nitrogen, *ARO4* transcript levels dropped by a factor of 2 compared with mid-log phase. Other general control regulated genes as *HIS7*, for example, also showed decreased mRNA levels under nitrogen starvation (data not shown). Repression of *ARO4* by nitrogen starvation depended on either *GCN2* or *GCN4*, because *ARO4* transcript levels remained unchanged under this condition in both *gcn2*Δ and *gcn4*Δ mutant strains. Thus, starvation for nitrogen only represses the Gcn4p-dependent transcription of *ARO4*. To confirm this finding, we also measured expression of an *ARO4* mutant gene (*ARO4m*), whose single GCRE site present in its promoter is inactivated by two point mutations and whose expression therefore is independent of Gcn4p (22). Under non-starvation conditions, *ARO4m* mRNA levels were decreased 2-fold when compared with expression of wild-type *ARO4* (Fig. 2*B*). In addition, *ARO4m* expression was no longer inducible by 3AT. Thus, expression of *ARO4m* is comparable to expression of wild-type *ARO4* in a *gcn4*Δ mutant background. Importantly, starvation for nitrogen did not affect expression of the *ARO4m* mutant gene, corroborating that the drop of *ARO4* expression in nitrogen-starved cells is due to a loss of the Gcn4p-dependent transcription of the gene.

In summary, the results in this section show that starvation for nitrogen increases *GCN4* transcription but represses transcription of *ARO4*, an amino acid biosynthetic gene that is induced by amino acid starvation. Moreover, repression affects specifically the Gcn4p-dependent transcription of *ARO4*, as no repression by nitrogen starvation can be observed in strains lacking elements of the general control system, such as *GCN2* or *GCN4*, or when the GCRE element of the *ARO4* promoter was inactivated. In contrast, the amino acid catabolic gene *DUR1,2* is not induced by amino acid starvation but is strongly

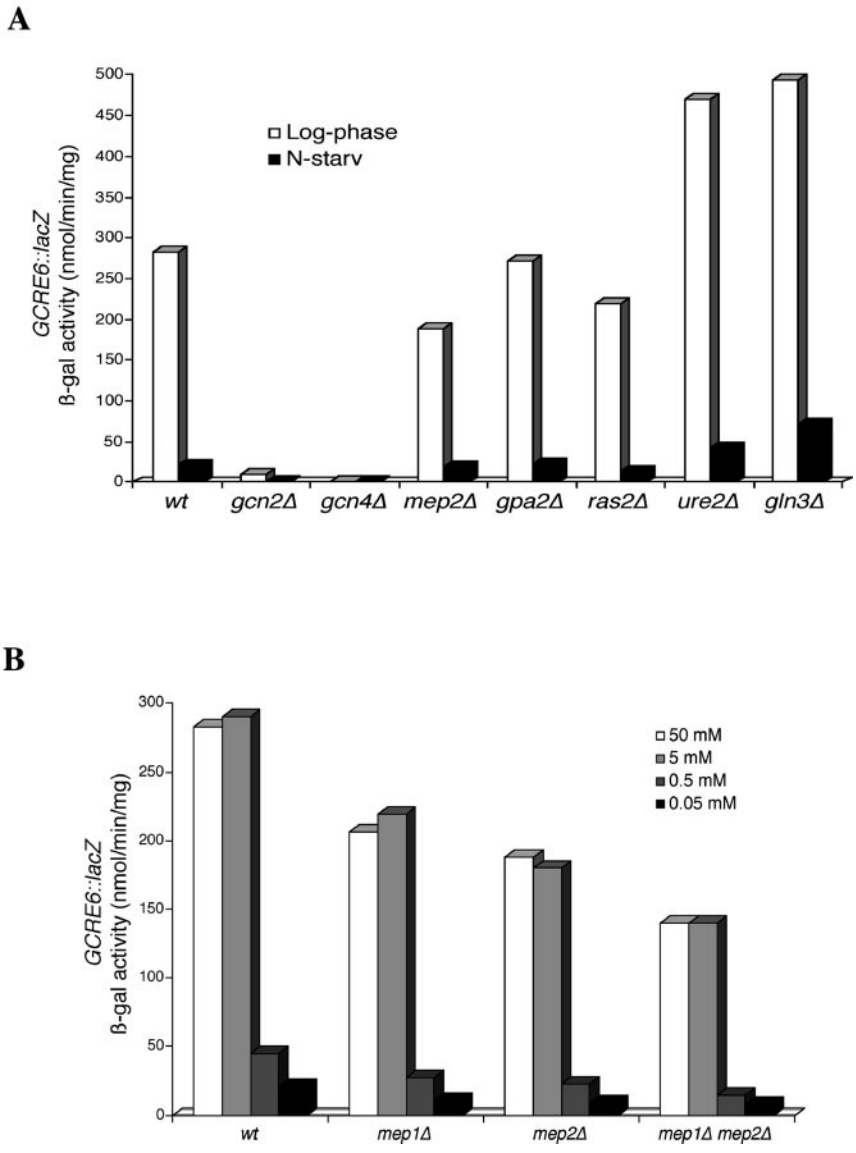


FIG. 3. **Regulation of Gcn4p-dependent gene expression by ammonium.** *A,* repression of Gcn4p-dependent gene expression by nitrogen starvation is independent of *MEP2*, *GPA2*, *RAS2*, *URE2,* and *GLN3*. Expression of the *GCRE6*::*lacZ* reporter gene was measured in yeast strains RH2396 (*wt*), RH2397 (*gcn2*Δ), RH2398 (*gcn4*Δ), RH2406 (*mep2*Δ), RH2404 (*gpa2*Δ), RH2400 (*ras2*Δ), RH2489 (*ure2*Δ), and RH2490 (*gln3*Δ) carrying plasmid pRS314 under non-starvation (log phase) or under nitrogen starvation (*N-starv*) conditions. *B,* Gcn4p-regulated gene expression depends on ammonium uptake by Mep1p and Mep2p. Expression of *GCRE6*::*lacZ* was measured in yeast strains RH2396 (*wt*), RH2405 (*mep1*Δ), RH2406 (*mep2*Δ), and RH2445 (*mep1*Δ *mep2*Δ) carrying plasmid pRS314 in minimal medium containing 50, 5, 0.5, or 0.05 mM ammonium sulfate as the sole nitrogen source. *A* and *B*, bars depict means of at least three independent measurements of β-galactosidase activities with a standard deviation not exceeding 15%.

inducible by nitrogen starvation in a general control-independent manner.

Repression of Gcn4p-regulated Transcription by Nitrogen Starvation Is Independent of the Ammonium Sensing and Utilization System Controlled by MEP2, GPA2, URE2, or GLN3—We tested whether repression of Gcn4p-dependent transcription by nitrogen starvation depends on elements of the general control system like the sensor kinase Gcn2p or whether the ammonium sensing and signaling system that includes the Mep2 high affinity ammonium permease and the G-α-subunit Gpa2p (41) is involved. We also investigated the role of the two regulators for the ammonium utilization, Ure2p and Gln3p (24). For this purpose, the *GCRE6*::*lacZ* reporter was integrated into the *URA3* locus of *gcn2*Δ, *mep2*Δ, *gpa2*Δ, *ure2*Δ, and *gln3*Δ mutant strains, respectively. We also measured *GCRE6*::*lacZ* reporter activity in a *ras2*Δ mutant strain, because the Mep2p/Gpa2p system has been postulated to exert its effects via cAMP. Expression of *GCRE6*::*lacZ* was reduced 28-fold in a strain lacking *GCN2* in non-starvation medium, yet was still repressible to a certain extent by nitrogen starvation (Fig. 3*A*). This indicates that nitrogen starvation-induced repression of Gcn4p-dependent transcription might involve loss of Gcn2p activity. In contrast, inactivation of neither *MEP2*, *GPA2,* nor *RAS2* significantly affected expression of *GCRE6*::*lacZ* under conditions of high or low ammonium concentrations (Fig. 3*A*), indicating that these signaling components are not directly involved in mediating nitrogen starvation-induced repression of Gcn4p-dependent transcription. Under nitrogen starvation in *ure2*Δ or *gln3*Δ mutant strains, a comparable decrease of Gcn4p activity to the wild-type cells was detected, suggesting that Ure2p and Gln3p are also not involved in the observed repression of *GCN4*. Nevertheless, both mutant strains, which are impaired in the ammonium regulation, showed an increase of the β-galactosidase activity of the *GCRE6*::*lacZ* reporter of about 60% in log phase even under non-nitrogen starvation conditions. This increase might be caused by the poor ability of the cells to utilize ammonium resulting in amino acid starvation.

Expression of *GCRE6*::*lacZ* was further determined in dependence on different ammonium sulfate concentrations and on the presence of different combinations of the ammonium transporters Mep1p and Mep2p to test whether intracellular ammonium concentrations might be a trigger for repression of Gcn4p-dependent gene expression. Double mutant strains carrying deletions of both *MEP1* and *MEP2* have been shown to exhibit reduced ammonium uptake (21, 41). We postulated that *mep1*Δ *mep2*Δ double mutant strains were more sensitive to a lack of ammonium in the growth medium with respect to loss of

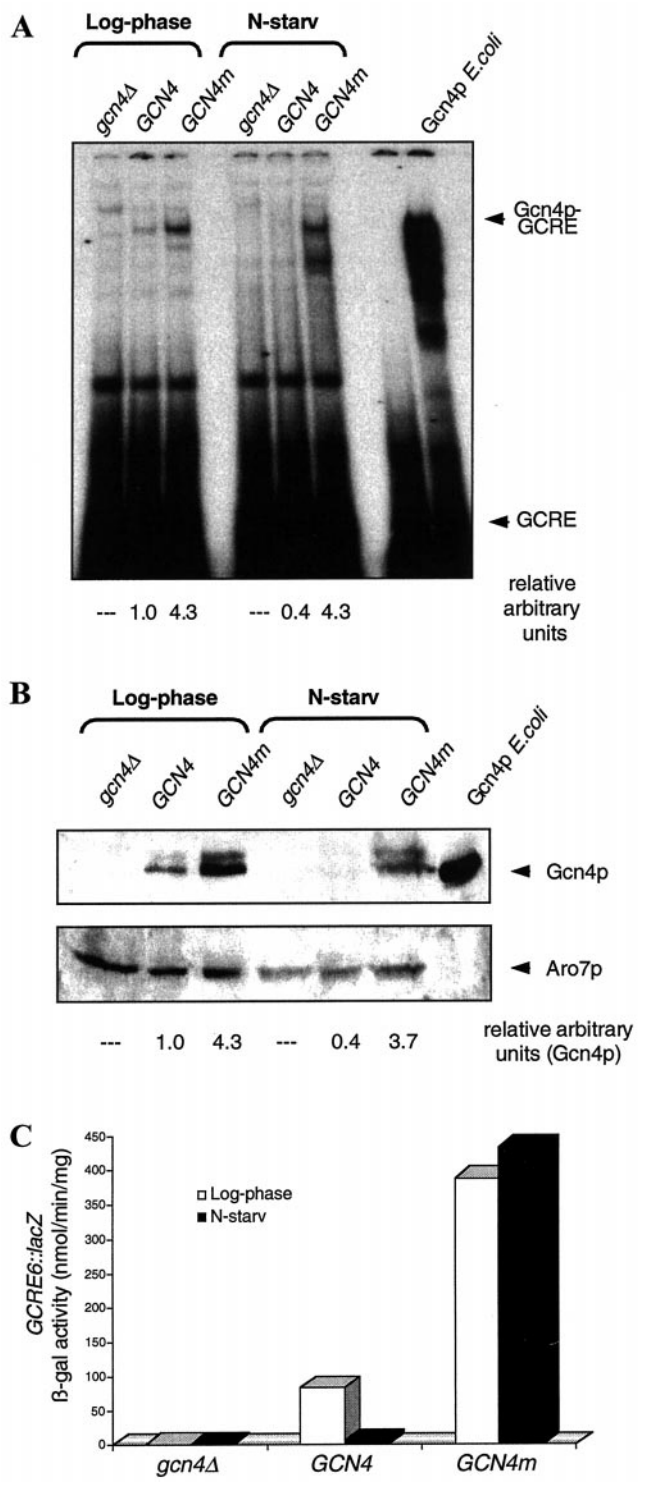


FIG. 4. **Regulation of Gcn4p DNA binding activity, Gcn4p protein levels, and Gcn4p transcriptional activity by nitrogen starvation.** *A,* gel retardation assays. Crude protein extracts were prepared from yeast strain RH2398 (*gcn4*Δ) carrying either the control plasmid pRS314 (*gcn4*Δ), plasmid pME1092 expressing wild-type *GCN4* (*GCN4*), or plasmid pME1098 expressing the *GCN4m* mutant allele with inactivated uORFs in the *GCN4* leader sequence grown under non-starvation (log phase) or under nitrogen starvation (*N-starv*) conditions. *In vitro* DNA binding activity of Gcn4p present in crude protein extracts was measured by mixing 10 μg of protein extracts from each sample with 10 fmol of a synthetic ^{32}P-end-labeled DNA fragment carrying a single GCRE site and separation of protein-DNA complexes on a native 6% acrylamide gel. Gcn4p purified from *E. coli* was used as positive control. Specific Gcn4p-GCRE complexes are indicated (bound), and signals were quantified using a BAS-1500 PhosphorImaging scanner (Fuji). Numbers represent the amount of ^{32}P-end-labeled DNA fragment bound to Gcn4p in arbitrary units with a value for the strain carrying the *GCN4* wild-type plasmid grown under non-starvation conditions set to 1. *B,* amount of Gcn4p present in protein extracts isolated in *A* was analyzed by Western blot analysis using a polyclonal anti-Gcn4p antibody. As an internal control, protein levels of Aro7p were measured in the same extracts using a polyclonal anti-Aro7p antibody. Signals for Gcn4p and Aro7p were quantified using the Molecular Analyst software from Bio-Rad. Numbers represent Gcn4p levels relative to Aro7p with a value for a *wt GCN4* expressing strain set to 1. *C,* expression of the *GCRE6*::*lacZ* reporter gene was measured in strains described in *A* under non-starvation (log phase) and nitrogen starvation (*N-starv*) conditions. β-Galactosidase activities are given in nmol/min/mg. *Bars* depict means of at least three independent measurements with a standard deviation not exceeding 15%.

GCRE6::*lacZ* reporter activity, if intracellular ammonium was a trigger for repression of Gcn4p transcriptional activity. Accordingly, we found that expression of *GCRE6*::*lacZ* was significantly lower in the *mep1*Δ *mep2*Δ double mutant when compared with a wild-type strain in the presence of both high and low amounts of ammonium sulfate (Fig. 3*B*). Expression in the single *mep1*Δ or *mep2*Δ mutants was also reduced to a certain extent. Our results indicate that reduced ammonium uptake and consequently reduced intracellular ammonium concentrations are a trigger for repression of Gcn4p-dependent transcription under nitrogen starvation conditions.

Gcn4p DNA Binding Activity Is Not Affected by Nitrogen Starvation—Our finding that Gcn4p-dependent transcription via GCRE elements is repressed 11-fold by nitrogen starvation, whereas *GCN4* mRNA levels increase by a factor of 2, prompted us to examine more closely the mechanism of repression. For this purpose, we measured both Gcn4p DNA binding activity and the amount of Gcn4 protein in cells grown in medium containing either high or low concentrations of ammonium. *gcn4*Δ mutant strains with a chromosomally integrated *GCRE6*::*lacZ* reporter gene and carrying either a control plasmid or *GCN4* on a centromeric vector were grown in non-starvation or in nitrogen starvation medium. Crude protein extracts were prepared and analyzed for Gcn4p DNA binding activity by gel mobility shift assays with a synthetic GCRE fragment (Fig. 4*A*). In addition, the amount of Gcn4p present in the extracts was determined by Western hybridization analysis using a specific anti-Gcn4p antibody (Fig. 4*B*). Purified Gcn4p heterologously expressed in *E. coli* was used as a control in both cases. We found a roughly 2.5-fold decrease in both Gcn4 protein levels and the amount of synthetic GCRE fragment bound by Gcn4p present in crude extracts when cells were starved for ammonium. *GCRE6*::*lacZ* reporter activity was reduced 14-fold in these strains (Fig. 4*C*). These results indicate that repression of Gcn4p-dependent transcription induced by nitrogen starvation is in part due to a decrease in the amount of intracellular Gcn4p. In addition, the DNA binding activity of Gcn4p does not appear to be affected by ammonium in the medium, because loss of Gcn4p DNA binding activity is paralleled by a decrease of intracellular Gcn4 protein levels.

Translational Repression of GCN4 mRNA by Nitrogen Starvation Requires the uORFs in the GCN4 Leader Sequence—We determined whether the decrease in intracellular amounts of Gcn4p upon ammonium limitation might be due to a change in the translational efficiency of *GCN4* mRNA, because translational control of *GCN4* mRNA is a well documented mechanism for regulation of *GCN4* expression (4, 12, 42, 43). Mutations in the start codons of the uORFs in the *GCN4* leader sequence are known to uncouple *GCN4* mRNA from translational control by the general control system upon amino acid starvation. We reasoned that if translation of *GCN4* was affected by nitrogen starvation, translation of a *GCN4* mRNA with inactivated uORFs in the upstream leader sequence should be unaffected

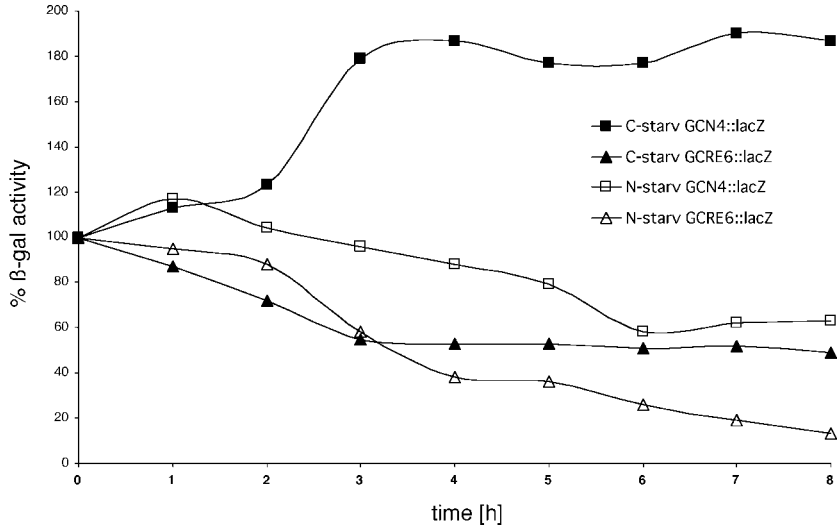


FIG. 5. **Time course of different β-galactosidase reporter constructs under carbon and nitrogen starvation conditions.** The yeast strains were incubated overnight, transferred into fresh medium, and collected after 4 h of growth. These cells served as inoculation for the starvation media (time point, 0 h). At time 0, the β-galactosidase activity was set to 100% for each strain. In the strain RH2399 the *GCN4*::*lacZ* reporter activity was tested under glucose limitation (■) and nitrogen limitation (□), respectively. Additionally the *GCRE6*::*lacZ* fusion gene was measured in the strain RH2396 under glucose limitation (▲) and nitrogen limitation (△). All values result from at least three independent measurements with a standard deviation not exceeding 15%.

by ammonium concentrations in the growth medium. As a consequence, Gcn4 protein levels and *GCRE6*::*lacZ* reporter gene expression should be identical in strains grown with or without limitation for ammonium. We measured Gcn4p DNA binding activity, Gcn4 protein levels, and *GCRE6*::*lacZ* reporter activity in a strain expressing a *GCN4* mutant allele (*GCN4m*) carrying point mutations that inactivate all four uORFs in the *GCN4* upstream leader under high and low ammonium conditions (Fig. 4). We found that in a strain expressing the *GCN4m* allele both Gcn4p binding to synthetic GCRE-DNA fragments and intracellular Gcn4p levels were increased 4.3-fold under non-nitrogen starvation conditions, when compared with *GCN4* with intact uORFs. As a consequence, expression of *GCRE6*::*lacZ* reporter was induced 4.6-fold. Most important, a significant decrease in neither Gcn4p binding to GCREs, intracellular Gcn4p levels, nor expression of *GCRE6*::*lacZ* was detected under nitrogen starvation conditions when Gcn4p was translated from the *GCN4m* mRNA. Thus, mutations in the uORFs not only uncouple *GCN4* expression from translational control by the general control system but also from repression by nitrogen starvation. This indicates that ammonium regulates intracellular Gcn4p levels by a translational control mechanism involving the upstream open reading frames of the *GCN4* mRNA.

Since Yang *et al*. (44) recently found that glucose starvation in *S288c* cells resulted in an activation of the *GCN4* expression 4-fold or more, we investigated whether this effect exists also in Σ*1278b* cells. We used the same *GCN4*::*lacZ* fusion construct as described previously (44), and we found that glucose starvation activates *GCN4* expression only 2-fold in Σ*1278b* cells (Fig. 5) but much more (4-fold) in *S288c* yeast cells (data not shown). Therefore, our *S288c* data confirmed the results of Yang *et al*. (44). In contrast to glucose starvation, nitrogen starvation only slightly decreased *GCN4*::*lacZ* expression but drastically reduced *GCRE6*::*lacZ* expression by a factor of 10. Therefore, nitrogen and glucose starvation affects *GCN4* expression in opposite ways. Surprisingly, the increased *GCN4* expression during glucose starvation does not result in an increased expression of Gcn4p-regulated target promoters. The *GCRE6*::*lacZ* reporter decreases in a time course after glucose starvation by a factor of 2. This suggests that the expressed Gcn4p might be modified or destabilized under glucose starvation conditions.

We further measured expression of *GCN4*::*lacZ* fusion constructs in more detail to corroborate the model that starvation for nitrogen affected translational efficiency of *GCN4* mRNA. *GCN4*::*lacZ* fusions are an accurate measure for translational efficiency of *GCN4* mRNA when compared with *GCN4* transcript levels (42). Wild-type strains as well as mutant strains lacking either *GCN2* (*gcn2*Δ) or *GCN4* (*gcn4*Δ) or both (*gcn2*Δ/*gcn4*Δ) were transformed with plasmids carrying either a *GCN4*::*lacZ* fusion construct or a *GCN4m*::*lacZ* fusion with mutated uORFs. β-Galactosidase activities of resulting strains were measured under both non-starvation and nitrogen starvation conditions (Fig. 6). In a wild-type strain with an intact general control system, expression levels of *GCN4*::*lacZ* decreased 2-fold when cells were starved for ammonium. When *GCN2* was lacking from strains (*gcn2*Δ or *gcn2*Δ/*gcn4*Δ double mutants), *GCN4*::*lacZ* expression decreased by a factor of 9.6 under nitrogen non-starvation conditions and 2.1-fold under starvation in comparison to the wild type. However, no significant differences between non-starvation and nitrogen starvation conditions were measured in *gcn2*Δ strains. Interestingly, deletion of *GCN4* itself led to a 5.9-fold induction of *GCN4*::*lacZ* expression. This induction is due to the lack of any amino acids in the medium used (SD medium). It is known that Gcn4p is required for basal expression of genes involved in different amino acid biosynthetic pathways like arginine. A lack of the Gcn4p transcription factor in the cell and amino acid starvation in the medium induces the general control system. In strains lacking both *GCN4* and *GCN2*, no induction of *GCN4*::*lacZ* could be measured, corroborating the interpretation that the 5.9-fold higher *GCN4*::*lacZ* expression levels in *gcn4*Δ mutants are due to an activated general control system. However, nitrogen starvation repressed *GCN4*::*lacZ* expression by a factor of 4 even in the *gcn4*Δ mutant background with an activated general control system. This indicates that nitrogen starvation efficiently counteracts amino acid starvation-induced activation of *GCN4* expression. Moreover, the repression mechanism takes place at the level of *GCN4* translation. Measurements with the *GCN4m*::*lacZ* construct corroborated this interpretation. Expression of this construct was no longer dependent on the presence of either *GCN2* or *GCN4*, because any of the strains used show comparable expression levels of *GCN4m*::*lacZ*. More importantly, no repression but even a roughly 2-fold induction of *GCN4m*::*lacZ* was detected when strains were starved for nitrogen. This corresponds to the increase in *GCN4* mRNA levels as shown in Fig. 2. Thus, repression of *GCN4*::*lacZ* expression by nitrogen starvation requires the uORFs present in the *GCN4* upstream leader, again suggesting a translational mechanism of repression.

Nitrogen Starvation Blocks GCN4 mRNA Translation Under

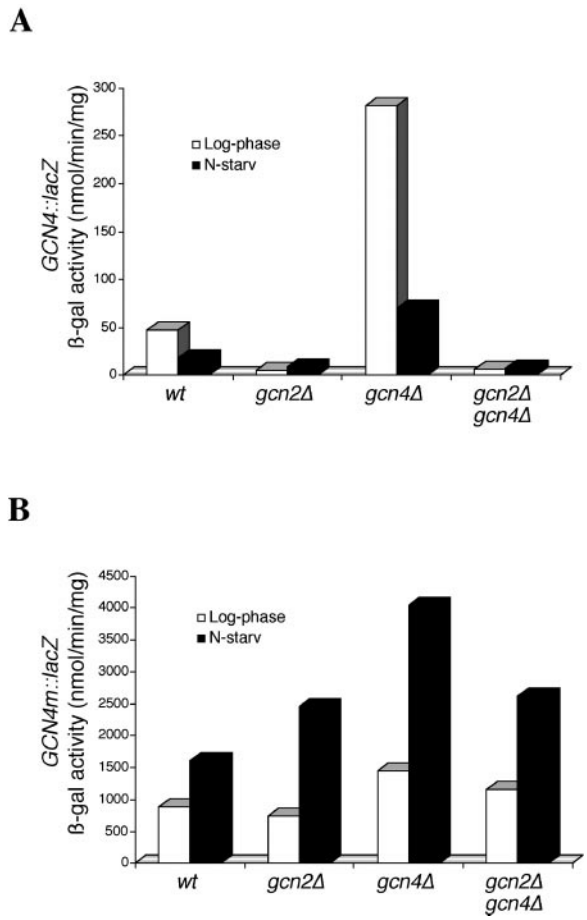


FIG. 6. **Repression of *GCN4* expression by nitrogen starvation depends on the uORFs in the *GCN4* leader sequence.** *A,* expression of a *GCN4*::*lacZ* fusion gene was measured in yeast strains RH2399 (wt), RH2401 (*gcn2*Δ), RH2402 (*gcn4*Δ), and RH2402 (*gcn2*Δ *gcn4*Δ) under non-starvation (log phase) and nitrogen starvation (*N-starv*) conditions after 8 h. *B,* expression of a *GCN4m*::*lacZ* fusion gene with inactivated uORFs in the *GCN4* leader sequence was measured in identical strains as described in *A*. *A* and *B*, bars depict means of at least three independent measurements of β-galactosidase activities with a standard deviation not exceeding 15%.

Simultaneous Amino Acid Starvation Conditions in an eIF-2α~P-independent Manner—Gcn2p and eIF-2α~P are positive trans-acting factors of general translational control mechanism of the *GCN4* mRNA (14). Nitrogen starvation might act via these factors to block translation. To test these possibilities, we measured the protein levels of Gcn2p and the relation between eIF-2α and eIF-2α~P. In addition, we determined the Gcn4p and *GCN4*::*lacZ* levels (Fig. 7). We found that nitrogen starvation does not affect the Gcn2p level (Fig. 7*A*). Surprisingly, a strong increase of eIF-2α phosphorylation could be detected in a nitrogen-starved *S288c* strain after 24 h (Fig. 7*B*). In contrast, only a weak increase was found in the Σ*1278b* strain (Fig. 7*A*). An explanation for this finding may be the partially induced general control of Σ*1278b* strains (38), which can be observed by comparing the β-galactosidase activity of a *gcn2*Δ and a wild-type strain measured by the *GCRE*::*lacZ* reporter construct (Fig. 3*A*). Accordingly, the phosphorylation level of eIF-2α is higher in log phase in a Σ*1278b* strain than in an *S288c* strain (Fig. 7), resulting in a weaker increase of eIF-2α~P levels under nitrogen starvation. Expression of the *GCN4*::*lacZ* reporter construct was identical in strains grown to log phase or starved for nitrogen (Fig. 7*B*). Under 3AT conditions, however, *GCN4*::*lacZ* expression is 13-fold higher than under nitrogen starvation, whereas the phosphorylation levels of eIF-2α are comparable. Maximal phosphorylation of eIF-2α is achieved significantly faster under amino acid starvation than under nitrogen starvation conditions (Fig. 7*C*). Thus, under nitrogen starvation conditions, phosphorylation of eIF-2α does not correlate with the expression of the *GCN4*::*lacZ* reporter gene, implicating an additional regulatory mechanism for the translational control of *GCN4*. This conclusion is further supported by the fact that under simultaneous nitrogen and amino acid starvation conditions, the phosphorylation level of eIF-2α was inducible nearly 2-fold, whereas the expression of *GCN4*::*lacZ* remained constant (Fig. 7*B*). Furthermore, the effect caused by nitrogen starvation appears to overrule that of amino acid starvation.

We also measured the Gcn4p levels under all different conditions (Fig. 7*B*) to test the correlation between the amount of Gcn4p in the cells and the expression of the *GCN4*::*lacZ* reporter construct. In non-nitrogen starvation medium, Gcn4p levels and β-galactosidase activities correlate well. Under nitrogen starvation conditions, however, where comparable β-galactosidase activities as in log phase were measured, only very low levels of Gcn4p were detectable, indicating a destabilization of Gcn4p. In addition, expression of the *GCN4m*::*lacZ* reporter construct increased under nitrogen starvation conditions (data not shown), suggesting that both a higher instability of Gcn4p and the translational control are involved in the decrease of the Gcn4p levels under nitrogen starvation.

We further tested whether *GCN3* might be involved in translational repression of *GCN4* under nitrogen starvation conditions. Gcn3p is part of the regulatory eIF-2B subcomplex that is inhibited in its guanine nucleotide exchange activity of eIF-2α by phosphorylated eIF-2α (26). Expression of *GCN4*::*lacZ* was measured in strains lacking *GCN3* or carrying *GCN3* on a high copy vector (Fig. 8), because nitrogen starvation might lower expression of *GCN3*, an effect that should be corrected by high copy expression of *GCN3*. However, even in strains carrying *GCN3* on a high copy number plasmid, expression of *GCN4*::*lacZ* under nitrogen starvation conditions was as low as in the control strain (Fig. 8). This suggests that *GCN3* is not required in the repression of *GCN4* mRNA translation by nitrogen starvation.

In summary, we detected a translational block of *GCN4* mRNA resulting in a decrease of the Gcn4p amount under nitrogen starvation conditions. This translational control mechanism appears to be independent of the known trans-acting elements Gcn2p, eIF-2α, or Gcn3p but still requires the four uORFs of the *GCN4* mRNA. Furthermore, nitrogen starvation conditions block an activation of the general control caused by 3AT without interfering with the phosphorylation of eIF-2α.

DISCUSSION

Yeast cells integrate many nutritional signals to adapt their metabolism for optimal growth and development. Nitrogen starvation requires the expression of genes for enzymes able to degrade nitrogen-containing compounds. Simultaneously, cells adapt morphologically by switching to a filamentous growth mode. In this study, we investigated how starvation for nitrogen affects the "general control" regulatory network that induces amino acid biosynthetic gene expression when yeast cells are starved for amino acids. Because amino acid biosynthesis requires at least one aminotransferase reaction, this network is an important part of the general nitrogen metabolism. Yeast cells utilize ammonia exclusively by incorporation into glutamate and glutamine (24). This prompted us to analyze whether the general signal "lack of nitrogen" includes the more specific signal "lack of amino acid" and subsequently activates the general control system and its transcriptional activator Gcn4p.

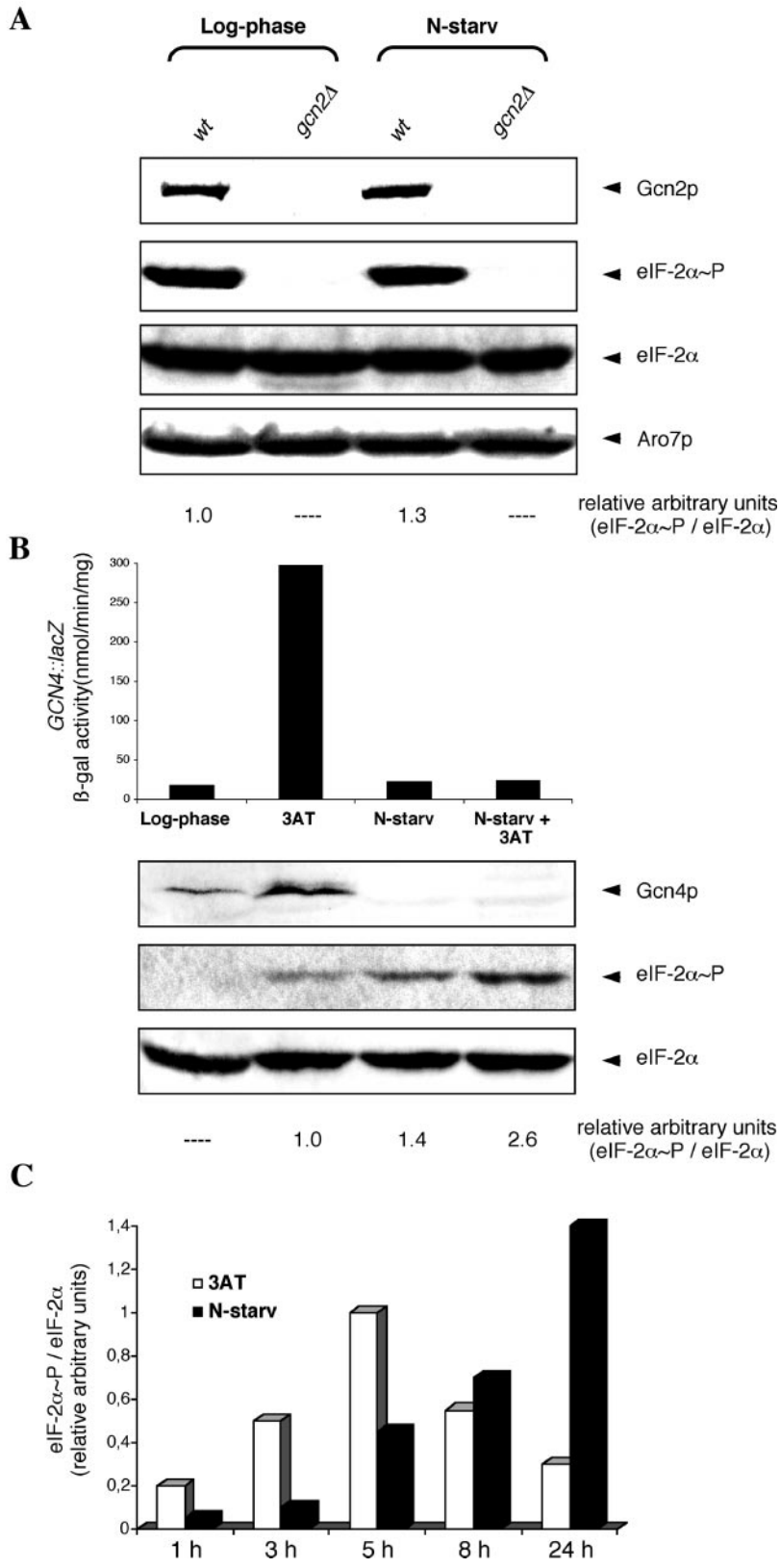


FIG. 7. **Regulation of Gcn2p and eIF-2α~P protein levels by nitrogen starvation.** *A,* crude protein extracts were prepared from yeast Σ*1278b* strains RH2396 (wt) and RH2397 (*gcn2*Δ) carrying plasmid pRS314 grown under non-starvation (log phase) or under nitrogen starvation conditions (*N-starv*). The amount of Gcn2p, eIF-2α, and eIF-2α~P protein present in isolated protein extracts were analyzed by Western blot analysis using a polyclonal anti-Gcn2p antibody, a polyclonal anti-eIF-2α antibody, or a phosphorylation-specific polyclonal anti-eIF-2α~P antibody. As an internal control, protein levels of Aro7p were measured in the same extracts using a polyclonal anti-Aro7p antibody. Signals for eIF-2α~P and eIF-2α were quantified, and their relation is given below in relative arbitrary units. *B,* crude protein extracts from the strain RH2520 (haploid, *S288c*), which carried the p180 plasmid, were prepared. Cells from an overnight culture were diluted and incubated for 5 h under non-starvation conditions (log phase) or amino acid starvation conditions by adding 10 mM 3AT. For nitrogen starvation (*N-starv*) or nitrogen starvation with additional 3AT (*N-starv + 3AT*), yeast cells were incubated under nitrogen starvation for 24 h and then divided up in two cultures. One culture was mixed with 10 mM 3AT, whereas the other remained unchanged, and cultures were further incubated for 5 h before harvesting. From all cultures the β-galactosidase activities were measured. Bars depict the means of at least three independent measurements of β-galactosidase activities with a standard deviation not exceeding 15%. From the same cultures the protein levels of Gcn4p, eIF-2α, and eIF-2α~P were analyzed by Western hybridization using specific antibodies. The ratio between eIF-2α~P and eIF-2α was determined and is shown at the *bottom*. *C,* a time course of the eIF-2α phosphorylation status was performed under amino acid starvation (*3AT*) and under nitrogen starvation conditions (*N-starv*) using strain RH2520 and the cultivation procedures described in *B*. Bars represent the ratio between eIF-2α~P and eIF-2α after 1, 3, 5, 8, and 24 h of starvation, respectively. Arbitrary units were chosen with a value of 1 for the ratio measured after 5 h of amino acid starvation.

In contrast, we found the opposite effect that a general lack of any nitrogen source specifically and efficiently inhibits Gcn4p-mediated gene expression.

Several lines of evidence indicate that inhibition of general control-regulated gene expression by nitrogen starvation occurs at the level of *GCN4* mRNA translation. (i) We find that Gcn4 protein levels decrease in the absence of nitrogen, whereas *GCN4* transcript levels increase. (ii) Reduction of Gcn4p levels or *GCN4*::*lacZ* fusion expression can been observed only when translated from *GCN4* mRNA species carrying the uORFs in the leader sequence. (iii) Nitrogen starvation completely blocks amino acid starvation-dependent induction of *GCN4*::*lacZ* expression and subsequently increased Gcn4p levels, depending on the presence of the uORFs. However, phosphorylation of eIF-2α by Gcn2p is not blocked by nitrogen starvation. To the contrary, we find that nitrogen starvation

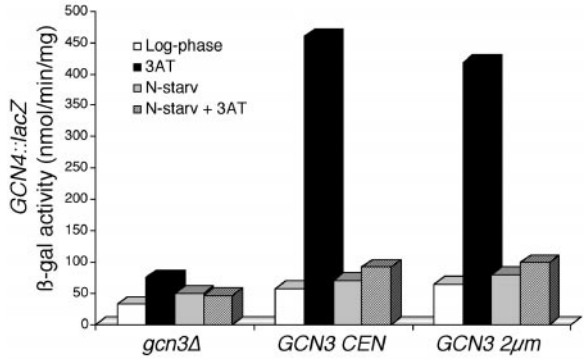


FIG. 8. **Influence of copy number variation of *GCN3* on expression of *GCN4*::*lacZ*.** Expression of the *GCN4*::*lacZ* reporter gene was measured in yeast strain GP3153 carrying a control vector (*gcn3*Δ), *GCN3* on a CEN plasmid (*GCN3 CEN*), or *GCN3* on a 2-μm plasmid (*GCN3 2 μm*) under different nutritional conditions. Strains were cultivated as described in Fig. 7*B*, and β-galactosidase activities were measured from all cultures. Bars depict means of at least three independent measurements of β-galactosidase activities with a standard deviation not exceeding 15%.

leads to an even stronger increase in eIF-2α phosphorylation than amino acid starvation. Yet starvation for nitrogen completely blocks efficient translation of *GCN4* mRNA, even under simultaneous amino acid starvation conditions. This suggests that nitrogen starvation blocks *GCN4* mRNA translation by an additional yet undiscovered mechanism. Because nitrogen starvation does not affect Gcn2p or eIF-2α protein levels nor the ratio of eIF-2α~P/eIF-2α, a component of the general control system that acts downstream of eIF-2α might be altered in function or expression. This result indicates an additional translational regulation mechanism using the same *cis*-elements.

Our study further suggests that nitrogen starvation, apart from repressing translation of *GCN4* mRNA, also down-regulates the Gcn4 protein on a posttranslational level. This conclusion is based on two observations. (i) Whereas in the absence of *GCN2* expression of *GCN4*::*lacZ* is not repressible by nitrogen starvation, expression of the *GCRE6*::*lacZ* reporter is still down-regulated to a certain extent. (ii) When uORFs are absent in the *GCN4* leader, expression of *GCN4m*::*lacZ* (reflecting translation of *GCN4*) is induced 2-fold by nitrogen starvation, but both Gcn4 protein levels and expression of the *GCRE6*::*lacZ* reporter are not further induced. A simple explanation might be that nitrogen starvation reduces Gcn4 protein stability. Whereas amino acid starvation inhibits Gcn4p degradation by the ubiquitin-mediated proteolytic system (45), nitrogen starvation might have opposite effects and activate proteolysis of Gcn4p.

A nitrogen sensing and signaling system has to induce translational repression of *GCN4*. At least two distinct developmental programs are known that are negatively regulated by the presence of ammonium, pseudohyphal development, and meiosis. A signaling system that consists of Mep2p, Gpa2p, and the cAMP-dependent protein kinase is thought to regulate positively pseudohyphal development when cells are starved for nitrogen (22, 46). However, neither Mep2p nor Gpa2p seems to be involved in translational control of *GCN4*. In the presence of ammonium, yeast turns off the utilization of poor nitrogen sources, such as urea and proline. This phenomenon is referred as nitrogen catabolite repression (47). The genetic system involves the function of the positively acting GATA transcription factor Gln3p that is negatively regulated by the repressor protein Ure2p in the presence of ammonium. However, neither *ure2*Δ nor *gln3*Δ strains show effects which indicate that these proteins are involved in *GCN4* repression under nitrogen starvation. The reduction of the intracellular ammonium concentration by deleting the ammonium permeases *MEP1* and *MEP2* led to a decrease of Gcn4p activity. This suggests the existence of an additional sensor system for the intracellular ammonium concentration, which is able to induce a signal for repression of the *GCN4* mRNA translation.

In summary, our studies show that yeast cells up-regulate amino acid biosynthesis only when enough nitrogen-containing precursors are available. Nitrogen starvation specifically represses activity of the general control system but does not affect the basal expression of amino acid biosynthetic genes. This demonstrates that amino acid biosynthesis is not completely shut down even under severe starvation conditions. The importance of amino acid biosynthesis under nutrient limiting conditions is also reflected by the regulatory mechanism that we found in this study. Although yeast cells abrogate up-regulation of amino acid biosynthesis when nitrogen sources are absent, mRNA levels for the transcriptional activator Gcn4p are kept at a higher level in order to rapidly induce translation of *GCN4* mRNA when nitrogen becomes available.

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