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Translation of the Yeast Transcriptional Activator *GCN4* Is Stimulated by Purine Limitation: Implications for Activation of the Protein Kinase GCN2

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The transcriptional activator protein GCN4 is responsible for increased transcription of more than 30 different amino acid biosynthetic genes in response to starvation for a single amino acid. This induction depends on increased expression of *GCN4* at the translational level. We show that starvation for purines also stimulates *GCN4* translation by the same mechanism that operates in amino acid-starved cells, being dependent on short upstream open reading frames in the *GCN4* mRNA leader, the phosphorylation site in the α subunit of eukaryotic translation initiation factor 2 (eIF-2α), the protein kinase GCN2, and translational activators of *GCN4* encoded by *GCN1* and *GCN3*. Biochemical experiments show that eIF-2α is phosphorylated in response to purine starvation and that this reaction is completely dependent on GCN2. As expected, derepression of *GCN4* in purine-starved cells leads to a substantial increase in *HIS4* expression, one of the targets of GCN4 transcriptional activation. *gcn* mutants that are defective for derepression of amino acid biosynthetic enzymes also exhibit sensitivity to inhibitors of purine biosynthesis, suggesting that derepression of *GCN4* is required for maximal expression of one or more purine biosynthetic genes under conditions of purine limitation. Analysis of mRNAs produced from the *ADE4*, *ADE5,7*, *ADE8*, and *ADE1* genes indicates that GCN4 stimulates the expression of these genes under conditions of histidine starvation, and it appeared that *ADE8* mRNA was also derepressed by GCN4 in purine-starved cells. Our results indicate that the general control response is more global than was previously imagined in terms of the type of nutrient starvation that elicits derepression of *GCN4* as well as the range of target genes that depend on GCN4 for transcriptional activation.

The general amino acid control response of *Saccharomyces cerevisiae* is a regulatory mechanism for the induction of more than 30 genes involved in 11 amino acid biosynthetic pathways (reviewed in references 22 and 23). When *S. cerevisiae* is starved for amino acids (23) or when the activity of tRNA synthetases is impaired (34, 43), expression of the transcriptional activator protein GCN4 is increased and GCN4 stimulates the transcription of amino acid biosynthetic genes which are subject to the general control.

Expression of *GCN4* is regulated in response to amino acid availability by both positive and negative regulators. When amino acids are abundant, four short open reading frames (uORFs) in the *GCN4* mRNA leader act in *cis* to repress *GCN4* expression at the translational level by preventing ribosomes scanning the leader from reaching the *GCN4* AUG codon. According to a recently proposed model (1), ribosomes will translate the first uORF encountered (uORF1) and resume scanning. Under nonstarvation conditions, essentially all of these ribosomes will reinitiate translation at one of the remaining uORFs, uORF2 to uORF4, and fail to reinitiate translation again at the *GCN4* start codon. Products of the *SUI2*, *SUI3*, and multiple *GCD* genes are *trans*-acting negative regulators that act to repress *GCN4* translation in concert with the uORFs (22, 23, 60). *SUI2* (8) and *SUI3* (15) encode, respectively, the α and β subunits of eukaryotic translation initiation factor 2 (eIF-2α and eIF-2β), which binds the initiator $tRNA^{Met}$ to the small ribosomal subunit during the initiation process (38). The *GCD1*, *GCD2*, *GCD6*, and *GCD7* genes also encode essential proteins involved in translation initiation (4, 7, 17, 22, 55). Recent work indicates that these GCD proteins are subunits of the yeast equivalent of mammalian eIF-2B (4, 6, 7), which recycles eIF-2 by exchanging bound GDP for GTP following each round of translation initiation (28, 47, 54). These results suggest that high levels of eIF-2 and eIF-2B activities present under conditions of amino acid sufficiency restrict translation initiation on *GCN4* mRNA to the uORFs and prevent initiation at the *GCN4* start codon.

Positive regulators encoded by *GCN1*, *GCN2*, and *GCN3* are required under amino acid starvation conditions to overcome the inhibitory effects of the uORFs on translation initiation at *GCN4* (23). GCN2 is a serine/threonine protein kinase that stimulates *GCN4* translation by phosphorylating eIF-2α (14). eIF-2α is phosphorylated on a serine residue at position 51, and replacement of this serine residue with alanine prevents the derepression of *GCN4* in amino acid-starved cells (14). By analogy with mammalian systems, Dever et al. (14) proposed that the phosphorylated form of eIF-2 inhibits the guanine nucleotide exchange activity of eIF-2B, thus reducing the levels of active eIF-2. As a result, many ribosomes which have translated uORF1 and resumed scanning will not rebind the ternary complex consisting of eIF-2, GTP, and initiator $tRNA^{Met}$ until after bypassing the start codons at uORF2 to uORF4. Most of these ribosomes will reacquire this key initiation factor before scanning past the *GCN4* start site and will reinitiate translation at *GCN4* instead (1, 14). It is thought that the GCN3 protein, which resides with GCD factors in the yeast eIF-2B complex (6, 7), mediates the inhibitory effect of phosphorylated eIF-2 on the recycling function of eIF-2B (7, 13). The GCN1 protein is also required for the derepression of *GCN4* and functions in

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stimulating the phosphorylation of eIF-2α by GCN2 (22, 23, 31).

There is evidence that the GCN2 protein has an important role in sensing amino acid starvation. In addition to the protein kinase domain, GCN2 contains a carboxy-terminal segment of about 530 amino acids that is similar in sequence to histidyl-tRNA synthetases (HisRSs; 56). This carboxy-terminal domain is required in vivo for GCN2 positive regulatory function and for the association between GCN2 and ribosomes (45). The sequence similarity between GCN2 and HisRSs, combined with the fact that a reduction in tRNA aminoacylation signals derepression of the general control system, led Wek et al. (56) to propose that the HisRS-like region is a regulatory domain that detects uncharged tRNA and activates the protein kinase moiety in amino acid-starved cells. This hypothesis is consistent with the isolation of dominant mutations mapping in the HisRS-like region of GCN2 that lead to activation of the protein kinase domain in the absence of an amino acid starvation signal (46).

There are several reasons to suspect that GCN4 is involved in transcriptional control of purine biosynthetic genes in addition to its role in regulating the levels of amino acid biosynthetic enzymes. Consensus GCN4 binding sites are found in the promoters of all of the genes required for the synthesis of purine nucleotides that have been sequenced thus far, including *ADE1* (41), *ADE2* (52), *ADE3* (51), *ADE4* (39), *ADE5,7* (20), and *ADE8* (58). Binding of the GCN4 protein to two of three potential binding sites in the *ADE4* promoter was demonstrated by Mösch et al. (39), and mutations in these sequences that eliminate binding of GCN4 in vitro decrease *ADE4* transcription in vivo. Moreover, it was reported that *ADE4* expression is substantially higher in a *gcd2-1* mutant, which constitutively expresses GCN4 at elevated levels, compared with a *gcn4* mutant; this difference in *ADE4* expression was dependent on the GCN4 binding sites in the *ADE4* promoter (39). These findings suggest that GCN4 plays a direct role in transcriptional activation of *ADE4*.

Complicating the interpretation of these results is the finding that the BAS1 protein recognizes the TGACTC core sequence present in the GCN4 binding sites in the *HIS4*, *ADE2*, and *ADE5,7* promoters (11). Mutations within this core sequence that prevent BAS1 binding to oligonucleotide probes in a gel retardation assay also decrease the expression of an *ADE2-lacZ* fusion in vivo. Together, these findings suggest that BAS1 and GCN4 compete for binding of the TGACTC sequences at these genes and that flanking sequences affect the relative affinities of these proteins for binding. Furthermore, mutations within the TGACTC sequence probably affect binding of both BAS1 and GCN4, making it difficult to determine which of these proteins is essential for transcriptional activation of a given gene.

In view of the results suggesting that GCN4 activates *ADE4* transcription and the possibility that GCN4 may function at other *ADE* genes, we decided to investigate whether purine starvation would increase expression of *GCN4* and genes under its control. Our investigation has shown that GCN4 synthesis is efficiently derepressed in response to purine limitation and that this derepression requires the activity of the known positive regulators of *GCN4* translation. Thus, purine limitation and amino acid starvation increase *GCN4* expression by the same regulatory mechanism. As expected, derepression of GCN4 synthesis in purine-starved cells leads to increased transcription of *HIS4*. In addition, we present in vivo evidence that translational derepression of *GCN4* is required for wild-type levels of purine biosynthesis under conditions of purine limitation. Our results have significant implications for the molecular mechanism of signal recognition and activation of the protein kinase domain of GCN2. They also suggest that the translational control mechanism governing *GCN4* expression is an important component of the cellular response to limitation for purines as well as amino acids.

MATERIALS AND METHODS

Strains and plasmids. The strains used in this study are listed in Table 1. The bradytrophic strain H2029 was produced by crossing a leaky *ade1* allele into our wild-type background by the following crosses. X2928-3D-1A (**a** *ade1 gal1 leu1 his2 ura3 trp1 met14*) was crossed to H1513 (α *ura3-52 leu2-3 leu2-112 trp1-Δ63 ino1-13*), producing RRX1.5C (**a** *ade1 his2 ura3 trp1 ino1-13*). RRX1.5C was backcrossed to H1513, producing RRX7.15C (α *ade1 leu2-3 leu2-112 ura3 trp1 ino1-13*). RRX7.15C was crossed to H1515 (**a** *ura3-52 leu2-3 leu2-112 trp1Δ63*), producing H2029 (**a** *ade1 ura3 leu2-3 leu2-112 trp1*). H2030 is isogenic to H2029 but carries a *LEU2*-marked deletion allele of *gcn2*. It was produced by transformation (26) of H2029 with the 4.25-kb *Bam*HI fragment of p500 (57), selecting for Leu^+ transformants that are sensitive to 3-aminotriazole (3AT). 3AT causes histidine starvation by inhibiting the *HIS3* product; thus, strains with mutations in *gcn* genes are sensitive to 3AT because of their inability to derepress *GCN4* and thereby increase *HIS3* expression under starvation conditions (22). Strains H1303-H1306 and H1307-H1310 were obtained in two tetrads from a cross between AGH601-1B (α *ino1-1 ura3-52 GCN1*) and H1049 (**a** *gcn1-1 ura3-52*). The *gcn1-1* ascospore clones were identified by their $3AT^s$ phenotype. Strain H1049 was produced in the process of backcrossing a strain containing the *gcn1-1* allele (49), provided by Ralf Hütter, with several strains in our collection. Strain H2061 carries a *URA3*-marked deletion allele of *gcn2* and was produced by transformation of strain H1305 with the *Sna*BI-*Bgl*II fragment of plasmid p638 (19) containing the marked-deletion allele and selecting for Ura^+ colonies. The *gcn2Δ::URA3* allele in the resulting transformant was identified by its $3AT^s$ phenotype.

Plasmids p180 and p227 contain, respectively, the *GCN4-lacZ* fusion with and without the four uORFs described previously (21, 40). Plasmid p925 is a high-copy-number plasmid carrying the *SUI2* allele (14). Plasmid p164 carries the wild-type *GCN4* allele (40), and p238 carries the *GCN4* allele with point mutations eliminating the AUG codons of all four uORFs (40). Plasmid p665 contains the wild-type allele of *GCN1* on a low-copy-number *URA3* vector (31).

Growth media and conditions. Strains were grown in synthetic glucose medium (SD) (50) containing only the required supplements: tryptophan, 0.4 mM; leucine, 4 mM; isoleucine, 1 mM; valine, 1 mM; inositol, 0.2 mM; and arginine, 0.5 mM. Adenine was added to 0.6 mM where indicated. Synthetic complete medium containing all amino acids, inositol, and *p*-aminobenzoic acid (PABA) was prepared as described previously (50). Analogs were added as follows: 10 mM 3AT, 50 μg of 8-azaadenine (azA) per ml, 50 μg of 8-azaguanine (azG) per ml. azA at 50 μg/ml was added to solid medium (SD) containing tryptophan, leucine, isoleucine, valine, and inositol at the above-mentioned concentrations and 1 mM histidine, or azA was added to solid medium (SC) containing all of the amino acids, PABA, and inositol.

Strains selected for analysis of enzyme activities were

TABLE 1. Strains

Strain	Genotype	Reference or source
H1049	a *gcn1-1 ura3-52*	This study
H1149	α *leu2-3 leu2-112 ino1-13 ura3-52*::[*HIS4-lacZ ura3-52*] *gcn2*::*LEU2*	57
H1303	a *gcn1-1 ura3-52*	This study
H1304	α *GCN1 ura3-52 ino1-13*	This study
H1305	a *GCN1 ura3-52*	This study
H1306	α *gcn1-1 ura3-52 ino1-13*	This study
H1307	a *GCN1 ura3-52 ino1-13*	This study
H1308	α *gcn1-1 ura3-52 ino1-13*	This study
H1309	α *GCN1 ura3-52*	This study
H1310	a *gcn1-1 ura3-52*	This study
H1513	α *ura3-52 leu2-3 leu2-112 trp1-Δ63 ino1-13*	A. M. Cigan
H1515	a *ura3-52 leu2-3 leu2-112 trp1-Δ63*	A. M. Cigan
H1720	a *gcn4Δ1 ura3-52 leu2-3 leu2-112 ino1-13*	C. Moehle
H1816	a *ura3-52 leu2-3 leu2-112 trp1-Δ63*::[*GCN4-lacZ TRP1*] *sui2Δ gcn2Δ* [*SUI2 LEU2*]	13
H1896	a *ura3-52 leu2-3 leu2-112 trp1-Δ63*::[*GCN4-lacZ TRP1*] *sui2Δ* [*SUI2 LEU2*]	13
H2029	a *ade1 ura3 leu2-3 leu2-112 trp1*	This study
H2030	a *ade1 ura3 leu2-3 leu2-112 trp1 gcn2Δ*::*LEU2*	This study
H2061	a *GCN1 ura3-52 gcn2Δ*::*URA3*	This study
TD323	a *ura3-52 leu2-3 leu2-112 trp1-Δ63 sui2Δ* [*SUI2-S51A LEU2*]	T. Dever
TD367	a *ura3-52 leu2-3 leu2-112 trp1-Δ63 sui2Δ gcn2Δ* [*SUI2- LEU2*]	T. Dever
TD392	a *ura3-52 leu2-3 leu2-112 trp1-Δ63 sui2Δ gcn3Δ* [*SUI2 LEU2*]	T. Dever
RRX1.5C	a *ade1 his2 ura3 trp1 ino1-13*	This study
RRX7.15C	α *ade1 leu2-3 leu2-112 ura3 trp1 ino1-13*	This study
AGH601-1B	α *ino1-13 ura3-52 GCN1*	This study
X2928-3D-1A	a *ade1 gal1 leu1 his2 ura3 trp1 met14*	YGSC[a]

[a] YGSC, Yeast Genetic Stock Center.

grown overnight in a 5-ml culture that was used in toto to inoculate a second overnight culture of 25 ml. Thirty milliliters of culture medium was inoculated at a 1/60 dilution and incubated at 30°C with constant shaking. Analogs were added after 2 h of growth, and cells were harvested after a total growing time of 8 h. Cells were pelleted by centrifugation in a J6B rotor at 4,000 × *g* for 5 min, and excess medium was removed by aspiration. Cell pellets were frozen at −20°C prior to enzyme assay. Strains chosen for isoelectric focusing slab gel electrophoresis were grown for 2 days in SD and were inoculated at a 1/50 dilution in 50 ml of medium. Cells were grown for 6 h, with analog addition occurring 1 h prior to harvesting. Cells were pelleted by centrifugation as described above, washed once with ice-cold water, repelleted, and frozen at −20°C. Strains selected for analysis of RNA by Northern blot analysis were grown for 2 days in SD and were inoculated at a 1/50 dilution into 50 ml of medium. Cells were grown as described above for enzyme analysis and pelleted by centrifugation. Cell pellets were rinsed in 1 ml of ice-cold water, transferred to a 1.5-ml tube, repelleted, and frozen at −80°C.

Isoelectric focusing electrophoresis. Cell pellets were thawed in breaking buffer containing 40 mM piperazine-1,4-bis(2-ethanesulfonic acid), pH 6.0; 100 mM sodium chloride; 2 mM phenylmethylsulfonyl fluoride; 1 mM dithiothreitol; 2 mg of sodium fluoride per ml; 8 mg of β-glycerolphosphate per ml; and 10 mg of ammonium sulfate per ml. Samples were prepared and subjected to isoelectric focusing slab gel electrophoresis, followed by immunoblot analysis with antibodies specific for yeast eIF-2α and ^{125}I-labelled protein A to detect antigen-antibody complexes, all as described previously (14).

Enzyme assays. Cell pellets prepared for β-galactosidase assay were thawed in breaking buffer containing 100 mM Tris-hydrochloride, pH 8.0; 20% glycerol; and 1 mM β-mercaptoethanol. Extracts were prepared with glass beads and a Braun homogenizer as described previously (37). Assays were conducted with 96-well microtiter plates (33, 35), and results are expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute per milligram of total protein. Protein was assayed by the Bradford method (3) with Bio-Rad reagent.

Northern blots. Total RNA was prepared from the frozen cell pellets as previously described (24). Ten micrograms of each RNA sample was electrophoretically separated on a 1.5% formaldehyde gel (48) and blotted to GeneScreen Plus membranes by capillary action (48). Random-primed, radiolabelled DNAs (48) were used to probe the Northern blots. Experimental probes were prepared from DNA fragments found completely within the coding region for each gene. Each probe hybridized to a single RNA species of the appropriate size. The *ADE1* probe was a 0.56-kb *Xba*I-*Nde*I fragment prepared from a triple digest of plasmid pHSS6/f1/*ADE1*.13 (12) with *Xba*I, *Nde*I, and *Cla*I. The *ADE2* probe was a 1.2-kb *Hin*dIII fragment prepared from pASZ10 (52). The *ADE4* probe was a 1.3-kb *Eco*RV fragment from plasmid pR122, which was produced by subcloning the 4.5-kb *Bam*HI fragment of plasmid pPM13 (30) containing *ADE4* into pUC19. The *ADE5,7* probe was a 2.1-kb *Hin*dIII fragment from plasmid pYEADE5,7(5.2R) (20). The *ADE8* probe was a 0.3-kb *Xho*I-*Nru*I fragment from plasmid pR102 (58). A probe corresponding to the 1.4-kb *Eco*RV-*Sal*I fragment of *HIS4* on plasmid p19 (16) was used as a positive control for a gene under GCN4 regulation. A 6.7-kb *Hin*dIII fragment containing the *PYK1* gene, encoding pyruvate kinase, was used as the probe for an unregulated transcript, as described previously (21). Probes were stripped from filters in 50% formamide–1% sodium dodecyl sulfate at 65°C for several hours, rinsed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and exposed to X-ray film to

TABLE 2. Expression of β-galactosidase from *GCN4-lacZ* fusions in wild-type and *gcn* strains under conditions of purine limitation

Strain/plasmid (genotype)	Analog	β-Galactosidase activity[a] (U) during growth in:			
		SD	SDade[b]	SC	SCade[c]
H1515/p180 (*GCN*)	None	7.3	8.0	8.6	9.0
	3AT	100	48	8.6	9.2
	azA	130	8.9	190	12
	azG	90	9.3	120	8.8
H1515/p227 (*GCN*)	None	630	790	760	640
	3AT	1,100	780	840	710
	azA	1,000	590	1,100	640
	azG	730	950	730	610
TD367/p180 (*gcn2Δ*)	None	11	11	12	12
	3AT	38	36	13	14
	azA	17	12	29	12
	azG	16	11	24	ND[d]
TD323/p180 (*sui2-S51A*)	None	11	9.9	11	12
	3AT	37	33	13	12
	azA	14	10	26	10
	azG	14	11	15	ND
TD392/p180 (*gcn3Δ*)	None	20	21	18	10
	3AT	54	55	21	12
	azA	25	20	41	10
	azG	28	24	30	ND

[a] Samples were assayed in duplicate with two to three transformants of each strain, except for the azG and SCade (see below) samples from the *gcn* mutants, for which assays were performed in duplicate with a single transformant. Standard errors were less than 15% except for seven samples (H1515/p227 in SDade with both no analog and azG, TD367 in SC with azG, and TD392 in SDade and SC with both no analog and azA) for which the standard error was between 16 and 25%.

[b] SDade, SD with minimal supplements (see Materials and Methods) and containing 0.6 mM adenine.

[c] SCade, SC (SD containing all 20 amino acids; see Materials and Methods) containing 0.6 mM adenine.

[d] ND, not determined.

verify that the probe had been removed. Blots were hybridized sequentially with several probes, and an attempt to detect mRNA in order of ascending length was made. Hybridized blots were quantified with a Molecular Dynamics Phosphorimager.

RESULTS

Starvation for purine nucleotides leads to increased expression of a *GCN4-lacZ* fusion. To investigate the possibility that *GCN4* expression is regulated by purine levels, we introduced plasmid p180 bearing a *GCN4-lacZ* fusion containing all four uORFs into wild-type strain H1515 and subjected the resulting transformants to starvation for either histidine or purines. The analog 3AT, an inhibitor of the *HIS3* gene product, was used to starve cells for histidine (22). The analogs azA and azG, which cause pseudofeedback inhibition of the first enzyme of purine biosynthesis (32, 42), were used to starve cells for purines. Table 2 shows that starvation with 3AT in SD had the expected effect in producing a 14-fold derepression of *GCN4-lacZ* expression from p180 (40). Similarly, purine starvation imposed with either azA or azG led to a 12- to 18-fold increase in *GCN4-lacZ* expression. The derepression of *GCN4-lacZ* elicited with purine analogs occurred equally whether cells were grown in minimally supplemented medium (SD) or in medium supplemented with all 20 amino acids (SC). The latter result indicates that the purine analogs did not derepress *GCN4* expression by causing an amino acid imbalance or limitation. As expected, the derepression by purine analogs was overcome completely by the addition of adenine to the medium.

Derepression of *GCN4-lacZ* elicited by purine analogs occurs at the translational level. The increase in *GCN4-lacZ* expression seen in response to purine starvation could occur at the transcriptional or translational level. To distinguish between these two possibilities, strain H1515 was transformed with plasmid p227 containing the *GCN4-lacZ* fusion in which all four uORFs had been removed by point mutations (40). Removal of the uORFs eliminates translational regulation of *GCN4*; thus, any increase in expression from p227 would be attributable to transcriptional induction. Table 2 shows that histidine or purine starvation imposed by the same analogs described above caused less than a twofold derepression of β-galactosidase activity from p227. This small increase compared with the 12- to 18-fold derepression observed under the same conditions for the p180 fusion strongly suggests that purine starvation stimulates *GCN4* expression at the translational level.

To determine which elements of the general control system are required for regulation of *GCN4* translation by purine availability, mutants isogenic to H1515 that fail to derepress *GCN4* when starved for amino acids were examined for their response to purine limitation imposed by azA and azG. The wild-type *GCN4-lacZ* fusion on p180 was introduced into the *gcn2Δ* mutant TD367, the *gcn3Δ* mutant TD392, and strain TD323 containing a mutant form of eIF-2α in which serine 51 was changed to alanine. As expected, derepression of *GCN4-lacZ* expression from p180 in response to histidine starvation by 3AT was severely impaired in each of these mutants (Table 2). Derepression of *GCN4* was also impaired when azA or azG was added to elicit purine nucleotide starvation. Because an isogenic *gcn1*

TABLE 3. Expression of β-galactosidase from a *GCN4-lacZ* fusion in *gcn1-1* and *GCN1* ascospore clones from the same tetrad under conditions of purine limitation

Strain/plasmid	*GCN1* allele	Analog	β-Galactosidase activity[a] (U) during growth in:		
			SD	SDade[b]	SC
H1307/p180	*GCN1*	None	8.6	10	5.6
		3AT	90	ND[c]	ND
		azA	68	10	98
		azG	70	13	80
H1308/p180	*gcn1-1*	None	5.0	6.2	3.2
		3AT	11	ND	ND
		azA	8.2	6.8	16
		azG	6.6	7.7	6.4
H1309/p180	*GCN1*	None	5.9	6.8	6.4
		3AT	96	ND	ND
		azA	74	8.0	82
		azG	62	6.8	68
H1310/p180	*gcn1-1*	None	4.4	5.0	2.7
		3AT	17	ND	ND
		azA	9.7	4.6	18
		azG	6.6	3.8	7.1

[a] Assays were performed in duplicate with a single transformant of each strain. Similar activities were obtained with transformants of a second tetrad (data not shown).
[b] See Table 2, footnote *b*.
[c] ND, not determined.

mutant was unavailable, two *gcn1-1* and two *GCN1* ascospore clones from a single tetrad were transformed with p180 and assayed for the ability to derepress *GCN4-lacZ* in response to histidine or purine starvation. As shown in Table 3, the *gcn1-1* allele prevented the derepression of *GCN4* under both starvation conditions. Thus, each of the gene products that is required for translational derepression of *GCN4* under amino acid starvation conditions is also required for this response in cells limited for purines.

Starvation of an *ade1* bradytroph derepresses *GCN4-lacZ* and elicits the general control response. It has been proposed that uncharged tRNA is the signal for activation of the protein kinase GCN2 and the consequent increase in *GCN4* translation (56). Thus, it was important to address the possibility that the purine analogs azA and azG were being incorporated into tRNA in place of bona fide purines and reducing the efficiency of aminoacylation. We reasoned that if this hypothesis were correct, purine starvation imposed by growing a strain containing a leaky *ade* mutation (an *ade* bradytroph) on limiting amounts of purines would not lead to derepression of *GCN4*. On the contrary, we observed efficient derepression of the *GCN4-lacZ* fusion by starving the *ade1* bradytrophic strain H2029 for purine nucleotides. H2029 was grown on minimal medium containing 15 or 150 μM adenine. An adenine concentration of 150 μM is sufficient to restore a wild-type growth rate of 110 min to this strain, whereas the doubling time in the presence of 15 μM adenine is 130 min (data not shown). The results in Table 4 indicate that growth on limiting amounts of adenine increased *GCN4-lacZ* expression from the p180 construct by a factor of 4.5, which is comparable to the sevenfold increase observed in the same strain starved for histidine with 3AT in the presence of nonlimiting amounts of adenine (150 μM). By contrast, we observed less than a twofold increase in *GCN4-lacZ* expression from the construct lacking uORFs (p227) in strain H2029 when grown at the low adenine concentration. These results indicate that purine limitation imposed by starving an *ade1* bradytroph for adenine stimulates *GCN4* expression at the translational level. To determine whether this derepression requires the protein kinase GCN2, the *gcn2Δ* mutation was introduced into the *ade1* bradytroph (generating H2030) and expression of *GCN4-lacZ* from the p180 construct was measured after growth at the same concentrations of adenine mentioned above. As shown in Table 4, strain H2030 failed to derepress *GCN4-lacZ* expression at the low adenine concentration (15 μM), establishing a clear requirement for GCN2 in the response to purine limitation imposed in the *ade1* bradytroph.

In the course of conducting the experiments just described, we realized that *GCN4* expression increases in response to very severe purine deprivation by a mechanism that does not require the uORFs or GCN2 and thus probably occurs at the transcriptional level. The results shown in Fig.

TABLE 4. Expression of β-galactosidase from a *GCN4-lacZ* fusion in an *ade1* bradytroph under conditions of limiting adenine

Strain/plasmid (*GCN2* allele)	Growth condition	β-Galactosidase activity[a] (U)	Fold derepression
H2029/p180 (*GCN2*)	150 μM ade[b]	10 (0.9)	
	15 μM ade	50 (5.3)	5.0
	150 μM ade, 3AT	70 (3.1)	7.0
H2029/p227 (*GCN2*)	150 μM ade	840 (71)	
	15 μM ade	1,300 (64)	1.7
	150 μM ade, 3AT	980 (83)	1.1
H2030/p180 (*gcn2Δ*)	150 μM ade	8.0 (0.5)	
	15 μM ade	9.4 (0.8)	1.2
	150 μM ade, 3AT	21 (2.5)	2.6

[a] Assays were performed in duplicate with two to six transformants of each strain. Standard errors are in parentheses.
[b] ade, adenine.

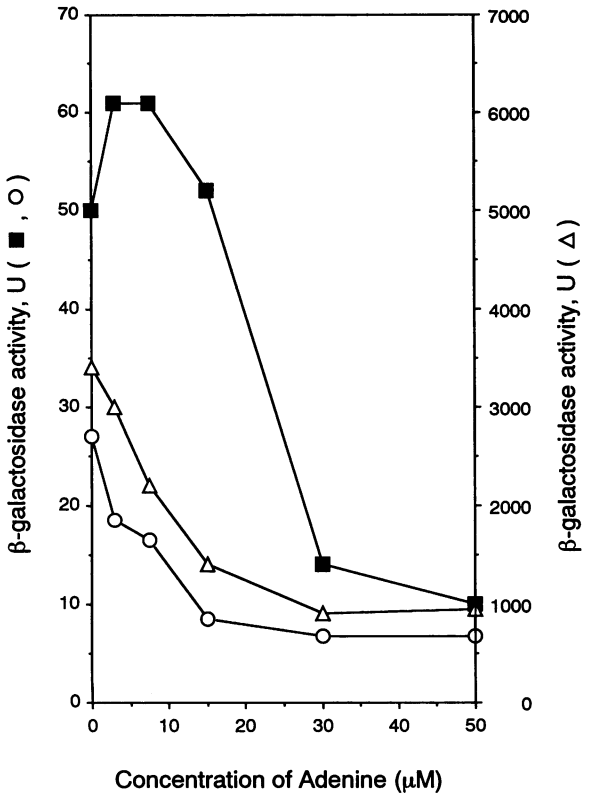

FIG. 1. Expression of β-galactosidase from a *GCN4-lacZ* fusion in an *ade1* bradytroph. Strains were grown for 2 days in SD supplemented with leucine, isoleucine, valine, tryptophan, and 150 μM adenine and diluted 1/60 into fresh medium containing the same amino acid supplements and adenine at the following concentrations: 0, 3, 7.5, 15, 30, 50, 150, and 600 μM. β-Galactosidase activities are approximately the same from 50 to 600 μM, and these values are omitted from the graph. Symbols: ■, *GCN2* strain H2029 transformed with p180, bearing all four uORFs; ○, *gcn2Δ* strain H2030 transformed with p180; △, strain H2029 transformed with p227, lacking all four uORFs.

1 indicate that expression of β-galactosidase from the p180 *GCN4-lacZ* construct containing all four uORFs increased substantially when the adenine concentration in the medium was decreased from 30 to 15 μM. Under the same conditions, expression from the p227 fusion lacking the uORFs increased by a much smaller amount, and the same was true for the p180 fusion in the *gcn2Δ* mutant (Fig. 1). These results are very similar to those shown in Table 4. However, at the lower adenine concentrations of 7.5 and 3 μM, expression from the p180 and p227 fusions increased comparably and was independent of GCN2 (Fig. 1). On the basis of results shown for the p180 construct in the *gcn2Δ* strain, it appears that a GCN2-independent mechanism increases *GCN4* expression by approximately threefold. Because roughly the same amount of derepression was observed for the p227 construct lacking translational control elements, the GCN2-independent mechanism is probably a transcriptional response to severe purine limitation.

Purine nucleotide starvation increases phosphorylation of eIF-2α by the protein kinase GCN2. It was shown previously that histidine starvation stimulates the protein kinase activity of GCN2 and leads to increased phosphorylation of eIF-2α on serine 51 (14). The results just described indicate

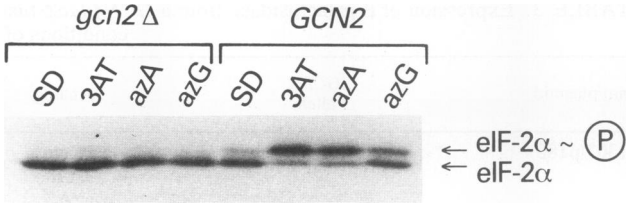

FIG. 2. Phosphorylation of eIF-2α during purine starvation. Extracts were prepared from strains H1816 (*gcn2Δ*) and H1896 (*GCN2*). eIF-2α was separated on a one-dimensional isoelectric focusing gel and visualized as described in Materials and Methods. Strains were grown in nonstarvation medium (SD) or were starved for histidine with 3AT or for purines with either azA or azG. H1816 (*gcn2Δ*) extracts and H1896 (*GCN2*) extracts were used as indicated above the lanes. The top arrow indicates the GCN2-dependent phosphorylated species, and the bottom arrow indicates eIF-2α in its basal phosphorylation state (14).

that GCN2 and serine 51 of eIF-2α are both required for translational derepression of *GCN4* in response to purine limitation imposed by the purine analogs azA and azG. Thus, we predicted that the level of eIF-2α phosphorylation would increase in response to azA or azG treatment, dependent on both GCN2 and serine 51 in eIF-2α. To test this prediction, we used isoelectric focusing gel electrophoresis to separate differentially phosphorylated forms of eIF-2α and immunoblot analysis with antisera against eIF-2α to visualize the amount of eIF-2α which is phosphorylated in the presence and absence of the purine analogs. As seen in Fig. 2, the addition of 3AT or the purine analogs led to increased phosphorylation of eIF-2α in a *GCN2* strain. The extent of phosphorylation was comparable in cells grown in the presence of 3AT and azA; somewhat less phosphorylation was seen in the azG-treated cells. The latter may reflect the fact that azG generally leads to less derepression in this strain than does azA or 3AT (Table 2). We observed no phosphorylation of eIF-2α in an isogenic *gcn2Δ* strain under any of the conditions examined (Fig. 2) or when serine 51 was mutated to alanine (data not shown). These results establish that eIF-2α is phosphorylated on serine 51 in response to purine limitation and that GCN2 is absolutely required for this modification.

Positive regulatory domains of GCN2 are required for the response to purine starvation. The GCN2 protein kinase contains several domains in addition to the kinase catalytic moiety which are required in vivo for derepression of *GCN4* translation but which are dispensable in vitro for kinase catalytic activity as assayed by autophosphorylation (57). These additional regions of GCN2 are thought to be regulatory in nature, functioning to stimulate protein kinase activity in vivo under conditions of amino acid starvation. The carboxy terminus is related in sequence to HisRSs and is required for ribosome association which may provide GCN2 with access to uncharged tRNA or to the substrate eIF-2α. The amino terminus is not related in sequence to other known proteins, and its mode of action in stimulating GCN2 function is unclear. To determine whether each of these separate domains is required for derepression of *GCN4* in response to histidine and purine starvation, we analyzed 15 two-codon insertion mutations located throughout GCN2 for their effects on the general control response to 3AT and azA. Expression of a *HIS4-lacZ* fusion was measured as the indicator of derepression of the general control pathway. *HIS4*, encoding three enzymatic activities in the histidine

TABLE 5. Expression of β-galactosidase from a *HIS4-lacZ* fusion in *GCN2* mutants under conditions of histidine or purine starvation

GCN2 allele[a]	Plasmid	Affected domain[b]	β-Galactosidase activity[c] (U) during growth in:		
			SD	3AT	azA
GCN2	p222	wt	130 (4.1)	460 (28)	710 (57)
gcn2Δ	YCp50	Δ	82 (5.1)	81 (6.1)	240 (6.4)
437-SS	p570	NH_2	84 (0.5)	88 (11)	240 (6.3)
941-AR	p553	HisRS	84 (1.1)	99 (4.7)	240 (13)
1092-EL	p559	HisRS	74 (4.8)	96 (4.9)	220 (12)
1177-EL	p561	HisRS	88 (1.0)	110 (6.8)	280 (19)
1502-SS	p558	COOH	88 (1.2)	100 (4.1)	260 (8.5)
1587-EL	p563	COOH	89 (2.9)	110 (9.3)	240 (12)
469-AR	p568	NH_2	140 (2.5)	560 (52)	710 (54)
503-SS	p551	NH_2	130 (7.1)	500 (32)	700 (41)
677-SS	p565	PK	140 (2.9)	520 (38)	780 (56)
1467-SS	p560	COOH	120 (2.9)	510 (19)	790 (35)
255-AR	p564	NH_2	93 (4.4)	440 (35)	410 (19)
360-SS	p569	NH_2	100 (3.2)	500 (36)	550 (43)
871-SS	p562	PK	110 (3.5)	560 (38)	590 (47)
1329-SS	p552	HisRS	97 (1.8)	440 (13)	500 (24)
1332-SS	p556	HisRS	110 (0.0)	460 (34)	520 (25)

[a] *GCN2* alleles are named according to the location in the protein coding sequence of the two-codon insertion and the identity of the inserted amino acids, using the single-letter code for amino acids.

[b] The domain of GCN2 affected by each insertion mutation is indicated as follows: wt, wild-type protein; NH_2, amino-terminal domain; HisRS, domain with homology to histidyl-tRNA synthetases; COOH, the extreme carboxy terminus; PK, the protein kinase domain.

[c] Assays were performed in duplicate with a single transformant of the *gcn2Δ* mutant strain H1149 for each construct. Standard errors are in parentheses.

biosynthetic pathway, is one of the target genes of GCN4 transcriptional activation (22).

In the presence of the wild-type *GCN2* allele, the addition of 3AT had the expected result (29) of derepressing *HIS4-lacZ* expression by a factor of 3.5 (Table 5). Similarly, the addition of azA led to a 5.5-fold derepression of the *HIS4-lacZ* fusion. Deletion of *GCN2* abolished the derepression response to 3AT and substantially reduced the degree of derepression elicited when azA was added. The origin of the GCN2-independent increase in *HIS4-lacZ* expression observed with azA addition is not known but may be related to the adenine regulation of *HIS4* transcription described previously (2). In a separate experiment, we confirmed that the derepression of *HIS4-lacZ* expression that occurs in purine-starved cells is similarly impaired in a *gcn4Δ* mutant (data not shown). These results are significant in showing that expression of *HIS4*, a transcriptional target of GCN4, is derepressed in response to purine limitation and that a large component of this derepression is dependent on the translational activator GCN2, shown above to mediate increased GCN4 protein synthesis under purine starvation conditions.

Analysis of the two-codon insertion mutations in *GCN2* suggested that they could be divided into three groups. Six of the mutants were defective for the induction of *HIS4-lacZ* expression in response to either 3AT or azA addition: 437-SS, 941-AR, 1092-EL, 1177-EL, 1502-SS, and 1587-EL (two-codon insertions are listed by the residue number at the position of the insertion followed by the identity of the inserted amino acids). These six mutants respond as though there is no GCN2 protein in the cell. Four of the insertion mutations have no significant effect on *HIS4-lacZ* expression under either starvation condition (469-AR, 503-SS, 677-SS, and 1467-SS) and behaved like wild-type GCN2. The remainder of the mutants appeared to be partially defective for derepression in response to azA but wild type in their response to 3AT. The five alleles in this third group (255-AR, 360-SS, 871-SS, 1329-SS, and 1332-SS) collectively affect the amino-terminal, protein kinase, and HisRS domains, suggesting that the entire GCN2 protein is required for activation of the protein kinase function in response to purine limitation. The identification of mutations that selectively impair derepression of *HIS4* in purine-starved cells could indicate the existence of more stringent requirements in GCN2 for activation by purine limitation versus histidine starvation. Alternatively, these mutations may simply lower the level of the GCN2 protein: if the activation of GCN2 kinase function is less efficient under the conditions of purine versus histidine starvation, such mutations would appear to selectively impair the response to purine limitation.

Evidence that translational derepression of *GCN4* is required for wild-type levels of resistance to purine analogs. Our results show that *GCN4* translation increases under purine starvation conditions and that *HIS4*, one of the target genes of GCN4, is efficiently derepressed in these circumstances. The findings of Mösch et al. (39) suggested that GCN4 is required for efficient transcriptional activation of *ADE4*; however, it was not determined whether basal or induced levels of GCN4 are required for wild-type levels of *ADE4* expression. We wished to determine whether transcription of purine biosynthetic genes under conditions of purine limitation requires the high levels of the GCN4 protein produced by derepression of *GCN4* translation. Toward this end, we tested isogenic sets of wild-type and *gcn* strains for differential sensitivity to purine analogs. One set of three strains contained a *gcn4Δ* allele, wild-type *GCN4*, or the dominant *GCN4^c* allele that constitutively expresses high levels of the GCN4 protein because of the absence of all four uORFs in the *GCN4* mRNA leader (40). A second set of strains contained a *gcn2Δ* allele or wild-type *GCN2*, and the third set contained the *gcn1-1* allele, wild-type *GCN1*, or a combination of *gcn1-1* and the *GCN4^c* allele. The *GCN4^c* allele completely overcomes the requirement for the positive regulator GCN1 for derepression of the general control system (53). Figure 3 shows the ability of these strains to form colonies on minimally supplemented (SD) and complete (SC) medium containing azA. All of the *gcn* strains grew poorly on media containing azA. *GCN4^c* strains clearly grew better than the wild-type strains on SD with azA,

FIG. 3. Growth of wild-type and *gcn* strains on azA-containing medium. Three plates are shown: the upper left plate is amino acid complete medium (SC) with no analog, the upper right plate is SC medium containing azA, and the lower left plate is minimally supplemented medium (SD) containing azA (media are described in Materials and Methods). The lower right circle contains the code for strains, starting from the top left sector and proceeding counterclockwise: *gcn4*Δ, strain H1720 containing a *gcn4*Δ allele and vector YCp50; *GCN4*, *gcn4*Δ strain H1720 carrying the wild-type *GCN4* allele on plasmid p164; *GCN4*^{c}, *gcn4*Δ strain H1720 carrying the constitutively derepressed allele of *GCN4* on plasmid p238; *gcn2*Δ, strain H2061, which is isogenic to H1305 except for the *URA3*-marked deletion allele of *gcn2*; *GCN2*, strain H1305 carrying vector YCp50; *gcn1-1*, strain H1303 carrying vector YCp50; *GCN1*, strain H1303 carrying the *GCN1* allele on plasmid p665; *GCN4*^{c} *gcn1-1*, strain H1303 carrying plasmid p238 with the constitutively derepressed allele of *GCN4*. Strains were streaked for single colonies and incubated for several days at 30°C.

although this difference was diminished on SC containing azA. Similar results were obtained when azG was added to either medium (data not shown).

The poor growth of the *gcn1-1* and *gcn2*Δ mutants relative to their wild-type counterparts on media containing azA suggests that the derepressed levels of *GCN4* translation, which depend on GCN1 and GCN2, are required for efficient purine biosynthesis under conditions of purine limitation. This interpretation is supported by the fact that the azA sensitivity of the *gcn1-1* mutant was overcome completely by the *GCN4*^{c} allele. It is also consistent with the greater azA resistance conferred by the *GCN4*^{c} allele versus wild-type *GCN4*, as the former produces higher levels of the GCN4 protein than are present in wild-type strains under starvation conditions (compare expression from p180 and p227 in Table 2). The pattern of azA sensitivity shown in Fig. 3 is identical to that observed with analogs such as 3AT which inhibit amino acid biosynthesis (22). The fact that the *gcn* mutants are sensitive to azA in the presence or absence of all 20 amino acids in the medium indicates that azA does not inhibit the growth of these strains by causing an amino acid imbalance or limitation. Therefore, our results suggest that the expression of one or more purine biosynthetic genes is dependent on the derepressed levels of the GCN4 protein produced under conditions of purine limitation by increasing the translational efficiency of *GCN4* mRNA.

Involvement of GCN4 in *ADE* gene expression. Our findings that GCN4 expression increases under conditions of purine limitation and that *gcn1*, *gcn2*, and *gcn4* mutants are more sensitive than wild-type strains to purine analogs are both consistent with the idea that GCN4 binds to the TGACTC sequences in the promoters of *ADE* genes and activates their transcription in response to purine limitation. In an effort to identify specific *ADE* genes whose transcription is regulated by GCN4, we used RNA blot hybridization analysis to measure the effects of purine and histidine starvation on the steady-state levels of the mRNAs encoded by five different *ADE* genes. RNA was isolated from three isogenic strains

bearing wild-type *GCN4*, a *gcn4Δ* allele, or the *GCN4^c* allele lacking uORFs, grown under nonstarvation conditions in the presence or absence of adenine or in medium containing azA or 3AT to induce purine or histidine starvation, respectively. The levels of the *ADE* gene transcripts were quantified by analyzing the blots with a Phosphorimager and were normalized to the levels of the *PYK1* mRNA (encoding pyruvate kinase) detected on the same blots. *PYK1* mRNA has been used previously for normalization of transcript levels under nonstarvation and histidine starvation conditions (21), and we verified that its level did not vary significantly in total RNA or relative to actin mRNA under the conditions of our experiments (data not shown).

As shown in Fig. 4, the level of *HIS4* mRNA was derepressed under both histidine and purine starvation conditions in the wild-type *GCN4* strain (lanes labelled 3AT and azA, respectively). As expected, *HIS4* expression was very high and essentially constitutive in the *GCN4^c* strain and was low and unregulated in the *gcn4Δ* strain. Quantitation of these data indicated that *HIS4* mRNA levels were derepressed 7.2-fold under histidine starvation conditions in the *GCN4* strain (Table 6), whereas only 1.6- and 1.1-fold derepression occurred in the *gcn4Δ* and *GCN4^c* strains, respectively. Under purine starvation conditions, *HIS4* expression was derepressed 7.0-fold in the *GCN4* strain but only ca. 2-fold in the *gcn4Δ* and *GCN4^c* strains (Table 6). These results are in agreement with those shown in Table 5, indicating that increased expression of GCN4 mediates the derepression of *HIS4* transcription in response to either purine or histidine starvation.

The regulation of the *ADE* transcripts is more complex than that seen for *HIS4* mRNA. One obvious difference was that all five *ADE* transcripts were repressed two- to fourfold by the addition of adenine to the medium, and this repression occurred equally in the *gcn4Δ*, *GCN4*, and *GCN4^c* strains. Under conditions of histidine starvation, expression of the *ADE4*, *ADE1*, *ADE8*, and *ADE5,7* mRNAs increased by a small amount in the wild-type *GCN4* strain (Fig. 4, compare lanes 6 and 8). Quantitation of the results from several experiments indicated that the addition of 3AT to the medium increased the levels of these four transcripts by a factor of 1.3 to 2.0 (Table 6). The amounts of these transcripts either remained the same or decreased in the *gcn4Δ* strain in response to histidine starvation (Fig. 4, compare lanes 1 and 3), such that two- to threefold-higher levels of the mRNAs were observed in histidine-starved *GCN4* versus *gcn4Δ* strains (Table 6). In contrast to these results, the level of *ADE2* mRNA increased by ca. twofold in response to histidine starvation in both the *GCN4* and *gcn4Δ* strains. These results are consistent with a requirement for GCN4 in the transcriptional activation of *ADE4*, *ADE1*, *ADE8*, and *ADE5,7* in histidine-starved cells. In accord with this hypothesis, less induction of these four *ADE* transcripts was seen in response to 3AT treatment of the *GCN4^c* strain compared with the *GCN4* strain (Table 6). Unlike the situation at *HIS4*, however, the levels of these mRNAs were not significantly higher in the *GCN4^c* strain than in the wild-type strain under nonstarvation conditions (Fig. 4, compare lanes 6 and 11). One explanation for the latter results could be that GCN4 activates the *ADE* genes under histidine starvation conditions but is prevented from doing so under nonstarvation conditions by the binding of another transcription factor, such as BAS1.

Similar to the results seen under histidine starvation conditions, the levels of the *ADE1*, *ADE5,7*, *ADE8*, and *ADE4* transcripts increased by about twofold in response to azA treatment in the *GCN4* strain (Fig. 4, compare lanes 6 and 9). In contrast to what was seen with histidine-starved cells, however, comparable increases were observed in response to azA treatment in the *gcn4Δ* and *GCN4^c* strains for *ADE1*, *ADE5,7*, and *ADE4*. Only in the case of *ADE8* did we observe a greater derepression response to azA treatment in the *GCN4* strain than in both the *gcn4Δ* and *GCN4^c* mutants (Table 6). As in the case of histidine starvation, the *ADE2* mRNA increased by roughly the same amount in all three strains. Thus, of the five *ADE* mRNAs examined, only the levels of *ADE8* mRNA fluctuated in the same pattern seen for *HIS4*, which is indicative of GCN4-dependent derepression under conditions of purine limitation. It is still

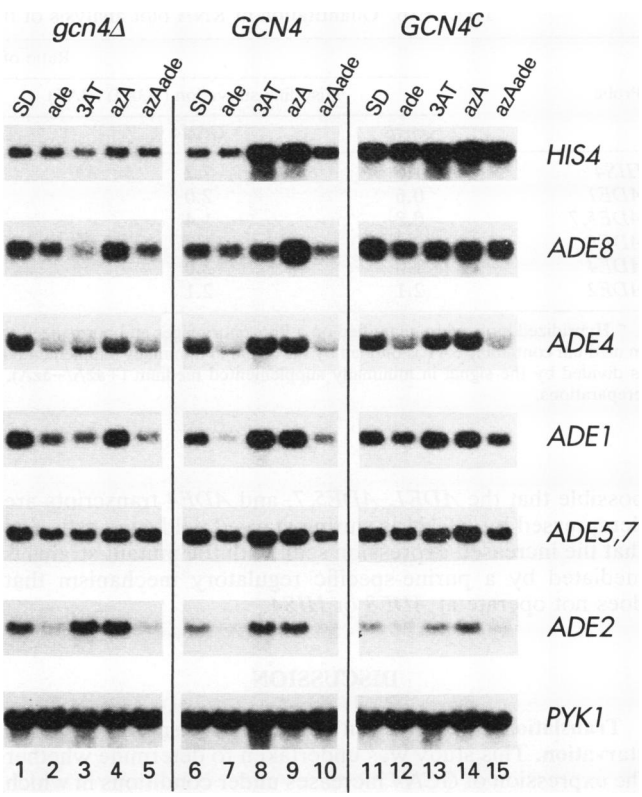

FIG. 4. Northern analysis of the role of GCN4 in *ADE* gene expression. Total RNA was prepared from cultures grown under conditions that repress or derepress *GCN4* expression. Three strains were analyzed: H1720/YCp50, which carries the *gcn4Δ* allele in the chromosome and was transformed with vector alone (lanes 1 to 5); H1720/p164, which carries the wild-type allele of *GCN4* on a YCp50-derived vector (lanes 6 to 10); and H1720/p238, which carries the *GCN4^c* allele from which GCN4 is expressed constitutively at very high levels because of elimination of the uORFs (lanes 11 to 15). Strains were grown in minimally supplemented medium (lanes 1, 6, and 11), minimally supplemented medium containing adenine (lanes 2, 7, and 12), histidine starvation medium containing 3AT (lanes 3, 8, and 13), adenine starvation medium containing azA (lanes 4, 9, and 14), and nonstarvation medium in which adenine was added in the presence of azA (lanes 5, 10, and 15). RNA was resolved by agarose gel electrophoresis and blotted as described in Materials and Methods. Blots were hybridized sequentially with radiolabelled probes for *ADE1*, *ADE2*, *ADE4*, *ADE5,7*, *ADE8*, *HIS4*, and *PYK1* mRNAs and subjected to autoradiography with X-ray film. The results shown here are essentially identical to the data obtained with two other sets of RNA prepared from replicate cultures of the same strains.

TABLE 6. Quantitation of RNA blot analysis of the effects of *GCN4* mutations on *ADE* gene transcription

Probe	Ratio of hybridization signals under condition[a]					
	Histidine starvation (+3AT/−3AT)			Purine starvation (+azA/−azA)		
	gcn4Δ	*GCN4*	*GCN4*^{c}	*gcn4Δ*	*GCN4*	*GCN4*^{c}
HIS4	1.6	7.2	1.1	1.4	7.0	2.2
ADE1	0.6	2.0	1.6	1.4	2.0	1.9
ADE5,7	0.8	1.4	1.0	1.2	1.7	2.3
ADE8	0.5	1.3	0.8	1.5	2.5	1.3
ADE4	1.0	2.0	1.2	1.2	1.6	1.9
ADE2	2.1	2.1	1.4	2.0	1.8	2.1

[a] Hybridized blots were quantified on a Phosphorimager and normalized to the *PYK1* message for each blot. For histidine starvation, the hybridization signal in medium containing 3AT is divided by the signal in minimally supplemented medium (+3AT/−3AT); for purine starvation, the signal in medium containing azA is divided by the signal in minimally supplemented medium (+azA/−azA). Values are the averages of ratios calculated from two independent sets of RNA preparations.

possible that the *ADE1*, *ADE5,7*, and *ADE4* transcripts are derepressed by GCN4 in purine-starved wild-type cells and that the increased expression seen with the mutant strains is mediated by a purine-specific regulatory mechanism that does not operate at *ADE8* or *HIS4*.

DISCUSSION

Translational derepression of *GCN4* in response to purine starvation. This study was undertaken to determine whether the expression of *GCN4* increases under conditions in which purines are limiting for the growth of yeast cells. These experiments were motivated by the fact that consensus GCN4 binding sites have been identified in the promoters of the *ADE1*, *ADE2*, *ADE3*, *ADE4*, *ADE5,7*, and *ADE8* genes (20, 39, 41, 52, 51, 58) and a report that GCN4 contributes directly to the transcriptional activation of *ADE4* (39). We have shown that starvation for purines in any of several different ways leads to increased *GCN4* expression at the translational level by a mechanism that depends on the uORFs in the *GCN4* mRNA leader, phosphorylation of eIF-2α by the protein kinase GCN2, and the products of *GCN3* and *GCN1*. These are the same regulatory components that are required for translational derepression of *GCN4* in response to amino acid starvation.

The derepression of *GCN4* in purine-limited cells leads to a large increase in the expression of *HIS4*, one of the many targets of GCN4 transcriptional activation in *S. cerevisiae*. We obtained evidence that GCN4 mediates a modest increase in transcription at the *ADE1*, *ADE4*, *ADE5,7*, and *ADE8* genes under conditions of histidine starvation and at *ADE8* under conditions of purine limitation. Transcription of the other *ADE* genes, including *ADE2*, appears to be stimulated in response to purine starvation by a mechanism that is independent of GCN4. In spite of our inability to observe a strong effect of GCN4 on the transcription of *ADE* genes in purine-starved cells, we found that *gcn1*, *gcn2*, and *gcn4* mutants were more sensitive to purine analogs than were isogenic wild-type strains and that the *GCN4*^{c} allele overcame the purine analog sensitivity associated with the *gcn1* allele. The sensitivity of the *gcn1* and *gcn2* mutants to amino acid analogs is an in vivo indication that the derepression of *GCN4* mediated by GCN1 and GCN2 is required for increased expression of amino acid biosynthetic genes in amino acid-starved cells. Likewise, we presume that the purine analog sensitivity of these *gcn* mutants indicates likewise that derepression of *GCN4* is required for increased expression of one or more purine biosynthetic genes in purine-starved cells.

It is possible that the small increase in *ADE8* transcription mediated by GCN4 under purine starvation conditions is responsible for the sensitivity of *gcn* mutants to purine analogs. There are several cases in which only one or a small subset of genes in a given amino acid biosynthetic pathway shows significant regulation by GCN4. In addition, there are examples of amino acid biosynthetic genes that are derepressed by GCN4 by a factor of 2 or less, such as the *ILV* genes (23, 25, 44). Of course, it is also possible that one of the six purine biosynthetic genes not analyzed here is regulated by GCN4 to a greater extent than was seen for *ADE8*.

Our results suggest that the binding and interaction of the GCN4 and BAS1 proteins at *ADE* genes are quite complex. Daignan-Fornier and Fink (11) have shown that the BAS1 protein will protect a sequence which contains the TGACTC core in potential GCN4 binding sites found at the *ADE2* and *ADE5,7* genes from DNase I digestion. Mutations within this sequence decrease expression of *ADE2* in vivo. The results of our Northern analyses indicate that *ADE2* is not regulated by GCN4. On the other hand, GCN4 does play a role in regulating the expression of *ADE5,7* under histidine starvation conditions, presumably through an interaction with the same TGACTC sequence. We obtained evidence that GCN4 also stimulates transcription of *ADE4* in response to histidine starvation. This finding is in accord with the results of Mösch et al. (39), indicating that GCN4 binding sites at this gene mediate a ca. threefold-higher level of expression in a *gcd2-1* mutant, which constitutively overexpresses GCN4, versus that in a wild-type strain. In contrast with these previous findings, however, we were unable to detect significant derepression of *ADE4* mRNA in our *GCN4*^{c} strain versus the isogenic *GCN4* strain (Fig. 4). We suggested above that binding of another factor at *ADE4* may prevent GCN4 from activating *ADE4* transcription in our strain, except under histidine starvation conditions. Perhaps this interfering factor is less active or even absent in the *gcd2* mutant employed by Mösch et al. (39), such that increased GCN4 synthesis in that strain leads to elevated *ADE4* transcription under all conditions.

Derepression of *HIS4* transcription by GCN4 under conditions of purine limitation was clearly evident in our strains (Tables 5 and 6 and Fig. 4). Perhaps other target genes of GCN4 in different amino acid biosynthetic pathways (22) are likewise induced under these starvation conditions. A pos-

sible rationale for an increase in amino acid biosynthesis under conditions of purine limitation might be to provide precursors for purine biosynthesis, which requires incorporation of aspartate, glycine, and the amide group of glutamine at different points in the pathway (27). The expression of *GLN1*, encoding glutamine synthetase, is under the general control (36). However, because *gcn* mutants are sensitive to purine analogs in the presence of amino acids in the medium (Fig. 3), it seems unlikely that increased amino acid biosynthesis is the only reason why GCN4 synthesis is stimulated by purine starvation. Therefore, we favor the idea that GCN4 also stimulates transcription from *ADE8* and perhaps other purine biosynthetic genes under these conditions. It is interesting that the purine, histidine, and trytophan biosynthetic pathways compete for phosphoribosyl-pyrophosphate (PRPP) (27), suggesting that PRPP synthetase may be derepressed in response to starvation for amino acids or purines in order to provide the PRPP needed to fuel these separate pathways.

The mechanism of GCN2 protein kinase activation under conditions of purine limitation. A fully functional GCN2 protein is required for translational derepression of *GCN4* in response to purine starvation. Mutations in *GCN2* that impair the general control response to amino acid or purine starvation include alleles mapping outside of the protein kinase domain (Table 5), at least some of which have been shown to produce a protein that retains in vitro autophosphorylation activity (45, 57). These mutations are thought to be regulatory in nature, affecting signal recognition and activation of the protein kinase domain of GCN2 under starvation conditions. The fact that mutations mapping in the amino-terminal, HisRS-related, or carboxy-terminal domain of GCN2 impair the derepression response to either purine or amino acid starvation may indicate that all three of these domains are required to activate GCN2 kinase function in response to either starvation signal.

The involvement of the HisRS-related domain of GCN2 in the derepression response to either purine or amino acid limitation could be explained most easily if purine starvation affects the charging of tRNA. This could occur if some step in tRNA maturation, such as the addition of CCA at the 3′ end (18) or the guanylation of histidyl-tRNA (10, 59), is inhibited under conditions of purine depletion. We have found that an increase in the level of eIF-2α phosphorylation can be detected within 30 min following the addition of purine analogs (unpublished observations). This response time may be too short for a mechanism which requires an accumulation of newly synthesized, unmodified tRNA to generate a starvation signal. An alternate possibility is that the aminoacylation reaction itself is impaired as the means of generating uncharged tRNA in response to purine starvation. For example, the aminoacylation of all tRNAs could be inhibited by a reduction in ATP levels under conditions of diminished adenine biosynthesis. Alternatively, specific synthetases could be inhibited by purine limitation.

A fundamentally different mechanism of GCN2 activation by purine starvation could be proposed, in which the HisRS-related domain is not specifically required for the derepression response to purine limitation and another signal besides uncharged tRNA is recognized by GCN2 in purine-starved cells. This model is ostensibly at odds with our finding that partially functional *gcn2* mutations in the HisRS-related domain impair the response to purine limitation to an even greater extent than under conditions of histidine starvation. However, these results could be explained by proposing that a higher level of GCN2 function is required to achieve adequate eIF-2α phosphorylation under purine versus histidine starvation conditions and that all of the mutations in the HisRS-like region simply reduce the level of the GCN2 protein. Alternatively, the mutations in the HisRS-related domain could disrupt an interaction between GCN2 and another molecule that is required to detect the purine starvation signal, e.g., the positive regulator GCN1. Finally, the mutations in the HisRS-like region could weaken the ribosome association of GCN2 and thereby prevent access to the substrate eIF-2. In fact, the HisRS-related sequences have been implicated in ribosome binding by GCN2 (46). Additional experiments will be required to determine the nature of the purine starvation signal and the regions of GCN2 that are specifically required to detect and transduce this signal to the GCN2 kinase domain.

Our results demonstrate that the general amino acid control system of *S. cerevisiae* responds to starvation for purines in addition to amino acids. This finding raises the possibility that starvation for other nutrients such as pyrimidines, lipids, or vitamins also stimulates expression of *GCN4* and the large number of biosynthetic genes under its control. Mutations in the *cpc-1* gene (the *GCN4* homolog) of *Neurospora crassa* lead to increased sensitivity to analogs of amino acids, purines, and pyrimidines (5), suggesting that the general (cross-pathway) control system of *N. crassa* similarly extends beyond the regulation of amino acid biosynthetic genes. Translational derepression of *GCN4* requires activation of the protein kinase GCN2 that phosphorylates the general translation initiation factor eIF-2α. Phosphorylation of eIF-2α on serine 51 is thought to inhibit translation initiation by a mechanism conserved between *S. cerevisiae* and humans (14). Clemens et al. (9) have shown that reduced charging of $tRNA^{Ile}$ in mammalian cells leads to increased phosphorylation of eIF-2α, prompting the suggestion that mammalian cells contain a homolog of GCN2 (14). If so, eIF-2α phosphorylation and translational control of gene expression may be elicited by purine limitation in mammalian cells, just as in *S. cerevisiae*.

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