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Mutations in *GCD11*, the structural gene for eIF-2γ in yeast, alter translational regulation of *GCN4* and the selection of the start site for protein synthesis

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Translation initiation factor 2 (eIF-2) in eukaryotic organisms is composed of three non-identical subunits, α, β and γ. In a previous report, we identified *GCD11* as an essential gene encoding the γ subunit of eIF-2 in the yeast *Saccharomyces cerevisiae*. The predicted amino acid sequence of yeast eIF-2γ displays remarkable similarity to bacterial elongation factor Tu, including the presence of sequence elements conserved in all known guanine nucleotide binding proteins. We have identified the molecular defects present in seven unique alleles of *GCD11* characterized by a partial loss of function. Three of these mutations result in amino acid substitutions within the putative GTP binding domain of eIF-2γ. We show that the *gcd11* mutations specifically alter regulation of *GCN4* expression at the translational level, without altering the scanning mechanism for protein synthesis initiation. Six of the mutant alleles presumably alter the function of eIF-2γ, rather than its abundance. A single allele, *gcd11-R510H*, suppresses a mutant *his4* allele that lacks a functional AUG start codon. The latter result indicates that the γ subunit of eIF-2 participates in recognition of the start site for protein synthesis, a role previously demonstrated in yeast for eIF-2α and eIF-2β.

Key words: eIF-2γ/general control/protein synthesis

Introduction

Initiation factor 2 (eIF-2) plays a critical role in the initiation of protein synthesis in eukaryotic organisms. eIF-2 forms a ternary complex with GTP and aminoacylated $tRNA_i^{Met}$ that delivers Met-$tRNA_i^{Met}$ to the 40S ribosomal subunit (reviewed in Hinnebusch and Liebman, 1991; Merrick, 1992). Based upon the available data, it is believed that a 43S pre-initiation complex consisting of the 40S subunit, ternary complex and additional initiation factors binds near the 5′-capped end of an mRNA and scans in a 5′→3′ direction until a start codon is recognized (Kozak, 1989). The GTP bound to eIF-2 is then hydrolyzed in a reaction that requires eIF-5, and an eIF-2−GDP binary complex is released. A 60S ribosomal subunit then attaches to the 40S−mRNA−Met-$tRNA_i^{Met}$ complex to form an 80S complex that enters into the elongation phase of protein synthesis.

In order to bind Met-$tRNA_i^{Met}$, eIF-2 must be in the GTP-bound state. Thus eIF-2−GDP produced during the initiation step must exchange GDP for GTP in order to participate in a subsequent round of protein synthesis initiation. The GDP/GTP exchange reaction on eIF-2 is catalyzed by eIF-2B, a factor that is composed of five non-identical subunits in both humans and yeast (Cigan *et al.*, 1993). The five structural genes encoding subunits of yeast eIF-2B, denoted *GCD1*, *GCD2*, *GCD6*, *GCD7* and *GCN3*, have been isolated and sequenced, along with human cDNAs encoding homologs of the yeast *GCD2* (eIF-2Bδ) and *GCD6* (eIF-2Bε) genes (Hannig and Hinnebusch, 1988; Hill and Struhl, 1988; Paddon *et al.*, 1989; Bushman *et al.*, 1993; Price *et al.*, 1994). The exchange reaction catalyzed by eIF-2B can be regulated to control the rate of protein synthesis initiation. One well-characterized mechanism involves phosphorylation of Ser51 on eIF-2α by specific protein kinases. In mammalian cells, this phosphorylation event increases the affinity of eIF-2 for eIF-2B and reduces eIF-2B function by sequestering limiting amounts of the exchange factor, thereby reducing the level of ternary complex in the cell (Siekierka *et al.*, 1984; Merrick, 1992). Mammalian eIF-2α kinases are activated by conditions that lead to cellular stress, including heat shock, heme deprivation, amino acid starvation and the presence of low amounts of double-stranded RNA that occur during certain viral infections (Merrick, 1992). GCN2, an eIF-2α kinase in *Saccharomyces cerevisiae*, is activated in response to amino acid starvation by increased levels of uncharged tRNAs that may be recognized by a domain of GCN2 that is similar to histidyl-tRNA synthetase (Krupitza and Thireos, 1990; Dever *et al.*, 1992; Hinnebusch, 1993).

GCN2 was originally identified as a positive effector in the general control pathway that acts to increase transcription of at least 30 unlinked amino acid biosynthetic genes, in multiple pathways, in response to starvation of a single amino acid (Wolfner *et al.*, 1975; Hinnebusch and Liebman, 1991; Hinnebusch, 1993). Transcriptional activation of the amino acid biosynthetic genes requires the transcriptional activator protein GCN4 (Hinnebusch and Liebman, 1991; Hinnebusch, 1993). *GCN4* is itself regulated at the translational level by *cis*-acting elements and *trans*-acting effectors. The *cis*-acting elements include four short upstream open reading frames (uORFs) present in the 5′-leader region of the *GCN4* mRNA. *Trans*-acting effectors are the products of *GCD* (negative effectors) and additional *GCN* (positive effectors) genes. In unstarved cells, the uORFs present in the *GCN4* mRNA leader inhibit synthesis of the GCN4 protein. Initiation and subsequent termination at the first uORF is believed to generate a fraction of 40S subunits that resume scanning in the absence of eIF-2. Dever *et al.* (1992) proposed that unstarved cells contain a high level of ternary complex that allows these 40S subunits to re-acquire the ternary

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Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source or reference
EY159	*MATα leu2-3 leu2-112 ura3-52 gcn3::LEU2*	Hannig and Hinnebusch, 1988
EY250	*MATa gcd11-508 gcn2-101 gcn3-101 ura3-52*	Hannig *et al.*, 1993
EY305	*MATα gcd11-505 leu2-3 leu2-112 ura3-52 gcn3::LEU2*	This study
EY555	*MATa gcd11-507 gcn2-101 gcn3-101 his1-29 ura3-52 (HIS4::lacZ ura3-52)*	This study
EY558	*MATa gcd11-506 gcn2-101 gcn3-101 his1-29 ura3-52 (HIS4::lacZ ura3-52)*	This study
EY582[a]	*MATa gcd11::hisG leu2-3 leu2-112 ura3-52* <Ep293(*GCD11*)>	This study
EY585	*MATα gcd11::hisG leu2-3 leu2-112 ura3-52* <Ep293(*GCD11*)>	This study
EY606[a]	*MATa gcd11::hisG leu2-3 leu2-112 ura3-52 gcn3::hisG* <Ep293(*GCD11*)>	This study
EY636[a]	*MATa gcd11::hisG leu2-3 leu2-112 ura3-52* <Ep552(*GCD11*)>	This study
EY640[d]	*MATa his4-303*(AUU) *ino1-13 ura3-52*	This study
EY641[d]	*MATa his4-303*(AUU) *ino1-13 ura3-52 SUI3-2*	This study
EY647	*MATα gcd11::hisG his4-303*(AUU) *ino1-13 leu2-3 leu2-112 ura3-52* <Ep293(*GCD11*)>	This study
F35	*MATa ino1 can1 ura3-52 (HIS4::lacZ URA3$^+$)*	Lucchini *et al.*, 1984
H4	*MATa leu2-3 leu2-112 ura3-52*	Harashima and Hinnebusch, 1986
H57[b]	*MATα GCD11-501 gcn2-101 gcn3-101 his1-29 ura3-52 (HIS4::lacZ ura3-52)*	Harashima and Hinnebusch, 1986
H59[b]	*MATα GCD11-502 gcn2-101 gcn3-101 his1-29 ura3-52 (HIS4::lacZ ura3-52)*	Harashima and Hinnebusch, 1986
H61[b]	*MATα GCD11-503 gcn2-101 gcn3-101 his1-29 ura3-52 (HIS4::lacZ ura3-52)*	Harashima and Hinnebusch, 1986
H79[b]	*MATα GCD11-504 gcn2-101 gcn3-101 his1-29 ura3-52 (HIS4::lacZ ura3-52)*	Harashima and Hinnebusch, 1986
H96[b]	*MATα gcn2-101 gcn3-101 his1-29 ura3-52 (HIS4::lacZ ura3-52)*	Harashima and Hinnebusch, 1986
H117[c]	*MATa gcn2-101 gcn3-101 his1-29 ino1 ura3-52 (HIS4::lacZ URA3$^+$)*	Harashima and Hinnebusch, 1986
H236[c]	*MATa gcd11-505 gcn2-101 gcn3-101 his1-29 ino1 ura3-52 (HIS4::lacZ URA3$^+$)*	Harashima and Hinnebusch, 1986
H245[c]	*MATa gcd11-506 gcn2-101 gcn3-101 his1-29 ino1 ura3-52 (HIS4::lacZ URA3$^+$)*	Harashima and Hinnebusch, 1986
H271[c]	*MATa gcd11-507 gcn2-101 gcn3-101 his1-29 ino1 ura3-52 (HIS4::lacZ URA3$^+$)*	Harashima and Hinnebusch, 1986
H272[c]	*MATa gcd11-508 gcn2-101 gcn3-101 his1-29 ino1 ura3-52 (HIS4::lacZ URA3$^+$)*	Harashima and Hinnebusch, 1986
H1402	*MATα leu2-3 leu2-112 ino1 ura3-52 (HIS4::lacZ ura3-52)*	Hannig *et al.*, 1990
TD117-1A[d]	*MATa his4-303*(AUU) *ino1-13 ura3-52 (his4*(AUU)*::lacZ URA3$^+$)*	Castilho-Valavicius *et al.*, 1990
TD117-1AR7[d]	*MATa his4-303*(AUU) *ino1-13 ura3-52 (his4*(AUU)*::lacZ URA3$^+$) SUI3-2*	Castilho-Valavicius *et al.*, 1990

[a]Isogenic strains.
[b]Isogenic strains.
[c]Isogenic strains.
[d]Isogenic strains.

complex and re-initiate at uORFs 2–4. Re-initiation and termination events at uORFs 2–4 preclude the scanning of sufficient numbers of 40S ribosomal subunits to the authentic *GCN4* start codon (Miller and Hinnebusch, 1989; Grant and Hinnebusch, 1994). However, activation of the GCN2 kinase by amino acid starvation reduces the level of ternary complex, allowing a fraction of scanning 40S ribosomes that initiate and terminate at uORF1 to scan through uORFs 2–4 prior to re-acquiring the ternary complex. 40S ribosomes that re-acquire the ternary complex in the interval between uORF4 and the *GCN4* start codon initiate protein synthesis at the *GCN4* start codon, leading to a 5- to 10-fold increase in GCN4 protein in starved cells (Grant *et al.*, 1994). The model also predicts that partial loss-of-function mutations in genes encoding subunits of eIF-2 or eIF-2B which reduce the level of ternary complex should mimic starvation conditions and lead to constitutive derepression of *GCN4* translation.

In the yeast *Saccharomyces cerevisiae* the essential genes *SUI2*, *SUI3* and *GCD11* encode the α (37 kDa), β (36 kDa) and γ (58 kDa) subunits of eIF-2 respectively (Donahue *et al.*, 1988; Cigan *et al.*, 1989; Hannig *et al.*, 1993). Mutant alleles of *SUI2* and *SUI3* were originally isolated in a genetic selection for suppressors of an initiation codon mutation in the yeast *HIS4* gene (Donahue *et al.*, 1988; Cigan *et al.*, 1989) and were subsequently shown to confer constitutive derepression of *GCN4* expression (Willliams *et al.*, 1989). Mutations in *GCD11* were isolated as suppressors of the inability to derepress the yeast general control pathway in a *gcn2 gcn3* double mutant (Harashima and Hinnebusch, 1986). Eight mutant alleles of *GCD11* were originally described and partially characterized (Harashima and Hinnebusch, 1986; Harashima *et al.*, 1987). Four of the alleles are dominant (*GCD11-501, GCD11-502, GCD11-503* and *GCD11-504*) and four are recessive (*gcd11-505, gcd11-506, gcd11-507* and *gcd11-508*) for the suppressor phenotype. The predicted 527 amino acid sequence of yeast eIF-2γ is remarkably similar to bacterial elongation factor EF-Tu (Hannig *et al.*, 1993), an extensively studied GTP binding protein that forms a ternary complex with aminoacylated elongator tRNAs. Yeast eIF-2γ contains a complete set of consensus sequence elements that are required for guanine nucleotide binding in EF-Tu and other GTP binding proteins (Bourne *et al.*, 1991). In addition, residues C-terminal to the GTP binding domain (G domain) in eIF-2γ that correspond to residues in domains II and III in the EF-Tu crystal structure are similar in both protein species and compatible with a similar domain structure for the yeast protein (Hannig *et al.*, 1993; Gaspar *et al.*, 1994).

In this report we have identified the molecular defect present in each of the eight original *gcd11* alleles. Seven of the alleles contain a single amino acid substitution (six are unique) and a single allele (*gcd11-508*) contains a transposable (Ty) element inserted near the C-terminus that extends the *GCD11* ORF by 30 amino acids. Strains harboring mutant alleles of *GCD11* exhibit characteristics consistent with global defects in protein synthesis initiation and all constitutively increase the translation of *GCN4* mRNA. A single allele, *gcd11-R510H*, alters the start site for protein synthesis when tested using a mutant *his4* allele that lacks a functional AUG codon. R510 maps to

domain III of the modeled eIF-2γ structure, suggesting that this region of eIF-2γ may function in monitoring interactions at the ribosomal P (peptidyl) site during the initiation process.

Results

Identification of the molecular defects in gcd11 alleles

Eight mutant alleles of *GCD11* were originally isolated as suppressors of the 3-AT-sensitive phenotype conferred by mutations in positive regulators of the general control encoded by *GCN2* and *GCN3* (Table I; Harashima and Hinnebusch, 1986; Harashima *et al.*, 1987). The *gcd11* mutations in the original isolates conferred varying degrees of both slow growth (slg$^-$) and constitutive derepression of general control-regulated genes, suggesting that multiple alleles were represented. We cloned each allele by a two-step integration/excision method (Rothstein, 1991) and determined the molecular defect in each allele by DNA sequence analysis of a 2.1 kb *Hin*dIII−*Sna*BI restriction fragment that includes the complete *GCD11* open reading frame. The mutations and their corresponding allele assignments are as follows: *GCD11-501* (R127L, CGU→CUU); *GCD11-502* and *-504* (H108N, CAU→AAU); *GCD11-503* (R510H, CGU→CAU); *gcd11-505* (E383K, GAA→AAA); *gcd11-506* (Y142H, UAC→CAC); *gcd11-507* (G397A, GGU→GCU). *gcd11-508* contains a Ty element (Clare and Farabaugh, 1985) inserted at codon 525 (out of 527). The insertion alters the encoded amino acid sequence, beginning with codon 526, to VGIEINYHLL-TSIYITSILSYTVLEDDANDEKstop. The six missense mutations alter residues that are conserved in three eIF-2γ homologs from *S.cerevisiae*, *Schizosaccharomyces pombe* and humans (Figure 1).

Effects of mutations on steady-state levels of eIF-2γ

We first determined if the *gcd11* alleles altered the steady-state levels of eIF-2γ, since reduced levels of eIF-2 would provide a simple explanation for both the general control and the growth phenotypes in the mutant strains. We measured the steady-state levels of eIF-2γ by immunoblot analysis of protein extracts prepared from exponentially growing cultures of the original *gcn2 gcn3 gcd11* strains and their isogenic *GCD11*$^+$ parents (Figure 2A). Quantitation of the immunoblot by laser densitometry and direct counting revealed that the level of eIF-2γ in five of the six strains harboring a point mutation in *GCD11* differed reproducibly by <1.5-fold from the isogenic wild-type strains. This suggests that the phenotypes in these mutant strains are due to a functional defect in the eIF-2γ protein, rather than a defect in the expression of *GCD11*. The level of eIF-2γ was reduced ~2-fold in the *gcd11-E383K* strain. In contrast, the level of the elongated form of eIF-2γ in the *gcd11-508* strain was reduced by ~10-fold compared with the isogenic wild-type strain. Thus the phenotypes exhibited by *gcd11-508* strains may result from reduced levels of eIF-2, rather than an intrinsic biochemical defect in the encoded eIF-2γ protein.

Construction of isogenic gcd11 GCN3 strains

We wished to examine phenotypes conferred by *gcd11* mutations in *GCN*$^+$ strains in order to rule out effects that might be due to the presence of the *gcn2* and *gcn3* mutations present in the original isolates. Toward this goal, we constructed an isogenic set of *GCD11*$^+$ and *gcd11* strains in the *GCN*$^+$ strain EY582 using the plasmid shuffle technique. In these strains, the sole copy of *GCD11* is harbored on the centromere-containing, low copy plasmid pSB32. To insure that the plasmid-borne alleles did not alter the relative steady-state levels of eIF-2γ in these strains, we measured the steady-state level of eIF-2γ in each strain by immunoblot analysis. Results presented in Figure 2B indicate that the levels eIF-2γ are equivalent in the *GCD11*$^+$ and *gcd11* plasmid shuffle strains (<1.5-fold difference) except for the *gcd11-508* strain, in which the level of eIF-2γ is reduced to one-third that in the *GCD11*$^+$ strain.

During the course of these experiments, we were consistently unable to construct strains in the *GCN3*$^+$ EY582 background harboring pSB32-borne versions of *gcd11-R127L* and *gcd11-E383K* as the sole copy of *GCD11*. Phenotypes conferred by certain alleles of *GCD11* are known to be exacerbated by the presence of wild-type *GCN3* (Harashima *et al.*, 1987), which encodes a non-essential subunit of eIF-2B (Hannig and Hinnebusch,

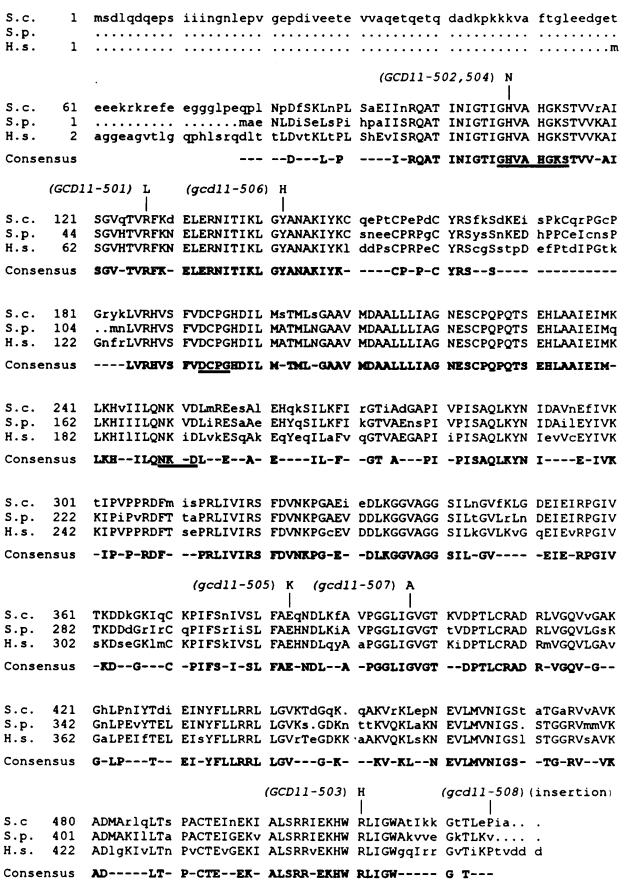

Fig. 1. Location of mutations in eIF-2γ. The amino acid changes in seven missense alleles and the site of the insertion of a Ty element in *gcd11-508* are shown above the amino acid sequence for eIF-2γ from *S.cerevisiae* (S.c.; Hannig *et al.*, 1993). The deduced amino acid sequences of eIF-2γ from *Schizosaccharomyces pombe* (S.p.; Erickson and Hannig, in preparation) and humans (H.s.; Gaspar *et al.*, 1994) are shown below the *S.cerevisiae* sequence. A consensus sequence of residues conserved in all three homologs is shown in bold. Three sequence elements conserved in GTP binding proteins are underlined.

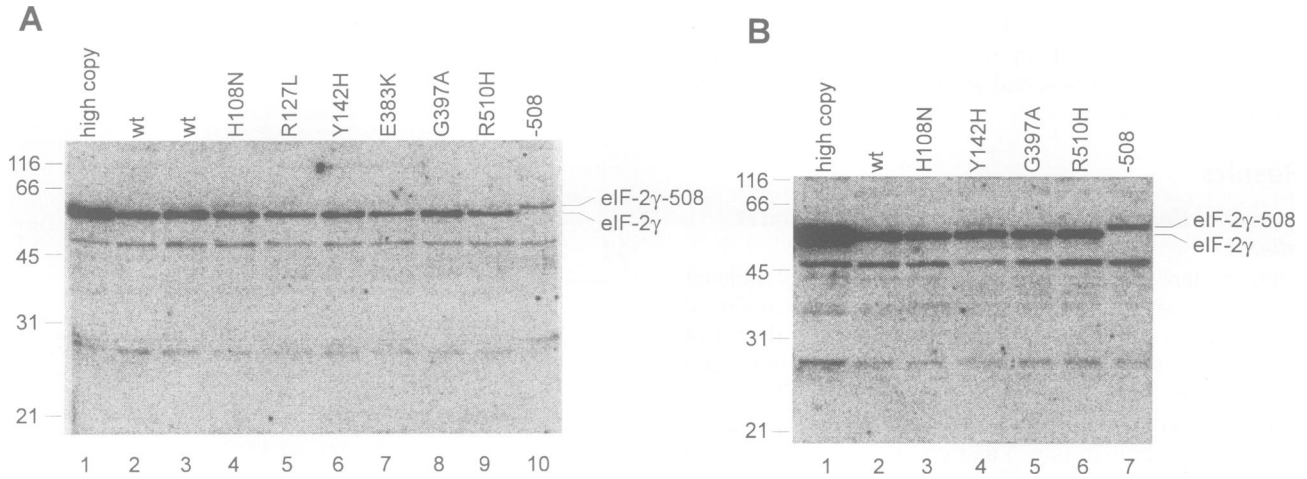

Fig. 2. Phosphorimage of immunoblots showing the steady-state levels of eIF-2γ in exponentially growing *GCD11* and *gcd11* strains. (**A**) Whole cell extracts were prepared from exponentially growing cultures of the original *gcd11* isolates and their wild-type parents. Equal amounts of protein from each preparation were analyzed by immunoblot analysis using polyclonal antisera specific to eIF-2γ, then detected using [^{125}I]protein A. The extracts were prepared from the following strains: lane 1, H96 + Ep362 (Ep362 contains *GCD11* in the high copy plasmid YEp24); lane 2, H96; lane 3, H117; lane 4, H59; lane 5, H57; lane 6, H245; lane 7, H236; lane 8, H271; lane 9, H61; lane 10, H272. The strains used in lanes 1, 2, 4, 5 and 9 are isogenic, as are the strains used in lanes 3, 6, 7, 8 and 10. (**B**) Whole cell extracts were prepared from the isogenic strains, constructed in EY582 by plasmid shuffling, and examined as in (A). The strain used in lane 1 harbors wild-type *GCD11* on the high copy 2μ plasmid YEp13, while those used in lanes 2–7 harbor low copy plasmids with the indicated *GCD11* alleles. The mobilities of molecular weight markers (in kDa) are listed in both panels on the left.

1988; Cigan *et al.*, 1993). To confirm that our inability to construct certain *gcd11 GCN* strains was due to the presence of *GCN3* rather than a result of some undefined element in our genetic background, we used the plasmid shuffle technique to construct *GCD11*$^+$ and *gcd11* strains in EY606, a Δ*gcn3* strain that is isogenic to EY582. As expected, the *gcd11-508* allele conferred a reduced growth rate in EY606 relative to EY582 (Hannig *et al.*, 1993). As shown in Table II, all of the mutant alleles functioned as the sole copy of *GCD11* in strain EY606. Introduction of a low copy plasmid containing wild-type *GCN3* into the plasmid shuffle strains constructed in EY606 exacerbated the slow growth defect in the *gcd11-R127L*, *-Y142H* and *-E383K* strains, but almost completely alleviated the growth defect in the *gcd11-508* derivative. Our results extend the studies of Harashima *et al.* (1987) and suggest further that our inability to construct certain *gcd11 GCN3* strains by the plasmid shuffle technique may result from antagonism of the encoded eIF-2γ protein by *GCN3*.

Table II. Relative growth rates of isogenic *GCN3* or Δ*gcn3* strains harboring different *gcd11* alleles

GCD11 allele	Relative growth rate of single colonies at 30°C		
	EY582[a]	EY606[a] (Δ*gcn3*)	EY606 + Ep69[b] (*GCN3*)
GCD11	+++++	+++++	+++++
High copy *GCD11*	+++++	+++++	n.d.[c]
H108N	+	+	+
R127L	−	++	+/−
Y142H	+	++	+
E383K	−	+++	+
G397A	++++	++++	n.d.
R510H	+++	+++	n.d.
508 (Ty insertion)	++++	++	++++

[a]Isogenic strains.
[b]Ep69 is a low copy, autonomously replicating plasmid that contains the wild-type *GCN3* gene.
[c]Not determined.

Altered monosome:polysome ratios in gcd11 strains

Mutations in the structural gene for eIF-2γ might be expected to confer global defects in translation initiation. Strains defective in translation initiation often exhibit altered polysome profiles obtained following centrifugation of extracts prepared from cycloheximide-treated cells (to inhibit translation elongation) through sucrose gradients. In particular, such strains can show increased monosome:polysome (M/P) ratios due to the accumulation of 40S and 60S ribosomal subunits that interact in the absence of mRNA to form 80S 'couples' (Cigan *et al.*, 1991). Under the conditions used, these 80S couples co-fractionate with monosomes bound to mRNA.

We asked if the isogenic *gcd11 GCN3* strains that we had constructed above showed increased M/P ratios consistent with a global defect in translation initiation. The polysome profiles depicted in Figure 3 show increased M/P ratios in the *gcd11* strains relative to that in an isogenic *GCD11*$^+$ strain. The data in Figure 3 also show a correlation between the magnitude of the increase in the M/P ratio and the severity of the growth defect in each mutant strain. However, increased M/P ratios in the *gcd11* strains are not due solely to the decreased growth rates of these strains, since the relative M/P ratio from an isogenic petite *GCD11*$^+$ strain was nearly identical to that of its grande *GCD11*$^+$ parent (1.2 versus 1.0), even though the doubling time of the petite strain was almost 2-fold greater (3.2 versus 1.8 h). In addition, the doubling time of the *gcd11-R510H* strain is equivalent to that of the petite *GCD11*$^+$ strain, but nevertheless shows a 2.3-fold increase in M/P ratio relative to the petite strain. The polysome profiles of the strains harboring missense mutations in *GCD11* are therefore consistent with a global defect in

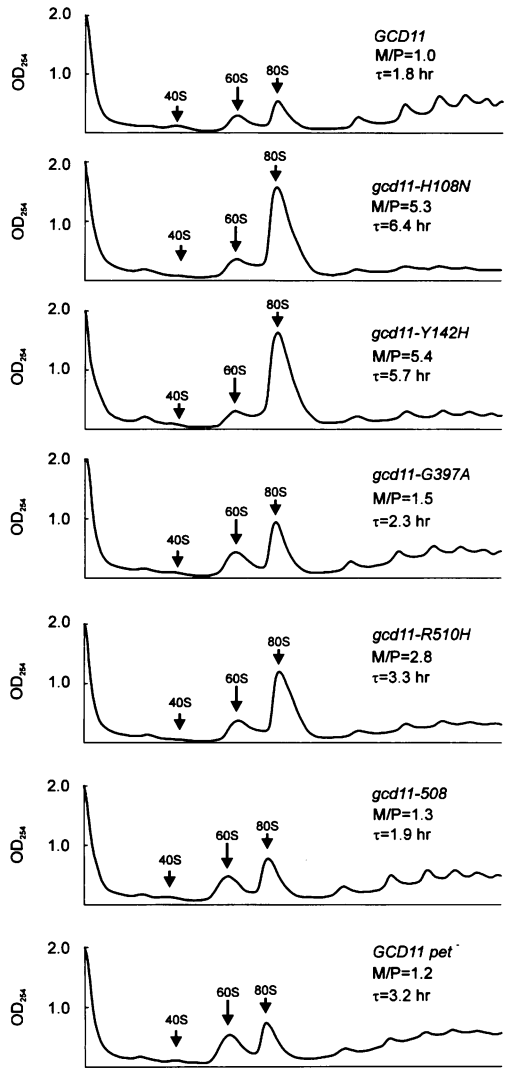

Fig. 3. Polysome profile analysis of *GCD11* and *gcd11* strains. Cell extracts were prepared from cycloheximide-treated (50 μg/ml) exponentially growing cultures of isogenic plasmid shuffle strains harboring the listed *GCD11* allele as its sole copy. Sixteen OD_{260} units from each extract were layered on 7–47% sucrose gradients, centrifuged for 2 h at 200 000 *g* and fractions were collected with constant monitoring at OD_{254}. The monosome:polysome ratio (M/P) was calculated by dividing the area under the 80S peak by the area under the 2mer−5mer peaks and normalized to the ratio in the $GCD11^+$ strain, which was set to 1. The peaks corresponding to the 40S, 60S and 80S are labeled accordingly. OD_{254} is the optical density at 254 nm. The doubling time (τ) for each strain was determined in rich medium by monitoring the optical density at 600 nm.

protein synthesis initiation. The *gcd11-508 GCN3*$^+$ strain shows an M/P ratio similar to that of the wild-type control (1.2 versus 1.0), but an isogenic *gcd11-508 Δgcn3* strain has a M/P ratio of 2.6 (data not shown). The difference in the polysome profiles and growth rates between the two *gcd11-508* strains suggests that *GCN3* can contribute to the function of eIF-2γ in *gcd11-508* strains.

Mutations in GCD11 alter the translational regulation of GCN4 expression

Harashima and Hinnebusch (1986) demonstrated previously that *GCN4* expression increased constitutively in a *GCD11-501 gcn2 gcn3* yeast strain. This increased expression was later shown to be due to increased translation of

β-Galactosidase activity (U) from *GCN4-lacZ* fusions

Plasmid-borne *GCD11* allele	p180 R	p180 DR	p226 R	p226 DR	p227 R	p227 DR
H108N	921	1012	104	121	2335	2326
Y142H	804	788	84	90	2465	1652
G397A	273	424	11	37	1321	1357
R510H	277	518	25	67	1518	1437
-508	116	316	20	38	1387	1470
wt	23	415	10	47	936	1316
high copy wt	52	249	13	46	888	1110

Fig. 4. β-Galactosidase activities in *gcd11* strains containing *GCN4−lacZ* fusions. Plasmids p180, p226 or p227, which contain a *GCN4−lacZ* fusion with all four upstream open reading frames (uORFs), uORF4 only or no uORFs respectively are represented by the diagrams. A box represents an open reading frame while an X represents a point mutation which removes the start codon of the corresponding open reading frame. These plasmids were introduced into strains harboring the different *GCD11* alleles (shown in the first column) on a low copy plasmid. Cultures from 3–5 independent transformants were grown under repressing (R) or derepressing (DR) conditions and analyzed as described in Materials and methods. Units of β-galactosidase activity are given in nmol *o*-nitrophenyl-β-D-galactopyranoside (ONPG) cleaved/min/mg total protein. The activities given are the average activities from 3–5 transformants, each of which differed by <30% from the average.

the *GCN4* mRNA (Mueller *et al.*, 1987). We have extended these analyses to show that mutations in *GCD11* specifically alter *GCN4* expression at the translational level in *GCN*$^+$ strains and that the altered expression in mutant strains requires the four short ORFs present in the *GCN4* mRNA leader. For these experiments we used isogenic *GCD11*$^+$ and *gcd11* strains constructed by the plasmid shuffle technique in the *GCN*$^+$ strain EY582. We introduced into these strains a low copy plasmid containing one of three *GCN4−lacZ* alleles that differ only in the region encoding the *GCN4* mRNA leader. These alleles contained either all four uORFs (p180), uORF4 only (p226) or no uORFs (p227). In each case, the missing ORFs (indicated by an X in Figure 4) were removed by introducing point mutations in the AUG codon of the uORF (Mueller and Hinnebusch, 1986).

Figure 4 shows the results of assays for β-galactosidase activity used to model *GCN4* expression under both repressing (R) and amino acid starvation (DR, derepressing) conditions. β-Galactosidase activity in p180 transformants of the *GCD11*$^+$ strain increased ~20-fold under amino acid starvation conditions. β-Galactosidase activity expressed from a *GCN4−lacZ* allele that lacks the four uORFs (p227), used to model changes in the level of the *GCN4−lacZ* transcript (Mueller and Hinnebusch, 1986), increased only 1.4-fold in starved cells. Taken together, these data indicate that the increased β-galactosidase activity expressed from p180 in starved cells is due primarily to an increase in translation of the *GCN4−lacZ* mRNA. The constitutively low levels of β-galactosidase activity in the p226 (uORF4 only) transformants suggests that the scanning mechanism for translation initiation is not altered under starvation conditions. These results are in agreement with previous results using related wild-type strains (Mueller and Hinnebusch, 1986).

p180 transformants of the *gcd11* strains show constitutively increased *GCN4−lacZ* activity. In these cases,

Relevant allele	*HIS4*^{AUG}-*lacZ* β-gal activity (U)	AUG ratio	*his4*^{UUG}-*lacZ* β-gal activity (U)	UUG ratio	UUG ratio / AUG ratio	His +/ - in *his4*^{AUU} strain
gcd11-H108N [a]	5030	5.6	449	18.7	3.3	-
gcd11-Y142H [a]	4990	5.5	508	21.1	3.8	-
gcd11-E383K [a]	nd[c]	nd	nd	nd	nd	-
gcd11-G397A [a]	2220	2.5	129	5.3	2.2	-
gcd11-R510H [a]	2607	2.9	724	30.1	10.4	+
gcd11-508 [a]	1915	2.1	59	2.4	1.1	-
wt [a]	901	1.0	24	1.0	1.0	-
high copy *GCD11* [a]	1110	1.2	39	1.6	1.3	-
SUI3 [b]	528	1.0	7	1.0	1.0	-
SUI3-2 [b]	1346	2.5	637	89.9	35.3	+

[a] Isogenic strains.
[b] Isogenic strains.
[c] nd - not determined.

Fig. 5. β-Galactosidase activities in *gcd11* strains harboring low copy plasmids containing either *HIS4*^{AUG}−*lacZ* or *his4*^{UUG}−*lacZ*. Isogenic plasmid shuffle strains harboring the various alleles of *GCD11* were transformed with low copy plasmids containing either *HIS4*^{AUG}−*lacZ* or *his4*^{UUG}−*lacZ*. The two constructs are identical except for a single base change in the *his4*^{UUG}−*lacZ* fusion that alters the start codon from AUG to UUG. β-Galactosidase activities were determined from 3–5 independent transformants as described in the legend to Figure 4. The AUG ratio is the β-galactosidase activity in strains harboring the *HIS4*^{AUG}−*lacZ* plasmid divided by the activity in the isogenic wild-type strain. Likewise, the UUG ratio is the ratio of β-galactosidase activity in a strain harboring the *his4*^{UUG}−*lacZ* plasmid to the activity in the wild-type strain harboring the same plasmid. The UUG ratio/AUG ratio is the UUG ratio divided by the AUG ratio. The last column shows the ability of each allele to suppress a *his4*^{AUU} allele and thereby allow growth on media lacking histidine. Plasmids containing each of the *gcd11* alleles listed in the first column were the sole copy of *GCD11* in EY647 (*his4*^{AUU}) after plasmid shuffling. The last two rows of the right column show the results following introduction of a low copy plasmid containing either *SUI3* or *SUI3*-2 into the *GCD11*$^{+}$ EY647 derivative.

expression from p180 increases 5- to 40-fold in non-starved cells relative to the *GCD11*$^{+}$ strain. Conversely, expression of *GCN4*−*lacZ* from p227 in the *gcd11* strains shows a small increase of 1.5- to 2.6-fold. Thus the constitutive expression of *GCN4* in *gcd11* strains is primarily due to a constitutive increase in translation of *GCN4* mRNA. Qualitatively, the degree of derepression in unstarved cells (Figure 4, p180, R) correlates well with the growth rate defect and increased M/P ratios in the mutant strains. Note that the smallest increase in *GCN4*−*lacZ* expression occurs in the *gcd11-508* strain, yet this strain exhibits the greatest change in level of the eIF-2γ protein (a reduction to one-third of wild-type; Figure 2B). This further supports our notion that the defects seen in the *gcd11* missense strains result from functional defects in the encoded eIF-2γ proteins. p226 transformants of the *gcd11* strains show greatly reduced levels of *GCN4*−*lacZ* expression, suggesting that the scanning mechanism remains intact in *gcd11* strains. Expression of *GCN4*−*lacZ* from p226 is greatest in the *gcd11-H108N* and *gcd11-Y142H* strains, which are also the most highly derepressing of the *gcd11* alleles examined. Elevated expression from p226 suggests that the defective eIF-2 complexes in these latter strains may be prone to increased 'leaky scanning' that allows the scanning ribosomes to bypass the highly inhibitory uORF4 more frequently than in other strains.

Initiation at non-AUG codons

Donahue and colleagues used *his4* alleles lacking a functional AUG codon to isolate mutations in genes that act in *trans* to alter the selection of the start site for protein synthesis (Donahue *et al.*, 1988; Cigan *et al.*, 1989; Castilho-Valavicius *et al.*, 1990, 1992). Using these alleles in a genetic selection for His^{+} revertants, they identified mutations in genes encoding the α (*SUI2*) and β (*SUI3*) subunits of eIF-2 that were shown to allow translation initiation at an in-frame UUG codon (Donahue *et al.*, 1988; Cigan *et al.*, 1989; Yoon and Donahue, 1992). These alleles also constitutively derepress *GCN4* expression (Williams *et al.*, 1989). We asked if mutations in *GCD11*, isolated based upon their general control phenotype, could also alter start site selection and allow expression of β-galactosidase from a *his4*^{UUG}−*lacZ* allele in which the normal *HIS4* AUG codon is replaced by a UUG codon. In this *his4*^{UUG}−*lacZ* construct, initiation at downstream AUG codons cannot give rise to a protein product with β-galactosidase activity (Donahue and Cigan, 1988). As a control, we measured expression from *HIS4*^{AUG}−*lacZ*. This was necessary since both of these *HIS4*−*lacZ* translational fusions are expressed from the yeast *HIS4* promoter and are therefore subject to transcriptional regulation by the general control pathway.

Figure 5 shows the results of β-galactosidase assays using extracts prepared from isogenic *GCD11*$^{+}$ and *gcd11* strains harboring a low copy plasmid containing either *HIS4*^{AUG}−*lacZ* or *his4*^{UUG}−*lacZ*. All of the *gcd11* strains showed increased β-galactosidase activity in the *HIS4*^{AUG}−*lacZ* transformants when compared with the *GCD11*$^{+}$ strains. The increased activity is a reflection of an increase in the level of the *HIS4*−*lacZ* transcript due

to constitutive derepression of the general control in the *gcd11* strains. When normalized to activity in the wild-type strain (Figure 5, AUG ratio), the derepression response is seen to vary from 2- to 5.6-fold in unstarved cells, in agreement with previous studies (Harashima and Hinnebusch, 1986). In the *his4^{UUG}−lacZ* transformants, the increased β-galactosidase activity in the *gcd11* strains is presumably a combined effect of transcriptional activation from the *HIS4* promoter and increased initiation at a non-AUG start codon. Initiation at non-AUG codons can be estimated as the ratio of the normalized β-galactosidase expression from *his4^{UUG}−lacZ* divided by that from *HIS4^{AUG}−lacZ* (Figure 5, UUG ratio/AUG ratio). As a control, we performed a similar analysis using isogenic *SUI3* and *SUI3-2* strains (Donahue *et al.*, 1988; Castilho-Valavicius *et al.*, 1990). The *SUI3-2* strain showed a 35-fold increase (UUG ratio/AUG ratio) in expression from *his4^{UUG}−lacZ*, which represents initiation at non-AUG codons.

Among the *gcd11* strains, the *gcd11-R510H* strain showed the largest increase (10.4-fold) in expression of β-galactosidase activity from *his4^{UUG}−lacZ* relative to *HIS4^{AUG}−lacZ*. Five additional *gcd11* alleles showed a <4-fold increase in expression from *his4^{UUG}−lacZ* that may not be accounted for by the increased level of *his4−lacZ* transcript (note that, except for *gcd11-R510H*, the more highly derepressing *gcd11* alleles show the higher UUG ratio/AUG ratio values). Northern blot analysis (data not shown) revealed that for an individual *gcd11* allele, the steady-state level of *his4−lacZ* mRNA was equivalent in the *his4^{UUG}−lacZ* and the *HIS4^{AUG}−lacZ* transformants. As expected, the *lacZ* transcript levels were increased in the *gcd11* mutants compared with the *GCD11^{+}* strain. In addition, the level of the *his4^{UUG}−lacZ* transcript was ~2-fold higher in the *gcd11-H108N* strain relative to the *gcd11-R510H* strain, yet the *his4^{UUG}−lacZ* activity was reduced by 40% in the former strain. This supports the hypothesis that the increase in *lacZ* expression from the *his4^{UUG}−lacZ* fusion in the *gcd11-R510H* strain is due to increased translation initiation at non-AUG codons.

To examine the physiological significance of increased expression of *his4^{UUG}−lacZ* in the *gcd11* strains, we constructed a set of isogenic *GCD11^{+}* and *gcd11* strains by the plasmid shuffle technique in the His^{-} yeast strain EY647. This strain harbors the *his4^{AUU}* allele at the *HIS4* locus. In this background, *GCD11^{+}* strains are histidine auxotrophs. As a control, the dominant *SUI3-2* allele, when introduced on a low copy plasmid (p364) into yeast strain EY647 (*GCD11^{+}*), suppressed the *his4^{AUU}* mutation and allowed growth in the absence of histidine (Figure 5, right column). As expected, introduction of wild-type *SUI3* on a low copy plasmid failed to suppress the *his4^{AUU}* mutation. Strains harboring the *gcd11-H108N*, *gcd11-Y142H*, *gcd11-E383K*, *gcd11-G397A* or *gcd11-508* alleles were histidine auxotrophs. However, the *gcd11-R510H* allele suppressed the *his4^{AUU}* mutation and allowed growth on media lacking histidine. This strain was His^{-} prior to plasmid shuffling (to remove the *GCD11^{+}* allele), suggesting that the suppressor phenotype is recessive. These results indicate that eIF-2γ participates in recognition of the start codon by eIF-2 during initiation of protein synthesis. These results also demonstrate that neither partial loss-of-function in the γ subunit of eIF-2 nor strong derepression of *GCN4* expression is sufficient to alter recognition of the start site for protein synthesis.

Discussion

We have identified the molecular defects present in eight mutant alleles of *GCD11* (Harashima and Hinnebusch, 1986; Harashima *et al.*, 1987). The defects include six unique point mutations and a single Ty element insertion. With the exception of *gcd11-508*, none of the *gcd11* alleles leads to a significant reduction in the steady-state level of eIF-2γ, suggesting that the phenotypes in these latter strains are not simply due to an overall reduction in the level of eIF-2. Collectively, the *gcd11* alleles confer an unconditional slow growth phenotype and constitutively derepress the general control pathway. The *gcd11* strains examined also exhibit increased monosome:polysome ratios consistent with a general defect in protein synthesis initiation and specifically alter the translational regulation of *GCN4* expression.

The analysis of mutations in *GCD11* is facilitated by similarities between the predicted amino acid sequences of eIF-2γ and bacterial elongation factor EF-Tu. Structures for EF-Tu and p21^{ras} in both the GDP- and GTP-bound states have been determined based upon X-ray crystallographic studies (de Vos *et al.*, 1988; Pai *et al.*, 1989; Tong *et al.*, 1989; Kjeldgaard and Nyborg, 1992; Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993). Although the G domains of EF-Tu and p21^{ras} share only 16% amino acid sequence identity, the α-carbon backbones of the respective GTP binding domains are nearly superimposable (Valencia *et al.*, 1991). eIF-2γ exhibits an even higher degree of sequence similarity with the EF-Tu G domain (34% identical, 49% similar) and considerable similarity in domains II and III (19% identical, 33% similar) that are C-terminal to the G domain (domain I) in both proteins (Hannig *et al.*, 1993; Gaspar *et al.*, 1994). We have used the structure of EF-Tu−GDP (Kjeldgaard and Nyborg, 1992) as a model to illustrate the location of residues in eIF-2γ that are altered by the *gcd11* alleles described in this report (Figure 6). The *gcd11* mutations map to regions corresponding to domain I (*gcd11-H108N*, *gcd11-R127L* and *gcd11-Y142H*), domain II (*gcd11-E383K*, *gcd11-G397A*) and domain III (*gcd11-R510H* and *gcd11-508*) of the EF-Tu structure. None of the eight alleles alter residues in the 88 amino acid N-terminal extension of yeast eIF-2γ that is absent in EF-Tu. This N-terminal extension is not conserved in the human and *S.pombe* eIF-2γ homologs (Figure 1) and may therefore serve some species-specific function.

His108 in yeast eIF-2γ is located within the first conserved motif (G-X_4-G-K-S/T) found in GTP binding proteins and is conserved at the second position of this motif in all GTP binding translation factors (Bourne *et al.*, 1991). This sequence element is located in a loop region and the side chains of residues in this loop contact the β-phosphate of the guanine nucleotide in EF-Tu−GDP. The analogous residue in EF-Tu is H19, which interacts with Q115 (conserved as Q228 in eIF-2γ) in the α3-helix of domain I in the EF-Tu−GDP structure (Kjeldgaard and Nyborg, 1992). Mutations that alter amino acids surrounding H19 in EF-Tu and the analogous residue in p21^{ras} (A11) reduce the ability of these proteins to hydrolyze GTP

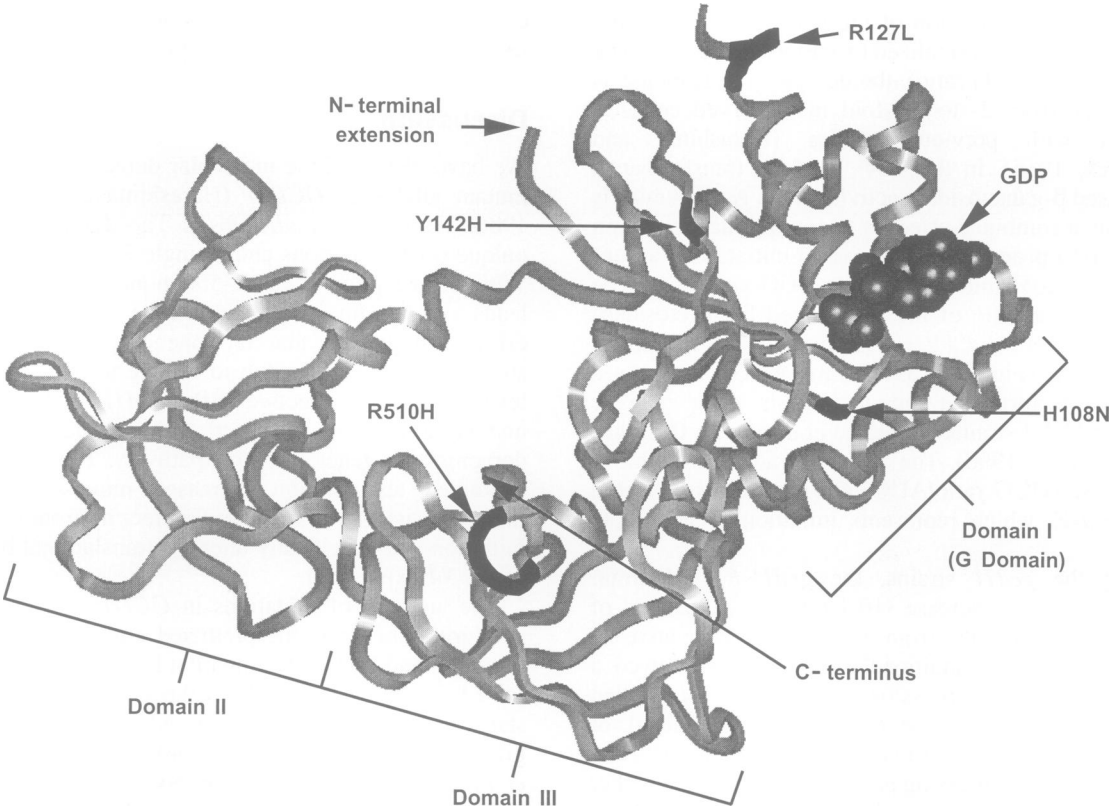

Fig. 6. Ribbon model of EF-Tu−GDP showing the relative locations of amino acid residues in eIF-2γ altered in the *gcd11* strains. The three structural domains of EF-Tu and the bound GDP molecule are shown. The location of the N-terminal insertion in eIF-2γ (relative to EF-Tu) is indicated. The positions of the amino acid substitutions in eIF-2γ are shown in black. The mutations in domain II are not shown, due to a greater degree of uncertainty in aligning residues in this region. Note the absence of the L2 loop, as the coordinates for this structure were obtained from trypsin-treated EF-Tu (Kjeldgaard and Nyborg, 1992).

(Gibbs *et al.*, 1984; Jacquet and Parmeggiani, 1988). In the initiation reaction, GTP hydrolysis on eIF-2 is required for formation of 80S complexes (Merrick, 1992). In this respect, the increased expression of *GCN4−lacZ* from both p180 (all four uORFs) and p226 (uORF4 only) in *gcd11-H108N* strains could be explained by leaky scanning at the uORFs due to inefficient initiation by pre-initiation complexes containing eIF-2 [eIF-2γ(H108N)]. Alternatively, the accumulation of ribosomes stalling at AUG codons could reduce the overall level of ternary complex in these strains and thereby lead to increased *GCN4* translation.

The *gcd11-Y142H* mutation alters a tyrosine residue in the β2-strand that immediately follows the putative effector loop [loop L2, (136)ITIKLG**Y**AN(144)]. The corresponding amino acid residue in EF-Tu is His66 [(60)ITINTS**H**VE(68)], which is located at the bottom of the cleft between domains I and III in the EF-Tu−GTP crystal structure (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993) and can be cross-linked to tRNA (Duffy *et al.*, 1981; Metz-Boutigue *et al.*, 1989). This histidine residue is not conserved in EF-1α, the functional homolog of EF-Tu in eukaryotes, and therefore the possibility remains that this residue is in close proximity to the tRNA rather than in direct contact (Kinzy *et al.*, 1992). We have observed a slight reduction in the relative amount of eIF-2γ(Y142H) that is ribosome bound as an eIF-2 complex (data not shown), consistent with the possibility that eIF-2γ(Y142H) might affect the binding of Met-tRNA$_i^{Met}$. *gcd11-R127L* alters a residue [(124)QTV**R**FKDELERNITI(138)] in a region near the junction of the C-terminal end of the α1-helix of the G domain and loop L2 (the putative effector loop) in the EF-Tu structure (Gaspar *et al.*, 1994; our unpublished observations). The eIF-2γ sequence is 13 amino acids shorter than the EF-Tu sequence in this region (Kjeldgaard and Nyborg, 1992) and thus our assignment of R127 to the α1-helix is tentative. It is possible that this helix is shorter in eIF-2γ compared with EF-Tu, as is the corresponding α-helix in p21^{ras} (Pai *et al.*, 1989; Valencia *et al.*, 1991). In this case, R127 might form a portion of loop L2. It is of interest to note that sequences within the putative α1-helix of the human eIF-2γ G domain can be cross-linked to the initiator Met-tRNA$_i^{Met}$ (Gaspar *et al.*, 1994). *gcd11-R510H*, which is discussed further below, alters a residue [(510)**R**LIGWATIKK(519), EF-Tu residues (381)**R**TVGAGVVAK(390)] that maps near the loop connecting the β5- and β6-strands in domain III at the C-terminus of EF-Tu. As shown in Figure 6, we have modeled this loop on the same side of eIF-2γ as is Y142. The corresponding face of EF-Tu and EF-1α (toward the viewer in Figure 6) has been implicated in tRNA binding using cross-linking and chemical protection studies (Kinzy *et al.*, 1992). In eIF-2γ these residues (Y142 and R510) may be involved in tRNA binding or, perhaps, interactions with other proteins such as eIF-2β, which can also be cross-linked to GTP and Met-tRNA$_i^{Met}$ (Anthony *et al.*, 1990). We are attempting to purify eIF-2 containing altered γ subunits for biochemical analyses to provide further

insight into the importance of these residues for eIF-2 function.

Previous studies have provided genetic evidence that the α (SUI2) and β (SUI3) subunits of eIF-2 (Donahue *et al.*, 1988; Cigan *et al.*, 1989; Castilho-Valavicius *et al.*, 1990, 1992), the initiation factor SUI1 (Yoon and Donahue, 1992), and $tRNA_i^{Met}$ (Cigan *et al.*, 1988) function in recognition of the start site for protein synthesis. Our results indicate that eIF-2γ is also involved in selection of the start site by eIF-2. A single *gcd11* allele (*gcd11-R510H*) alters recognition of the start site for protein synthesis. Additional alleles that confer similar phenotypes have recently been isolated (S.Rippel and E.M.Hannig, unpublished data). The mutant *sui1* and *SUI3* alleles have been shown to allow initiation at UUG codons (Yoon and Donahue, 1992). The mechanism by which *gcd11-R510H* suppresses *his4* alleles that lack a functional AUG codon is currently under study. Relative to the other *gcd11* alleles examined, *gcd11-R510H* only moderately derepresses *GCN4* translation (Figure 4) and therefore the ability to initiate at non-AUG codons is not simply related to the degree of partial loss of function (as judged also by growth rates and monosome:polysome ratios).

Our analyses of *gcd11* alleles in isogenic *GCN3* and Δ*gcn3* strains (Table II) confirm and extend previous observations that *GCN3* (encoding a non-essential subunit of eIF-2B) exacerbates the slow growth phenotype in *gcd11-R127L*, *gcd11-Y142H* and *gcd11-E383K* strains. Conversely, the introduction of *GCN3* into a Δ*gcn3 gcd11-508* strain ameliorates the slow growth phenotype in this strain, perhaps by stabilizing the gcd11-508 protein (but not to wild-type levels; see Figure 2). In contrast, the presence of *GCN3* has no observable effect on the growth of *gcd11-H108N*, *gcd11-G397A* and *gcd11-R510H* strains. Various explanations could account for these genetic interactions. *gcd11* alleles affected by the presence/absence of *GCN3* might directly affect interactions between eIF-2γ and eIF-2B or, alternatively, might alter the quaternary structure of eIF-2 and thereby indirectly affect interactions of eIF-2 with the exchange factor. Hinnebusch and colleagues have proposed that the presence of GCN3 increases the stability of eIF-2(α-PO_4)−eIF-2B complexes (Vasquez de Aldana *et al.*, 1993). In a similar fashion, some altered eIF-2γ proteins might act to stabilize eIF-2−eIF-2B complexes (mimicking eIF-2α phosphorylation), while others hinder the formation of an active eIF-2−eIF-2B complex. In either scenario, *GCN3* could enhance the defect associated with eIF-2γ(R127L), eIF-2γ(Y142H) and eIF-2γ(E383K).

Studies of the translational regulation of *GCN4* expression have revealed that inhibition of *GCN4* expression by the uORFs in the 5′-leader sequences of its mRNA can be overcome by controlling the level of ternary complex in the cell. A number of eukaryotic mRNAs from yeast to mammals contain uORFs in the 5′-leader sequences that may also be subject to regulation by a *GCN4*-like translational control mechanism (Kozak, 1989; Geballe and Morris, 1994). In support of this idea, both yeast and mammals possess eIF-2α kinases that, when activated, are thought to decrease the level of ternary complex by stabilizing the eIF-2−eIF-2B complex (Koromilas *et al.*, 1992; Barber *et al.*, 1993). Further genetic and biochemical analysis of the structural genes encoding eIF-2 and eIF-2B subunits in yeast will provide information critical for understanding the molecular basis for the essential and regulatory interactions between eIF-2 and eIF-2B.

Materials and methods

Strains and genetic techniques

Escherichia coli strain DH5α was used to propagate plasmid DNAs that were introduced by standard methods (Sambrook *et al.*, 1989). Plasmid DNA was introduced into yeast strains by the alkali cation method (Ito *et al.*, 1983). 5-Fluoroorotic acid (5-FOA) was purchased from PCR Incorporated. 3-Amino-1,2,4-triazole (3-AT) was purchased from Sigma. Additional media used for the growth of yeast strains were prepared as described (Sherman *et al.*, 1979).

Yeast strains used for these experiments are listed in Table I. All are derived from strain S288C using standard genetic techniques (Sherman *et al.*, 1979). EY305 was derived from H236 by crossing with EY159. EY555 and EY558 were obtained as ascospores from crosses of H96 with H271 and H245 respectively. EY582 was used to construct isogenic *gcd11* strains by the plasmid shuffle technique and was obtained as follows. A 6.4 kb *Eco*RI−*Sal*I restriction fragment containing the *gcd11::hisG-URA3-hisG* (*Δ1–527*) allele was purified from plasmid Ep523 (see below) and introduced into a Ura^- diploid strain formed by mating strains H4 and H1402. DNA was purified from Ura^+ yeast transformants, digested with *Eco*RI and analyzed by Southern blot analysis using a radiolabeled 4.3 kb *Bam*HI−*Bgl*II restriction fragment from Ep264, containing the *GCD11* gene, as probe (Feinberg and Vogelstein, 1983; Hannig *et al.*, 1993). Diploid strains containing a single copy of the disrupted allele at one of the two chromosomal *GCD11* loci were plated onto media containing 5-FOA to select for Ura^- strains that had lost the *URA3* gene as a result of recombination between the *hisG* direct repeats (5-FOA is toxic to *URA3*$^+$ cells; Boeke *et al.*, 1987). The recombination event was confirmed by Southern blot analysis of the Ura^- derivatives (data not shown). The YCp50/*GCD11*(*URA3*$^+$) plasmid Ep293 (Hannig *et al.*, 1993) was then introduced into the Ura^- diploid strain and Ura^+ transformants were selected and sporulated. EY582 and EY585 were obtained as Ura^+ ascospores that were inviable when tested on 5-FOA medium, indicating that the plasmid Ep293 harbored the sole copy of *GCD11* in this strain. EY636 harbors a wild-type GCD11 allele on pSB32 (Rose and Broach, 1991) and was constructed from EY582 using the plasmid shuffle technique (see below).

EY606 lacks the entire *GCN3* open reading frame and was constructed by introducing a 7 kb *Eco*RI−*Pvu*II fragment containing the *gcn3::hisG-URA3-hisG* (*Δ1–305*) allele from Ep545 into EY636. Chromosomal DNA prepared from Ura^+ transformants of EY636 was digested with *Eco*RI and examined by Southern blot analysis, using a radiolabeled 4 kb *GCN3* fragment from Ep186, to identify strains in which the *GCN3* locus had been disrupted. Ura^+ transformants were then patched onto 5-FOA media and a Ura^- derivative was selected and then analyzed by Southern blot analysis as above. Plasmid Ep293 was introduced into this strain and Ura^+ transformants were selected. Following growth on media lacking uracil, EY606 was obtained as a Ura^+ Leu^- colony that had lost the resident pSB32(*LEU2*$^+$)/GCD11 plasmid Ep552. EY606 is sensitive to 30 mM 3-AT (a Gcn^- phenotype), whereas the parent strain EY636 is resistant to 3-AT.

EY640 and EY641 are Ura^- derivatives of the isogenic strains TD117-1A and TD117-1AR7 respectively and were obtained as Ura^- papillae on 5-FOA medium. EY647 was obtained as an ascospore from a cross of EY640 with EY585.

Plasmids

Ep264 is a low copy YCp50-based plasmid which contains a 4.5 kb genomic fragment harboring the *GCD11* gene (Hannig *et al.*, 1993). A 4.3 kb *Bam*HI−*Bgl*II fragment from Ep264 was subcloned into the unique *Bam*HI site of the yeast integration vector YIp5 (Struhl *et al.*, 1979) to create Ep487. A 1.5 kb *Hpa*I restriction fragment encoding GCD11 amino acid residues 1–487 (out of 527 residues) was removed from Ep487 and the plasmid was then resealed to create Ep488. A 4.5 kb *Sal*I−*Eco*RI fragment from Ep264 was subcloned into the *Sal*I/*Eco*RI sites of YIp5 to create Ep467. Ep467 was digested with *Hin*dIII and *Sna*BI, blunt-ended with Klenow enzyme and recircularized following the addition of *Bam*HI linkers to create Ep520. Ep520 lacks the 2.1 kb *Hin*dIII−*Sna*BI fragment that contains the entire *GCD11* open reading frame, along with 250 and 235 bp of 5′- and 3′-flanking sequences

respectively. The 3.8 kb *Bam*HI–*Bgl*II fragment from pNKY51 (Alani *et al.*, 1987), containing the yeast *URA3* gene flanked by direct repeats of the *E.coli hisG* gene, was inserted into the *Bam*HI site of Ep520 to create Ep523. Ep552 is a low copy pSB32-based plasmid that contains the *GCD11*$^+$ allele isolated from yeast strain H96.

Ep543 was created from Ep308, which harbors the *gcn3::LEU2* (*Δ1–305*) allele (Hannig and Hinnebusch, 1988), by digestion with *Bam*HI, followed by flush-ending with Klenow enzyme and recircularization to remove the unique *Bam*HI site present in this Ep308. Ep543 was digested with *Hin*dIII to remove the *LEU2* gene and the plasmid was recircularized following the addition of *Bam*HI linkers. A 3.8 kb *Bam*HI–*Bgl*II fragment from pNKY51 containing the *hisG-URA3-hisG* sequences was ligated into the new *Bam*HI site to create Ep545.

p180, p226 and p227, kindly provided by A.G.Hinnebusch, are low copy plasmids harboring *GCN4–lacZ* alleles that contain all four upstream open reading frames (uORFs), uORF4 alone or no uORFs respectively. uORFs were removed by introducing point mutations into the AUG codon of the respective reading frames (Mueller and Hinnebusch, 1986). p367 and p391 (gifts from T.F.Donahue) are low copy plasmids containing the *HIS4*^{AUG}*–lacZ* and *his4*^{UUG}*–lacZ* alleles respectively (Donahue and Cigan, 1988). p364 is a YCp50 plasmid containing the dominant *SUI3-2* allele (Donahue *et al.*, 1988).

Recovery of chromosomal alleles

Ep488 was linearized with *Sna*BI and introduced into Ura$^-$ strains harboring wild-type (H96) or mutant (EY250, EY305, EY555, EY558, H57, H59, H61 and H79) *GCD11* alleles. The *GCD11* sequences in Ep488 lack 92% of the *GCD11* open reading frame, thereby reducing the probability of gene conversion events. Integration of a single copy of linearized Ep488 at the *GCD11* locus was confirmed by Southern blot analysis of *Hin*dIII-cleaved genomic DNA purified from Ura$^+$ transformants (data not shown). An 8.7 kb genomic *Hin*dIII fragment in the transformants contains the entire *GCD11* ORF and flanking sequences, along with YIp5 vector sequences necessary for selection and propagation in *E.coli*. This 8.7 kb fragment was circularized using DNA ligase and recovered following introduction into $CaCl_2$-treated *E.coli* DH5α cells (Sambrook *et al.*, 1989). For each allele, the 2.1 kb *Hin*dIII–*Sna*BI *GCD11* fragment was sequenced using a Sequenase version 2.0 kit (US Biochemical) and synthetic oligonucleotide primers. The sequence of the region containing each mutation was verified by DNA sequence analysis of plasmids recovered from two additional independent yeast transformants.

Construction of isogenic strains by plasmid shuffling

A 3.7 kb *Hin*dIII–*Bam*HI restriction fragment was isolated from the YIp plasmids recovered from the wild-type and mutant strains above and cloned into the low copy *LEU2* plasmid pSB32 (Rose and Broach, 1991). This genomic fragment contains the entire *GCD11* open reading frame along with 0.25 and 1.6 kb of 5′- and 3′-flanking sequences respectively. The pSB32-borne alleles were introduced into the Leu$^-$ yeast strains EY582, EY606 or EY647 and Leu$^+$ prototrophs were selected. These strains also harbor *GCD11*$^+$ on the *URA3*$^+$ plasmid Ep293. The doubly-transformed strains were patched onto solid media containing 5-FOA to obtain derivatives that have lost Ep293 and therefore contain the pSB32-borne allele as the sole copy of *GCD11*. Petite derivatives of the *GCD11*$^+$ strain were isolated following growth in the presence of ethidium bromide as described (Sherman *et al.*, 1979).

β-Galactosidase assays

Cultures for β-galactosidase assays were grown in liquid SD medium supplemented appropriately to maintain selection for plasmids. A saturated overnight culture grown at 23°C was diluted into fresh medium and grown for 8 h at 30°C with aeration [repressing (R) conditions]. For derepressing (DR) conditions, 3-AT was added to a final concentration of 10 mM at 2 h and the cultures were grown for an additional 6 h. 3-AT inhibits the *HIS3* gene product and elicits starvation for histidine (Klopotowski and Wiater, 1965). Strain F35 contains a single copy of *HIS4–lacZ* integrated at *ura3-52* and was used as an internal control for the β-galactosidase assays. β-Galactosidase assays were performed as described (Hannig and Hinnebusch, 1988). Protein concentrations were determined by the dye binding method of Bradford (Bradford, 1976), using bovine serum albumin as a standard.

Analysis of steady-state levels of eIF-2γ

Protein extracts were prepared from log phase cells essentially as described (Hannig *et al.*, 1993), except that we found the inclusion of protease inhibitors to be unnecessary. Aliquots containing approximately equal amounts of total protein, as determined in preliminary experiments, were fractionated on denaturing 10% SDS–polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose paper and stained with Ponceau S to ensure equal transfer. Immunoblot analysis was performed using polyclonal rabbit antisera specific for yeast eIF-2γ as described (Hannig *et al.*, 1993). Bound antibodies were detected using [^{125}I]protein A (ICN; >30 mCi/mg) at 0.3 μCi/ml. Blots were analyzed by autoradiography and densitometric scanning of multiple lightly exposed autoradiographs and also by direct counting using a phosphorimager (Molecular Dynamics). Phosphorimages were labeled using CorelDraw version 3.0.

Analysis of polysome profiles

Cultures were grown in YEPD media at 30°C and harvested at an OD_{600} of 1.0. Cycloheximide was added to the cultures at 50 μg/ml immediately prior to harvesting and cell extracts were prepared in the presence of 50 μg/ml cycloheximide as described (Cigan *et al.*, 1991). The breaking buffer was supplemented with 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A and 1 mM phenylmethylsulfonyl fluoride. Sixteen A_{260} units of an 11 000 *g* supernatant were layered onto 11.6 ml 7–47% linear sucrose gradients and centrifuged at 39 000 r.p.m. in a Beckman SW41 rotor at 4°C for 2 h. Fractions were collected using an ISCO Model 640 gradient fractionator and monitored continuously at 254 nm.

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References

Alani,E., Cao,L. and Kleckner,N. (1987) *Genetics*, **116**, 541–545.
Anthony,D.D.,Jr, Kinzy,T.G. and Merrick,W.C. (1990) *Arch. Biochem. Biophysics*, **281**, 157–162.
Barber,G.N., Wambach,M., Wong,M.-L, Dever,T.E., Hinnebusch,A.G. and Katze,M.G. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 4621–4625.
Berchtold,H.L. Reshetnikova,C.O. Reiser,A, Schirmer,N.K., Sprinzl,M. and Hilgenfeld,R. (1993) *Nature*, **265**, 126–132.
Boeke,J.D., Trueheart,J., Natsoulis,G. and Fink,G.R. (1987) *Methods Enzymol.*, **154**, 164–175.
Bourne,H.R., Sanders,D.A. and McCormick,F. (1991) *Nature*, **349**, 117–127.
Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
Bushman,J.L., Asuru,A.I., Matts,R.L. and Hinnebusch,A.G. (1993) *Mol. Cell. Biol.*, **13**, 1920–1932.
Castilho-Valavicius,B., Yoon,H. and Donahue,T.F. (1990) *Genetics*, **124**, 483–495.
Castilho-Valavicius,B., Thompson,G.M. and Donahue,T.F. (1992) *Gene Expression*, **2**, 297–309.
Cigan,A.M., Feng,L. and. Donahue,T.F. (1988) *Science*, **242**, 93–97.
Cigan,A.M., Pabich,E.K., Feng,L. and Donahue,T.F. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 2784–2788.
Cigan,A.M., Foiani,M., Hannig,E.M. and Hinnebusch,A.G. (1991) *Mol. Cell. Biol.*, **11**, 3217–3228.
Cigan,A.M., Bushman,J.L., Boal,T.R. and Hinnebusch,A.G. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 5350–5354.
Clare,J. and Farabaugh,P. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 2829–2833.
Dever,T.E., Feng,L., Wek,R.C., Cigan,A.M., Donahue,T.F. and Hinnebusch,A.G. (1992) *Cell*, **68**, 585–596.
de Vos,A.M., Tong,L, Milburn,M.V., Matias,P.M., Jancarik,J., Noguchi,S., Nishimura,S., Miura,K., Ohtsuka,E. and Kim,S.-H. (1988) *Science*, **239**, 888–893.
Donahue,T.F. and Cigan,A.M. (1988) *Mol. Cell. Biol.*, **8**, 2955–2963.
Donahue,T.F., Cigan,A.M., Pabich,E.K. and Castillo-Valavicius,B. (1988) *Cell*, **54**, 621–632.
Duffy,L.K., Gerber,L., Johnson,A.E. and Miller,D.L. (1981) *Biochem*istry, **20**, 4663–4666.
Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13 (addendum, **137**, 266–267, 1984).
Gaspar,N.J., Kinzy,T.G., Scherer,B.J., Hümbelin,M., Hershey,J.W.B. and Merrick,W.C. (1994) *J. Biol. Chem.*, **269**, 3415–3422.

Geballe,A.P. and Morris,D.R. (1994) *Trends Biol. Sci.*, **19**, 159–164.
Gibbs,J.B., Sigal,I.S., Poe,M. and Scolnick,E.M. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 5704–5708.
Grant,C.M. and Hinnebusch,A.G. (1994) *Mol. Cell. Biol.*, **14**, 606–618.
Grant,C.M., Miller,P.F. and Hinnebusch,A.G. (1994) *Mol. Cell. Biol.*, **14**, 2616–2628.
Hannig,E.M. and Hinnebusch,A.G. (1988) *Mol. Cell. Biol.*, **8**, 4808–4820.
Hannig,E.M., Williams,N.P., Wek,R.C. and Hinnebusch,A.G. (1990) *Genetics*, **126**, 549–562.
Hannig,E.M., Cigan,A.M., Freeman,B.A. and Kinzy,T.G. (1993) *Mol. Cell. Biol.*, **13**, 506–520.
Harashima,S. and Hinnebusch,A.G. (1986) *Mol. Cell. Biol.*, **6**, 3990–3998.
Harashima,S., Hannig,E.M. and Hinnebusch,A.G. (1987) *Genetics*, **117**, 409–419.
Hill,D.E. and Struhl,K. (1988) *Nucleic Acids Res.*, **16**, 9253–9265.
Hinnebusch,A.G. (1993) *Mol. Microbiol.*, **10**, 215–223.
Hinnebusch,A.G. and Liebman,S.W. (1991) In Broach,J., Pringle,J. and Jones,E. (eds), *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis, and Energetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 627–735.
Ito,H., Fukada,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163–168.
Jacquet,E. and Parmeggiani,A. (1988) *EMBO J.*, **7**, 2861–2867.
Kinzy,T.G., Freeman,J.P., Johnson,A.E. and Merrick,W.C. (1992) *J. Biol. Chem.*, **267**, 1623–1632.
Kjeldgaard,M. and Nyborg,J. (1992) *J. Mol. Biol.*, **223**, 721–742.
Kjeldgaard,M., Nissen,P., Thirup,S. and Nyborg,J. (1993) *Structure*, **1**, 35–50.
Klopotowski,T. and Wiater,A. (1965) *Arch. Biochem. Biophys.*, **112**, 562–566.
Koromilas,A.E., Roy,S., Barber,G.N., Katze,M.G. and Sonenberg,N. (1992) *Science*, **257**, 1685–1689.
Kozak,M. (1989) *J. Cell Biol.*, **108**, 229–241.
Krupitza,G. and Thireos,G. (1990) *Mol. Cell Biol.*, **10**, 4375–4378.
Laemmli,U. (1970) *Nature*, **227**, 680–685.
Lucchini,G.A., Hinnebusch,A.G., Chen,C. and Fink,G.R. (1984) *Mol. Cell. Biol.*, **4**, 1326–1333
Metz-Boutigue,M.H., Reinholt,J., Ebel,J., Ehersmann,C. and Ehersmann,B. (1989) *FEBS Lett.*, **245**, 194–200.
Merrick,W.C. (1992) *Micobiol. Rev.*, **56**, 291–315.
Miller,P.F. and Hinnebusch,A.G. (1989) *Genes Dev.*, **3**, 1217–1225.
Mueller,P.P. and Hinnebusch,A.G. (1986) *Cell*, **45**, 201–207.
Mueller,P.P., Harashima,S. and Hinnebusch,A.G. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 2863–2867.
Paddon,C.J., Hannig,E.M. and Hinnebusch,A.G. (1989) *Genetics*, **122**, 551–559.
Pai,E.F., Kabash,W., Krengel,U., Holmes,K.C., John,J. and Wittinghofer,A. (1989) *Nature*, **341**, 209–214.
Price,N.T., Francia,G., Hall,L. and Proud,C.G. (1994) *Biochim. Biophys. Acta*, **1217**, 207–210.
Rose,M.D. and Broach,J.R. (1991) *Methods Enzymol.*, **194**, 195–230.
Rothstein,R. (1991) *Methods Enzymol.*, **194**, 281–301.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sherman,F., Fink,G.R. and Lawrance,C.W. (1979) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Siekierka,J., Manne,V. and Ochoa,S. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 352–356.
Struhl,K., Stinchcomb,D.T., Scherer,S. and Davis,R.W. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 1035–1039.
Tong,L., Milburn,M.V., de Vos,A.M. and Kim,S.-H. (1989) *Science*, **245**, 244.
Valencia,A., Kjeldgaard,M., Pai,E.F. and Sander,C. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5443–5447.
Vasquez de Aldana,C.R., Dever,T.E. and Hinnebusch,A.G. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 7215–7219.
Williams,N.P., Hinnebusch,A.G. and Donahue,T.F. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 7515–7519.
Wolfner,M., Yep,D., Messenguy,F. and Fink,G.R. (1975) *J. Mol. Biol.*, **96**, 273–290.
Yoon,H. and Donahue,T.F. (1992) *Mol. Cell. Biol.*, **12**, 248–260.

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