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Molecular Analysis of *GCN3*, a Translational Activator of *GCN4*: Evidence for Posttranslational Control of *GCN3* Regulatory Function

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GCN4 **encodes a transcriptional activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. The *GCN3* product is a positive regulator required for increased synthesis of GCN4 protein in amino acid-starved cells. GCN3 appears to act indirectly by antagonizing *GCD*-encoded negative regulators of *GCN4* expression under starvation conditions; however, GCN3 can also suppress the effects of *gcd1* and *gcd12* mutations under nonstarvation conditions. These results imply that the *GCN3* product can promote either repression or activation of *GCN4* expression depending on amino acid availability. We present a complete physical description of the *GCN3* gene and its transcript, plus measurements of *GCN3* expression at the transcriptional and translational levels under different growth conditions. *GCN3* encodes a 305-amino-acid polypeptide with no significant homology to any other known protein sequence. *GCN3* mRNA contains no leader AUG codons, and no potential *GCN4* binding sites were found in *GCN3* 5′ noncoding DNA. In accord with the absence of these regulatory sequences found at other genes in the general control system, *GCN3* mRNA and a *GCN3-lacZ* fusion enzyme are present at similar levels under both starvation and nonstarvation conditions. These data suggest that modulation of GCN3 regulatory function in response to amino acid availability occurs posttranslationally. A *gcn3* deletion leads to unconditional lethality in a *gcd1-101* mutant, supporting the idea that *GCN3* is expressed under normal growth conditions and cooperates with the *GCD1* product under these circumstances to carry out an essential cellular function. We describe a point mutation that adds three amino acids to the carboxyl terminus of GCN3, which inactivates its positive regulatory function required under starvation conditions without impairing its ability to promote functions carried out by GCD12 under nonstarvation conditions.**

Expression of amino-acid-biosynthetic genes in the yeast *Saccharomyces cerevisiae* is regulated by at least two mechanisms. The first involves pathway-specific repression by the amino acid end products of certain pathways. A second mechanism, known as general amino acid control, leads to increased transcription of at least 30 genes encoding enzymes in nine different pathways in response to starvation for any single amino acid. The products of nine *GCN* genes are required for derepression of structural genes subject to general control under starvation conditions. The products of 12 *GCD* genes are required for repression of these genes under normal growth conditions. Studies of epistasis relationships among regulatory mutations suggest that the products of *GCN1*, *GCN2*, and *GCN3* act indirectly as positive effectors by negative regulation of *GCD* gene products (for a review, see reference 19). *GCN4*, identified by this genetic analysis as the most direct positive regulator in the general control system, was shown to function as a transcriptional activator by binding to regulatory sequences located upstream from structural genes subject to the general control (1, 22).

Expression of *GCN4* itself is regulated by amino acid availability. In wild-type cells, synthesis of GCN4 protein increases under amino acid starvation conditions. This response is dependent on the products of the positive regulators encoded by *GCN2* and *GCN3*. Mutations in *GCD1* and *GCD10* to *GCD13* lead to constitutive derepression of *GCN4* expression, showing that these *GCD* factors are required for repression of *GCN4* expression under nonstarvation conditions (12, 17). In contrast to the transcriptional regulation of amino acid biosynthetic genes subject to general control, regulation of *GCN4* expression has a significant translational component. Four short open reading frames (ORFs) are present in the 600-nucleotide leader of *GCN4* mRNA. Removal of these ORFs, either by deletion or by point mutations in the AUG codons, leads to constitutive derepression of *GCN4* expression, independent of amino acid starvation and other general control *trans*-acting factors. Mutations in the upstream ORFs have little effect on *GCN4* mRNA levels, indicating that these sequences regulate *GCN4* expression at the translational level (16, 31, 39). A ca. 240-nucleotide segment containing the four ORFs is sufficient to confer regulation typical of *GCN4* on translation of a heterologous transcript (30). These results identify the *GCN4* upstream ORFs as the regulatory targets of the products of *GCN2*, *GCN3*, *GCD1*, and *GCD10* to *GCD13*.

The fact that mutations in *GCD* genes restore derepression of *GCN4* expression in *gcn2* and *gcn3* mutants led to the aforementioned conclusion that *GCN2* and *GCN3* stimulate *GCN4* expression by negative regulation of *GCD* factors. Demonstration of allele specificity between the *gcd1-101* mutation and different *gcn3* mutant alleles raised the possibility that this antagonism involves a direct interaction between the *GCN3* and *GCD1* gene products (21). Analysis of allele-specific interactions was recently extended to include a deletion-insertion allele of *GCN3* (described herein), additional mutant alleles of *GCD1*, and mutations in the

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GCD12 gene. The results showed that the two mutant phenotypes associated with newly isolated *gcd1* mutations (constitutive derepression of *GCN4* expression and temperature sensitivity for growth) are exacerbated by a null allele of *GCN3*. In fact, expression of both phenotypes in *gcd12* mutants is absolutely dependent on a loss-of-function *gcn3* mutation. The fact that wild-type *GCN3* reduces the effects of *gcd1* and *gcd12* mutations indicates that the *GCN3* product can either promote or substitute for the functions of GCD1 and GCD12 needed for repression of *GCN4* expression and normal growth rates under nonstarvation conditions, even though GCN3 antagonizes the negative regulatory function of these *GCD* factors under amino acid starvation conditions. (At least for *GCD1*, GCN3 could promote rather than substitute for GCD1 function because Tsm$^-$ *gcd1* mutations are known to be leaky. The latter follows from the fact, discussed below, that a *GCD1* deletion is lethal.) The *gcn3-102* allele behaves like wild-type *GCN3* in suppressing both mutant phenotypes associated with *gcd1* and *gcd12* mutations; however, it is completely defective for *GCN3* positive regulatory function (11). This result suggested that the two modes of GCN3 action may depend on different domains of the GCN3 protein.

Because a *gcn3* deletion can lead to mutant phenotypes under both normal and starvation conditions, the *GCN3* product is expected to be present in both states. If so, it is necessary to account for the fact that GCN3 stimulates *GCN4* expression only under starvation conditions. One possibility is that *GCN3* expression is regulated at the transcriptional or translational levels and that different amounts of GCN3 protein have different effects on *GCN4* expression. Alternatively, a modification of the *GCN3* product may be required for activation of its positive regulatory function under starvation conditions. A third possibility is that the structure or activity of some other *GCN* or *GCD* factor that is functionally or physically associated with GCN3 is altered by amino acid availability, thereby activating GCN3 function indirectly. To initiate our analysis of this problem at the molecular level, we have carried out a complete physical characterization of the *GCN3* gene and its mRNA product. Our results suggest that *GCN3* encodes a protein that occurs at similar levels under both normal and starvation conditions, implying that modulation of *GCN3* positive regulatory function occurs posttranslationally.

We also show that a deletion of *GCN3* in wild-type cells leads to reduced growth rates only under amino acid starvation conditions, resulting from an inability to derepress the transcription of structural genes subject to the general control. By contrast, deletion of *GCN3* is unconditionally lethal in a *gcd1-101* strain, supporting the idea that *GCN3* is expressed under normal growth conditions and contributes to an essential cellular function carried out by GCD1. Furthermore, we show that an alteration of the carboxyl terminus of GCN3 inactivates its positive regulatory role without impairing the ability to enhance the essential and the negative regulatory functions of GCD12. This finding supports the notion that modulation of *GCN3* positive regulatory function in response to changes in amino acid availability requires a change in the structure or activity of a particular domain of the GCN3 protein.

MATERIALS AND METHODS

Strains and genetic techniques. *Escherichia coli* strains used for plasmid propagation were DH5α (9) or DB6507, which is HB101 (3) carrying a Tn*5* insertion at *pyrF* (obtained from D. Botstein). *E. coli* JM109 (40) was used for propagation of M13 bacteriophage derivatives. The *dam* strain GM33, a gift from D. Thiele, was obtained through New England BioLabs Inc., Beverly, Mass. The genotypes of all *S. cerevisiae* strains used are listed in Table 1.

TABLE 1. *S. cerevisiae* strains

Strain	Genotype
S288C	*MAT*α
H4	*MAT***a** *leu2-3 leu2-112 ura3-52*
H15	*MAT*α *leu2-3 leu2-112 ura3-52 gcn2-101*
H17	*MAT*α *leu2-3 leu2-112 ura3-52 gcn3-102*
H24	*MAT*α *ura3-52 gcn4-101*
F98	*MAT*α *ura 3-52 gcd1-101*
H211	*MAT***a** *gcn3-102 gcd1-101 ura3-52*
H221	*MAT*α *gcn3-102 ura3-52 inol GAL2 leu2-3 leu2-112 ura3-52*
H652	*MAT*α *gcn3::LEU2 gcd12-503 ura3-52 leu2-3 leu2-112*
EY45	*MAT*α *leu2-3 leu2-112*
EY47	*MAT*α *ura3-52 leu2-3 leu2-112*
EY48	*MAT***a** *gcn3-102 leu2-3 leu2-112*
EY51[a]	*MAT***a** *leu2-3 leu2-112 ura3-52 gcn3::LEU2*
EY93	*MAT*α *gcn3::LEU2 leu2-3 leu2-112 lys2*
EY94	*MAT***a** *gcn3::LEU2 leu2-3 leu2-112 lys2*
EY125[b]	*MAT*α *leu2-3 leu2-112 ura3-52 gcn3::LEU2*

[a] Isogenic with H4.
[b] Isogenic with H17.

Double-stranded plasmid and phage DNAs were introduced into bacteria by the method of Hanahan (8) or by the calcium chloride technique (29) for strain GM33. Yeast culture and genetic analysis were performed as described previously (36). Yeast transformations were done by the lithium acetate method (24). Complementation of *gcn3* mutations was scored by the ability to grow on SD medium (36) supplemented with all amino acids at 0.1 mg/ml, except for leucine (present at 40 mM) and histidine (omitted), plus 3-amino-1,2,4-triazole (AT) at 30 mM. (AT is a competitive inhibitor of the *HIS3* product and thus produces histidine starvation.) For complementation analysis of the *GAL1-5'*Δ constructs, the same medium was used with 2% galactose instead of 2% glucose.

Plasmid constructions. YCp50 is an *S. cerevisiae-E. coli* shuttle vector containing the yeast *URA3* gene for isolation of transformants and the yeast *ARS1* and *CEN4* sequences for autonomous low-copy-number propagation in *S. cerevisiae* (25). YEp24 is an *S. cerevisiae-E. coli* shuttle vector containing the yeast *URA3* gene and sequences from the yeast 2μm plasmid for autonomous high-copy-number propagation in *S. cerevisiae* (2). A 5.0-kilobase (kb) *Eco*RI fragment containing the *GCN3* gene was subcloned from pAH17 (21) in both orientations into the unique *Eco*RI restriction site of YCp50, producing mp116 and mp120. (In mp116, the 5'-3' orientations of *URA3* and *GCN3* are the same.) mp116 and mp120 were linearized at unique *Bam*HI and *Sph*I restriction sites present in the vector, and unidirectional deletions were generated from the *Bam*HI termini by using the exonuclease III procedure of Henikoff (14). After the plasmids were treated with S1 nuclease and Klenow fragment to generate blunt ends, *Bam*HI octameric linkers (New England BioLabs) were attached prior to recircularization of the resulting deletion plasmids.

Ep69 is one such mp116-derivative containing the 4-kb deletion fragment 3'Δ+1546 (see Fig. 1 and 2). Ep150 was derived from Ep69 by digestion at the unique *Cla*I restriction site in the *GCN3* sequence, formation of blunt termini with

Klenow fragment, and recircularization with T4 DNA ligase. The correct construct was identified by the formation of an *Nru*I site in place of the *Cla*I site. Construction of Ep177 began by insertion of the 1.48-kb *Dra*I-*Bam*HI *GCN3* fragment (see Fig. 2, +72 to +1546), isolated from Ep69, between the *Hin*cII and *Bam*HI sites of pSP64 (Promega Biotec, Madison, Wis.) to create Ep155. After Ep155 had been propagated in *dam E. coli* GM33, it was digested at the unique *Xba*I site in *GCN3*, and an *Xba*I-*Sma*I adapter (dCTAGCCCGGG; Pharmacia/PL Biochemicals) was inserted. The 0.88-kb *Cla*I-*Bam*HI fragment from the resulting plasmid (Ep176) was used to replace the corresponding fragment in Ep69, producing Ep177.

Ep179, Ep181, and Ep183 were constructed from mp120 derivatives containing the 5′ deletion constructs 5′Δ+102, 5′Δ+72, and 5′Δ+228, respectively, by insertion of a *Bam*HI fragment containing the *GAL1,10* transcriptional control element at the *Bam*HI site present at each deletion junction. A 0.69-kb *GAL1,10* fragment was obtained from a modified version of plasmid pBM150 (25) containing a *Bam*HI site adjacent to the *Eco*RI site at the *GAL10*-vector junction (Ep178). Addition of the *Bam*HI site to pBM150 involved a minor modification of the linker-tailing procedure (27), by using a *Bam*HI decameric linker obtained from New England BioLabs.

Plasmid Ep157, used in RNA blot hybridization analysis, was constructed by isolating the 0.97-kb *Dra*I-*Bam*HI *GCN3* fragment (+72 to +1044) from the mp116 derivative containing 3′Δ+1044 and inserting this fragment at the *Hin*cII and *Bam*HI sites in the polylinker of pSP64. Ep168, used in nuclease mapping of the 5′ ends of *GCN3* mRNA, was constructed by inserting the 1.02-kb *Bam*HI-*Cla*I *GCN3* fragment (positions −351 to +671) from the mp120-derived deletion plasmid containing 5′Δ−351 at the *Bam*HI and *Acc*I sites in the polylinker of pSP65 (Promega Biotec).

Plasmids Ep146 and Ep149, used in constructing *gcn3* deletion-disruption alleles, were generated as follows. Ep69 was digested with *Cla*I, and the unique *Cla*I site at position +671 in the *GCN3* sequence (see Fig. 2) was converted to a blunt end with Klenow fragment and modified by the attachment of a *Hin*dIII octameric linker (New England BioLabs). This linear DNA was then digested with *Hin*dIII to remove excess linkers and cleaved at the *Hin*dIII site at position −115 in the *GCN3* sequence. The resulting large DNA fragment, lacking the *GCN3* sequences at −115 to +671, was purified by agarose gel electrophoresis and recircularized to create Ep142. A 2.2-kb *Sal*I-*Xho*I *LEU2* fragment was isolated from YEp13 (4), made blunt ended with Klenow fragment, modified by the addition of *Hin*dIII octameric linkers, and inserted at the *Hin*dIII site in Ep142 to create Ep146. A 1.1-kb *URA3* fragment was isolated from Ylp26 (2) and inserted at the *Hin*dIII site of Ep142 to create Ep149.

Plasmid Ep174, used in making *gcd1*::*LEU2* disruptions, was constructed as follows. YCp50-Sc4014 was produced by insertion of a 2.4-kb *Bam*HI fragment containing the *GCD1* gene at the *Bam*HI site of YCp50 (D. Hill and K. Struhl, personal communication). A 1.1-kb *Hin*dIII fragment containing *GCD1* sequences −134 to +955 (where +1 is the 5′ end of *GCD1* mRNA) was replaced by the 2.2-kb *Hin*dIII *LEU2* fragment from Ep146.

The *GCN3*-*lacZ* fusion was constructed by first subcloning the 1.66-kb *Hin*dIII-*Bam*HI fragment (positions −115 through +1546) from Ep69 into M13mp19 (New England BioLabs). By using the two-primer method of Pielak et al. (33) and a synthetic 42-residue oligonucleotide, we altered the sequence in the interval from 5′-GATTAAAAAAAT CACAT-3′ to 5′-GATGGTCGACAATAACAT-3′ (+1019 to +1036), thereby removing the *GCN3* termination codon and inserting a new *Sal*I site (*GCN3-501*). The *GCN3* nucleotide sequence of the resulting single-stranded phage DNA was determined from +671 (*Cla*I site) to +1546 (*Bam*HI site) by using synthetic oligonucleotide primers and the dideoxyribonucleotide chain termination technique (35) to ensure the absence of unintended mutations. The 0.88-kb *Cla*I-*Bam*HI fragment containing the mutation was used to replace the corresponding fragment in Ep69. The resulting plasmid (Ep186) was digested with *Sal*I, and a 3.1-kb *Sal*I fragment containing an amino-terminally truncated *lacZ* coding sequence from pMC1871 (a gift from M. Casadaban) was inserted in frame with *GCN3* protein-coding sequences (plasmid Ep188).

The high-copy *GCN3*-*lacZ* fusion plasmid Ep225 was constructed by replacing the 0.7-kb *Nhe*I-*Eag*I fragment of YEp24 with a 5.3-kb *Nhe*I-*Eag*I fragment from Ep188 containing the fusion construct. The *Nhe*I restriction site in Ep188 is located upstream from the *GCN3* coding sequence at position −252; the *Eag*I site is located in vector sequences downstream from *GCN3*, approximately 0.38 kb from the *GCN3* insert-vector junction. A similar *Nhe*I-*Eag*I fragment from Ep69 that contains only *GCN3* sequences is sufficient to complement a *gcn3* mutation when inserted into YEp24 (data not shown).

DNA sequence analysis. Sequence analysis of *GCN3* was performed by the dideoxy-chain termination technique (35) with the exonuclease III-generated deletion plasmids derived from mp116 and mp120 described above. In some cases, double-stranded plasmid DNA was used directly (41). Otherwise, single-stranded DNA derived from M13 subclones of *GCN3* deletion constructs was used. For deletion constructs containing 3.4 kb of *GCN3* DNA or less, the M13 subclones were obtained by inserting the appropriate *Eco*RI-*Bam*HI fragments from mp116 and mp120 derivatives at the *Eco*RI and *Bam*HI sites of M13mp18 or M13mp19 (40). For deletion constructs containing larger amounts of *GCN3* DNA, *Hin*dIII-*Bam*HI fragments were subcloned into the same M13 vectors by using the *Hin*dIII site at position −115 in the *GCN3* sequence.

DNA sequences were analyzed with the Integrated Database and Extended Analysis System for Nucleic Acids and Proteins (IDEAS; M. Kanehisa, National Cancer Institute, Frederick Cancer Research Facility, Frederick, Md.) and the FASTP program (28).

RNA analysis. Yeast strains were cultured in SD medium (36) supplemented with 2 mM leucine, 0.5 mM isoleucine, 0.5 mM valine, and 0.2 mM uracil (when required). Starting from a 1:50 dilution of a saturated culture, cells were grown for 8 h at 30°C and 300 rpm to the mid-logarithmic phase for repressing growth conditions. For derepressing conditions, AT was added to 10 mM after 2 h and cells were grown for another 6 h. Cultures were harvested and total RNA was prepared as described (10), omitting bentonite. RNA blot hybridization was performed as described (17). The levels of pyruvate kinase (*PYK*) mRNA were first determined by hybridization with radiolabeled plasmid FR2 (provided by G. R. Fink). RNA samples containing equivalent amounts of *PYK* mRNA were then analyzed for *HIS4*, *GCN3*, or *GCN3*-*lacZ* mRNA levels. The *HIS4* probe was a 2.8-kb *Eco*RI fragment, containing the 3′ end of the *HIS4* transcription unit, isolated from pR5 (provided by P. Farabaugh). The probe used for *GCN3* and *GCN3*-*lacZ* mRNAs was the 0.6-kb *Dra*I-*Cla*I *GCN3* fragment isolated from plasmid Ep157 as a *Hin*dIII-*Cla*I fragment, with a *Hin*dIII site in the

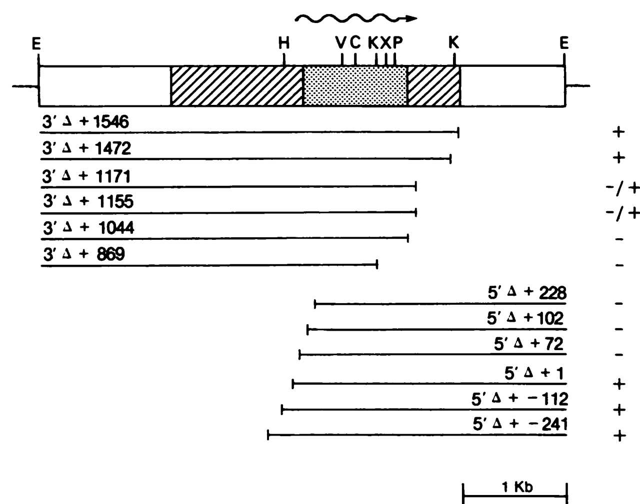

FIG. 1. Deletion analysis of *GCN3*. Symbols: ▨, *GCN3* ORF that falls within the 2.7-kb region (▨) for which the complete nucleotide sequence was determined (see Fig. 2); ⤳, position and orientation of the *GCN3* transcript. Unidirectional deletions of a 5.0-kb *Eco*RI *GCN3* fragment are indicated below the schematic, identified by the position of the deletion junction in the DNA sequence shown in Fig. 2. The lines indicate the DNA remaining for each deletion. Selected deletion plasmids were tested for their ability to complement the AT^s phenotype of *gcn3-102* in transformants of H17. Symbols: +, strong complementation; −/+, weak complementation; −, no complementation.

polylinker. All probes were radiolabeled with [α-^{32}P]dCTP by the random primer method (7). Strand-specific radiolabeled RNA probes were synthesized by SP6 RNA polymerase in vitro from Ep155 and Ep158, containing identical *GCN3* fragments inserted at the polylinker of pSP64 or pSP65, respectively (Promega Biotec). Transcription from *Bam*HI-cleaved Ep155 produces a 1.5-kb positive-polarity transcript from positions +72 to +1546 in the *GCN3* sequence. Ep158 contains the same *GCN3* fragment as Ep155 inserted at the *Hin*cII and *Bam*HI sites of pSP65. Transcription from *Sal*I-cleaved Ep158 produces a 1.5-kb RNA species of minus polarity with respect to *GCN3* mRNA.

For nuclease mapping experiments, poly(A)$^+$ RNA was isolated from total yeast RNA by oligo(dT)-cellulose chromatography as described previously (10). To prepare the probe for 5′-end mapping, Ep168 was digested with *Ssp*I (see Fig. 2, +121), and the 5′ termini were dephosphorylated with calf intestinal phosphatase and labeled with ^{32}P by using [γ-^{32}P]ATP and T4 polynucleotide kinase. Following digestion with *Bam*HI, the *Ssp*I-*Bam*HI C fragment (positions −351 to +121) was isolated, denatured, and subjected to polyacrylamide gel electrophoresis to obtain the labeled single strands used as the probe (29). For 3′-end mapping, Ep155 was digested with *Xba*I and the 3′ termini were labeled with Klenow fragment and [α-^{32}P]dCTP. After digestion with *Bam*HI, the *Xba*I-*Bam*HI B fragment (positions +893 to +1546) was isolated and processed as described above to obtain the labeled single strands used as the probe. RNA-DNA hybridization and nuclease digestions were performed as described previously (20). Primer extension experiments were conducted by the method of Nasmyth et al. (32) with the synthetic primer 5′-CTTAATAGCGTGAC CAATGCTTC-3′, complementary to positions +188 through +210 in the *GCN3* sequence. The primer was labeled at its 5′ terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Assay of *GCN3-lacZ* fusion constructs. Yeast strains containing plasmid-borne *GCN3-lacZ* fusions were cultured as described above for RNA isolation, omitting either uracil or leucine-isoleucine-valine as required to maintain selection for the plasmids. Cultures in the mid-logarithmic phase were divided and harvested for parallel measurements of RNA (see above) and fusion enzyme activity. β-Galactosidase activity was assayed as described previously (17) and expressed as nanomoles of ONPG (*o*-nitrophenyl-β-D-galactopyranoside) cleaved per minute per milligram of protein.

Construction of *gcn3* and *gcd1* deletion-insertion alleles. For a diagram of the fragments isolated from Ep149 and Ep146 for constructing *gcn3*::*URA3* and *gcn3*::*LEU2* disruptions, respectively, see Fig. 5. These fragments were purified by preparative gel electrophoresis and used to transform *ura3* or *leu2* yeast strains to Ura$^+$ or Leu$^+$, respectively (34). Ep174 was digested with *Bam*HI, and the resulting 3.5-kb fragment containing *gcd1*::*LEU2* was isolated and used to transform *leu2* yeast strains to Leu$^+$. All gene replacements were confirmed by DNA blot hybridization analysis (37) of total transformant DNA. Total yeast DNA was prepared essentially as described previously (38). DNA was digested with *Eco*RI prior to agarose gel electrophoresis and transfer to nitrocellulose. Blots from *gcn3* disruption strains were probed with the 4.0-kb *Eco*RI-*Bam*HI *GCN3* fragment from Ep69 (3′Δ+1546). The 2.37-kb *Bam*HI *GCD1* fragment from YCp50-Sc4024 was used to probe blots from *gcd1*::*LEU2* stains. All probes were radiolabeled by the random primer technique (7).

RESULTS

Deletion mapping of the *GCN3* complementation unit. *gcn3* mutations confer sensitivity to inhibitors of amino acid biosynthesis such as AT. The *GCN3* gene was cloned on the high-copy-number plasmid pAH17 by complementation of the AT sensitivity (AT^s) conferred by the *gcn3-102* mutation (21). A 5.0-kb *Eco*RI fragment was subcloned from pAH17 into the *Eco*RI site of the low-copy-number plasmid YCp50. Two plasmids (mp116 and mp120) containing the 5.0-kb *Eco*RI fragment inserted in different orientations were obtained and introduced by transformation into *S. cerevisiae* H17 containing the *gcn3-102* mutation. Both mp116 and mp120 complement the AT^s phenotype of H17 and therefore contain the entire *GCN3* gene.

To define more precisely the boundaries of the *GCN3* complementation unit, we constructed unidirectional deletions in mp116 and mp120 from a restriction site in the YCp50 sequences, generating nested deletions from each end of the 5.0-kb *Eco*RI fragment (Fig. 1). Selected deletion alleles were introduced into H17 by transformation and tested for complementation of the AT^s phenotype of *gcn3-102*. The result of this analysis localized the *GCN3* complementation unit to a 1.5-kb interval bounded by the endpoints of deletions 5′Δ+1 and 3′Δ+1472 (Fig. 1).

The *GCN3* complementation unit is coincident with a single long ORF. DNA sequence analysis of the *GCN3* region revealed the presence of a single long ORF of 305 amino acids, beginning at position +107 and extending to position +1021 in the sequence shown in Fig. 2. The smallest of the aforementioned 5′ deletions that complements *gcn3-102* removes all sequences upstream from position +1 corresponding to the 5′ end of *GCN3* mRNA, as determined below. The ability of 5′Δ+1 to complement *gcn3-102* could indicate that the *GCN3* promoter lies within the transcription unit. Alternatively, a gratuitous promoter could be present upstream

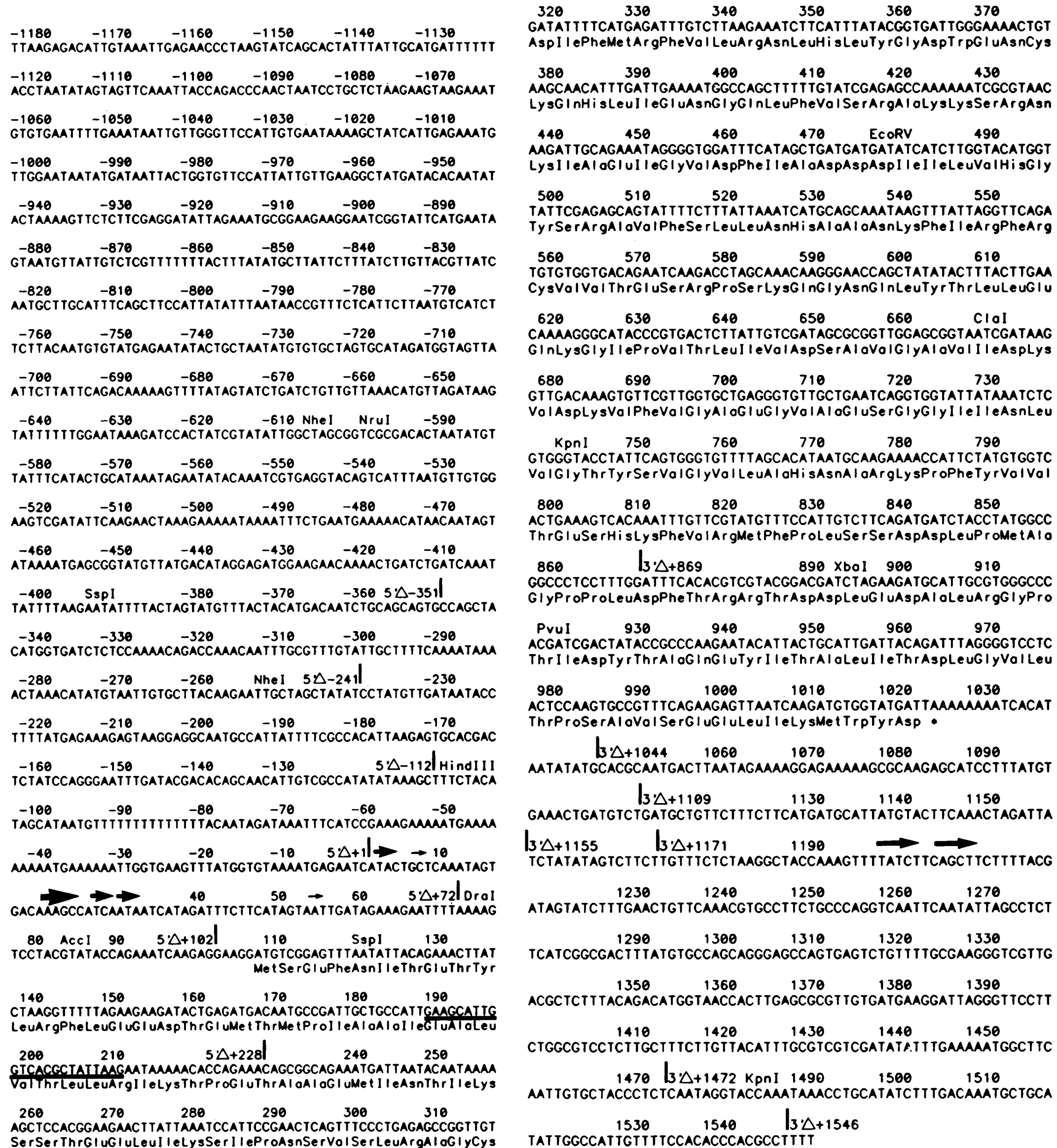

FIG. 2. DNA sequence analysis of *GCN3*. The complete sequence of 2,733 nucleotides in the *GCN3* region was determined on both strands. The predicted sequence of a 305-amino-acid polypeptide is listed beneath the nucleotide sequence, beginning at position +107 and extending to +1021. The junctions of deletions depicted in Fig 1 are shown above the sequence. Solid arrows indicate the positions and, by their size, the relative amounts of *GCN3* mRNA 5′ ends at positions +1, +6, +20, +26, +29, and +53, as determined by primer extension analysis. The complementary sequence of the oligonucleotide used for primer extension is underlined (positions +188 to +210). Arrowheads at positions +1201 and +1208 indicate approximate locations of the 3′ ends of *GCN3* mRNA.

from *GCN3* in the YCp50 sequences. The latter explanation is favored by the fact that the 5′ end of the *URA3* gene is adjacent to the deletion junction in each member of the 5′ deletion series. Additionally, a *Hin*dIII fragment containing *GCN3* but lacking all sequences upstream from −115 complements *gcn3-102* only if inserted into YCp50 in the same orientation used in the 5′ deletion plasmids (data not shown).

Larger 5′ deletions that additionally remove the mRNA start sites (5′Δ+72 and 5′Δ+102) and amino-terminal protein-coding sequences (5′Δ+228) fail to complement *gcn3-102*. A fragment from the *GAL1-GAL10* intergenic region containing transcriptional upstream activation sequences and the first 56 nucleotides of the *GAL1* mRNA leader region was inserted at the deletion junctions of 5′Δ+72, 5′Δ+102, and 5′Δ+228. The inserted sequences do not include the *GAL1* initiation codon. The *GAL1*-5′Δ+72 and *GAL1*-5′Δ+102 constructs should direct the synthesis of the entire *GCN3* polypeptide, whereas the *GAL1*-5′Δ+228 construct is expected to express an amino-terminally truncated protein lacking the first 44 codons of the *GCN3* ORF. The *GAL1* promoter insertion restored the ability of the 5′Δ+72 and 5′Δ+102 alleles to complement the AT^{s} phenotype of *gcn3-102* strain H221, provided that galactose rather than glucose was used as the carbon source. By contrast, no complementation was observed with either carbon source for the *GAL1* promoter insertion into 5′Δ+228. These results are consistent with our assignment of the beginning of the *GCN3* protein-coding sequences shown in Fig. 2 and suggest that the amino-terminal 44 residues are required for expression of active GCN3 protein.

All 3′ deletions that remove sequences from the *GCN3* transcription unit also fail to complement *gcn3-102*. This

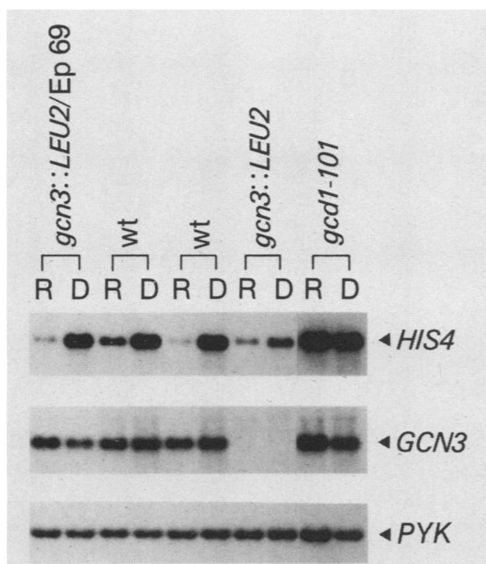

FIG. 3. RNA blot hybridization analysis of *GCN3* mRNA. Total RNA from the indicated yeast strains was fractionated by 1% agarose-formaldehyde gel electrophoresis, transferred to Gene-Screen Plus membranes (New England Nuclear Corp.), and hybridized with radiolabeled DNA probes for the indicated genes. (Exposures for the three probes were different). Strains used (from left to right): EY51 (*gcn3*::*LEU2*) transformed with Ep69, a low-copy plasmid containing the cloned *GCN3* gene; H4 (wt), examined in duplicate; EY51 (*gcn3*::*LEU2*); F98 (*gcd1-101*). (The *gcn3*::*LEU2* allele in EY51 was constructed by disruption of GCN3 in strain H4; see below.) R and D refer to conditions repressing or derepressing for genes under general control, respectively, as described in Materials and Methods. In a separate experiment, the size of *GCN3* mRNA was determined to be approximately 1.2 to 1.3 kb by comparison with RNA size markers (data not shown).

result was somewhat surprising, since four of these deletions ($3'\Delta+1044$, $3'\Delta+1109$, $3'\Delta+1155$, $3'\Delta+1171$) contain the complete *GCN3* protein-coding sequence. When the $3'\Delta+1044$ and $3'\Delta+1155$ alleles were introduced into a *gcn3* deletion strain described below, they failed to produce detectable amounts of *GCN3* mRNA (data not shown). Therefore, these deletions block *GCN3* expression at the level of transcription or mRNA stability.

Additional corroboration of our assignment of the *GCN3* ORF was provided by the construction of frameshift mutations at the *Cla*I and *Xba*I restriction sites in the *GCN3* coding sequences on the low-copy-number plasmid, Ep69, containing the complementing allele $3'\Delta+1546$ (Fig. 1). The mutation at the *Cla*I site (+668) shifts the *GCN3* ORF by +1 nucleotide at a position ca. 60% of the *GCN3* ORF downstream from the presumptive initiation codon. The *Xba*I mutation shifts the reading frame by +2 nucleotides at a position ca. 86% of the coding region downstream from the initiation codon. Both alleles produce normal levels of *GCN3* mRNA (data not shown) but fail to complement the AT^s phenotype of *gcn3-102* when introduced into strain H17.

The *GCN3* protein-coding sequence is transcribed into a 1.2-kb mRNA. We examined transcription of *GCN3* by RNA blot hybridization analysis with a 0.6-kb *Dra*I-*Cla*I *GCN3* fragment (+72 to +671) as the hybridization probe. This fragment lies completely within the *GCN3* complementation unit and spans 62% of the *GCN3* ORF. The results of this analysis indicate the presence of a 1.2-kb hybridizing species present in total RNA (Fig. 3). This transcript is enriched in a poly(A)$^+$ fraction of total RNA obtained by oligo(dT)-cellulose chromatography (data not shown). With this technique, no difference in the size of *GCN3* mRNA was observed in RNA isolated from cells grown under normal conditions versus amino acid starvation conditions (Fig. 3).

We mapped the 5' end of *GCN3* mRNA by S1 nuclease and exonuclease VII protection experiments with poly(A)$^+$ RNA isolated from wild-type strain S288C. The DNA probe used in these studies was the noncoding stand of the 0.47-kb *Bam*HI-*Ssp*I fragment (−351 to +121), isolated from the $5'\Delta-351$ construct and 5' end labeled at the *Ssp*I site (Fig. 4A). The results of this analysis suggest the occurrence of multiple 5' ends for *GCN3* mRNA mapping to an interval located 90 to 130 nucleotides upstream from the *Ssp*I site at +121 (Fig. 4B). The observed pattern of 5' ends is the same in RNA isolated from cells grown under normal conditions and amino acid starvation conditions. The 5' ends were mapped more precisely by primer extension analysis with a synthetic oligonucleotide complementary to positions +188 to +210 (Fig. 4C). All 5' ends mapped by primer extension fall within the interval defined by S1 nuclease and exonuclease VII mapping experiments. The 5' proximal start site detected by primer extension was designated +1; the major start site maps to position +20 in the DNA sequence shown in Fig. 2.

The 3' ends of *GCN3* mRNA were determined by S1 nuclease and exonuclease VII mapping (Fig. 4D) with a single-stranded probe 3' end labeled at the unique *Xba*I site in the *GCN3* coding sequence (+896) and extending in the 3' direction to a *Bam*HI site present at position +1546 in deletion plasmid $3'\Delta+1546$. *GCN3* mRNA protects probe fragments from exonuclease VII ranging in size from ca. 303 to ca. 325 nucleotides, with major protected fragments of ca. 308 and ca. 315 nucleotides. Combination of the results of the 5' and 3' mapping of *GCN3* mRNA suggests that the *GCN3* transcript is ca. 1,200 nucleotides in length, in agreement with approximate size measurements made from RNA blot hybridization analysis.

Features of the *GCN3* sequence. On the basis of results of the DNA sequence analysis and mapping the 5' end of *GCN3* mRNA, we conclude there are no out-of-frame upstream AUG codons in the leader of *GCN3* mRNA, such as occur in the *GCN4* transcript (16, 39). In addition, there are no sequences with strong similarity to the GCN4 binding site consensus sequence (5'-RRTGACTCATTT-3' [1, 15]) present in the 1.2 kb of 5' noncoding DNA shown in Fig. 2. Thus, *GCN3* is devoid of regulatory sequences found at certain other genes in the general control system.

The predicted amino acid sequence of *GCN3* exhibits no strong similarity to any other known protein sequence present in the National Biomedical Research Foundation or the European Molecular Biology Laboratory data bases. Interestingly, the amino-terminal portion of the predicted GCN3 polypeptide contains a potential N-linked glycosylation site at amino acid residue 5 (Asn-Ile-Thr [13, 23]). This sequence is followed by a hydrophobic region (residues 20 to 36) predicted to possess membrane-spanning capability (26). This region has both α-helix- and β-sheet-forming potential when analyzed by using the Chou and Fasman rules for predicting protein secondary structure (5). These features may be important for correct processing or localization of the GCN3 polypeptide. Alternatively, the hydrophobic region may act to stabilize complexes. To examine these possibilities, we are attempting to raise antibodies to the GCN3 protein.

Construction of deletion-insertion alleles of *GCN3*. We used

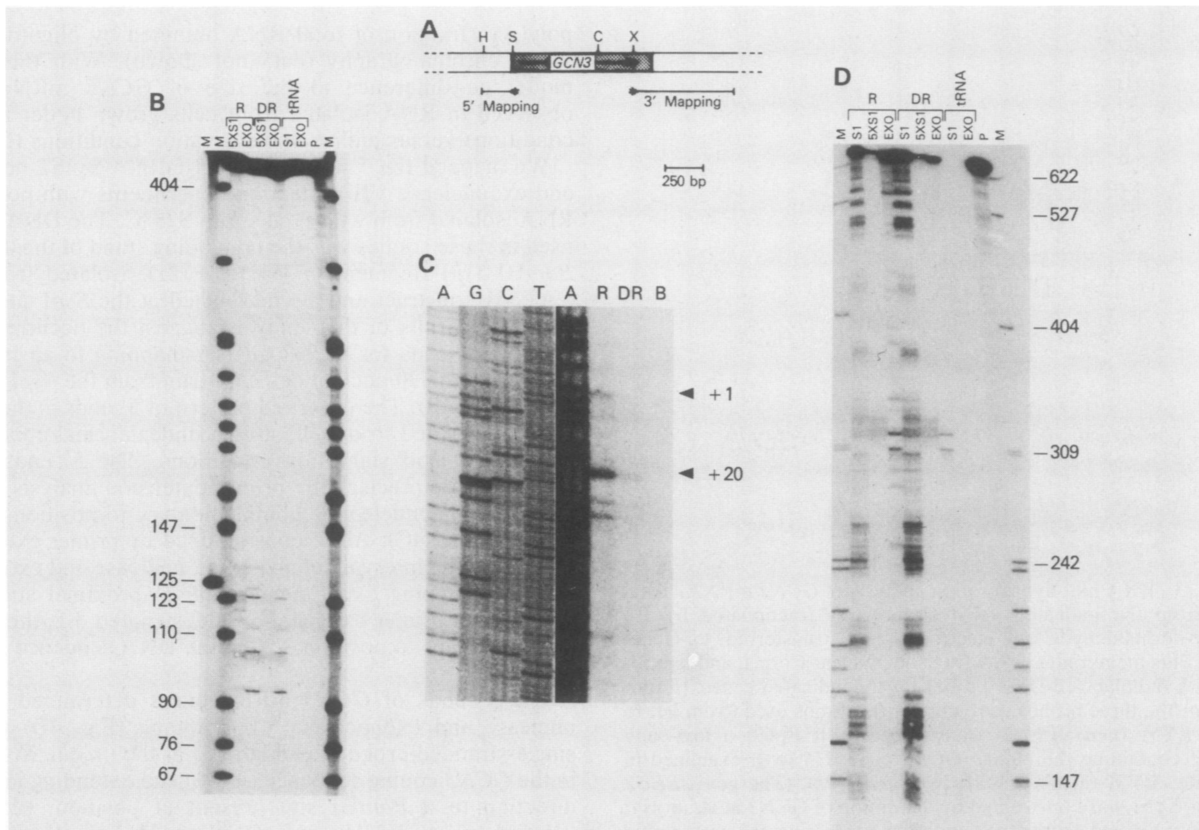

FIG. 4. Mapping the ends of *GCN3* mRNA. (A) Probes used for S1 nuclease and exonuclease VII mapping of the 5′ and 3′ ends of *GCN3* mRNA. Asterisks indicate the radiolabeled terminus of each single-stranded DNA probe. Shaded region and arrow indicate the extent and direction of the *GCN3* ORF; H, *Hin*dIII; S, *Ssp*I; C, *Cla*I; and X, *Xba*I. (B) 5′ end mapping. Hybrids formed with 30 μg of poly(A)$^+$ RNA isolated from wild-type strain S288C grown under repressing (R) or derepressing (DR) conditions, or with 30 μg of *E. coli* tRNA (tRNA), and the 5′ probe radiolabeled at the *Ssp*I site were digested with S1 nuclease at 1,000 U/ml (5×S1) or exonuclease VII at 400 U/ml (EXO). Resistant hybrids were denatured and electrophoresed through a 6% polyacrylamide gel containing 8 M urea. The lengths of protected probe fragments were determined by comparison with radiolabeled *Hin*dIII fragments of lambda DNA (125 bases) and *Msp*I fragments of pBR322 DNA (lane M). Lane P contains the undigested probe. (C) 5′ end mapping by primer extension analysis. A synthetic oligonucleotide complementary to positions +188 to +210 in the *GCN3* sequence was radiolabeled at its 5′ terminus and hybridized with 30 μg of poly(A)$^+$ RNA isolated from S288C or with 30 μg of tRNA (lane B), as described in panel B. Primer extension reactions were conducted with avian myeloblastosis virus reverse transcriptase in the presence of unlabeled nucleoside triphosphates. Extension products were analyzed as in panel B and compared with a sequencing ladder (lanes A, G, C, and T) generated with the same primer, with single-stranded *GCN3* DNA as a template in dideoxy-sequencing reactions. +1 marks the longest primer extension product; +20 marks the major primer extension product. (D) Hybrids formed with 30 μg of poly(A)$^+$ RNA from S288C or with 30 μg of tRNA, as described in panel B, and the 3′ single-stranded probe radiolabeled at the *Xba*I site were digested with S1 nuclease at 200 U/ml (S1) or 1000 U/ml (5×S1) or with exonuclease VII at 400 U/ml. The lengths of resistant hybrids were determined as in panel B by comparison with a radiolabeled *Msp*I digest of pBR322 DNA (lane M). Lane P contains the undigested probe.

our knowledge of the boundaries of the *GCN3* gene to construct *gcn3* deletion-insertion alleles. These constructions began with the low-copy-number plasmid Ep69 containing the complementing 3′Δ+1546 allele. The 0.79-kb *Hin*dIII-*Cla*I fragment containing 62% of the *GCN3* coding sequences and 0.22 kb of 5′ flanking DNA (Fig. 2) was deleted from Ep69 and replaced with a 2.2-kb fragment containing the *LEU2* gene (producing Ep146) or a 1.1-kb fragment containing the *URA3* gene (producing Ep149). Both Ep146 and Ep149 fail to complement *gcn3-102* when introduced into strain H17 by transformation. The wild-type *GCN3* gene was replaced with each of these mutant alleles by transforming *leu2 ura3* strain H4 with DNA fragments containing the *gcn3*::*LEU2* or *gcn3*::*URA3* allele, selecting for Leu$^+$ or Ura$^+$ colonies, respectively (34). DNA blot hybridization analysis of total DNA isolated from the resulting transformants showed that the expected gene replacements had occurred (Fig. 5).

Both the Leu$^+$ and the Ura$^+$ transformants exhibit the AT^s phenotype characteristic of *gcn* mutants. When mated to *gcn3-102* strain H17, they each gave rise to AT^s hybrids, demonstrating the expected noncomplementation between the *gcn3* disruptions and *gcn3-102*. When mated to *GCN3 ura3 leu2* strain EY47, they yielded AT-resistant (AT^r) hybrids. Sporulation of these hybrids produced tetrads showing exclusively 2 AT^s Leu$^+$ (or Ura$^+$):2 AT^r Leu$^-$ (or Ura$^-$) segregation. No difference in growth rate was observed among the segregants under nonstarvation conditions. These results indicate that the *gcn3*::*LEU2* and *gcn3*::*URA3* alleles lack *GCN3* positive regulatory function and, in this respect, behave like recessive *gcn3* point mutations isolated previously. Furthermore, they show that the *GCN3* product is dispensable in wild-type strains under normal growth conditions, being required only under starvation conditions for derepression of genes subject to general control. Results presented in the next selection document in

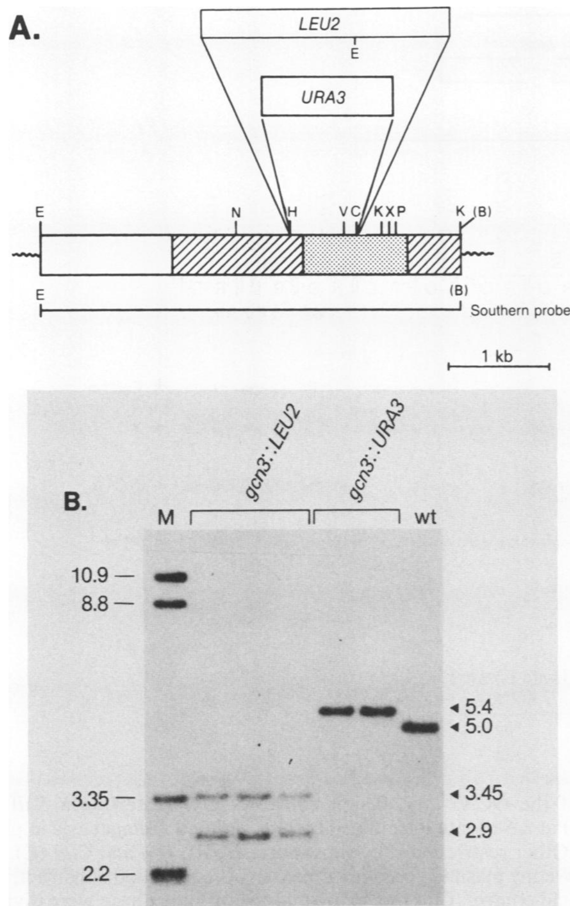

FIG. 5. *gcn3* deletion-insertion alleles. (A) The *GCN3* region is depicted schematically as in Fig. 1. A 0.79-kb *Hin*dIII (H)-*Cla*I (C) fragment from Ep69, containing 62% of the *GCN3* ORF, was replaced with a 1.1-kb fragment containing the yeast *URA3* gene or a 2.2-kb fragment containing the yeast *LEU2* gene to create Ep149 and Ep146, respectively. The 3.7-kb *Eco*RI (E)-*Pvu*I (P) fragment from Ep149 or the 3.9-kb *Nru*I (N)-*Bgl*I fragment from Ep146 (the *Bgl*I site is located in vector sequences 0.3 kb downstream from the insert-vector junction in Ep69) were used to replace the wild-type *GCN3* allele in strain H4 by transformation (34). Other restriction sites shown are *Eco*RV (V), *Kpn*I (K) and *Xba*I (X); (B) marks the *Bam*HI site present at the deletion junction of Ep69. The probe used for DNA blot hybridization analyses is the entire *GCN3* *Eco*RI-*Bam*HI fragment from Ep69. (B) DNA blot hybridization analysis of H4 (wt) derivatives containing gene replacements at the *GCN3* locus. Total yeast DNA, isolated from transformants containing putative *gcn3*::*LEU2* or *gcn3*::*URA3* gene replacements, was digested with *Eco*RI, fractionated by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with the radiolabeled probe shown in panel A. Marker lane M is a collection of *GCN3* restriction fragments, the sizes of which were determined in a separate experiment by comparison with a *Hin*dIII digest of lambda DNA.

greater detail the regulatory defects associated with these deletion mutations.

Characterization of *GCN3* expression at the transcriptional and translational levels. We wished to determine whether the level of *GCN3* positive regulatory function in the cell is controlled through regulation of the amount of GCN3 protein. Toward this end, we measured the amount of *GCN3* mRNA present under different growth conditions by blot hybridization analysis (Fig. 3). The levels of *HIS4* mRNA were examined in parallel as an indicator of transcriptional derepression mediated by the general control system: *PYK* mRNA was examined as an unregulated transcript. As expected, substantial derepression of *HIS4* mRNA was observed in response to histidine starvation in wild-type strain H4 and occurred constitutively in the *gcd1-101* strain F98. The *gcn3*::*LEU2* deletion strain EY51 showed much less derepression of *HIS4* mRNA under starvation conditions than the isogenic *GCN3* strain H4 did. This regulatory defect was overcome by transformation of EY51 with the low-copy-number plasmid Ep69 containing the wild-type *GCN3* gene. These results confirm the positive role of the *GCN3* product in transcriptional regulation of structural genes subject to general control. In contrast with the results obtained for *HIS4* mRNA, the data in Fig. 3 demonstrate little or no fluctuation in *GCN3* mRNA levels between repressing and derepressing conditions in wild-type strain H4. Likewise, the *gcd1-101* mutation has little effect on expression of the *GCN3* transcript. These data indicate that an increase in the level of *GCN3* mRNA is not required for *GCN3*-mediated positive regulation of *HIS4* transcription. On the basis of densitometric scans of lightly exposed autoradiograms of blots hybridized with probes of similar specific activity, we estimate the constitutive level of the *GCN3* transcript to be approximately 10 to 20% of the repressed level of the *HIS4* transcript.

We also investigated the regulation of *GCN3* expression at the translational level by measuring the activity of a *GCN3*-*lacZ* fusion enzyme in cells grown under repressing and derepressing conditions. Construction of the *GCN3-lacZ* fusion was begun by removing the stop codon from the *GCN3* ORF and replacing it with a *Sal*I restriction site. A *Sal*I fragment containing amino-terminally truncated *lacZ* coding sequences was then inserted at the new *Sal*I site to create an in-frame translational fusion containing all of the *GCN3* coding sequences. (The fusion was constructed in this fashion in an attempt to produce a bifunctional *GCN3-lacZ* allele that expresses *GCN3* positive regulatory function in addition to β-galactosidase activity; however, the fusion allele gives no complementation of the AT^s phenotype of H17 or EY51.) The *GCN3-lacZ* construct was introduced into *S. cerevisiae* by transformation on the low-copy replicating plasmid Ep188. In agreement with the RNA blot hybridization analysis of wild-type *GCN3* mRNA, we observed little change in fusion enzyme levels between repressing and derepressing growth conditions (Fig. 6B). In addition, the levels of fusion enzyme activity correlate well with the levels of fusion transcript present under the same circumstances (Fig. 6C). Thus, with this approach, there is no evidence for translational regulation of *GCN3* expression.

In the course of analyzing *GCN3-lacZ* fusion mRNA, we observed an unexpected transcript in all strains transformed with Ep188 (Fig. 6C, transcript X). Using strand-specific probes, we determined that this unexpected transcript is an antisense RNA originating from within *lacZ* sequences and extending into the *GCN3* coding sequences (data not shown). Since the levels of transcript X appeared to increase under starvation conditions in some strains and to be constitutively elevated in others, we were concerned that its presence might interfere with translation of *GCN3-lacZ* mRNA. The following observations suggest that no such interference occurs.

The *GCN3-lacZ* fusion was placed on high-copy plasmid YEp24 producing Ep225 and introduced into wild-type strain H4. Fusion enzyme and fusion transcript levels were measured under repressing and derepressing conditions as de-

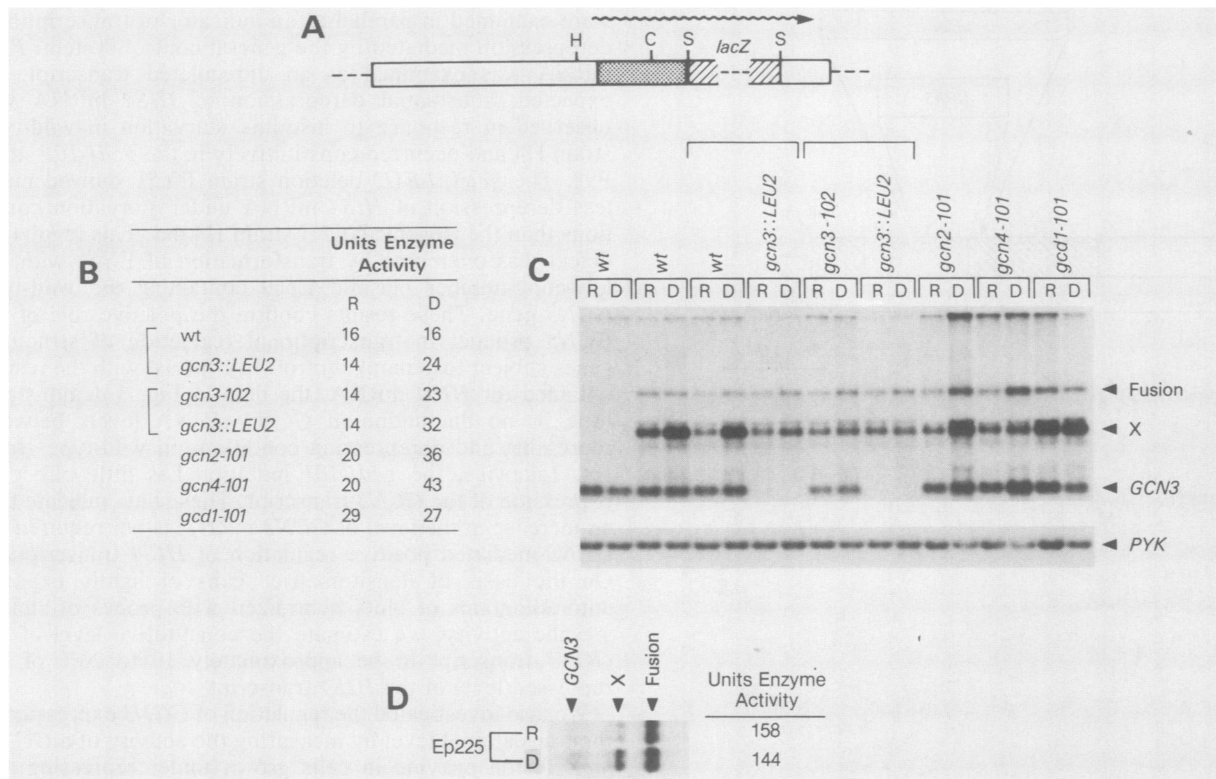

FIG. 6. Analysis of a *GCN3-lacZ* translational fusion. (A) Schematic of the *GCN3-lacZ* fusion constructed by inserting a *Sal*I (S) restriction fragment encoding an N-terminally truncated *lacZ* polypeptide (▨) at a *Sal*I site introduced by site-directed mutagenesis in place of the *GCN3* stop codon. ⇝, Direction of transcription of the fusion mRNA. Other restriction sites shown are *Hin*dIII (H) and *Cla*I (C). (B) Yeast strains with the indicated genotypes were transformed with Ep188, a low-copy plasmid containing the *GCN3-lacZ* fusion construct, and grown under conditions repressing (R) or derepressing (D) for general amino acid control. Cultures in the mid-logarithmic phase were divided and used to prepare protein extracts or total RNA (panel C), as described in Materials and Methods. Protein extracts were assayed for β-galactosidase specific activity; the values shown are the averages of at least three independent determinations that varied by 30% or less. The strains used are listed below in panel C. (C) Total RNA samples, predetermined to contain equivalent amounts of pyruvate kinase (*PYK*) mRNA, were fractionated by 1% agarose–formaldehyde gel electrophoresis and analyzed by blot hybridization for RNA species homologous to the 0.6-kb *Dra*I-*Cla*I *GCN3* fragment. Both the wild-type *GCN3* transcript (*GCN3*) and the plasmid-derived *GCN3-lacZ* fusion transcript (Fusion), in addition to an unexpected RNA species (X), hybridize with this probe. The first strain examined on the left is the wild-type strain H4 lacking fusion plasmid Ep188. The remaining strains contain Ep188 and (from left to right) are H4 examined in duplicate, EY51 (*gcn3*::*LEU2*; isogenic with H4), H17 (*gcn3-102*), EY125 (*gcn3*::*LEU2*; isogenic with H17), H15 (*gcn2-101*), H24 (*gcn4-101*), and F98 (*gcd1-101*). The upper brackets indicate isogenic strains. (D) Wild-type strain H4, transformed with high-copy plasmid Ep225 containing the *GCN3-lacZ* fusion. β-Galactosidase specific activity is shown adjacent to the corresponding results from RNA blot hybridization analysis of the fusion and the X transcripts. (The RNA samples contain equivalent amounts of pyruvate kinase mRNA; data not shown.)

scribed above. The results in Fig. 6D show that fusion enzyme activity was increased ca. 10-fold by the increased dosage of Ep225 compared with the low copy-number fusion construct Ep188. As observed for Ep188 transformants, fusion enzyme levels from Ep225 are essentially constitutive. Surprisingly, the levels of antisense transcript X increased much less than those of *GCN3-lacZ* mRNA when the fusion construct was placed on Ep225. Consequently, the *GCN3-lacZ*-to-X transcript ratios were substantially different between the high-copy and low-copy fusion constructs under both repressing and derepressing conditions. The fact that both constructs express the *GCN3-lacZ* fusion enzyme constitutively suggests that the antisense transcript has little influence on translation of *GCN3-lacZ* mRNA. On the basis of these results and those shown in Fig. 6, we conclude that the amount of GCN3 protein shows little or no regulation by amino acid availability or the *trans*-acting regulatory factors encoded by *GCN4*, *GCN2*, and *GCD1*. (In support of this conclusion, preliminary results with polyclonal antibodies against GCN3 indicate that the steady-state amount of GCN3 protein is unaffected by amino acid starvation in a wild-type strain.)

Deletion of *GCN3* exacerbates the growth defect in *gcd1-101* mutants. In a previous study, we were unable to construct viable double mutants containing the *gcn3-101* and *gcd1-101* mutations, whereas a *gcn3-102 gcd1-101* double mutant was isolated by partial reversion of the temperature-sensitive growth phenotype of a *gcd1-101* strain (21). Having acquired the ability to delete *GCN3* by transformation, we reexamined the hypothesis that *gcd1-101* mutations are unconditionally lethal in strains containing a null allele of *GCN3*. Wild-type *GCN3* was replaced by *gcn3*::*URA3* in a *gcn3-102 gcd1-101 ura3-52*/*GCN3 gcd1-101 ura3-52* diploid derived from a cross between strains H211 and F98. Ura$^+$ transformants of the H211-F98 bybrid were isolated and screened by DNA blot hybridization analysis for the presence of one copy of the 5.0-kb *Eco*RI restriction fragment derived from the *GCN3* or *gcn3-102* allele and a 5.4-kb *Eco*RI restriction fragment derived from the *gcn3*::*URA3* allele. Diploids with the correct hybridization pattern, along with the (untrans-

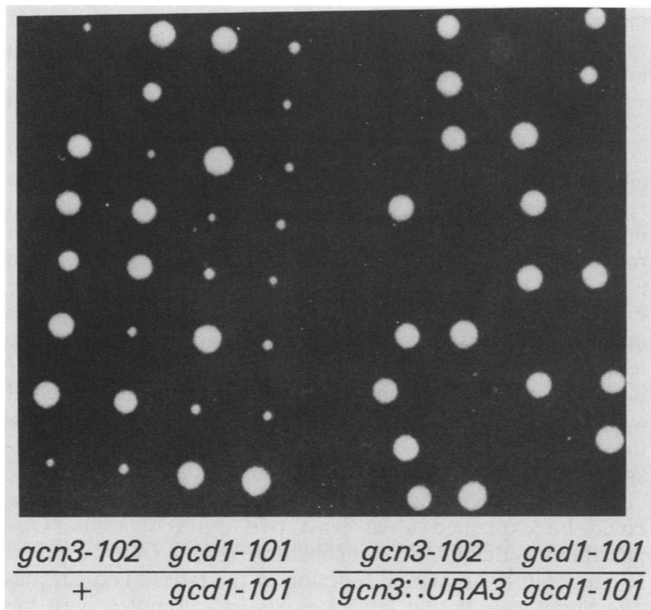

FIG. 7. *gcn3*::*URA3 gcdl-101* ascospores fail to germinate. A *gcn3-102 gcdl-101*/*GCN3 gcdl-101* diploid strain (left) and a *gcn3-102 gcdl-101*/*gcn3*::*URA3 gcdl-101* transformant (right) derived from the former by gene replacement of *GCN3* by *gcn3*::*URA3* were sporulated and subjected to tetrad analysis. Ascus dissection was conducted on complete medium YPD, and ascospores were incubated at 23°C for 5 days. The four spore clones from each of the asci are arranged in rows from top to bottom.

formed) parent diploid, were sporulated and subjected to tetrad analysis.

Tetrad analysis of the parent diploid (Fig. 7, left panel) showed 2+:2− segregation for rapid colony formation at 23°C. The faster-growing spore clones were shown to contain the *gcn3-102* allele by complementation testing with known *gcn3* mutant strains EY93 and EY94. These results confirm that *gcn3-102 gcdl-101* spores germinate more rapidly than *GCN3 gcdl-101* spores even at 23°C, the most permissive temperature for *gcdl-101* mutants. The tetrads shown dissected in the right panel of Fig. 7, produced by one of the Ura$^+$ transformants, demonstrate the segregation pattern for the *gcn3-102* and *gcn3*::*URA3* alleles in a homozygous *gcdl-101* background. In this case, two of the four spores from each tetrad either failed to germinate or required 2 to 3 weeks to form even a small colony. All spores that formed large colonies within 5 days were Ura$^-$ and failed to complement the *gcn3* tester strains, showing that they contain the *gcn3-102* allele. We conclude that spores with the genotype *gcn3*::*URA3 gcdl-101* are either inviable or extremely defective for germination, even at the most permissive temperature for *gcdl-101* mutants. Thus, it appears that *GCN3* function is unconditionally required for viability in a *gcdl-101* strain, supporting the idea that the *GCN3* product promotes the essential function of GCD1 under normal growth conditions, even though it opposes GCD1 regulatory function under starvation conditions. The *gcn3-102* allele retains the ability to promote the essential function of GCD1 in a *gcdl-101* mutant, even though it is completely defective for positive regulation of *GCN4* expression.

The *gcn3-102* mutation cannot suppress a *gcdl* deletion. The fact (illustrated in Fig. 7) that *gcn3-102 gcdl-101* strains grow better than *GCN3 gcdl-101* strains suggests that the *gcn3-102* product is more efficient at promoting or supplying *GCDl* function than the product of wild-type *GCN3* is. Knowing that deletion of *GCDl* is lethal in a *GCN3* strain (Hill and Struhl, personal communication), we wished to determine whether the inviability of a *gcdl* deletion can be suppressed by *gcn3-102*. A partial deletion of *GCDl* was constructed by substituting a 1.1-kb *Hin*dIII fragment of the *GCDl* gene, containing 59% of the predicted protein-coding sequence and 0.18 kb upstream from the presumptive ATG initiation codon, with a 2.2-kb *LEU2* fragment. This construct was used to disrupt one of the two *GCDl* alleles in two different *leu2*/*leu2 GCDl*/*GCDl* diploid strains. One diploid strain used in these experiments is homozygous for wild-type *GCN3* (H4 × EY45); the second is homozygous for *gcn3-102* (EY48 × H17). The *GCDl* disruptions were carried out by transformation with a DNA fragment containing the *gcdl*::*LEU2* allele, selecting for Leu$^+$ colonies. DNA blot hybridization confirmed that gene replacement had occurred for only one of the two *GCDl* alleles in each diploid (data not shown). Sporulation of Leu$^+$ transformants of each strain gave rise to only Leu$^-$ ascospore clones. We conclude that *gcn3-102* cannot suppress the inviability resulting from a deletion of *GCDl*.

Addition of three amino acids to the carboxyl terminus of *GCN3* inactivates positive regulation but not suppression of a *gcdl2* mutation. The *Sal*I mutation at the *GCN3* termination codon described above adds three amino acids to the carboxyl terminus of the *GCN3* ORF (Gly-Arg-Gln). This mutation (referred to henceforth as *GCN3-501*) completely destroys the ability to complement the AT^s phenotype of the *gcn3*::*LEU2* mutation in strain EY51. This fact was shown by transforming EY51 with the low-copy-number plasmid Ep186 containing *GCN3-501*, or with YCp50. Transformants containing either plasmid were equally AT^s. The *GCN3-501* allele produces normal amounts of *GCN3* mRNA (data not shown). In addition, when introduced on Ep186 into the *GCN3* strain H4, it leads to an AT^s phenotype nearly as severe as that conferred by the *gcn3*::*LEU2* disruption in strain EY51. The dominant negative phenotype of this allele suggests that it makes an altered protein that is inactive for positive regulation and that interferes with the function of wild-type GCN3. Interestingly, the *GCN3-501* product also retains the ability to suppress the phenotypes of a *gcdl2* mutation. This fact was demonstrated by transforming *gcn3*::*LEU2 gcdl2-503* strain H652 with Ep186 and YCp50. The YCp50 transformants exhibit the temperature-sensitive growth and AT^r phenotype associated with *gcdl2-503* (11). (The AT^r phenotype results from suppression of *gcn3*::*LEU2* by *gcdl2-503*). By contrast, the Ep186 transformants of H652 exhibit no temperature sensitivity and are AT^s. These results suggest that the *GCN3-501* mutation inactivates the positive regulatory function of GCN3 required under starvation conditions without impairing its ability to suppress the phenotypes of *gcdl2* mutations expressed under normal growth conditions. These characteristics are the same as those described above for *gcn3-102* with regard to suppression of *gcdl-101*. It was shown previously that *gcn3-102* also completely suppresses the mutant phenotypes associated with *gcdl2* mutations (11), making *gcn3-102* and *GCN3-501* identical in this respect. Close examination of the *gcn3-102* mutation suggests that this allele also exhibits a degree of dominance. When the *gcn3-102* strain H17 was transformed with plasmid Ep69 containing *GCN3*, the resulting transformants were significantly less AT^r than were closely related wild-type *GCN3* strains such as H4.

DISCUSSION

Genetic analysis has identified multiple positive regulatory factors involved in the general control of amino acid biosynthetic genes in *S. cerevisiae*. The GCN4 protein binds to *cis*-acting regulatory sequences found at structural genes subject to general control and thereby stimulates their transcription directly (1, 22). The products of *GCN1*, *GCN2*, and *GCN3* are believed to function indirectly by positive regulation of *GCN4* expression under amino acid starvation conditions (21). The positive role of the *GCN3* product was established unequivocally by the construction of strains containing a partial deletion of *GCN3* (Fig. 5). Such strains are unable to stimulate *GCN4* expression in response to starvation (11); consequently, they fail to derepress transcription of genes under *GCN4* positive control (Fig. 3). Both the *GCN2* and *GCN3* products regulate *GCN4* expression at the translational level by suppressing the inhibitory effects of multiple AUG codons present in the leader of *GCN4* mRNA (17, 31, 39).

The nonderepressible phenotype associated with a deletion of *GCN3* is completely suppressed by mutations in the negative regulatory genes *GCD1* and *GCD12*: such *gcd gcn3*::*LEU2* double mutants exhibit constitutive derepression of *GCN4* expression (11). This finding strongly suggests that the *GCN3* product functions as a positive regulator of *GCN4* by antagonism or repression of the products of *GCD1* and *GCD12* in amino-acid-starved cells. Interestingly, expression of the mutant phenotypes associated with *gcd1* and *gcd12* mutations (constitutive derepression of *GCN4* expression and temperature sensitivity for growth) is dependent on the allelic state of *GCN3*. The wild-type *GCN3* allele and the *gcn3-102* mutation were shown previously to overcome both phenotypes associated with *gcd1* and *gcd12* mutations (11). For *gcd12* mutations, this suppressive interaction is complete: *GCN4* expression is efficiently repressed under nonstarvation conditions, and no growth defect is detectable in *GCN3 gcd12* or *gcn3-102 gcd12* double mutants. In contrast to *GCN3* and *gcn3-102*, the *gcn3-101* and *gcn3*::*LEU2* mutations exacerbate the growth defect and the derepressed phenotype associated with *gcd1* and *gcd12* mutations (11). In fact, the *gcn3*::*URA3* mutation leads to unconditional lethality in *gcd1-101* mutants (Fig. 7). These observations suggest that the *GCN3* product enhances both the essential and the regulatory functions of GCD1 and GCD12 under normal growth conditions, even though it antagonizes repression of *GCN4* expression by the same *GCD* factors under starvation conditions.

The *gcn3-102* mutation eliminates the positive regulatory function of *GCN3*, destroying its ability to antagonize *GCD* factors under starvation conditions, without affecting its ability to restore *GCD* function under nonstarvation conditions in *gcd1* and *gcd12* mutants. The location of *gcn3-102* in the *GCN3* sequence remains to be determined, and the possibility exists that this mutation affects translation of *GCN3* mRNA rather than the structure or function of GCN3 protein. We showed here that addition of three amino acids to the carboxyl terminus of GCN3 is sufficient to elicit the same interesting phenotype displayed by *gcn3-102*. To explain this result, we suggest that an altered protein is produced by *GCN3-501* that functions properly under normal growth conditions to enhance GCD function but lacks a domain required for its positive regulatory role as an antagonist of GCD factors under starvation conditions. In this view, the positive, regulatory function of GCN3 would be activated under starvation conditions by a covalent modification of the protein or by an altered interaction with other regulatory factors whose structure or expression is modulated by amino acid availability.

The idea that the structure or activity of the GCN3 protein is altered under starvation conditions is consistent with our conclusion that the GCN3 protein level is unregulated by amino acid availability. RNA blot hybridization analysis reveals no change in the amount of *GCN3* mRNA present under repressing versus derepressing conditions. The same result applies to expression of β-galactosidase activity from a *GCN3-lacZ* translational fusion constructed to model the expression of the GCN3 protein. At present, we cannot rule out the possibility that *GCN3* expression is regulated at the level of protein stability and that *gcn3-102* and *GCN3-501* impair positive regulation by preventing the accumulation of a larger amount of GCN3 protein under starvation conditions. We hope to address this possibility by using GCN3-specific antiserum.

The requirement for *GCN3* for viability in *gcd1-101* strains could be explained in at least two different ways. One possibility is that GCN3 can substitute for the *GCD1* product to carry out the essential function of the latter. The alternative hypothesis is that GCN3 exists in a complex with the GCD1 protein and can stabilize the thermolabile product encoded by *gcd1-101*: if GCN3 is removed from the complex, the thermolability of the *gcd1-101* product is expressed unconditionally (11). The fact that *GCN3* cannot overcome the lethal effect of a *GCD1* deletion (Hill and Struhl, personal communication) is more consistent with the latter hypothesis in suggesting that a mutant gcd1 protein, presumably one capable of complex formation with GCN3, must be expressed for *GCN3* to restore GCD1 function. However, we cannot eliminate the possibility that GCN3 can compensate for only a partial loss of GCD1 function in temperature-sensitive *gcd1* mutants. The *gcn3-102* mutation was isolated as a suppressor of the temperature-sensitive phenotype of *gcd1-101* (21). Thus, the *gcn3-102* product might provide *GCD1* function more efficiently than wild-type GCN3. However, we found that a deletion of *GCD1* is also lethal in a *gcn3-102* strain. The available data are most closely consistent with the idea that *GCN3* and *GCD1* exist together in a complex and that removal of *GCN3* from this complex leads to unconditional inactivation of the thermolabile products of temperature-sensitive *gcd1* alleles. In this view, the *gcn3-102* mutant product stabilizes gcd1 mutant proteins more efficiently than wild-type *GCN3* (21). The dominant negative phenotype of *GCN3-501* provides additional support for complex formation in suggesting that GCN3 interacts with at least one other component of the regulatory system in a stoichiometric fashion. In this view, the product of *GCN3-501* competes with GCN3 and forms stable complexes inactive for derepression of *GCN4* expression. If, indeed, GCN3 forms a complex with one or more GCD proteins, alterations in GCN3 structure under starvation conditions could be responsible for antagonizing the function of these negative factors with which it interacts, thus accounting for the positive regulatory role of *GCN3* in controlling *GCN4* expression. In this view, the *GCN3-501* mutation prevents an alteration in GCN3 structure that is normally induced by starvation.

Although a protein-protein interaction model is consistent with the available data, we cannot rule out the possibility that GCN3 regulates transcription of *GCD1*. Under starvation conditions, GCN3 would repress *GCD1* transcription and thus antagonize its negative regulatory function. To explain the ability of *GCN3* to overcome the phenotype of

certain *gcd1* mutations, it must be proposed that *GCN3* acts as a positive regulator of transcription under nonstarvation conditions whose contribution to *GCD1* expression is detectable only in the presence of leaky *gcd1* mutations.

The fact that *gcd* mutations affect cell viability, in addition to their effects on translational control of *GCN4* mRNA, raises the possibility that *GCD* factors affect general protein synthesis. According to this interpretation, the role of GCN3 would be to antagonize or compete with certain factors involved in protein synthesis and thereby alter the translational efficiency of *GCN4* mRNA in amino-acid-starved cells. The predicted amino acid sequence of GCN3 shows no significant homology to any known protein in the data bases we examined and thus provides no clues about its biochemical activity. Characterization of the GCN3 protein with respect to its location, interactions with other regulatory factors, and possible functions in general protein synthesis is required to gain further insight into its role as a translational activator of *GCN4*. The experiments described here set the stage for these biochemical studies.

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