Overexpression of Eukaryotic Translation Elongation Factor 3 Impairs Gcn2 Protein Activation*

Jyothsna Visweswaraiah‡1,2, Su Jung Lee11, Alan G. Hinnebusch5, and Evelyn Sattlegger‡4‡3

From the ‡Institute of Natural Sciences, Massey University, Auckland 0745, New Zealand and the §Laboratory of Gene Regulation and Development, NICHD, National Institutes of Health, Bethesda, Maryland 20892

Received for publication, April 17, 2012, and in revised form, July 23, 2012 Published, JBC Papers in Press, August 10, 2012, DOI 10.1074/jbc.M112.368266

In eukaryotes, phosphorylation of translation initiation factor 2α (eIF2α) by the kinase Gcn2 (general control nonderepressible 2) is a key response to amino acid starvation. Sensing starvation requires that Gcn2 directly contacts its effector protein Gcn1, and both must contact the ribosome. We have proposed that Gcn2 is activated by uncharged tRNA bound to the ribosomal decoding (A) site, in a manner facilitated by ribosome-bound Gcn1. Protein synthesis requires cyclical association of eukaryotic elongation factors (eEFs) with the ribosome. Gcn1 and Gcn2 are large proteins, raising the question of whether translation and monitoring amino acid availability can occur on the same ribosome. Part of the ribosome-binding domain in Gcn1 has homology to one of the ribosome-binding domains in eEF3, suggesting that these proteins utilize overlapping binding sites on the ribosome and consequently cannot function simultaneously on the same ribosome. Supporting this idea, we found that eEF3 overexpression in Saccharomyces cerevisiae diminished growth on amino acid starvation medium (Gcn− phenotype) and decreased eIF2α phosphorylation, and that the growth defect associated with constitutively active Gcn2 was diminished by eEF3 overexpression. Overexpression of the eEF3 HEAT domain, or C terminus, was sufficient to confer a Gcn− phenotype, and both fragments have ribosome affinity. eEF3 overexpression did not significantly affect Gcn1-ribosome association, but it exacerbated the Gcn− phenotype of Gcn1-M7A that has reduced ribosome affinity. Together, this suggests that eEF3 blocks Gcn1 regulatory function on the ribosome. We propose that the Gcn1-Gcn2 complex only functions on ribosomes with A-site-bound uncharged tRNA, because eEF3 does not occupy these stalled complexes.

Protein synthesis requires specific soluble factors that cycle on and off the ribosome in an orderly fashion. Eukaryotic translation elongation factor 1A (eEF1A)4 delivers amino acyl tRNA (aa-tRNA) to the ribosomal acceptor site (A-site) in a codon-specific manner (1). Elongation factor 2 (eEF2) is essential for translocating the ribosome along the mRNA by one triplet codon. The delivery of aa-tRNA to the A-site is coupled with the release of deacylated tRNA (tRNA\textsuperscript{decacyl}) from the E-site (1). In yeast it has been shown that this coupled mechanism is triggered by a soluble ATPase called elongation factor 3 (eEF3) (2–4). For nonfungal eukaryotes the factor equivalent to eEF3 has not been found yet. However, the high conservation of the translational process implies that nonfungal organisms must have a factor with a function equivalent to that of eEF3. Supporting this idea, it was found that metazoan ribosomes have an intrinsic ATPase activity that is absent in yeast ribosomes (5), suggesting that in metazoans a ribosome bound factor executes the eEF3-like function rather than a soluble factor. Interestingly, in Escherichia coli the protein RbbA (ribosome-bound ATPase) was identified that exhibits ATPase activity and is tightly associated with ribosomes (6, 7) and thus may have an eEF3-like function.

eEF3 consists of several domains. The N-terminal domain contains repeats that were first found in the Huntingtin protein, eEF3, protein phosphatase 2A, and TOR and were therefore called HEAT repeats (1). HEAT repeats are predicted to be interaction surfaces for other proteins and/or nucleic acids (8), and in fact an eEF3 fragment encompassing this domain was reported to bind 18 S rRNA, and structural analyses of ribosome bound eEF3 revealed that the eEF3 HEAT repeats interact with rRNA and ribosomal proteins of the small ribosomal subunit (9, 10). Adjacent to the HEAT repeat domain is a four-helix bundle of unknown function. The eEF3 C terminus contains two ATP-binding cassettes (ABC) (1). Unique to eEF3 is the insertion of a chromodomain within the C-terminal ABC cassette II (10). The chromodomain, as well as the ABCII portion

* This work was supported in part by National Institutes of Health Intramural Research Program (to A. G. H.). This work was also supported by Marsden Fund Council Grant MAU0607 administered by the Royal Society of New Zealand, a Massey University Research Fund (to E. S.), and a Massey University Technicians Award (to E. S. for supporting this work). Both authors contributed equally to this work.

† Present address: Laboratory of Gene Regulation and Development, NICHD, National Institutes of Health, Bethesda, MD 20892.

‡ To whom correspondence should be addressed: Inst. of Natural Sciences, Massey University, P.O. Box 102 904, North Shore Mail Centre, Auckland, Albany 0745, New Zealand. Tel.: 64-9-414-0800; Ext. 9665; Fax 64-9-441-8142; E-mail: e.sattlegger@massey.ac.nz.

4 The abbreviations used are: eEF, eukaryotic translation elongation factor; aa-tRNA, amino acyl tRNA; 3AT, 3-amino-2,4-triazole; GAAC, general amino acid control; PKR, protein kinase R; SM, sulfometuron methyl; tRNA\textsuperscript{decacyl}, deacylated tRNA; ABC, ATP-binding cassette; 3AT⁎, 3AT sensitivity; CTD, C-terminal domain; TC, eEF1A-GTP-amino acyl tRNA ternary complex.
**eEF3 Impairs Gcn2 Activation**

N-terminal to this domain, contacts the small and large ribosomal subunit (10).

Constant and efficient protein synthesis is essential to life; hence a constant supply of amino acids must be maintained in the cell. For a timely response to amino acid shortages, cells need to constantly monitor amino acid availability, and studies in yeast suggested that this sensing occurs on the ribosome by the protein kinase Gcn2 (general control nonderepressible 2) and its effector protein Gcn1 (11–13). Gcn2 and Gcn1 are present in all eukaryotes, suggesting that they are universally essential for sensing and overcoming amino acid starvation. Upon sensing amino acid shortage, the Gcn2 kinase domain becomes activated, which subsequently phosphorylates the α subunit of translation initiation factor 2 (eIF2α) (14). eIF2 in its GTP-bound form is essential for initiating protein synthesis in that it delivers initiator methionyl tRNA (Met-tRNAi) bound form is essential for initiating protein synthesis in that it delivers initiator methionyl tRNA (Met-tRNAi) bound form (12). eIF2α phosphorylation evokes increased translation of specific genes containing unique upstream open reading frames in their mRNA. These genes encode for transcriptional activators, e.g., Gcn4 in yeast and ATF4 in mammals, that up-regulate the expression of many genes including those coding for key amino acid biosynthetic enzymes (14). Thus, increased Gcn4 or ATF4 protein levels lead to increased amino acid biosynthesis, thereby relieving cells from amino acid starvation or imbalance. Interestingly, starvation for any amino acid activates Gcn2 and stimulates the synthesis of all 20 amino acids; therefore the starvation transduction pathway governing Gcn2 was called general amino acid control (GAAC) (14).

There are important gaps in our knowledge about the molecular mechanisms underlying the perception of the amino acid starvation signal. Amino acid starvation leads to increased cellular levels of tRNA^{deacyl}, which is the direct signal for amino acid starvation (14, 15). Yeast studies suggest that tRNA^{deacyl} is detected by the histidyl tRNA synthetase-like domain in Gcn2, which leads to the activation of its adjacent kinase domain and to subsequent phosphorylation of its substrate, eIF2α (14). The effector protein Gcn1 is essential for Gcn2 function in vivo. A gcn1Δ strain is unable to activate Gcn2 upon amino acid starvation (Gcn− phenotype); however, Gcn2 kinase activity can be detected in the whole cell extract of a gcn1Δ strain, suggesting that Gcn1 is not required for the kinase activity per se but for transferring the starvation signal to Gcn2 (14). We and others showed that for signal perception Gcn2 must directly bind to its effector protein Gcn1 and that both proteins must associate with translating ribosomes (13, 14). The binding domains in the Gcn1 and Gcn2 proteins required for Gcn1-Gcn2 interaction are physically distinct from the ribosome-binding domains in either protein, supporting the idea that Gcn1 and Gcn2 form a trimeric complex with the ribosome and that the starvation signal is detected on the ribosome.

The Gcn1 middle portion binds the N terminus of Gcn20, a protein required for full Gcn2 activation (16). Gcn20 does not bind to ribosomes, but studies suggest that it may affect or regulate Gcn1-ribosome association and thus the GAAC system; however, the mechanism remains elusive (17). Interestingly, the Gcn1 middle portion has HEAT repeats and has homology to the N-terminal HEAT repeat domain of eEF3, and the Gcn20 C terminus has homology to the eEF3 C terminus including the ABC cassettes (17). This suggests that the Gcn1/20 complex has a similar function as eEF3, just that it is involved in delivering or releasing tRNA^{deacyl} from the ribosomal A-site instead of the E-site (17). This idea is also based on findings in prokaryotes where under amino acid starvation tRNA^{deacyl} binds in the A-site in a codon-specific manner (18, 19). Interestingly, it was shown that also in eukaryotes tRNA^{deacyl} can enter the A-site in a codon-specific manner (20), suggesting that the mechanism of the starvation signal occurring in the A-site may be conserved from prokaryotes to eukaryotes. Although in prokaryotes tRNA^{deacyl} binding to the A-site then leads to the activation of the ppGpp synthetase RelA and the stringent response (18, 19), in eukaryotes this leads to Gcn1-dependent Gcn2 stimulation and the activation of the GAAC. We have proposed a model for Gcn1-mediated Gcn2 activation in which under amino acid starvation tRNA^{deacyl} enters the ribosomal A-site in a codon-specific manner. Gcn1 is directly involved in the transfer of the starvation signal to Gcn2, by one or more of the following mechanisms: (a) delivery of tRNA^{deacyl} to the ribosomal A-site, (b) transfer of tRNA^{deacyl} from the A-site to Gcn2, and/or (c) being a scaffold protein that positions Gcn2 and potential additional factors in such a way that Gcn2 can detect the starvation signal in the A-site (12, 17). Several lines of evidence support our idea that Gcn1 can access the A-site, directly or indirectly. We found that overexpression of Gcn1 leads to hypersensitivity to paromomycin, a drug binding in the A-site. Conversely, Gcn1 deletion increased paromomycin resistance. Furthermore, internal deletions in Gcn1 affecting ribosome binding simultaneously increased paromomycin resistance, and so did deletion of Gcn20 (12).

Interestingly, we have recently discovered that eEF1A directly binds to the Gcn2 C terminus (21). Under amino acid starvation, this interaction is lost in vivo, tRNA^{deacyl} prevents eEF1A-Gcn2 interaction in vitro, and eEF1A prevents eIF2α phosphorylation in vitro but not Gcn2 autophosphorylation. Together, this strongly suggested that eEF1A prevents full Gcn2 activation under replete conditions and that under amino acid starvation uncharged tRNAs channeled to Gcn2 remove eEF1A from Gcn2 and thereby allow Gcn2 activation and subsequent eIF2α phosphorylation (21).

Structural studies of the eEF3-ribosome complex revealed that eEF3 binds close to the E-site (10); however, considering that Gcn1 is a very large protein, that the N-terminal 2052 amino acids in Gcn1 are involved in ribosome binding, and that it has homology to eEF3, it is likely that on the ribosome some of the eEF3-binding sites overlap with a subset of those for Gcn1. If this is true, then eEF3 and Gcn1 cannot function on the same ribosome. Here we present several lines of evidence supporting this prediction. We found that overexpression of eEF3 impairs Gcn1-mediated Gcn2 activation. The eEF3 HEAT domain was
sufficient for impairing Gcn1 function when overexpressed. Remarkably, we found that overexpressing the eEF3 C terminus (amino acids 910–1044) also was sufficient for impairing Gcn1 function and that this eEF3 segment also can bind to elongating ribosomes in vivo. Interestingly, eEF3 overexpression did not significantly affect Gcn1-ribosome or Gcn2-ribosome association; however, eEF3 overexpression exacerbated the Gcn1 phenotype associated with Gcn1-M7A, a Gcn1 mutant protein with reduced ribosome affinity. Together this supports the idea that eEF3 overexpression does not simply remove Gcn1 from the ribosome but instead affects Gcn1 function on the ribosome. We propose that eEF3 blocks a productive Gcn1-ribosome association, supporting the idea that Gcn1-Gcn2 detects the amino acid starvation signal on stalled elongating ribosomes lacking eEF3. Under replete conditions eEF3 may work in concert with eEF1A to prevent Gcn2 activation.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—Yeast strains and plasmids used in this study are summarized in Tables 1 and 2, respectively. The vectors used were pRS316 (22), pEG(KT) (22), and pES128-9 (12).

**Semiquantitative Growth Assays**—Yeast strains were grown to saturation in 4 ml of liquid medium, subjected to serial 10-fold dilutions, and then 5 μl of each dilution was transferred to solid medium containing substances to cover auxotrophies. If necessary, galactose was used as sole carbon source instead of glucose to induce the expression of genes under the galactose-inducible promoter. Amino acid analogues 3-amino-2,4-tria-}

---

### TABLE 1

**Strains used in this study**

| Strain | Genetic background | Genotype | Source |
|--------|-------------------|----------|--------|
| H1511  | H1515             | MATα ura3-52 trp1-63 leu2-3,112 GAL2+ | Ref. 36 |
| H2556  |                   | As H1511, and gen1Δ | Vazquez de Aldana and Hinnebusch |
| H1402  |                   | MATα insl ura3-52 leu2-3,112 (HIS4-lacZ ura3-52) | Ref. 37 |
| H1613  |                   | As H1402, and GCN2–E601K-E1591K | Ref. 38 |
| H1515  |                   | MATα ura3-52 leu2-3 leu2-112 trp1-Δ63 | Cigan and Hinnebusch |
| H2544  |                   | As H1515, and (GAL-CYC1-PKR LEU2) at leu2 | Romano and Hinnebusch |
| H1894  |                   | As H1515, and gen2Δ | Ref. 39 |

### TABLE 2

**Plasmids used in this study**

| Plasmid | Gene | Selectable marker (all Ampb) | Vector | Source |
|---------|------|------------------------------|--------|--------|
| pGST-YEF3 | GST-YEF3a,b | LEU2, CEN6 | pEG(KT) | Ref. 40 |
| pTKB705 | GST-YEF3a | LEU2, 2 μ | pTKB544 | Ref. 27 |
| pTKB706 | GST-YEF3[1–775]a,b,c | LEU2, 2 μ | pTKB544 | Ref. 27 |
| pTKB707 | GST-YEF3[100–367]a,b,c | LEU2, 2 μ | pTKB544 | Ref. 27 |
| pTKB708 | GST-YEF3[775–910]a,b,c | LEU2, 2 μ | pTKB544 | Ref. 27 |
| pTKB709 | GST-YEF3[910–1044]a,b,c | LEU2, 2 μ | pTKB544 | Ref. 27 |
| pTKB710 | GST-YEF3[775–1044]a,b,c | LEU2, 2 μ | pTKB544 | Ref. 27 |
| pES340–EF3–2–8 | GST–YEF3a | LEU2, 2 μ | pES128–9 | This study |
| p2245 | HA–E3L | LEU2, 2 μ | pEG(KT) | Ref. 28 |

**Yeast genes, under their own promoter**

| Plasmid | Gene | Selectable marker (all Ampb) | Vector | Source |
|---------|------|------------------------------|--------|--------|
| p1852 | GCN1-qt | LEU2, CEN6/ARSH4 | pRS16 | Ref. 16 |
| pES239–4–4a | gcn1–M7A–qt | LEU2, CEN6/ARSH4 | pRS16 | This study |

---

a This construct lacks the first 10 N-terminal amino acids of eEF3.
b Epitope tag at the N terminus of the ORF.
c Epitope tag at the C terminus of the ORF.
min, and finally the protein pellets were resuspended in 2× Laemmli loading dye.

Assaying for eIF2α Phosphorylation—Exponentially growing cells were subjected to amino acid starvation by adding either 3AT or SM and then subjected to formaldehyde cross-linking as done for polyribosome co-sedimentation assays. Whole cell extracts were generated as published elsewhere (23), and equal amounts of cell extract (5 μg) were subjected to SDS-PAGE and immunoblotting as described below.

Protein Techniques—Proteins were separated by SDS-PAGE using 4–12% gradient gels and transferred to PVDF membranes (Millipore) according to the manufacturer’s protocol. Proteins were detected by the chemiluminescence detection system (Pierce) using antibodies against Gcn1 (HL1405, 1:1000 (16)), Gcn2 (1:1000 (21)), eIF2α (1:2000 (24)), eIF2α phosphorylated on Ser-51 (1:5000; Bio-Source International, Inc.), Pgk1 (1:5000; Life Technologies), RPL39 (1:2500 (25)), RPS22 (1:2000; from Dr. Jan van’t Riet), or GST (1:2000; Santa Cruz). Immune complexes were visualized using horseradish peroxidase conjugated to donkey anti-rabbit antibodies (for the detection of Gcn1, GST, eIF2α, eIF2α-P, and RPS22 antibodies), goat anti-guinea pig (Gcn2) (Santa Cruz), or to sheep anti-mouse antibodies (Pgk1 and RPL39) (Pierce).

RESULTS

eEF3 Overexpression Elicits Sensitivity to 3-Amino-2,4-triazole (3AT)—If Gcn1 binds to the same or overlapping regions of the ribosome as eEF3, then eEF3 overexpression should affect Gcn1 function and consequently Gcn2 activation. Strains with impaired Gcn2 activity can easily be scored by their inability to grow on medium containing 3AT, a drug causing histidine starvation by inhibiting the histidine biosynthetic enzyme encoded by HIS3 (26). To test our prediction that eEF3 affects Gcn1 function and thus impairs Gcn2 activation, we introduced into the yeast wild-type strain H1511 a plasmid expressing GST-tagged eEF3 from a galactose-inducible promoter or a plasmid harboring GST alone. The resulting transformants were subjected to semiquantitative growth assays using solid medium containing galactose as carbon source, and 120 mM 3AT if indicated, and incubated at 30 °C for 3–5 days.

E3 did reduce the Slg+ phenotype to a slow growth (Slg−) phenotype (14). Gcn2+ still requires Gcn1 for its activity, presumably because it still needs to receive tRNA deacylated to become constitutively active. If overexpressed eEF3 affects Gcn1 function, then it should simultaneously diminish the constitutive kinase activity of Gcn2+, visible by a reversion of the Slg− phenotype conferred by Gcn2-. To test this, we introduced a plasmid expressing GST-tagged eEF3 under a galactose-inducible promoter or a plasmid harboring GST alone into isogenic strains harboring either the Gcn2+ allele or the Gcn2- wild-type gene on its chromosome, and the resulting transformants were subjected to semiquantitative growth assays. As expected, on medium containing glucose, i.e., no eEF3 overexpression, the strain harboring the Gcn2+ allele showed reduced growth compared with the wild-type control (Fig. 2A, left panel, rows 1–4 versus rows 5–8). On galactose, however, the Gcn2+ strain overexpressing eEF3 showed improved growth as compared with the Gcn2- strain overexpressing GST alone (Fig. 2A, rows 7 and 8 versus rows 5 and 6), supporting the idea that GAAC response is impaired by eEF3 overexpression.

Similarly to Gcn2+, expression of the mammalian double-stranded RNA-dependent eIF2α kinase PKR in yeast leads to a severe growth defect caused by high levels of eIF2α phosphorylation (28). In contrast to Gcn2, PKR does not require Gcn1 for function. If eEF3 impairs GAAC function by inhibiting Gcn1, and not by, for example, stimulating an eIF2α-P phosphatase or preventing eIF2α kinase domains from accessing their substrate, then, in contrast to Gcn2-, the Slg− phenotype elicited by PKR should not be diminished by eEF3 overexpression. To test this, we repeated the semiquantitative growth assay using a gcn2Δ strain overexpressing PKR from a chromosomally integrated PKR construct driven by a galactose-inducible promoter and also harboring a high copy plasmid containing a galactose-inducible gene coding for GST-eEF3, GST alone, or the PKR inhibitor from vaccinia virus, E3 (28). We found that, in contrast to E3, GST–eEF3 overexpression did not mitigate the growth defect elicited by PKR (Fig. 2B, galactose, rows 1 and 2 versus rows 3 and 4 versus rows 5 and 6). The fact that E3 did reduce the Slg− phenotype elicited by PKR indicated that a PKR inhibitor can successfully inhibit PKR activity even when its expression was induced at the same time as that of PKR. We verified that eEF3 overexpression inhibits GAAC in this strain background (Fig. 2B, galactose, SM, row 8 versus row 9).
for histidine by adding 3AT and then were cross-linked with formaldehyde to prevent phosphorylation or de-phosphorylation events that may occur during the harvesting or processing of samples. Whole cell extracts were subjected to SDS-PAGE and immunoblotting analysis to determine the relative amount of eIF2α phosphorylation (Fig. 3). As expected, amino acid starvation led to increased eIF2α-P levels (Fig. 3, A, lanes 3 and 7 versus lanes 4 and 8, and B); however, when eEF3 was overexpressed in starved cells, the amount of eIF2α-P was reduced by ~50% (Fig. 3, A, lanes 2 and 6 versus lanes 4 and 8, and B). Interestingly, we found that even under nutrient-replete conditions, eEF3 overexpression reduced eIF2α-P levels, by a factor of 4.5, to 22% of that of the wild-type control overexpressing GST alone (Fig. 3, C and D).

Next we repeated the above experiment but instead of 3AT we used SM, a drug causing starvation for branched chain amino acids by inhibiting acetylactate synthase, the first common enzyme in the branched chain amino acid biosynthetic pathway (29). As expected, the addition of SM led to increased eIF2α-P levels (Fig. 3, E, lane 4 versus lanes 5 and 6, and F). As found above, under starvation conditions eEF3 overexpression significantly reduced the amount of eIF2α-P levels by a factor of about 5 (Fig. 3, E, lanes 2 and 3 versus lanes 5 and 6, and F). We again found that eEF3 overexpression reduced eIF2α-P levels even under replete conditions, by a factor of 6.7, to 15% of that of the wild-type control overexpressing GST alone (Fig. 3, E, lane 1 versus lane 4, and F). Taken together, our results strongly suggest that eEF3 overexpression inhibits Gcn2 activation in response to amino acid limitation but also under replete conditions. Thus, overexpressed eEF3 impairs the GAAC pathway (Gcn− phenotype, general control nonderepressible).

**eEF3 Overexpression Does Not Simply Remove Gcn1 from the Ribosome**—The above results support the idea that the Gcn− phenotype associated with eEF3 overexpression is due to eEF3 affecting Gcn1-ribosome interaction. This may be facilitated by eEF3 removing Gcn1 from the ribosome. Alternatively, considering that three-quarters of the 2672-amino acid-long Gcn1 is involved in ribosome binding, eEF3 may not remove Gcn1 from the ribosome but instead may affect only a subset of the Gcn1 interacting with eEF3 (Fig. 4, A and B). We did verify that the strains overexpressed eEF3 (data not shown). Considering the fact that on one hand eEF3 overexpression lead to a drastic—at least 4.5-fold—reduction of Gcn2 function in phosphorylating eIF2α (Fig. 3, C and D), but on the other hand eEF3 overexpression barely affected Gcn1-ribosome or Gcn2-ribosome interaction, this suggests that inhibition of Gcn2 function is not simply
due to eEF3 removing Gcn1 from the ribosome. Taken together, this supports the idea that eEF3 may only affect a few of the many ribosome contact points in Gcn1 and that Gcn1 is still able to bind to the ribosome via the remaining unaffected ribosome contact points, but that the affected sites are crucial for Gcn1 function in Gcn2 activation.

Overexpression of the eEF3 HEAT Domain, or the eEF3 C-terminal Domain (CTD), Is Sufficient for Impairing Gcn2 Activation—If eEF3 overexpression impairs Gcn1 function on the ribosome, then the ribosome binding activity of eEF3 must be essential for affecting Gcn1 function in activating Gcn2. To test this prediction, we aimed to map the domain in eEF3 responsible for affecting Gcn1 function and to compare it with the ribosome-binding domains in eEF3. For eEF3, several ribosome-binding sites have been mapped so far (9, 10) (Fig. 5A).

One binding site is the chromodomain that is situated within the ABC cassette II in the eEF3 C terminus. The part of ABC cassette II N-terminal to the chromodomain also contacts the ribosome. Finally, the HEAT domain in the eEF3 N terminus has ribosome binding activities. To map the domain in eEF3 responsible for affecting Gcn1 function, various eEF3 fragments (Fig. 5A), and full-length eEF3 as control, fused to GST, were overexpressed in wild-type yeast cells, and the cells were scored for 3ATs as described above (Fig. 5B). In addition, we determined whether the eEF3 fragments were overexpressed as well as full-length GST-eEF3 (Fig. 5C). As reported above, in contrast to GST alone, overexpression of full-length eEF3 elicited a growth defect in presence of 3AT (Fig. 5B, compare second and third rows). We found that the eEF3 fragment encompassing amino acids 1–775, GST-eEF3[1–775], containing the HEAT domain, the ABC cassette I, and part of the ABC cassette II, conferred 3AT°. This phenotype was not as strong as that found for full-length eEF3, possibly because GST-eEF3[1–775] was not overexpressed as well as GST-eEF3 (Fig. 5C). Interestingly, a smaller fragment only harboring the HEAT domain, in GST-eEF3[100–367], conferred the same degree of 3AT° as
GST-eEF3[1–775], suggesting that the HEAT domain was sufficient to elicit 3AT\(^{-}\). A fragment harboring only the chromodomain, in GST-eEF3[775–910], did not elicit 3AT sensitivity. This is most likely not due to its expression levels, because GST-eEF3[100–367] was expressed almost as well as GST-eEF3[775–910] and was able to cause 3AT\(^{-}\), suggesting that overexpressing the eEF3 CTD is not sufficient to impair Gcn1 function. Remarkably, we found that overexpression of the eEF3 CTD encompassing amino acids 910–1044 was sufficient for causing a 3AT\(^{-}\) phenotype (Fig. 5B). Because the expression levels of GST-eEF3[100–367] and GST-eEF3[910–1044] were similar and both fragments elicited 3AT\(^{-}\) phenotypes of similar strength, this suggests that the HEAT domain and the CTD are equally important for affecting Gcn1 function. A fragment containing the CTD and the chromodomain, in GST-eEF3[775–1040], caused an even stronger phenotype than the CTD alone; in GST-eEF3[910–1040], however, we cannot exclude the possibility that this is due to its 2.5-fold higher expression level as compared with GST-eEF3[910–1040] (Fig. 5C).

The eEF3 C-terminal amino acids 775–1044, encompassing the CTD and part of the chromodomain, were reported previously to have ribosome binding activity in vitro, and it was proposed that the four distinct blocks of Arg and Lys residues found between amino acids 987 and 1044 mediate ribosome binding (31). In the eEF3-ribosome co-crystal structure and cryoEM studies, the chromodomain was found to contact the ribosome; however, no data for the C terminus were available because the eEF3 used in this study lacked the C-terminal charged amino acids (amino acids 981–1044) (10). Therefore, we next aimed to investigate whether the eEF3 CTD has ribosome binding activity by conducting ribosome co-sedimentation assays as described above. As expected, we found that GST-eEF3[775–1044] harboring some of the ribosome contact points co-sedimented with polyribosomes but GST alone did not, suggesting that it binds ribosomes in vivo as reported previously under in vitro conditions (31) (Fig. 6, top panel versus bottom panel). Interestingly, the eEF3 CTD encompassing only amino acids 910–1044 co-sedimented with polyribosomes as well, suggesting that this fragment does contain ribosome binding activity (Fig. 6, middle panel).
eEF3 Impairs Gcn2 Activation

Taken together, our results suggest that the eEF3 HEAT domain and eEF3-CTD, respectively, are sufficient for impairing Gcn2 function when overexpressed. Because both proteins have ribosome affinity, this supports the idea that Gcn1 function is impaired on the ribosome, thereby preventing Gcn2 activation.

The Gcn1 M7A Mutation Exacerbates the Gcn− Phenotype Associated with eEF3 Overexpression—If eEF3 affects only a subset of the many ribosome contact points in Gcn1, then eEF3 should be more efficient in inhibiting a Gcn1 protein that has impaired ribosome binding activity. The Gcn1-M7A mutant protein is known to have reduced ribosome affinity, and this is associated with a reduced ability to activate Gcn2 visible by a slight 3AT+ phenotype and reduced eIF2α phosphorylation (13). If eEF3 overexpression affects a subset of ribosome contact points in Gcn1, then eEF3 overexpression should exacerbate the 3AT+ phenotype of Gcn1-M7A. To test this, we generated cells that expressed plasmid borne Gcn1 or gcn1-M7A from its own promoter and over-expressed from a second plasmid GST alone or GST-eEF3 from a galactose-inducible promoter. The resulting transformants were then subjected to semiquantitative growth assays. As published previously, the gcn1-M7A strain is somewhat more sensitive to 3AT than the Gcn1+ strain (Fig. 7A, rows 3 and 4 versus rows 7 and 8). As reported above, overexpression of GST-eEF3 in the Gcn1+ strain elicited a 3AT+ phenotype (Fig. 7A, rows 5 and 6 versus rows 7 and 8). However, we found that in a gcn1-M7A strain, eEF3 overexpression elicited a 3AT+ that was much stronger than in a Gcn1+ strain (Fig. 7A, rows 1 and 2 versus rows 3 and 4); in particular eEF3 overexpression led to a more than 10-fold reduction in growth as compared with overexpression of GST alone (Fig. 7A, 2 mM 3AT, rows 1 and 2 versus rows 3 and 4).

Considering that the combined effect of the M7A mutation and eEF3 overexpression on cell growth in the presence of 3AT was clearly stronger than that of M7A or eEF3 overexpression alone, this indicates that the M7A mutation exacerbated the 3AT+ phenotype associated with eEF3 overexpression, strongly supporting the idea that eEF3 overexpression impairs Gcn1 function on the ribosome.

To verify that the exacerbation of the 3AT+ phenotype was due to Gcn2 inhibition, we scored the levels of eIF2α phosphorylation. As shown above, eEF3 overexpression in a Gcn1+ strain reduced the amount of eIF2α phosphorylation under replete as well as starved conditions (Fig. 7, B and C, lanes 5 and 6 versus lanes 7 and 8; D and E), and as reported previously the M7A mutation reduced the levels of eIF2α phosphorylation (Fig. 7, B and C, lanes 3 and 4 versus lanes 7 and 8; D, and E) (13). Under starvation conditions, eEF3 overexpression in the
GCN1+ strain reduced eIF2α phosphorylation 2.05-fold; however, in a gcn1-M7A strain, eIF2α phosphorylation was reduced even further, 2.58-fold as compared with the gcn1-M7A strain overexpressing GST alone (Fig. 7E), and this difference was even more pronounced under replete conditions (Fig. 7D). Together this supports the idea that eEF3 overexpression exacerbated the Gcn− phenotype of the Gcn1 M7A mutation. Considering that the M7A mutation affects Gcn1-ribosome association but no other known Gcn1 function, such as Gcn20 binding and Gcn2 binding, this strongly supports our idea that eEF3 overexpression affects Gcn1 function on the ribosome.

**DISCUSSION**

Continuous protein synthesis is essential to life, and so is a constant supply of amino acids. Eukaryotes possess the kinase Gcn2, which enables them to monitor amino acid availability and to cope with and overcome amino acid starvation. In our current working model, the starvation signal is uncharged tRNAs binding in the ribosomal A-site in a codon-specific manner (12, 17). The proteins Gcn1 and Gcn2 bind to ribosomes, and Gcn1 is involved in transfer of the starvation signal to the kinase Gcn2, which then becomes activated (14). Gcn20 binds to Gcn1 and is not essential for Gcn2 activation, and it was proposed that it may be required to regulate Gcn1-ribosome association under yet unknown conditions (16, 17). Gcn1 and Gcn2 are large proteins, and thus far it is unknown whether these proteins can function productively on ribosomes in all stages of the elongation cycle. Because eEF3 has homology to Gcn1, this question was of particular interest for elongating ribosomes that harbor eEF3.

In this study we show several lines of evidence supporting the idea that Gcn1 cannot function properly when eEF3 is overexpressed in the cell. eEF3 overexpression in an otherwise wild-type strain disabled growth on starvation medium (Gcn− phenotype), and this correlated with reduced phosphorylation of eIF2α, the substrate of Gcn2. Furthermore, eEF3 overexpression diminished the growth defect associated with constitutively active Gcn2 that still requires Gcn1 for function but did not suppress the growth defect associated with PKR expression. Interestingly, eEF3 overexpression also reduced basal eIF2α phosphorylation levels. In line with our model, this would suggest that a low amount of uncharged tRNAs occurs in the A-site even under replete conditions, leading to a basal eIF2α phosphorylation level and thus basal levels of amino acid biosynthesis. This observation coincides with the fact that Gcn2′ proteins still require Gcn1 for their hyperactive kinase function in nutrient-replete cells (14). Under replete conditions Gcn1 would transfer the rarely occurring uncharged tRNA to Gcn2′, which then becomes turned on inappropriately.

The ribosome-binding region in Gcn1 includes the middle portion of Gcn1 that has HEAT repeats, and these HEAT repeats have homology to the eEF3 HEAT repeat domain that also has ribosome binding activity. Thus, it was possible that these HEAT repeats utilize the same or overlapping binding sites on the ribosome and that eEF3 and Gcn1 compete for ribosome association. Supporting this idea, we found that overexpression of the eEF3 HEAT domain was sufficient for impairing GAAC. Remarkably, we found that overexpression of the eEF3 CTD, encompassing amino acids 910–1044, was also sufficient for impairing GAAC, and we demonstrated that this fragment can co-sediment with polysomes in vivo. Indeed, the eEF3 C terminus is Lys-rich, and it has been predicted previously that these amino acids may be involved in ribosome binding (31). Because eEF3 is not known to dimerize, the interaction of the eEF3 C terminus with the ribosome most likely is not mediated by endogenous eEF3. Thus, we propose that binding of the eEF3 HEAT domain and CTD to the ribosome are involved in its ability to impair Gcn1-activated Gcn2 activation by tRNAdeacyl. The eEF3 HEAT domain or the eEF3 CTD were not overexpressed as strongly as full-length eEF3, and the eEF3 fragments did not impair the GAAC as strongly as full-length eEF3; therefore we could not determine whether the HEAT domain and CTD work in concert to inhibit GAAC or whether their function in inhibiting GAAC is redundant.

Gcn1-ribosome and Gcn2-ribosome interactions were not significantly affected by eEF3 overexpression, suggesting that on the ribosome the binding sites for Gcn1/Gcn2 and eEF3 are not identical. Indeed, the ribosome binding area in Gcn1 is very large, 2052 amino acids, suggesting that it has multiple ribosome-binding sites (13). Thus, eEF3 overexpression might compete with only one or a few ribosome interaction site(s) in Gcn1 and thereby impair Gcn1 function, whereas other ribosome-binding sites in Gcn1 would still support efficient Gcn1-ribosome interaction. The ribosome contact competed by eEF3 might be required for the ability of the Gcn1/20-Gcn2 to properly sense uncharged tRNAs in the A-site.

We have obtained evidence supporting the idea that eEF3 does in fact affect Gcn1 function on the ribosome and not via an indirect mechanism. The M7A substitution in Gcn1—alanine replacements of 12 basic amino acids located in a predicted amphipathic α helix (between amino acids 754 and 796)—is known to weaken Gcn1-ribosome association by 25% but not to affect the association with other known Gcn1 binding partners such as Gcn2 or Gcn20 (13). The weakened ribosome binding affinity of Gcn1-M7A is correlated with a weak Gcn− phenotype and a modest reduction in eIF2α phosphorylation, indicative of its reduced ability to activate Gcn2 (13). We found that eEF3 overexpression exacerbated the Gcn− phenotype of Gcn1-M7A and further reduced the eIF2α phosphorylation levels of a gcn1-M7A strain, supporting the idea that eEF3 inhibits Gcn1 function on the ribosome. It is known that overexpression of a protein may lead to unspecific effects; however, the facts that not all eEF3 fragments impaired Gcn2 function when overexpressed, and that eEF3 overexpression exacerbated the Gcn− phenotype associated with point mutations in gcn1-M7A that specifically affect Gcn1-ribosome association, support the idea that the effect of eEF3 overexpression on Gcn2 activation is specific.

If eEF3 association with translating ribosomes interferes with the ability of the Gcn1/20 complex to activate Gcn2, how then can Gcn2 be activated on elongating ribosomes? One possibility is that eEF3 and Gcn1 bind to different conformational states of the ribosome, allowing Gcn1/20-Gcn2 to access the conformational state that lacks eEF3. An attractive model would be that eEF3 does not bind with high affinity to an elongating ribosome containing tRNAdeacyl in the A-site, which is the relevant sub-
strate for the Gcn1/20-Gcn2 complex. In fact, there is evidence that eEF3 interacts with eEF1A to help catalyze delivery of the eEF1A-GTP-amino acyl tRNA ternary complex (TC) to the ribosomal A-site (1, 27). Thus, eEF3 and Gcn1/20-Gcn2 would not normally occupy the same ribosomal complexes, whereas overexpressing eEF3 would force it to bind to the stalled complexes containing tRNA\textsuperscript{deacyl} in the A-site, by mass action, and thereby interfere with Gcn2 activation. In this view, the productive TC-eEF3 interaction at the A-site, in addition to the scarcity of tRNAs\textsuperscript{deacyl} entering the A-site, plays an important role in blocking activation of Gcn2 in nutrient-replete conditions.

Interestingly, we have just recently found several lines of evidence strongly suggesting that eEF1A inhibits Gcn2 activation under amino acid replete conditions (21). eEF1A directly binds to the Gcn2 C terminus, and in vivo the eEF1A-Gcn2 interaction is lost when cells are starved for amino acids. In vitro, eEF1A inhibits Gcn2-mediated eIF2α phosphorylation but not Gcn2 autophosphorylation. Considering that eEF3 interacts with eEF1A to help catalyze delivery of the TC to the A-site, it is tempting to speculate that eEF1A and eEF3 work in concert to ensure that Gcn2 stays in its latent state under amino acid replete conditions.

Considering that Gcn1 and Gcn2 can directly interact with each other (12), it is likely that Gcn1 and Gcn2 form a permanent complex that shuttles on and off the ribosome. In view of the fact that ribosomes are 15 or 350 times more abundant in the cell than Gcn1 or Gcn2 (32), stable Gcn1-Gcn2 association would be necessary to ensure that Gcn1 and Gcn2 reside on the same ribosome at any given time to allow Gcn1-mediated transfer of tRNA\textsuperscript{deacyl} to Gcn2.

We showed previously that Gcn1 overexpression leads to increased amounts of Gcn1 binding to polyribosomes, and this is associated with reduced cell growth, which could be due to interference with eEF3 or eEF1A function (12). Thus, the cellular Gcn1 levels are probably low to minimize its possible interference with elongation (12). Our data led to a refined model for sensing amino acid starvation as shown in Fig. 8. When during each round of the elongation cycle the A-site is available for accommodating the next tRNA in a codon-specific manner, either under replete conditions aa-tRNA is delivered by eEF1A (Fig. 8A, arrow 1), or under starvation conditions tRNA\textsuperscript{deacyl} enters the A-site (Fig. 8B, arrow 5). If aa-tRNA is delivered, then eEF3 triggers the con-
comitant release of tRNA\textsuperscript{deacyl} from the E-site (Fig. 8B, arrow 2). Subsequently translation elongation can proceed to the next steps involving peptide bond formation and translocation (Fig. 8A, arrows 3 and 4). In such productive elongation complexes, the HEAT domain and C terminus of eEF3 would prevent productive Gcn1-ribosome interactions and thereby prevent Gcn2 from detecting tRNAs\textsuperscript{deacyl}, e.g., tRNAs\textsuperscript{deacyl} leaving the E-site. Furthermore, eEF1A binding to the Gcn2 C terminus would prevent Gcn2 from substrate phosphorylation (21). Because eEF1A is abundant in the cell, it remains to be determined whether this inhibition is mediated by the eEF1A delivering aa-tRNA to the A-site or by another eEF1A molecule. For simplicity, the nonfunctional Gcn1/20-Gcn2 complex was drawn separately from the ribosome and associated with an eEF1A molecule not involved in the translation elongation cycle (Fig. 8A, bottom part). As suggested above, eEF1A and eEF3 might cooperate to maintain Gcn2 latency under amino acid replete conditions. Although eEF3 would prevent Gcn1-mediated delivery of tRNAs\textsuperscript{deacyl}, for example the ones naturally occurring during the translation elongation cycle, to Gcn2, eEF1A would ensure that Gcn2 does not phosphorylate its substrate. If under amino acid starvation tRNA\textsuperscript{deacyl} enters the A-site (Fig. 8B, arrow 5), and in line with the model predicted for the \textit{E. coli} stringent response, the E-site tRNA\textsuperscript{deacyl} does not leave the ribosome (33). The affinity of eEF3 for these stalled complexes would be lower, possibly because of the absence of eEF1A at the A-site, allowing Gcn1/20 to interact with the A-site, directly or indirectly, and mediate the transfer of tRNA\textsuperscript{deacyl} to Gcn2 for kinase activation (Fig. 8B, arrow 6). This tRNA\textsuperscript{deacyl} leads to eEF1A dissociating from Gcn2 and consequently allowing Gcn2-mediated eIF2\alpha phosphorylation. It is possible that apart from triggering the GAAC response, the Gcn1/20-Gcn2 complex may be necessary to vacate the A-site to prevent premature peptide chain termination triggered by A-site bound tRNA\textsuperscript{deacyl} (34, 35) and to enable the "post-starved" ribosome to enter again the translation elongation cycle (Fig. 8B, arrow 7). It still remains to be determined how tRNA\textsuperscript{deacyl} enters the A-site, via diffusion or via delivery by another protein; however, the model can accommodate both mechanisms.

Under replete conditions, if in a rare event tRNA\textsuperscript{deacyl} enters the A-site, one could envision that eEF3 makes way for the formation of a productive Gcn1-ribosome interaction, which then mediates the removal of tRNA\textsuperscript{deacyl} from the A-site and its delivery to Gcn2. At the same time eEF1A-mediated Gcn2 inhibition would cease and thereby allow eIF2\alpha phosphorylation by Gcn2, accounting for the basal eIF2\alpha levels in unstarved yeast cells. eEF3 is a fungal specific elongation factor, and it has been proposed that in nonfungal eukaryotes a factor permanently attached to the ribosome executes the function of eEF3 (5). We believe that our model may also be applicable to nonfungal eukaryotes. Gcn1 would be able to attach to a ribosome that harbors an integral eEF3-like protein. Conformational changes in the ribosome or of the integral eEF3-like protein then may allow or prevent Gcn1 function in transferring the starvation signal to Gcn2. Further studies will be necessary to test this model in nonfungal eukaryotes. Such studies may be of high interest in regards to finding specific anti-fungal agents.

We cannot exclude the possibility that eEF3 overexpression prevents Gcn2 function by a mechanism different to that proposed above. For example eEF3 could prevent the Gcn2 kinase domain from accessing and phosphorylating eIF2\alpha, or eEF3 could promote eIF2\alpha dephosphorylation by activating a phosphatase. However, these scenarios may be less likely considering that overexpression of eEF3 did not mitigate the toxicity of overexpressing the eIF2\alpha kinase PKR when expressed in yeast instead of Gcn2. Taken together, our data provide strong evidence that eEF3 negatively regulates Gcn2 activation in a manner requiring the HEAT domain and/or requiring an accessory ribosome-binding domain at the eEF3 C terminus that is dispensable for eEF3 essential function in translation elongation (1, 27). These findings provide added support for the model that Gcn2 is activated by uncharged tRNAs on elongating ribosomes and reveal an unanticipated role for a general elongation factor, in addition to eEF1A, in controlling this process. Mapping the binding sites for Gcn1 and the eEF3 C terminus on the ribosome will be necessary to provide a full understanding of how eEF3 blocks Gcn2 activation. Together with our recent findings that eEF1A inhibits eIF2\alpha phosphorylation by Gcn2, we propose that eEF3 works in concert with eEF1A to keep Gcn2 in its latent state when amino acids are plentiful.

Acknowledgments—We are grateful to Tracey Waller for technical support; Kalpana Chakraburtty and Terri Kinzy for plasmids; Thomas Dever, Maurice Swanson, and Jan van’t Riet for antibodies; Kalpana Chakraburtty for helpful comments during the start-up of this project; and Terri Kinzy for helpful discussions.

REFERENCES

1. Taylor, D. R., Frank, I., and Kinzy, T. G. (ed) (2006) \textit{Structure and Function of the Eukaryotic Ribosome and Elongation Factors}, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

2. Dasmahapatra, B., and Chakraburtty, K. (1981) Protein synthesis in yeast. I. Purification and properties of elongation factor 3 from \textit{Saccharomyces cerevisiae}. \textit{J. Biol. Chem.} 256, 9999–10004

3. Skogerson, L., and Wakatama, E. (1976) A ribosome-dependent GTPase from yeast distinct from elongation factor 2. \textit{Proc. Natl. Acad. Sci. U.S.A.} 73, 73–76

4. Triana-Alonso, F. J., Chakraburtty, K., and Nierhaus, K. H. (1995) The elongation factor 3 unique in higher fungi and essential for protein biosynthesis is an E site factor. \textit{J. Biol. Chem.} 270, 20473–20478

5. El'skaya, A. V., Ovcharenko, G. V., Palchevski, S. S., Petrushenko, Z. M., Triana-Alonso, F. J., and Nierhaus, K. H. (1997) Three tRNA binding sites in rabbit liver ribosomes and role of the intrinsic ATPase in 80S ribosomes from higher eukaryotes. \textit{Biochemistry} 36, 10492–10497

6. Kiel, M. C., Aoki, H., and Ganoza, M. C. (1999) Identification of a ribosomal ATPase in \textit{Escherichia coli} cells. \textit{Biochimie} 81, 1097–1108

7. Kiel, M. C., and Ganoza, M. C. (2001) Functional interactions of an \textit{Escherichia coli} ribosomal ATPase. \textit{Eur. J. Biochem.} 268, 278–286

8. Andrade, M. A., Petosa, C., O'Donoghue, S. I., and Prescott, C. D. (1998) The N terminus of eukaryotic translation elongation factor 3 interacts with 18S rRNA and 80S ribosomes. \textit{J. Biol. Chem.} 273, 10249–10252

9. Andersen, C. B., Becker, T., Blau, M., Andan, M., Mielke, T., Mielke, T., Boesen, T., Pedersen, J. S., Spahn, C. M., Kinzy, T. G., Andersen, G. R., and Beckmann, R. (2004) Structure of eEF3 and the mechanism of transfer RNA release from the E-site. \textit{Nature} 443, 663–668

10. Marton, M. J., Crouch, D., and Hinebusch, A. G. (1993) GCN1, a trans-
eEF3 Impairs Gcn2 Activation

...ational activator of GCN4 in Saccharomyces cerevisiae, is required for phosphorylation of eukaryotic translation initiation factor 2 by protein kinase Gcn2. Mol. Cell. Biol. 13, 3541–3556
12. Sattlegger, E., and Hinnebusch, A. G. (2000) Separate domains in GCN1 for binding protein kinase Gcn2 and ribosomes are required for Gcn2 activation in amino acid-starved cells. EMBO J. 19, 6622–6633
13. Sattlegger, E., and Hinnebusch, A. G. (2005) Polyribosome binding by GCN1 is required for full activation of eukaryotic translation initiation factor 2a kinase Gcn2 during amino acid starvation. J. Biol. Chem. 280, 16514–16521
14. Hinnebusch, A. G. (2005) Translational regulation of GCN4 and the general amino acid control of yeast. Annu. Rev. Microbiol. 59, 407–450
15. Zaborske, J. M., Wu, X., Wek, R. C., and Pan, T. (2010) Selective control of amino acid metabolism by the Gcn2 eIF2 kinase pathway in Saccharomyces cerevisiae. BMC Biochem. 11, 29
16. Vazquez de Aldana, C. R., Marton, M. J., and Hinnebusch, A. G. (1995) GCN20, a novel ATP binding cassette protein, and GCN1 reside in a complex that mediates activation of the eIF-2α kinase GCN2 in amino acid-starved cells. EMBO J. 14, 3184–3199
17. Marton, M. J., Vazquez de Aldana, C. R., Qiu, H., Chakraburtty, K., and Hinnebusch, A. G. (1997) Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2α kinase Gcn2. Mol. Cell. Biol. 17, 4474–4489
18. Cashel M, R. K. (1987) Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., Ingraham, J. L., Magasanik, B., Low, K. B., Schaechter, M., and Umbarger, H. E., eds), pp. 1410–1438, American Society for Microbiology, Washington, D.C.
19. Goldman, E., and Jakubowski, H. (1990) Uncharged tRNA, protein synthesis, and the bacterial stringent response. Mol. Microbiol. 4, 2035–2040
20. Murchie, M. J., and Leader, D. P. (1978) Codon-specific interaction of uncharged transfer-RNA with eukaryotic ribosomes. Biochim. Biophys. Acta 520, 233–236
21. Visweswariah, J., Lageix, S., Castilho, B. A., Izotova, L., Kinzy, T. G., Hinnebusch, A. G., and Sattlegger, E. (2011) Evidence that eukaryotic translation elongation factor 1A (eEF1A) binds the Gcn2 protein C terminus and inhibits Gcn2 activity. J. Biol. Chem. 286, 36568–36579
22. Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27
23. Visweswariah, J., Dautel, M., and Sattlegger, E. (2011) Generating highly concentrated yeast whole cell extract using low-cost equipment. Nat. Protoc. 6, 1039–1045
24. Dever, T. E., Feng, L., Wek, R. C., Cigan, A. M., Donahue, T. F., and Hinnebusch, A. G. (1992) Phosphorylation of initiation factor 2α by protein kinase Gcn2 mediates gene-specific translational control of GCN4 in yeast. Cell 68, 585–596
25. Anderson, J. T., Paddy, M. R., and Swanson, M. S. (1993) PUB1 is a major nuclear and cytoplasmic polyadenylated RNA-binding protein in Saccharomyces cerevisiae. Mol. Cell. Biol. 13, 6102–6113
26. Hilton, J. L., Kearney, P. C., and Ames, B. N. (1965) Mode of action of the herbicide, 3-amino-1,2,4-triazole(amitrole): inhibition of an enzyme of histidine biosynthesis. Arch. Biochem. Biophys. 112, 544–547
27. Anand, M., Balar, B., Ulloque, R., Gross, S. R., and Kinzy, T. G. (2006) Domain and nucleotide dependence of the interaction between Saccharomyces cerevisiae translation elongation factors 3 and 1A. J. Biol. Chem. 281, 32318–32326
28. Romano, P. R., Zhang, F., Tan, S. L., Garcia-Barrio, M. T., Katze, M. G., Dever, T. E., and Hinnebusch, A. G. (1998) Inhibition of double-stranded RNA-dependent protein kinase PKR by vaccinia virus E3. Role of complex formation and the E3 N-terminal domain. Mol. Cell. Biol. 18, 7304–7316
29. LaRossa, R. A., and Schloss, J. V. (1984) The sulfonlurea herbicide sulfonyluron methyl is an extremely potent and selective inhibitor of acetolactate synthase in Salmonella typhimurium. J. Biol. Chem. 259, 8753–8757
30. Ramirez, M., Wek, R. C., and Hinnebusch, A. G. (1991) Ribosome association of GCN2 protein kinase, a translational activator of the GCN4 gene of Saccharomyces cerevisiae. Mol. Cell. Biol. 11, 3027–3036
31. Kambampati, R., and Chakraburtty, K. (1997) Functional subdomains of yeast elongation factor 3. Localization of ribosome-binding domain. J. Biol. Chem. 272, 6377–6381
32. Caskey, C. T., Beaudet, A. L., Scolnick, E. M., and Rosman, M. (1971) Hydrolysis of fMet-tRNA by peptidyl transferase. Proc. Natl. Acad. Sci. U.S.A. 68, 3163–3167
33. Zavialov, A. V., Mora, L., Buckingham, R. H., and Ehrenberg, M. (2002) Release of peptide promoter by the GGQ motif of class 1 release factors regulates the GTPase activity of RF3. Mol. Cell 10, 789–798
34. Foiani, M., Cigan, A. M., Paddon, C. J., Harashima, S., and Hinnebusch, A. G. (1991) GCD2, a translational repressor of the GCN4 gene, has a general function in the initiation of protein synthesis in Saccharomyces cerevisiae. Mol. Cell. Biol. 11, 3203–3216
35. Hannig, E. M., Williams, N. P., Wek, R. C., and Hinnebusch, A. G. (1990) The translational activator GCN3 functions downstream from GCN1 and GCN2 in the regulatory pathway that couples GCN4 expression to amino acid availability in Saccharomyces cerevisiae. Genetics 126, 549–562
36. Han, E. M., Williams, N. P., Wek, R. C., and Hinnebusch, A. G. (1992) Mutations activating the yeast eIF-2α kinase Gcn2. Isolation of alleles altering the domain related to histidyl-tRNA synthetases. Mol. Cell. Biol. 12, 5801–5815
37. Kawagishi-Kobayashi, M., Silverman, J. B., Ung, T. L., and Dever, T. E. (1997) Regulation of the protein kinase PKR by the vaccinia virus pseudosubstrate inhibitor K3L is dependent on residues conserved between the K3L protein and the PKR substrate eIF2α. Mol. Cell. Biol. 17, 4146–4158
38. Kambampati, R., and Chakraburtty, K. (1997) Overexpression and purification of elongation factor 3 from Saccharomyces cerevisiae. Protein Expr. Purif. 10, 209–213