MOLECULAR AND CELLULAR BIOLOGY, Apr. 2000, p. 2505–2516 Vol. 20, No. 7
0270-7306/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

Defects in tRNA Processing and Nuclear Export Induce *GCN4* Translation Independently of Phosphorylation of the α Subunit of Eukaryotic Translation Initiation Factor 2

HONGFANG QIU,[1] CUIHUA HU,[1] JAMES ANDERSON,[1] GLENN R. BJÖRK,[2] SRIMONTI SARKAR,[3] ANITA K. HOPPER,[3] AND ALAN G. HINNEBUSCH[1]*

Laboratory of Eukaryotic Gene Regulation, National Institute of Child Health and Human Development, Bethesda, Maryland 20892[1]; Department of Microbiology, Umeå University, Umeå, Sweden[2]; and Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033[3]

Received 13 October 1999/Returned for modification 24 November 1999/Accepted 30 December 1999

Induction of *GCN4* translation in amino acid-starved cells involves the inhibition of initiator tRNA^{Met} binding to eukaryotic translation initiation factor 2 (eIF2) in response to eIF2 phosphorylation by protein kinase GCN2. It was shown previously that *GCN4* translation could be induced independently of GCN2 by overexpressing a mutant tRNA$^{Val}_{AAC}$ (tRNA^{Val*}) or the RNA component of RNase MRP encoded by *NME1*. Here we show that overexpression of the tRNA pseudouridine 55 synthase encoded by *PUS4* also leads to translational derepression of *GCN4* (Gcd$^-$ phenotype) independently of eIF2 phosphorylation. Surprisingly, the Gcd$^-$ phenotype of high-copy-number *PUS4* (hc*PUS4*) did not require PUS4 enzymatic activity, and several lines of evidence indicate that *PUS4* overexpression did not diminish functional initiator tRNA^{Met} levels. The presence of hc*PUS4* or hc*NME1* led to the accumulation of certain tRNA precursors, and their Gcd$^-$ phenotypes were reversed by overexpressing the RNA component of RNase P (*RPR1*), responsible for 5′-end processing of all tRNAs. Consistently, overexpression of a mutant pre-tRNA^{Tyr} that cannot be processed by RNase P had a Gcd$^-$ phenotype. Interestingly, the Gcd$^-$ phenotype of hc*PUS4* also was reversed by overexpressing *LOS1*, required for efficient nuclear export of tRNA, and *los1*Δ cells have a Gcd$^-$ phenotype. Overproduced PUS4 appears to impede 5′-end processing or export of certain tRNAs in the nucleus in a manner remedied by increased expression of RNase P or LOS1, respectively. The mutant tRNA^{Val*} showed nuclear accumulation in otherwise wild-type cells, suggesting a defect in export to the cytoplasm. We propose that yeast contains a nuclear surveillance system that perceives defects in processing or export of tRNA and evokes a reduction in translation initiation at the step of initiator tRNA^{Met} binding to the ribosome.

Starvation of yeast cells for amino acids or purines leads to increased expression of GCN4, a transcriptional activator of amino acid biosynthetic enzymes (general amino acid control). *GCN4* expression is stimulated at the translational level by a mechanism involving four short upstream open reading frames (uORFs) in its mRNA leader. During growth on amino acid-replete medium, scanning ribosomes translate the first uORF (uORF1) and reinitiate downstream at uORF2, uORF3, or uORF4 but cannot reinitiate again at the *GCN4* start codon. In amino acid-starved cells, eukaryotic translation initiation factor 2 (eIF2) is phosphorylated on its α subunit by protein kinase GCN2, and the phosphorylated eIF2 inhibits the guanine nucleotide exchange factor for eIF2, known as eIF2B. Consequently, formation of the ternary complex containing eIF2, GTP, and initiator methionyl-tRNA (Met-tRNA$_i^{Met}$) is reduced, impairing delivery of tRNA$_i^{Met}$ to the ribosome. In *GCN4* mRNA, the ensuing delay in rebinding of ternary complex to 40S ribosomes which have translated uORF1 allows them to scan past uORF2 to uORF4 and reinitiate downstream at the *GCN4* start codon instead (25, 26). Thus, *GCN4* translation is induced under conditions of diminished ternary-complex formation.

It is thought that GCN2 is activated in amino acid-starved cells by uncharged tRNAs (34, 42, 43, 54) which accumulate under these conditions and bind to a regulatory domain in GCN2 homologous to histidyl-tRNA synthetases (55, 57, 58). Because starvation for any of several amino acids elicits activation of GCN2 and attendant derepression of *GCN4* (34, 57), it is probable that most uncharged tRNAs can bind to GCN2 and activate its kinase function. Two positive regulators of GCN2, encoded by *GCN1* and *GCN20* (41, 53), show sequence similarity to translation elongation factor eEF3 and have ribosome binding activities (40). It has been proposed that the GCN1-GCN20 complex functions at the ribosome in promoting activation of GCN2 by uncharged tRNAs which have entered the decoding site (40).

There are several instances where *GCN4* translation is stimulated in a manner dependent on the uORFs but independent of GCN2 and eIF2 phosphorylation. Mutations in subunits of eIF2 or eIF2B appear to reduce the functions of these two factors and mimic the effects of eIF2 phosphorylation in restricting ternary-complex formation. These mutations constitutively derepress *GCN4* translation and the amino acid biosynthetic enzymes subject to general amino acid control (Gcd$^-$ phenotype). The same phenotype is observed for deletions that reduce the number of *IMT* genes encoding tRNA$_i^{Met}$ (14) and thereby decrease the steady-state level of this component of the ternary complex. Mutations in *GCD10* (18, 22) and *GCD14* (10, 13), whose products are required for methylation of adenosine-58 in tRNA$_i^{Met}$ (1), also have *GCN2*-independent Gcd$^-$ phenotypes. Lack of m^{1}A58 specifically impairs 5′-end processing and stability of tRNA$_i^{Met}$. In these instances, the Gcd$^-$ phenotype can be explained by a reduction

* Corresponding author. Mailing address: Laboratory of Eukaryotic Gene Regulation, National Institute of Child Health and Human Development, Bldg. 6A, Rm. B1A-13A, Bethesda, MD 20892. Phone: (301) 496-4480. Fax: (301) 496-6828. E-mail: ahinnebusch@nih.gov.

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
H113	*MAT*α *his1-29 ura3-52 leu2-3 leu2-112 gcn2-1* [*HIS4-lacZ*, *URA3*]	27
H1816	*MAT***a** *ura3-52 leu2-3 leu2-112 trp1-Δ63 gcn2Δ sui2Δ* [*GCN4-lacZ*, *TRP1*] [*SUI2 LEU2*]	13a
H1817	*MAT***a** *ura3-52 leu2-3 leu2-112 trp1-Δ63 gcn2Δ sui2Δ* [*GCN4-lacZ*, *TRP1*] [*SUI2-S51A LEU2*]	13a
H1894	*MAT***a** *ura3-52 leu2-3 leu2-112 trp1-Δ63 gcn2Δ*	T. Dever
H1895	*MAT***a** *ura3-52 leu2-3 leu2-112 trp1-Δ63 gcn2Δ* [*GCN4-lacZ*, *TRP1*]	T. Dever
H1897	*MAT***a** *ura3-52 leu2-3 leu2-112 trp1-Δ63 GCN2 sui2Δ* [*GCN4-lacZ*, *TRP1*] [*SUI2-S51A LEU2*]	13a
H1937	*MAT*α *gcn2::LEU2 ura3-52 leu2-3 leu2-112 [HIS4-lacZ, ura3-52]*	54
HQY316	*MAT***a** *ura3-52 leu2-3 leu2-112 trp1-Δ63 gcn2Δ los1Δ::hisG::URA3::hisG* [*GCN4-lacZ*, *TRP1*]	This work

in the ternary-complex level independently of eIF2 phosphorylation by GCN2.

Previously, we observed *GCN2*-independent derepression of *GCN4* translation in cells overexpressing tRNAs under conditions where it was presumed that the excess tRNA was not aminoacylated efficiently. This occurred most notably with a mutant form of $tRNA^{Val}_{AAC}$ harboring an A-to-G substitution in the 3′-terminal nucleotide ($tRNA^{Val*}$), which is expected to impair aminoacylation by valyl-tRNA synthetase. Overexpression of $tRNA^{Val*}$ did not lead to eIF2 phosphorylation in strains containing GCN2; however, it exacerbated the growth defect of a *GCN2*^{c} mutant (expressing a constitutively active kinase) in which eIF2 is hyperphosphorylated and thus impaired in general translation initiation. The latter findings suggested that excess $tRNA^{Val*}$ leads to reduced eIF2 function by a mechanism independent of eIF2 phosphorylation (54). To explain these findings, we proposed that yeast cells have a second sensor of uncharged tRNA besides GCN2 that also constrains eIF2 activity. Moreover, because $tRNA^{Val*}$ overexpression did not activate GCN2, it seemed possible that this defective tRNA was physically sequestered from GCN2 (54).

GCN2-independent derepression of *GCN4* translation also was elicited by overexpression of *NME1* (51), encoding the RNA component of RNase MRP (48). RNase MRP is involved in processing rRNA, and it was suggested that defects in ribosome biogenesis caused by *NME1* overexpression could impair *GCN4* translational control. Partial derepression of *GCN4* translation additionally occurred during growth on rich medium in mutant strains with constitutively high levels of protein kinase A (PKA) function (*RAS2*^{Val19} and *bcy1*Δ mutants) (16). It is unknown how elevated PKA function impairs translational control of *GCN4*.

In this report we show that overexpression of the tRNA pseudouridine 55 synthase encoded by *PUS4* (7) stimulates *GCN4* translation independently of GCN2 and its phosphorylation site on eIF2. We present several lines of evidence that PUS4 overexpression does not reduce the amount of functional $tRNA_i^{Met}$ as the means of limiting ternary-complex formation or its utilization in translation initiation. Instead, it appears that excess PUS4 impedes the 5′-end processing and export of certain tRNAs in the nucleus. The same mechanism seems to apply to overexpressed NME1; moreover, overproduction of a mutant pre-tRNA that cannot be processed by RNase P elicits a *GCN2*-independent Gcd$^-$ phenotype. These and other findings strongly suggest that yeast contains a surveillance system that perceives defects in tRNA processing or transport in the nucleus and reduces the efficiency of translation initiation in the cytoplasm in response.

MATERIALS AND METHODS

Identification of *PUS4* as a high-copy-number suppressor of *gcn2-1*. Plasmid pAH14 was isolated previously as a high-copy-number suppressor of a *gcn2-1* mutant (27). Sequencing the ends of the DNA insert and comparison with the complete yeast genome sequence showed that the insert is ~5 kb and contains three genes from chromosome XIV: *RFC3* (36), *MID1* (30), and *PUS4* (7). Subclones pHQ536 carrying *MID1* and *PUS4*, pHQ537 carrying *PUS4*, and pHQ538 carrying *RFC3* and *MID1* were constructed from pAH14 (see below) and introduced into *gcn2-1* mutant H113, and the resulting transformants were tested for growth on medium containing 3-aminotriazole (3-AT). Only plasmids pHQ536 and pHQ537 conferred 3-AT resistance, indicating that *PUS4* is a high-copy-number suppressor of *gcn2-1*. To confirm this conclusion, 5′ and 3′ deletions (pHQ546 and pHQ545, respectively) and an internal frameshift mutation (pHQ575) in *PUS4* were constructed (see below). None of these plasmids conferred 3-AT resistance in strain H113, and neither did the single-copy-number *PUS4* plasmid pHQ543 that we constructed.

Yeast strains and plasmid construction. All yeast strains except HQY316 used in this study were described previously and are listed in Table 1. HQY316 was constructed from H1895 by replacing *LOS1* with the *los1Δ::hisG::URA3::hisG* allele using a ~4.8-kb *Eco*RI-*Bgl*II fragment from plasmid pHQ871. The resulting *los1Δ* strain was identified by PCR and further confirmed by complementation of its Gcd$^-$ phenotype by *LOS1* plasmid pHQ860. The plasmids used in this work were constructed as follows. Plasmid pHQ536 containing *MID1* (30) and *PUS4* was constructed by inserting an ~4-kb *Sal*I fragment from pAH14 into YEplac181 (20) at the *Sal*I site. An ~2.0-kb *Bgl*II-*Asp*718 fragment containing *PUS4* (7) from pHQ536 was inserted into YEplac181 between the *Bam*HI and *Asp*718 sites to produce plasmid pHQ537. An ~3.8-kb *Nhe*I-*Xba*I fragment containing *RFC3* (36) and *MID1* from pAH14 was inserted into YEplac181 at the *Xba*I site to produce pHQ538. To construct the C-terminal deletion of *PUS4*, an ~1.5-kb *Xba*I fragment from pHQ537 was subcloned into YEplac181 at the *Xba*I site to produce pHQ545, encoding PUS4 amino acids 1 to 342. The N-terminal deletion of *PUS4* was constructed by removing the *Bam*HI fragment from pHQ537 to produce plasmid pHQ546, in which the 5′ noncoding region and first 74 codons of *PUS4* were deleted. A frameshift mutation in *PUS4* was constructed by digesting pAH14 with *Bam*HI, filling in the ends, and religating to produce pHQ575. Single-copy-number plasmid pHQ543 bearing *PUS4* was constructed by inserting an ~1.8-kb *Bgl*II-*Nae*I fragment from pHQ536 into YCplac111 (20). High-copy-number *PUS4* plasmid pHQ547 was constructed by inserting the *Bgl*II-*Sph*I fragment containing *PUS4* from pAH14 into YEp24 between the *Bam*HI and *Sph*I sites. The single-copy-number *GCN2* plasmid pHQ548 was created by inserting the *Xba*I-*Sal*I fragment from p722 (56) into YCplac111. To add the hemagglutinin (HA) epitope to PUS4, *Nru*I and *Mlu*I sites were first introduced into pHQ537 immediately 5′ to the *PUS4* stop codon by site-directed mutagenesis, using the Quik-Change site-directed mutagenesis kit (Stratagene), producing plasmid pHQ732. An ~100-bp PCR fragment encoding three copies of HA with *Ecl*136II and *Mlu*I ends was then inserted between the *Nru*I-*Mlu*I sites of pHQ732 to produce plasmid pHQ753 encoding PUS4-HA. An ~2-kb *Sal*I fragment encoding PUS4-HA from pHQ753 was inserted into YCplac111 and pHQ583 to produce single-copy-number and high-copy-number plasmids pHQ771 and pHQ839, respectively. pHQ583 is a derivative of YEplac181 in which the polycloning sites *Sac*I to *Bam*HI were deleted by filling in the *Eco*RI and *Xba*I sites and religating. pHQ853 and pHQ857, encoding *pus4-1-HA* and *pus4-2-HA*, respectively, were derived from pHQ839 by site-directed mutagenesis using the Quik-Change site-directed mutagenesis kit.

Plasmid pHQ731, used for in vitro synthesis of $tRNA^{Asp}$ mut#2 (8), was constructed by inserting a 52-bp double-stranded oligonucleotide encoding the T7 promoter and $tRNA^{Asp}$ mut#2 into pUC18 at the *Sma*I site. To construct hc*NME1* and hc*NME1*/*RPR1* plasmids, an *Eco*RI linker was first added to the filled-in *Bam*HI site of pDK45, an *NME1*-bearing plasmid obtained from Lasse Lindahl, to produce pHQ859. The ~0.7-kb *Eco*RI fragment containing *NME1* from pHQ859 was then inserted into YEplac181 and pHQ682 at their respective *Eco*RI sites to produce high-copy-number plasmids pHQ862 and pHQ863 containing *NME1* and *NME1*/*RPR1*, respectively. pHQ682 was constructed by inserting the ~1.3-kb *Eco*RI-*Hin*dIII fragment containing *RPR1* from pDK42, an *RPR1*-bearing plasmid obtained from Lasse Lindahl, into YEplac181 between the *Eco*RI and *Hin*dIII sites. An *Eco*RI linker was also added to the filled-in *Hin*dIII site of pDK42, so that an ~1.3-kb *Eco*RI fragment bearing *RPR1* could be isolated from the resulting plasmid (pHQ858) and inserted into pHQ839 at the *Eco*RI site, producing high-copy-number plasmid pHQ864 bearing *PUS4-HA* and *RPR1*.

LOS1-bearing plasmids YEpLOS1 and YCpLOS1 were described previously (29). To construct the *los1*Δ plasmid, a *Bam*HI linker was first added at the *Pvu*II site of YCpLOS1 to produce plasmid pHQ868 and a *Bam*HI fragment containing *hisG::URA3::hisG* was inserted at the *Bam*HI site to produce plasmid pHQ871. hc*LOS1* plasmid pHQ860 was constructed by inserting a 5.3-kb *Sph*I fragment from YCpLOS1 into the *Sph*I site of YEplac181. Plasmids pHQ982 and pHQ985 encoding wild-type and mutant pre-$tRNA^{Tyr}_{GUA}$, respectively, were constructed by inserting PCR-synthesized genomic DNA fragments with *Eco*RI and *Bam*HI sites at the 5′ and 3′ ends, respectively, between the corresponding sites in high-copy-number plasmid YEplac181. The genomic DNA fragments containing 170 and 18 bp of 5′ and 3′ noncoding DNA, respectively, were synthesized using the following oligonucleotide primers: 5′-CCGGAATTCCTGTATTAGT CGATATACCACC-3′ (forward primer), 5′-CGCGGATCCGCAAGATTTAA AAAAATATCTCCCGGGGGCGA-3′ (reverse primer for the wild-type 3′ trailer), and 5′-CGCGGATCCGCAAGATTTAAAAAAATACGACTCCCGGGG GCGA-3′ (reverse primer for the mutant 3′ trailer).

Assay of *HIS4-lacZ* and *GCN4-lacZ* fusions. Assays were conducted using cell extracts prepared from cultures grown in SD medium containing only the required supplements as described previously (37). For repression conditions, saturated cultures were diluted 1:50 into fresh medium and harvested in mid-logarithmic phase after 6 h of growth. For derepression conditions, cultures were grown for 2 h under repression conditions and then for 6 h after adding 3-AT to 10 mM, 5-methyltryptophan (5-MT) to 2 mM, or sulfometuron methyl (SM) to 0.5 μg/ml.

Assay of yeast tRNA pseudouridine 55 synthase. Synthesis of pseudouridine is accompanied by the release of a proton from carbon 5 in the pyrimidine ring of the uridine base (12); therefore, release of tritium from [5-^{3}H]uridine-labeled tRNA can be used as a measure of pseudouridine 55 synthase activity (46). PUS4, the *S. cerevisiae* enzyme, can catalyze the formation of pseudouridine 55 in a model substrate corresponding to the acceptor stem and TψC stem-loop of $tRNA^{Asp}$ (mut#2 minihelix) (8). Accordingly, we assayed PUS4 activity in cell extracts by measuring the release of tritium (46) from mut#2 minihelix RNA synthesized in vitro in the presence of [5-^{3}H]UTP. $tRNA^{Asp}$ mut#2 RNA labeled with [5-^{3}H]uridine was synthesized in vitro as previously described (46). Briefly, 10 μg of *Mva*I-digested pHQ731 was mixed with 100 μCi of [5-^{3}H]UTP (14.5 Ci/mmol; Amersham), dried under vacuum, and resuspended in 100 μl of a reaction mixture containing 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 10 mM GMP, 1 mM each GTP, CTP, and ATP, 250 μM UTP, and 100 U of RNasin (Promega). The reaction was initiated by adding 100 U of T7 RNA polymerase (Promega), and the mixture was incubated at 37°C for 2 h. Afterwards, the mixture was extracted once with phenol-chloroform (1:1) and the [^{3}H]RNA was ethanol precipitated and resuspended in water pretreated with diethylpyrocarbonate.

The tritium release assay for pseudouridine synthase was conducted as described previously (46). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mg of bovine serum albumin per ml, 80 U of RNasin (Promega), and ^{3}H-labeled $tRNA^{Asp}$ mut#2 (2.5×10^6 cpm). The reaction was started by adding S100 yeast whole-cell extract to a total volume of 100 μl, and the mixture was incubated at 30°C for 30 min. The reaction was terminated by adding 0.3 ml of a suspension of Norit A (12% in 0.1 N HCl). After 2 min at room temperature, the mixture was centrifuged and the radioactivity in the supernatant was determined. The activity of pseudouridine 55 synthase in the whole-cell extract was expressed as cpm of ^{3}H released per microgram of protein. S100 yeast whole-cell extracts were prepared as described previously (4).

Analysis of tRNA modification and aminoacylation in vivo. The primer extension assay used for mapping pseudouridine residues was conducted as described previously (5–7). In this assay, RNA is treated with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMCT), a chemical that reacts with both pseudouridine and uridine residues. The presence of CMCT-coupled nucleosides in tRNA impedes reverse transcription primed by an oligonucleotide annealed 3′ to the CMCT-coupled base. Because CMCT-pseudouridine is more resistant than CMCT-uridine to alkali treatment, the locations of pseudouridine residues in a tRNA molecule can be deduced from the presence of alkali-insensitive blocks to reverse transcription (5, 6). Chromatography of aminoacylated $tRNA^{Met}_i$ and elongator $tRNA^{Met}$ ($tRNA^{Met}_e$) on RPC-5 resin was carried out as described previously (1). For Northern analysis of in vivo-aminoacylated tRNAs, total RNA was prepared under acidic conditions and resolved by electrophoresis on acid-urea gels as described previously (52). The following oligonucleotides were used to probe the Northern blots: 5′-TGGTA GCGCCGCTCGGTTTCGAATCC-3′ ($tRNA^{Met}_i$), 5′-TGCTCCAGGGGAGG TTCGAACTCTCGACC-3′ ($tRNA^{Met}_e$), 5′-CACTCACGATGGGGGTCGAA-3′ ($tRNA^{Arg}_{UCU}$), 5′-TGCTCGAGGTGGGGA/TTTGAACCCACGACGG-3′ ($tRNA^{Ile}_{UAU}$), 5′-GATTGCAGCACCTGAGTTTCGCGTTATGG-3′ (5S rRNA), and 5′-GGTGGGAGACTTTCAACCCAAAGC-3′ (*NME1*).

Fluorescence in situ hybridization. The fluorescence in situ hybridization procedure was conducted as described previously (47), except that transformants carrying plasmids were grown at 30°C to log phase. The oligonucleotides used were probe 04 (47), to detect $tRNA^{Ile}_{UAU}$, and 5′-CGCCCAGGATCGAACTG GGGACGTTCTGCGTGTTAAGCAGATGCCATAACCGACTAGACC-3′, to detect $tRNA^{Val}_{AAC}$.

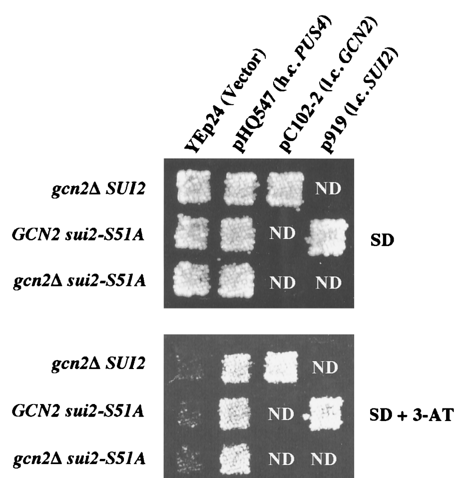

FIG. 1. High-copy-number plasmid encoding PUS4 derepresses histidine biosynthetic genes in the absence of GCN2 and Ser-51 of eIF2α. Isogenic strains H1816 (*gcn2*Δ *SUI2*), H1897 (*GCN2 sui2-S51A*), and H1817 (*gcn2*Δ *sui2-S51A*) were transformed with the indicated plasmids, replica-plated to SD medium or to SD medium containing 30 mM 3-AT, and incubated for 3 days at 30°C. pHQ547 is a high-copy-number (h.c.) plasmid containing *PUS4*; pC102-2 is a low-copy-number (l.c.) plasmid containing *GCN2*, and p919 is a low-copy-number (l.c.) plasmid containing *SUI2*.

RESULTS

Overexpression of *PUS4*, encoding tRNA pseudouridine 55 synthase, derepresses *GCN4* translation in the absence of eIF2 kinase GCN2. In an effort to identify a novel regulator of GCN2, we analyzed a previously described high-copy-number plasmid, pAH14, which suppresses the 3-AT-sensitive (3-AT^s) phenotype of a *gcn2-1* mutant (27). 3-AT is a competitive inhibitor of the histidine biosynthetic enzyme encoded by *HIS3*, and GCN4-mediated derepression of *HIS3* transcription is required for growth in the presence of this inhibitor. Accordingly, *gcn2* mutants are 3-AT^s because they fail to derepress *GCN4* translation in response to histidine starvation. Suppression of the 3-AT^s phenotype of *gcn2-1* by pAH14 suggested that *HIS3* derepression had been restored independently of GCN2. Sequencing the ends of the genomic DNA insert in pAH14 revealed that it contains three genes from chromosome XIV: *RFC3* (36), *MID1* (30), and *PUS4*, of which the last encodes tRNA pseudouridine 55 synthase (7). By analyzing subclones of pAH14, we determined that high-copy-number *PUS4* was sufficient for suppression of *gcn2-1* and that deletions removing the 5′ or 3′ end of the *PUS4* ORF or introduction of a frameshift mutation in *PUS4* abolished suppression (see Materials and Methods). Moreover, *PUS4* on a low-copy-number plasmid failed to suppress the *gcn2-1* allele (data not shown). Thus, we concluded that *PUS4* is a high-copy-number suppressor of *gcn2-1*.

We found that high-copy-number *PUS4* (hc*PUS4*) suppressed the 3-AT^s phenotypes of a *gcn2*Δ mutant and a strain containing an Ala substitution in the GCN2 phosphorylation site in eIF2α, Ser-51 (the *SUI2-S51A* allele) (Fig. 1). Thus, it appeared that hc*PUS4* derepresses *HIS3* expression independently of eIF2α phosphorylation by GCN2, an event required in wild-type cells for increased translation of *GCN4* mRNA. Analysis of a *HIS4-lacZ* fusion showed that expression of *HIS4*, another target of GCN4, was derepressed ca. threefold in *gcn2*Δ transformants bearing hc*PUS4* (Table 2). Similar degrees of *HIS4-lacZ* derepression were observed in *gcn2*Δ cells bearing hc*PUS4* in the presence or absence of inhibitors

TABLE 2. Effect of hc*PUS4* on *HIS4-lacZ* expression in a *gcn2*Δ strain starved for different amino acids

Plasmid	Gene[a]	β-Galactosidase activity (U)[b]			
		R	3-AT	5-MT	SM
YEp24	None	120	120	110	120
p585	*GCN2* (l.c.)	210	650	640	920
pHQ547	*PUS4* (h.c.)	330	370	290	450
p1362	$tRNA^{Val*}$ (h.c.)	360	530	280	510

[a] l.c., low copy number; h.c., high copy number.
[b] Transformants of strain H1937 (*gcn2*Δ) carrying the indicated plasmid were grown under repressing (R) nonstarvation condition or under derepressing conditions generated by adding 3-AT for histidine starvation, 5-MT for tryptophan starvation, or SM for leucine, isoleucine, and valine starvation. Extracts were prepared and assayed for enzyme activity, and the results shown are means from three transformants. The β-galactosidase activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein. The standard errors are less than 20%.

TABLE 4. Nonadditive effects of high-copy-number plasmids carrying $tRNA^{Val*}$ and *PUS4* on derepression of *GCN4-lacZ* expression in a *gcn2*Δ strain

Plasmid 1	Plasmid 2	β-Galactosidase activity (U)[a]	
		R	DR
YEp24 (vector)	YEplac181 (vector)	4	5
YEp24	pHQ537 (hc*PUS4*)	16	35
p1362 (hctRNA^{Val*})	YEplac181	23	45
p1362	pHQ537	22	55

[a] Transformants of strain H1895 (*gcn2*Δ) bearing the indicated plasmids were grown under repressing (R) nonstarvation conditions or under derepressing (DR) conditions produced by adding 3-AT for histidine starvation. Extracts were assayed for enzyme activity, and the results shown are means from three or more transformants. The standard errors are less than 20%.

of histidine (3-AT), tryptophan (5-MT), or isoleucine-valine (SM) biosynthesis. These findings suggested that *GCN4* expression was constitutively derepressed by hc*PUS4* independently of both amino acid starvation and eIF2 phosphorylation by GCN2. Supporting this conclusion, expression of a *GCN4-lacZ* fusion was derepressed five- to sixfold in *gcn2*Δ cells bearing hc*PUS4* in the presence or absence of 3-AT (Table 3). In contrast, expression of a *GCN4-lacZ* fusion lacking all four uORFs required for translational control was unaffected by hc*PUS4* (Table 3). These last results indicate that hc*PUS4* stimulates *GCN4* expression at the translational level. In agreement with this conclusion, the presence of hc*PUS4* had no effect on steady-state *GCN4* mRNA levels (data not shown).

A high-copy-number plasmid encoding the mutant $tRNA^{Val}_{AAC}$ described above (hctRNA^{Val*}) (54) led to slightly higher levels of *GCN4-lacZ* expression in a *gcn2*Δ strain than did hc*PUS4*; however, the presence of both plasmids in the same transformants did not increase *GCN4* expression in an additive fashion (Table 4). This nonadditivity suggests that overexpression of PUS4 or $tRNA^{Val*}$ leads to derepression of *GCN4* expression by a common mechanism.

hc*PUS4* leads to elevated pseudouridine 55 synthase activity in vivo. To show that cells bearing hc*PUS4* contain increased amounts of PUS4 protein, we assayed pseudouridine 55 synthase activity in cell extracts (see Materials and Methods). As shown in Table 5, extracts of transformants containing hc*PUS4* or a functional HA-tagged form of hc*PUS4* contained 15 to 16 times as much pseudouridine 55 synthase activity than did the corresponding extract from the vector transformant. This increase in enzyme activity was similar in magnitude to the increase in PUS4 protein levels measured in extracts from transformants bearing the HA-tagged *PUS4* allele (*PUS4-HA*) on high-copy-number versus single-copy-number plasmids, as judged by immunoblot analysis with anti-HA antibodies (Table 5). Based on these results, we conclude that hc*PUS4* leads to a large increase in the level of PUS4 enzyme activity in vivo.

Increased pseudouridine 55 synthase activity is not required for suppression of *gcn2* mutations by hc*PUS4*. The observations that overexpressing the mutant $tRNA^{Val*}$ derepressed *GCN4* translation independently of GCN2 (54), that hctRNA^{Val*} and hc*PUS4* had nonadditive effects on *GCN4* expression, and that PUS4 is a tRNA modification enzyme led us to consider that overexpression of PUS4 might lead to aberrant pseudouridine formation in tRNAs and impede aminoacylation by their cognate aminoacyl-tRNA synthetases. If this occurred with $tRNA^{Met}_{i}$, the only known tRNA in *Saccharomyces cerevisiae* that normally lacks this modification (49), it would lower ternary-complex levels and thereby derepress *GCN4* translation in *gcn2*Δ cells.

Several observations preclude the possibility of aberrant pseudouridine formation in $tRNA^{Met}_{i}$ or in any other tRNAs in hc*PUS4* transformants. First, we found no evidence for increased pseudouridine levels in total tRNA prepared from *gcn2*Δ transformants carrying hc*PUS4* versus vector alone.

TABLE 3. Derepression of *GCN4-lacZ* expression by hc*PUS4* in a *gcn2*Δ strain requires uORFs in *GCN4* mRNA

Plasmid (gene)	β-Galactosidase activity (U)[a]			
	p180		p227	
	R	DR	R	DR
YEplac181 (vector)	5	8	1,800	1,900
pHQ537 (hc*PUS4*)	25	48	1,900	1,900
pHQ548 (lc*GCN2*)	14	158	1,900	1,800

[a] The *gcn2*Δ strain H1894 was transformed with empty vector, high-copy-number plasmid pHQ537 carrying *PUS4*, or low-copy-number *GCN2* (lc*GCN2*) plasmid pHQ548 and cotransformed with p180 or p227 harboring *GCN4-lacZ* fusions containing all four uORFs or no uORFs, respectively, in the *GCN4* mRNA leader. Transformants were grown under repressing (R) conditions or under derepressing (DR) conditions imposed by adding 3-AT to elicit histidine starvation. Extracts of the transformants were assayed for enzyme activity, and the results are means from three transformants. The β-galactosidase activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein. The standard errors are less than 20%.

TABLE 5. Phenotypes and level of pseudouridine 55 synthase activity conferred by *PUS4* alleles[a]

Plasmid	Genotype	Growth on 3-AT	Relative ψ55 synthase activity	Relative protein level
Vector	*PUS4*	−	1	NA[b]
pHQ537	hc*PUS4*	+	16	NA
pHQ771	lc*PUS4-HA*	−	ND[c]	6
pHQ839	hc*PUS4-HA*	+	15	100
pHQ853	hc*pus4-1-HA*	+	1	66
pHQ857	hc*pus4-2-HA*	−	1	14

[a] Transformants of strain H1895 (*gcn2*Δ) bearing the indicated plasmids were tested for growth on medium containing 3-AT (30 mM). Extracts were assayed for pseudouridine 55 (ψ55) synthase activity and for levels of PUS4-HA by Western blot analysis. PUS4-HA proteins were detected using anti-HA antibodies and an enhanced chemiluminescence system (Amersham) to visualize immune complexes. The Western blot signals were quantified with a scanner (Silverscanner III) and NIH Image software (version 1.61).
[b] NA, not applicable.
[c] ND, not determined.

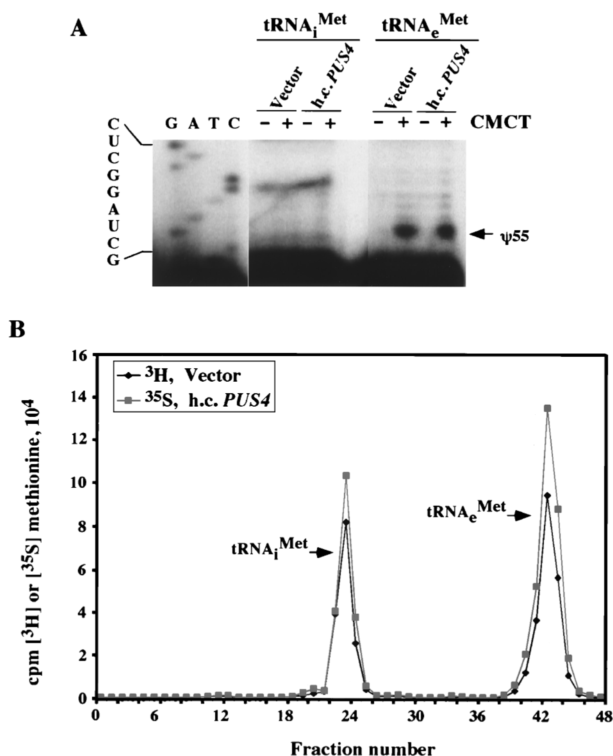

FIG. 2. High-copy-number *PUS4* does not alter base modification of $tRNA_i^{Met}$. (A) Samples of total tRNA (10 μg) prepared from *gcn2Δ* strain H1894 carrying empty vector (YEplac181) or hc*PUS4* plasmid (pHQ537) were treated (+) or not treated (−) with CMCT, and 1 μg was reverse transcribed using end-labeled primers complementary to $tRNA_i^{Met}$ or $tRNA_e^{Met}$ (nucleotides 60 to 76). Reverse transcription products were resolved in an 8% sequencing gel. The strong stops in reverse transcription of CMCT-treated tRNA correspond to pseudouridine-55 (ψ55), as indicated by the arrow. On the left of the gel is a sequence ladder of initiator $tRNA^{Met}$. The strong stops at position 52 for $tRNA_i^{Met}$ observed independently of CMCT presumably arise from strong secondary structure. (B) The same tRNA samples as in panel A were aminoacylated with [^{3}H]methionine or [^{35}S]methionine, and ca. 500,000 cpm was resolved on an RPC-5 column. Radioactivity in each fraction (2 ml) was measured by liquid scintillation and plotted against the fraction number. The elution positions of the methionine-accepting tRNAs are indicated at the appropriate positions.

When total tRNA isolated from these transformants was digested to nucleosides and resolved by high-pressure liquid chromatography (19), there was no significant difference in the amount of pseudouridine relative to other nucleosides, conventional or modified, between the two tRNA samples (data not shown). For example, the ratios of pseudouridine to t6A (N^6-threonylcarbamoyladenosine) in the vector and hc*PUS4* transformants were 6.65 and 6.82, respectively; the corresponding ratios of pseudouridine to $m^{22}G$ (N_2,N_2-dimethylguanosine; a modification at guanosine-26) were 2.61 and 2.68.

To determine whether overexpression of *PUS4* leads specifically to formation of pseudouridine-55 in $tRNA_i^{Met}$, total tRNA was isolated from *gcn2Δ* transformants containing hc*PUS4* or vector alone and subjected to a primer extension assay for mapping pseudouridine residues (see Materials and Methods). By applying this technique with primers that anneal 3′ to position 55 in $tRNA_i^{Met}$ or $tRNA_e^{Met}$, we observed the expected block to reverse transcription at pseudouridine 55 in $tRNA_e^{Met}$ from transformants containing hc*PUS4* or vector alone. In contrast, we observed no block at this location in $tRNA_i^{Met}$ that was enhanced by the presence of hc*PUS4* (Fig. 2A). Similar results were obtained using total tRNAs prepared from cultures starved for histidine by 3-AT treatment (data not shown). Thus, hc*PUS4* does not lead to detectable amounts of pseudouridine 55 in $tRNA_i^{Met}$ or diminish this modification in $tRNA_e^{Met}$.

In accordance with the above findings, we obtained strong evidence that pseudouridine 55 synthase activity is not required for the suppressor activity of hc*PUS4*. Using site-directed mutagenesis, we altered PUS4 residues 283 to 286 from TYIR to AAAA (producing *pus4-1-HA*) or altered residues 74 to 77 from LDPL to AAAA (*pus4-2-HA*) and introduced the mutant alleles into a *gcn2Δ* strain on high-copy-number plasmids. The residues altered by these mutations are conserved in pseudouridine 55 synthases among bacteria and yeast (32). As shown in Table 5, neither high-copy-number mutant allele led to pseudouridine 55 synthase activity in extracts above the background level produced by chromosomal *PUS4*. Immunoblot analysis showed that expression of *pus4-1-HA* was only slightly reduced, whereas *pus4-2-HA* expression was greatly decreased, compared to wild-type *PUS4-HA* (Table 5). Surprisingly, hc*pus4-1-HA* was indistinguishable from hc*PUS4-HA* in suppressing the 3-AT^s phenotype of the *gcn2Δ* mutant (Table 5), suggesting that hc*PUS4* suppressor activity does not require elevated pseudouridine 55 synthase activity. The fact that hc*pus4-2-HA* was inactive as a dosage suppressor can be explained by the fact that it was not highly expressed (Table 5).

Evidence that hc*PUS4* elicits derepression of *GCN4* partly by interfering with 5′-end processing of tRNA by RNase P. Although the pseudouridine 55 synthase activity of *PUS4* is not required for its suppressor activity, it was possible that increased binding of overexpressed PUS4 to one or more tRNAs would restrict the access of other enzymes involved in modification or processing of these tRNAs. As indicated above, we were particularly interested in possible differences in the structure or function of $tRNA_i^{Met}$ that could reduce ternary-complex formation. To investigate this last possibility, we first aminoacylated total tRNA from transformants containing hc*PUS4* or vector alone with [^{35}S]methionine or [^{3}H]methionine, respectively, and resolved the labeled tRNAs by RPC-5 column chromatography (31). tRNAs that differ by only a single methyl group can be resolved by RPC-5 chromatography (15). The results in Fig. 2B show that the elution positions of [^{35}S]methionine-charged $tRNA_i^{Met}$ and $tRNA_e^{Met}$ were identical between *gcn2Δ* transformants bearing hc*PUS4* and vector alone. These results suggest that mature methionine-accepting tRNAs are modified identically in cells overexpressing *PUS4* and wild-type cells; however, it is possible that certain modifications would not alter the behavior of methionyl-tRNAs on RPC-5 chromatography.

In a second approach, we investigated whether hc*PUS4* led to reductions in the efficiency of $tRNA_i^{Met}$ aminoacylation in vivo which might arise from a defect in one or more steps in the production of $tRNA_i^{Met}$. The degree of aminoacylation of a tRNA in vivo can be measured by isolating total tRNA at pH 4.5 to preserve the aminoacyl-tRNA linkage and resolving the aminoacylated and deacylated forms by gel electrophoresis followed by Northern blot hybridization (52). When this technique was carried out with tRNA isolated from transformants bearing hc*PUS4* versus vector alone, we observed no significant differences in the charged-to-uncharged ratios for $tRNA_i^{Met}$, $tRNA_e^{Met}$, $tRNA_{UCU}^{Arg}$, $tRNA_{UAU}^{Ile}$, and $tRNA_{CAA}^{Leu}$ (Fig. 3 and data not shown). These results suggest that $tRNA_i^{Met}$, as well as four other tRNAs analyzed by this technique, are aminoacylated with similar efficiencies in cells overexpressing *PUS4* and in wild-type cells.

We also measured the total steady-state levels of $tRNA_i^{Met}$, $tRNA_{UAU}^{Ile}$, and $tRNA_{CCA}^{Trp}$ by Northern analysis and observed no large differences for the mature forms of these tRNAs in *gcn2Δ* strains bearing hc*PUS4-HA* versus vector alone (Fig. 4,

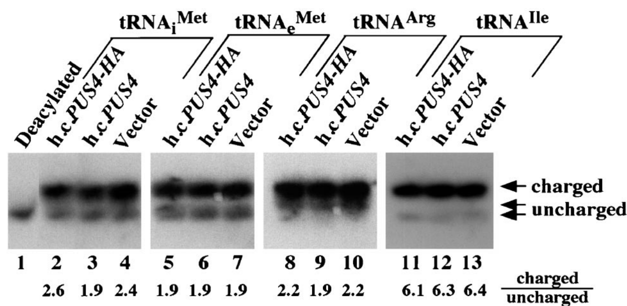

FIG. 3. Evidence that hc*PUS4* does not reduce in vivo aminoacylation of various tRNAs. Total RNAs prepared under acidic conditions (52) from strain H1894 (*gcn2*Δ) carrying empty vector (YEplac181) or high-copy-number plasmids carrying *PUS4* (pHQ537) or *PUS4-HA* (pHQ839) were resolved by electrophoresis on an acid-urea polyacrylamide gel and subjected to Northern blot analysis. The same blot was probed with radiolabeled oligonucleotides that specifically hybridized to the indicated tRNAs by stripping one probe from the blot before using the next. An aliquot of $tRNA_i^{Met}$ was deacylated in 2 M Tris-HCl (pH 8.0) and loaded in lane 1. The intensities of the hybridization signals corresponding to charged and uncharged tRNAs were quantified by phosphorimaging analysis, and the ratios of charged to uncharged tRNA signals are listed below each lane.

TABLE 6. Effect of overexpression of *NME1*, *PUS4*, and *RPR1* on 5′ processing of tRNA[a]

Plasmid	$tRNA_i^{Met}$ [b]			$tRNA^{Ile}_{UAU}$ [b]		
	Normalized amount		p/m ratio	Normalized amount		p/m ratio
	p	m		p	m	
Group 1						
Vector	100	100	0.50	100	100	0.13
hc*PUS4-HA*	194	93	1.05	138	97	0.19
hc*PUS4-HA/RPR1*	164	108	0.77	112	101	0.14
Group 2						
Vector	100	100	0.47	100	100	0.07
hc*NME1*	136	70	0.92	219	99	0.16
hc*NME1/RPR1*	127	105	0.57	150	105	0.10

[a] The intensities of the hybridization signals in the autoradiograms were quantified by phosphorimager analysis and normalized to the 5S rRNA signals. The data from Fig. 4 and from a replicate experiment not shown were averaged.

[b] The values for the $tRNA_i^{Met}$ precursors or the primary $tRNA^{Ile}_{UAU}$ precursor (p) in the transformants harboring hc*PUS4-HA* or hc*NME1* are expressed relative to the corresponding precursor levels in the vector transformants, which are assigned a value of 100. The levels of mature (m) tRNAs are similarly normalized to the levels observed in the vector transformants. p/m is the ratio of the absolute amounts of precursor and mature tRNAs (normalized for 5S rRNA content) for a given tRNA in each transformant.

lanes 1 and 2, and data not shown). Interestingly, the hc*PUS4-HA* transformants showed significant accumulation of the various precursors of $tRNA_i^{Met}$ containing both 5′ and 3′ extensions that are transcribed from different *IMT* genes (Fig. 4, lanes 1 and 2), leading to a precursor/mature $tRNA_i^{Met}$ ratio ca. twofold greater than that of the vector transformant (Table 6). After normalizing for the amounts of 5S RNA in the samples, we calculated that the hc*PUS4-HA* transformants contained 93% of the wild-type level of mature $tRNA_i^{Met}$. Thus, it appears that the hc*PUS4-HA* transformants process $tRNA_i^{Met}$ precursors more slowly than does the wild type but this defect does not substantially reduce the steady-state level of mature $tRNA_i^{Met}$. The hc*PUS4-HA* transformants also showed slight accumulation of the larger $tRNA^{Ile}_{UAU}$ precursor (Fig. 4), which corresponds to the primary transcript containing 5′ and 3′ extensions plus the intron (44); again, little or no reduction in the level of mature $tRNA^{Ile}_{UAU}$ was evident (Table 6). The presence of hc*PUS4* had no detectable effect on the levels of precursor or mature $tRNA^{Trp}_{CCA}$ precursor (data not shown). These findings suggest that *PUS4* overexpression decreases the rate at which 5′ and 3′ extensions are removed from a subset of tRNAs.

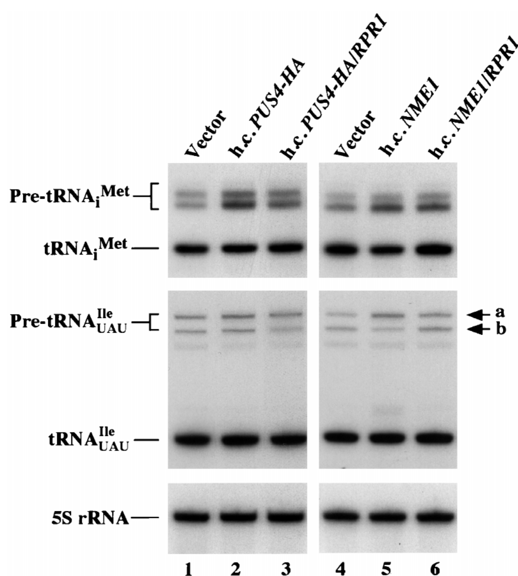

FIG. 4. Overexpressing *PUS4* or *NME1* leads to accumulation of untrimmed tRNA precursors. Total RNA (9 μg) prepared from *gcn2*Δ strains carrying empty vector YEplac181 or high-copy-number plasmids pHQ839 (h.c.*PUS4-HA*), pHQ864 (h.c.*PUS4-HA/RPR1*), (Vector) pHQ862 (h.c.*NME1*), and pHQ863 (h.c.*NME1/RPR1*) were subjected to Northern blot analysis and probed with a radiolabeled oligonucleotide complementary to $tRNA_i^{Met}$. The same blots were stripped and reprobed with radiolabeled oligonucleotides specific for $tRNA^{Ile}_{UAU}$ or 5S rRNA (see Materials and Methods). The positions of pre-$tRNA_i^{Met}$, mature $tRNA_i^{Met}$, pre-$tRNA^{Ile}_{UAU}$, mature $tRNA^{Ile}_{UAU}$, and 5S rRNA are indicated on the left. The primary transcript (upper band) and the 5′- and 3′-end-processed intron-containing pre-$tRNA^{Ile}_{UAU}$ (lower band) are indicated on the right by the letters a and b, respectively.

Although we observed only a small reduction in the steady-state level of mature $tRNA_i^{Met}$ in hc*PUS4-HA* transformants (Table 6), it was important to determine whether this defect was responsible for the suppressor phenotype of hc*PUS4*. We showed previously that a high-copy-number plasmid bearing *IMT4*, encoding $tRNA_i^{Met}$, overcame the Gcd^- phenotype of *gcd10* mutations that reduce steady-state levels of mature $tRNA_i^{Met}$ (1). In contrast, we saw little or no effect of hc*IMT4* on the phenotype of hc*PUS4* (Fig. 5A) even though it produced ca. fourfold-higher levels of mature $tRNA_i^{Met}$ (Fig. 5B). We conclude that the Gcd^- phenotype of hc*PUS4* does not arise from a reduction in the steady-state level of mature $tRNA_i^{Met}$.

The observation that hc*PUS4* leads to accumulation of untrimmed $tRNA_i^{Met}$ precursors suggested that an overabundance of these molecules in the nucleus might be a signal for activating *GCN4* translation by a GCN2-independent pathway. Because removal of the 5′ leader by RNase P appears to be required for subsequent removal of the 3′ trailer (44), we asked whether overexpression of the RNA component of RNase P, encoded by *RPR1*, would suppress the Gcd^- phenotype of hc*PUS4* in *gcn2*Δ cells. As shown in Fig. 6A, the presence of *RPR1* in the same high-copy-number plasmid bearing *PUS4-HA* overcame the 3-AT^r phenotype and partially suppressed the derepression of *GCN4* expression, conferred by

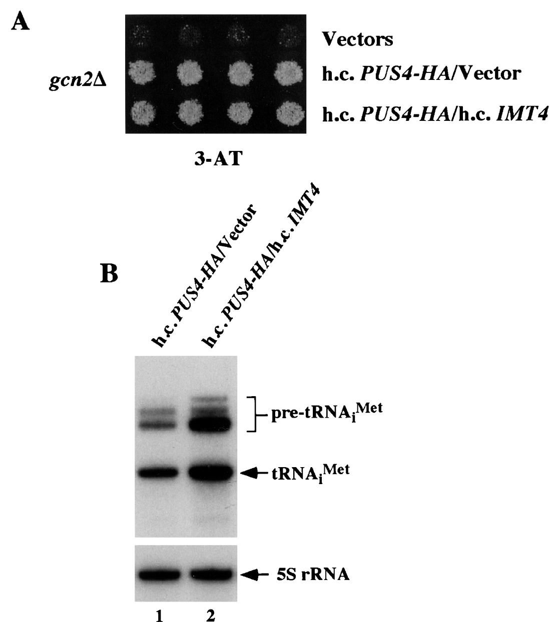

FIG. 5. Overexpression of initiator $tRNA^{Met}$ does not suppress the Gcd^- phenotype of hc*PUS4*. (A) Transformants of strain H1894 (*gcn2*Δ) bearing high-copy-number plasmids YEplac181 and YEp24 (Vectors), pHQ839 and YEp24 (h.c.*PUS4-HA*/vector), or pHQ839 and pC50 (h.c.*PUS4-HA*/h.c.*IMT4*) were replica-plated to SD medium containing 30 mM 3-AT and incubated for 3 days at 30°C. (B) Total RNA (6 μg) isolated from strains carrying the indicated high-copy-number plasmids were subjected to Northern blot analysis and probed with an oligonucleotide specific for initiator $tRNA^{Met}$.

hc*PUS4-HA*. Immunoblot analysis indicated that hc*RPR1* did not significantly affect PUS4-HA expression in these cells (Fig. 6B). Thus, overexpression of *RPR1* overrides the suppressor function of hc*PUS4* and does not simply reduce the extent of PUS4 overproduction. Northern blot analysis showed that the presence of *RPR1* with *PUS4-HA* in the same high-copy-number plasmid decreased the precursor/mature ratios for $tRNA_i^{Met}$ and $tRNA_{UAU}^{Ile}$ from 1.05 to 0.77 and 0.19 to 0.14, respectively (Fig. 4 and Table 6). These results are in agreement with the idea that hc*PUS4* elicits derepression of *GCN4*, at least in part, by interfering with 5′-end processing of certain tRNAs by RNase P.

Other evidence that accumulation of unprocessed pre-tRNAs stimulates *GCN4* translation by a GCN2-independent pathway relates to the previous observation that high-copy-number *NME1* also triggers this response (51). *NME1* encodes the RNA component of ribonuclease MRP, involved in pre-rRNA processing (48). Because RNases MRP and P are ribonucleoprotein complexes which share numerous protein subunits (11, 39), we considered that *NME1* overexpression might titrate protein subunits away from *RPR1* RNA. The ensuing reduction in RNase P levels would impair the processing of one or more pre-tRNAs, and the unprocessed precursors would trigger GCN2-independent derepression of *GCN4* translation. According to this hypothesis, simultaneous overexpression of *RPR1* and *NME1* should reverse the titration of subunits from RNase P and reduce the concentration of tRNA precursors, thereby restoring the repression of *GCN4* translation. As shown in Fig. 7A, the presence of hc*NME1* suppressed the $3\text{-}AT^s$ phenotype of a *gcn2*Δ mutant (Gcd^- phenotype) and the presence of *RPR1* on the same high-copy-number plasmid eliminated the suppressor activity of hc*NME1*. The antagonistic effect of hc*RPR1* on hc*NME1* suppressor activity did not involve a reduction in *NME1* expression (Fig. 7B).

Northern analysis revealed that the hc*NME1* transformants had increased amounts of precursors and decreased levels of mature $tRNA_i^{Met}$ compared to the vector transformants (Fig. 4, lanes 4 and 5), with a twofold increase in the precursor/mature ratio (0.92 versus 0.47 [Table 6]) for this tRNA. A twofold increase in the amount of unprocessed primary transcript for $tRNA_{UAU}^{Ile}$ also was observed in the hc*NME1* transformants with respect to the vector transformants (Fig. 4 and Table 6). The presence of hc*RPR1* together with hc*NME1* decreased the precursor/mature ratio from 0.92 to 0.57 for $tRNA_i^{Met}$ and from 0.16 to 0.10 for pre-$tRNA_{UAU}^{Ile}$ in the hc*NME1* transformant (Table 6). These data are consistent with the idea that hc*NME1* leads to derepression of *GCN4* by interfering with 5′-end processing of tRNAs by RNase P. Because introduction of hc*IMT4* did not reverse the Gcd^- phenotype of hc*NME1* (data not shown), it most probably results from accumulation of unprocessed pre-tRNAs rather than depletion of mature $tRNA_i^{Met}$.

To provide more direct evidence that untrimmed pre-tRNA elicits derepression of *GCN4* translation, we examined the consequences of overexpressing a mutant form of pre-$tRNA_{GUA}^{Tyr}$ that cannot be processed by yeast RNase P in vitro. Three base changes were introduced into wild-type pre-$tRNA_{GUA}^{Tyr}$ to extend the length of uninterrupted helix in the aminoacyl stem (35). In accordance with our hypothesis, the gene encoding the stem extension mutant of pre-$tRNA_{GUA}^{Tyr}$ on a high-copy-number plasmid conferred a Gcd^- phenotype in the *gcn2*Δ strain whereas the corresponding plasmid encoding wild-type pre-$tRNA_{GUA}^{Tyr}$ did not (Fig. 7C). The results of Northern analysis confirmed that the stem extension mutation impaired processing of the pre-$tRNA_{GUA}^{Tyr}$ in vivo (data not shown).

Evidence that *PUS4* overexpression elicits derepression of *GCN4* partly by interfering with nuclear export of tRNAs. It is

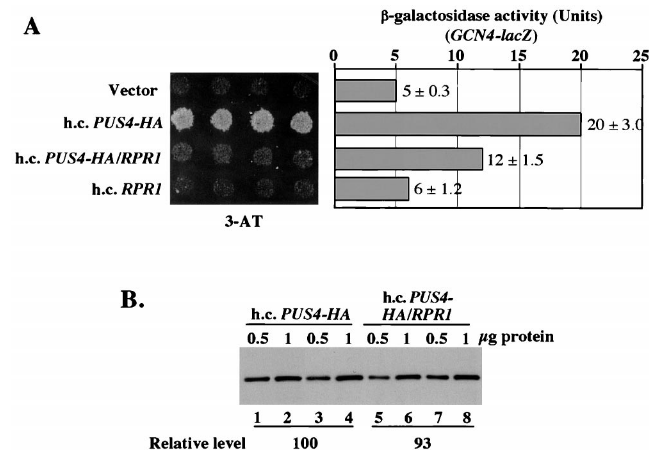

FIG. 6. Overexpression of *RPR1* reduces the Gcd^- phenotype of hc*PUS4*. (A) Transformants of strain H1895 (*gcn2*Δ) bearing high-copy-number plasmids YEplac181 (Vector), pHQ839 (h.c.*PUS4-HA*), pHQ864 (h.c.*PUS4-HA*/*RPR1*), or pHQ682 (h.c.*RPR1*) were replica-plated to SC medium containing 30 mM 3-AT and incubated for 3 days at 30°C (left panel). Extracts from the same transformants grown under repressing (nonstarvation) conditions were assayed for β-galactosidase activity, and the results shown in the right panel are the means and standard deviations from three individual transformants. (B) Expression of *PUS4-HA* in transformants carrying high-copy-number plasmids pHQ839 (h.c.*PUS4-HA*) or pHQ864 (h.c.*PUS4-HA*/*RPR1*) measured by Western blot analysis. PUS4-HA was detected by anti-HA antibody and visualized by enhanced chemiluminescence. The intensities of bands were calculated with a scanner (Silverscanner III) and NIH image software (version 1.61). The relative levels were calculated by averaging the band intensities from two independent extract preparations for each transformant (lanes 1 to 4, pHQ839 transformant; lanes 5 to 8, pHQ864 transformant).

thought that tRNA export in mammalian cells requires exportin-t (Xpo-t), which binds tRNA directly with high affinity (33). It also requires the GTP-bound form of Ran (RanGTP), which forms a complex with Xpo-t and tRNA (2, 33) involving extensive interactions with the backbone of the TψC and acceptor arms of the tRNA (3). LOS1 is a yeast homolog of Xpo-t (2, 33), and the nuclear accumulation of tRNA observed in a *los1*Δ mutant (47) plus the ability of LOS1 to interact with Ran-GTP in a tRNA-dependent fashion (23) have implicated LOS1 in tRNA export from the yeast nucleus. If LOS1 resembles Xpo-t in binding to the TψC and acceptor arms of tRNA, overexpressed PUS4 might compete with LOS1 for tRNA binding and interfere with tRNA export. This possibility is consistent with the findings that PUS4 can form stable complexes with tRNA in vitro (45), that a minimal substrate for enzymatic formation of pseudouridine 55 by PUS4 is a TψC stem-loop structure (8), and that pseudouridine 55 synthase in *Escherichia coli* requires the TψC stem-loop to catalyze pseudouridine formation (21). The accumulation of mature tRNA in the nucleus resulting from inhibition of LOS1 function by PUS4 might be a signal for derepression of *GCN4* translation. According to this hypothesis, overexpression of *LOS1* should reduce the derepression of *GCN4* elicited by hc*PUS4*.

In agreement with this prediction, *LOS1* on a high-copy-number plasmid partially overcame the ability of hc*PUS4-HA* to confer 3-AT^r and derepression of *GCN4-lacZ* translation in *gcn2*Δ cells without reducing the expression of PUS4-HA (Fig. 8A and B). In contrast, hc*LOS1* had little effect on these same phenotypes when conferred by hctRNA^{Val*} (Fig. 8A) or hc-*NME1* (Fig. 8C). This result is consistent with the idea that hc*PUS4* elicits derepression of *GCN4* in part by interfering with LOS1 function and producing the accumulation of mature tRNA in the nucleus. The hctRNA^{Val*} and hc*NME1* suppressors, by contrast, would derepress *GCN4* by producing defective or unprocessed tRNAs, respectively, without directly interfering with tRNA export. As expected, Northern analysis showed that hc*LOS1* did not reduce the accumulation of tRNA precursors in cells bearing hc*PUS4* (data not shown).

If nuclear accumulation of mature tRNA elicits GCN2-independent derepression of *GCN4*, inactivation of *LOS1* should increase *GCN4* expression in a *gcn2*Δ mutant. In agreement with this prediction, deletion of *LOS1* partially suppressed the 3-AT^s phenotype of the *gcn2*Δ strain (Fig. 8D). We also found that introduction of hc*PUS4* into the *los1*Δ *gcn2*Δ double mutant led to even greater 3-AT^r (Fig. 8D). This last observation can be explained by proposing that inhibiting LOS1 function in tRNA export is only one component of the derepression signal generated by hc*PUS4*. As indicated above, the fact that hc*RPR1* partially reversed the Gcd$^-$ phenotype of hc*PUS4* also points to a defect in tRNA 5′-end processing elicited by PUS4 overexpression.

In an effort to provide independent evidence that hc*PUS4* derepresses *GCN4* translation partly by interfering with LOS1-mediated tRNA export, we carried out fluorescence in situ hybridization to visualize the cellular distributions of various tRNAs in cells overexpressing PUS4. For tRNA$^{Ile}_{UAU}$, we consistently observed nuclear accumulation in most cells bearing hc*PUS4* versus vector alone (Fig. 9A and B). In addition, the presence of hc*LOS1* reduced the extent and frequency of tRNA$^{Ile}_{UAU}$ nuclear accumulation compared to the situation with hc*PUS4* alone (Fig. 9B and C). These results support the idea that PUS4 overexpression impedes nuclear export of tRNA$^{Ile}_{UAU}$ in a manner that can be overcome by increased expression of LOS1. Similar results were observed for tRNA$^{Val}_{AAC}$, although the extent of nuclear accumulation conferred by hc*PUS4* was less pronounced. No significant nuclear accumulation was detected for tRNA$^{Met}_{i}$, tRNA$^{Ile}_{AAU}$, tRNA$^{Tyr}_{GUA}$, and tRNA$^{Leu}_{CAA}$. Thus, it appears that PUS4 overexpression interferes with nuclear export of a subset of tRNAs. (The fact that hc*PUS4* did not produce detectable nuclear accumulation of tRNA$^{Met}_{i}$ despite accumulation of its untrimmed precursors in this strain [Fig. 4] may be explained by the fact that tRNA$^{Met}_{i}$ exhibits a more intense nuclear signal than the other tRNAs we examined in wild-type cells, presumably indicating a relatively large nuclear pool of mature tRNA$^{Met}_{i}$ under normal conditions.)

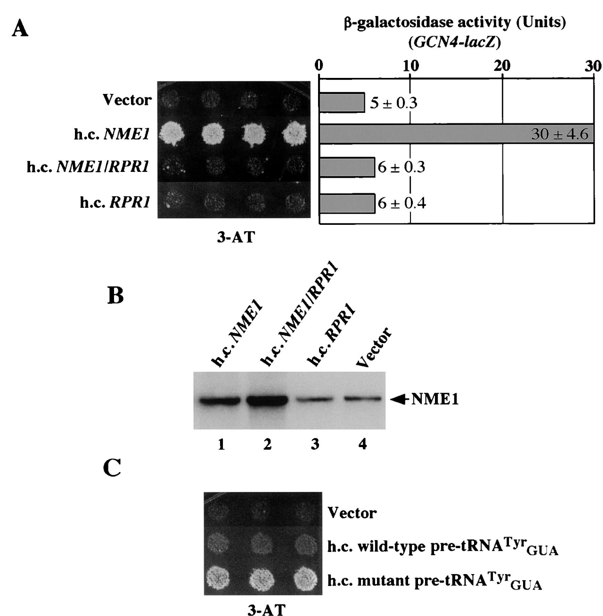

FIG. 7. Overexpression of *RPR1* reduces the Gcd$^-$ phenotype of hc*NME1*. (A) Transformants of strain H1895 (*gcn2*Δ) bearing high-copy-number plasmids YEplac181 (Vector), pHQ862 (h.c.*NME1*), pHQ863 (h.c.*NME1*/*RPR1*), or pHQ682 (h.c.*RPR1*) were replica-plated to SC medium containing 30 mM 3-AT and incubated for 3 days at 30°C (left panel). Extracts from the same transformants grown under repressing (nonstarvation) conditions were assayed for β-galactosidase activity, and the results shown in the right panel are the means and standard deviations of activities from three individual transformants. (B) Expression of *NME1* in transformants carrying high-copy-number plasmids YEplac181 (Vector), pHQ862 (h.c.*NME1*), pHQ863 (h.c.*NME1*/*RPR1*), and pHQ682 (h.c.*RPR1*) was measured by Northern blot analysis using radiolabeled oligonucleotide specific to *NME1*. (C) Transformants of strain H1895 (*gcn2*Δ) bearing high-copy-number plasmids YEplac181 (Vector), pHQ982 (h.c. wild-type pre-tRNA$^{Tyr}{}_{GUA}$) and pHQ985 (h.c. mutant pre-tRNA$^{Tyr}{}_{GUA}$) were replica-plated to SC medium containing 30 mM 3-AT and incubated for 3 days at 30°C.

Interestingly, we observed significant nuclear accumulation of tRNA^{Val*} in strains overexpressing this mutant tRNA versus the corresponding wild-type tRNA$^{Val}_{AAC}$ species (Fig. 10B and C). We previously proposed that the mutation in the 3′-terminal nucleotide of tRNA^{Val*} would impede aminoacylation in vivo because the same substitution reduced charging in vitro of a yeast tRNA$^{Val}_{AAC}$ model substrate (minihelix) (17) and of *E. coli* tRNA$^{Val}_{AAC}$ (50). To test this prediction, we used Northern analysis under acidic conditions to analyze the relative amounts of deacylated tRNA$^{Val}_{AAC}$ in cells overexpressing tRNA^{Val*} and in those overexpressing wild-type tRNA$^{Val}_{AAC}$. The results in Fig. 10D showed that essentially all of the overexpressed wild-type tRNA$^{Val}_{AAC}$ was acylated in vivo, since the vast majority of this sample (lane 6) comigrated with the acylated form of native tRNA$^{Val}_{AAC}$ rather than with the faster-migrating deacylated tRNA (lanes 2 and 1, respectively). Unexpectedly, it appeared that the mutant tRNA^{Val*} molecules in the deacylated sample (lane 3) migrated more slowly and were

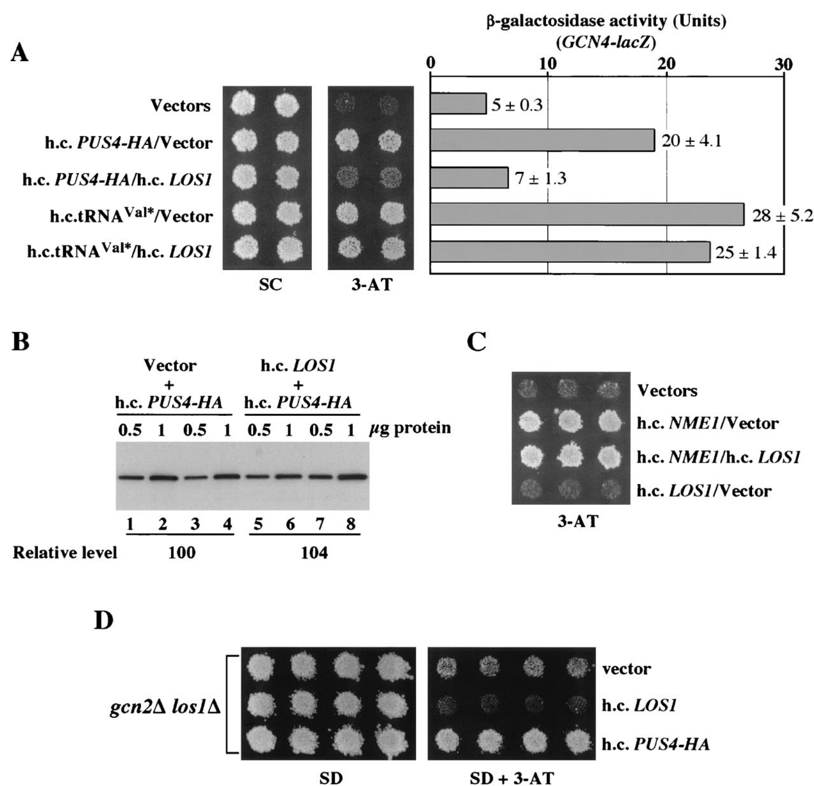

FIG. 8. Overexpression of *LOS1* reduces the Gcd$^-$ phenotype of hc*PUS4*. (A) Transformants of strain H1895 (*gcn2*Δ) bearing high-copy-number plasmids YEplac181/YEp24 (Vectors), pHQ839/YEp24 (h.c.*PUS4-HA*/Vector), pHQ839/YEpLOS1 (h.c.*PUS4-HA*/h.c.*LOS1*), p856/YEp24 (h.c.tRNA^{Val*}/Vector), and p856/YEpLOS1 (h.c.tRNA^{Val*}/h.c.*LOS1*) were replica-plated to SC medium containing 30 mM 3-AT and incubated for 3 days at 30°C (left panel). Extracts from the same transformants grown under repressing (nonstarvation) conditions were assayed for β-galactosidase activity, and the results shown in the right panel are the means and standard deviations from three individual transformants. (B) Expression of *PUS4-HA* in transformants carrying high-copy-number plasmids pHQ839/Vector (Vector + h.c.*PUS4-HA*) or pHQ839/YEpLOS1 (h.c.*LOS1* + h.c.*PUS4-HA*) measured by Western blot analysis. PUS4-HA was detected with an anti-HA antibody and visualized by enhanced chemiluminescence. Intensities of bands were calculated with a scanner (Silverscanner III) and NIH image software (version 1.61). The relative levels were calculated by averaging the band intensities from two independent extract preparations for each transformant (lanes 1 to 4, pHQ839/Vector transformant; lanes 5 to 8, pHQ839/YEpLOS1 transformant). (C) Transformants of strain H1895 (*gcn2*Δ) bearing high-copy-number plasmids YEplac181/YEp24 (Vectors), pHQ862/YEp24 (h.c.*NME1*/Vector), pHQ862/YEpLOS1 (h.c.*NME1*/h.c.*LOS1*), or YEplac181/YEpLOS1 (Vector/h.c.*LOS1*) were replica-plated to SC medium containing 30 mM 3-AT and incubated for 3 days at 30°C. (D) Deletion of *LOS1* has a Gcd$^-$ phenotype. Transformants of strain HQY316 (*gcn2*Δ*los1*Δ) bearing plasmids YEplac181 (vector), pHQ860 (h.c.*LOS1*), and pHQ839 (h.c.*PUS4-HA*) were replica-plated to SD medium containing the required supplements and 30 mM 3-AT and incubated for 3 days at 30°C.

more heterogeneous than the deacylated wild-type $tRNA^{Val}_{AAC}$ (lane 1), suggesting a defect in processing the mutant tRNA. The overexpressed mutant $tRNA^{Val*}$ in the acylated sample (lane 4) was only slightly more heterogeneous than the acylated wild-type $tRNA^{Val}_{AAC}$ (lane 6). This last observation, plus the fact that the aberrant species in deacylated $tRNA^{Val*}$ roughly comigrated with acylated wild-type $tRNA^{Val}_{AAC}$, led us to propose that the $tRNA^{Val*}$ molecules are aberrantly processed and aminoacylated inefficiently in vivo.

Recent findings indicate that tRNAs are aminoacylated in the nucleus and that this reaction stimulates their export to the cytoplasm both in mammalian cells (38) and in yeast (47a). This might explain our finding that mutant $tRNA^{Val*}$ accumulated in the nucleus (Fig. 10B). However, it is possible that the mutation in $tRNA^{Val*}$ also weakens its interaction with LOS1 (3), either directly or because of incomplete processing of the acceptor stem (Fig. 10D). In any case, the nuclear retention of $tRNA^{Val*}$ provides strong support for the idea that defects in the maturation of tRNA in the nucleus or in its export to the cytoplasm can trigger derepression of *GCN4* translation. Moreover, it can explain why hc$tRNA^{Val*}$ failed to stimulate eIF2α phosphorylation by GCN2, which are both presumably restricted to the cytoplasm (54).

DISCUSSION

Evidence that unprocessed tRNAs in the nucleus elicit derepression of *GCN4* translation independently of eIF2α phosphorylation. *GCN4* translation can be stimulated independently of GCN2 in mutants with lesions in subunits of eIF2 or eIF2B, in the genes encoding $tRNA^{Met}_i$, or in the *GCD10*- or *GCD14*-encoded proteins required for methylation of adenosine-58 in $tRNA^{Met}_i$. It is thought that all of these mutations mimic the effects of GCN2-mediated eIF2α phosphorylation by lowering the concentration of ternary complexes in the cytoplasm. It was suggested that a defect in ribosome biogenesis was responsible for derepressing *GCN4* translation in *gcn2* cells overexpressing *NME1* (51). Our finding that the Gcd$^-$ phenotype of hc*NME1* was suppressed by overexpressing *RPR1* points to a reduction in RNase P levels and diminished tRNA 5′-end processing as the cause of derepression. We propose that overexpression of *NME1* reduces RNase P levels by titrating from RPR1 one or more protein subunits shared between RNases MRP and P. Consistent with this hypothesis, we detected an ca. twofold increase in the precursor/mature ratio for $tRNA^{Met}_i$ in the hc*NME1* strain versus the wild type, and this phenotype was partially reversed by hc*RPR1*. The small reduction in mature initiator $tRNA^{Met}$ abundance caused by

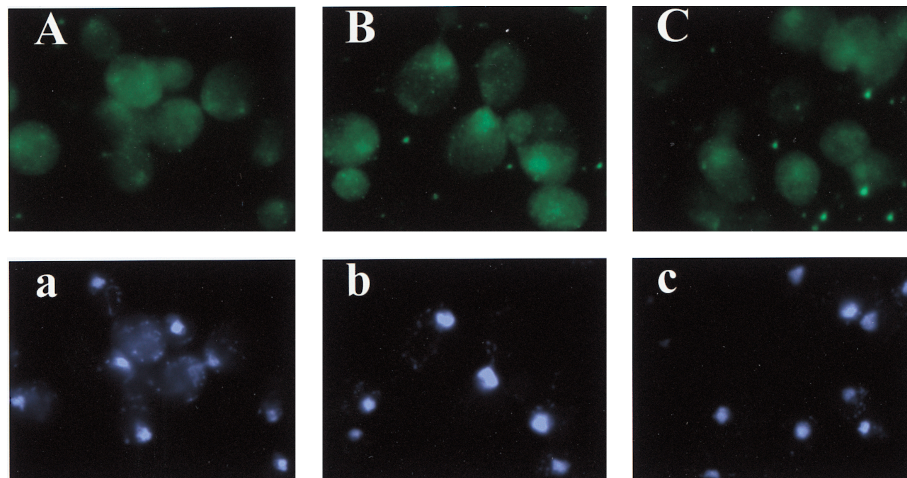

FIG. 9. hc*PUS4-HA* leads to nuclear accumulation of $tRNA^{Ile}_{UAU}$ detected by fluorescence in situ hybridization. Cells of transformants of strain H1895 (*gcn2*Δ) bearing high-copy-number plasmids YEplac181/YEp24 (vectors) (A and a), pHQ839/YEp24 (h.c.*PUS4-HA*/vector) (B and b), or pHQ839/YEpLOS1 (h.c.*PUS4-HA*/h.c.*LOS1*) (C and c) were subjected to fluorescence in situ hybridization using a probe specific for $tRNA^{Ile}_{UAU}$ (panels A, B, and C) or stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei (panels a, b, and c).

hc*NME1* cannot account for its Gcd⁻ phenotype, because it was not suppressed by hc*IMT4*. Instead, we propose that an increase in the levels of unprocessed tRNAs in the nucleus activates a regulatory mechanism that down-regulates ternary complex binding to 40S ribosomes by an amount sufficient to derepress *GCN4* translation. We cannot exclude the possibility that a defect in ribosome biogenesis also contributes to the derepression of *GCN4* conferred by hc*NME1*.

A more direct demonstration that unprocessed tRNAs trigger GCN2-independent derepression of *GCN4* was provided by our finding that an overexpressed mutant pre-$tRNA^{Tyr}_{GUA}$ that cannot be processed by RNase P also elicits a Gcd⁻ phenotype in *gcn2*Δ cells. Because unprocessed pre-tRNAs are not exported (9, 38, 47), this result provides strong evidence that the accumulated pre-tRNAs are recognized in the nucleus and send a signal to the cytoplasm, which leads to increased translation of *GCN4* mRNA. This signalling mechanism may additionally account for the Gcd⁻ phenotype of hc$tRNA^{Val*}$, because this mutant tRNA appeared to be processed aberrantly and was retained in the nucleus.

Evidence that overexpression of *PUS4* elicits GCN2-independent derepression of *GCN4* by impeding nuclear export and 5′-end processing of tRNAs. The derepression of *GCN4* translation in cells overexpressing *PUS4* also seems to be triggered partly by the accumulation of pre-tRNAs in the nucleus. The Gcd⁻ phenotype of hc*PUS4* was partially reversed by hc*RPR1*, suggesting that overexpressed PUS4 interferes with 5′-end processing by RNase P. Consistent with this model, we observed a ca. twofold increase in the precursor/mature ratio for $tRNA^{Met}_{i}$ in the hc*PUS4* transformant, which was reversed by cooverexpressing *RPR1*. The postulated interference with RNase P exerted by overexpressed PUS4 could involve direct competition between these two enzymes for binding to a subset of tRNA precursors. This idea is ostensibly at

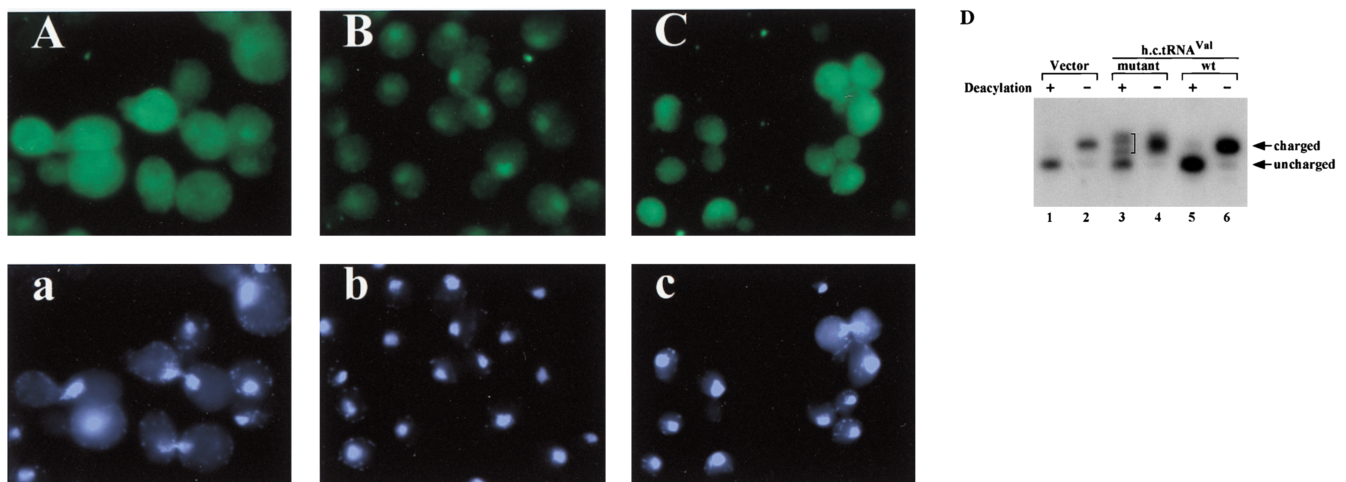

FIG. 10. Evidence that mutant $tRNA^{Val*}$ is defective for aminoacylation and processing and is retained in the nucleus. (A to C) Cells of transformants of strain H1937 (*gcn2*Δ) bearing empty vector YEp24 (A and a), p1362 ($tRNA^{Val*}$) (B and b), or p1308 (wild-type $tRNA^{Val}$) (C and c) were subjected to fluorescence in situ hybridization using a probe specific for $tRNA^{Val}_{AAC}$ (A, B, and C) or stained with DAPI to visualize nuclei (a, b, and c). (D) Total RNAs prepared under acidic conditions from the strains analyzed in panels A to C were resolved by electrophoresis on an acid-urea polyacrylamide gel and subjected to Northern blot analysis using a probe specific for $tRNA^{Val}_{AAC}$. In lanes 1, 3, and 5, the RNA samples were deacylated in 2 M Tris-HCl (pH 8.0) prior to electrophoresis.

odds with the fact that 5′-end processing of pre-$tRNA_i^{Met}$ was impaired by hc*PUS4* even though this tRNA is not a substrate for PUS4. When overexpressed 15-fold, however, PUS4 may bind tightly to pre-$tRNA_i^{Met}$ and block access of RNase P even though it fails to synthesize pseudouridine-55. Alternatively, PUS4 and RNase P may interact with a common tRNA chaperone that facilitates the activities of both enzymes, and overexpression of PUS4 could reduce the availability of this hypothetical chaperone for 5′-end processing of pre-$tRNA_i^{Met}$ by RNase P.

Unlike the situation with hc*NME1*, the Gcd^- phenotype of hc*PUS4* was partially suppressed by hc*LOS1* in addition to hc*RPR1*. Because LOS1 appears to be the yeast homologue of mammalian exportin-t, the suppression by hc*LOS1* could indicate that overexpression of PUS4 impedes tRNA export and that increased nuclear accumulation of one or more fully processed tRNAs contributes to the derepression of *GCN4* translation. Consistent with this interpretation, a *los1*Δ mutant had a Gcd^- phenotype, albeit weaker than that of hc*PUS4*, and we observed nuclear accumulation of $tRNA^{Ile}_{UAU}$ in strains bearing hc*PUS4* that was reversed by cooverexpressing LOS1. At the same time, we did not observe convincing nuclear accumulation of several other tRNAs examined in strains harboring hc*PUS4*. Thus, overexpressed PUS4 seems to inhibit LOS1-dependent nuclear export of only a subset of tRNAs. This inhibition might involve competition between LOS1 and PUS4 for binding to the affected tRNAs. Presumably, the selective nuclear retention of tRNAs is sufficient to trigger derepression of *GCN4* only when combined with the accumulation of certain pre-tRNAs which results from inhibition of RNase P by overexpressed PUS4.

Considering that removal of introns from pre-tRNAs is defective in *los1* mutants (28), it is conceivable that the Gcd^- phenotype of *los1*Δ cells results from accumulation of unspliced pre-tRNAs in the nucleus rather than from nuclear retention of fully processed tRNAs. Similarly, it could be argued that the $tRNA^{Ile}_{UAU}$ species retained in the nucleus of hc*PUS4* transformants (Fig. 9) are incompletely processed molecules rather than fully matured tRNAs. However, the latter possibility seems inconsistent with the fact that hc*PUS4* produces only a small increase in the relative abundance of pre-$tRNA^{Ile}_{UAU}$, which is a minor fraction of the combined pool of precursor and mature forms of this tRNA (Fig. 4). Thus, the increase in pre-$tRNA^{Ile}_{UAU}$ abundance seems insufficient to account for its considerable nuclear retention in hc*PUS4* transformants. Assuming that LOS1 is the tRNA exportin of yeast, it may be simpler to propose that hc*LOS1* overcomes the nuclear retention of mature $tRNA^{Ile}_{UAU}$ in cells overexpressing PUS4 rather than suggesting that it corrects a processing defect. Accordingly, we consider it likely that an overabundance of mature tRNA in the nucleus, as well as accumulation of unprocessed pre-tRNAs, can trigger derepression of *GCN4* by the GCN2-independent pathway.

It is thought that GCN2 is stimulated in the cytoplasm of amino acid-starved cells by uncharged tRNAs that interact with translating ribosomes. In view of recent findings that tRNAs are aminoacylated in the nucleus (38), we considered the possibility that uncharged tRNA in the nucleus could be a signal for GCN2-independent derepression of *GCN4*. Consistent with this model, we obtained evidence that $tRNA^{Val*}$ is retained in the nucleus and is aminoacylated inefficiently, possibly because of a processing defect. Either impeding 5′-end processing by overexpressing *NME1* or *PUS4*, or overproducing a mutant tRNA that cannot be processed by RNase P, should also produce an excess of pre-tRNAs in the nucleus that cannot be charged. Increased binding of overexpressed PUS4 to mature tRNAs in the nucleus might block their interaction with aminoacyl-tRNA synthetases or, by impeding export, generate increased nuclear pools of tRNA which outstrip the enzymatic capacity of synthetases in the nucleus. (The fact that hc*PUS4* did not perceptibly increase the proportion of total cellular tRNA that was uncharged could be explained by stipulating that only a small fraction of the mature tRNA is located in the nucleus.) Finally, this model could account for the GCN2-independent derepression of *GCN4* that accompanies overproduction of wild-type tRNAs under conditions of reduced aminoacylation (54). The idea that *GCN4* translation can be induced by uncharged tRNA in the nucleus is attractive; however, it seems equally possible that an excess of unprocessed or untransported tRNA in the nucleus, regardless of its aminoacylation status, is the primary signal for this derepression mechanism.

Under adverse environmental conditions where processing, modification or transport of tRNA is impaired, it could be advantageous to decrease the rate of protein synthesis. The inhibition of ternary-complex formation by phosphorylation of eIF2 is a widely employed mechanism to down-regulate translation under conditions of starvation or stress (24). Our results indicate that ternary-complex formation or utilization is reduced by a mechanism other than eIF2α phosphorylation in response to malfunctions in tRNA biogenesis. This may provide a useful strategy for coupling the rate of translation initiation in the cytoplasm with nuclear events involved in producing functional tRNA molecules that can participate in protein synthesis.

ACKNOWLEDGMENTS

We thank Lasse Lindahl for the *NME1* and *RPR1* plasmids and David Engelke for advice and gifts of plasmids. We thank Bobbie Felix for help in preparation of the manuscript and members of the Hinnebusch and Dever laboratories for discussion.

G.R.B. was supported by grants from the National Science Research Council (BU-2930) and the Swedish Cancer Society (project 680), and A.K.H. was supported by NIH grant GM27930.

REFERENCES

1. **Anderson, J., L. Phan, R. Cuesta, B. A. Carlson, M. Pak, K. Asano, G. R. Bjork, M. Tamame, and A. G. Hinnebusch.** 1998. The essential Gcd10p-Gcd14p nuclear complex is required for 1-methyladenosine modification and maturation of initiator methionyl-tRNA. Genes Dev. **12:**3650–3662.
2. **Arts, G. J., M. Fornerod, and I. J. Mattaj.** 1998. Identification of a nuclear export receptor for tRNA. Curr. Biol. **8:**305–314.
3. **Arts, G. J., S. Kuersten, P. Romby, B. Ehresmann, and I. W. Mattaj.** 1998. The role of exportin-t in selective nuclear export of mature tRNAs. EMBO J. **17:**7430–7441.
4. **Auxillien, S., P. F. Crain, R. W. Trewyn, and H. Grosjean.** 1996. Mechanism, specificity and general properties of the yeast enzyme catalysing the formation of inosine 34 in the anticodon of transfer RNA. J. Mol. Biol. **262:**437–458.
5. **Bakin, A., and J. Ofengand.** 1993. Four newly located pseudouridylate residues in *Escherichia coli* 23S ribosomal RNA are all at the peptidyltransferase center: analysis by the application of a new sequencing technique. Biochemistry **32:**9754–9762.
6. **Bakin, A., and J. Ofengand.** 1995. Mapping of the 13 pseudouridine residues in *Saccharomyces cerevisiae* small subunit ribosomal RNA to nucleotide resolution. Nucleic Acids Res. **23:**3290–3294.
7. **Becker, H. F., Y. Motorin, R. J. Planta, and H. Grosjean.** 1997. The yeast gene YNL292w encodes a pseudouridine synthase (Pus4) catalyzing the formation of ψ55 in both mitochondrial and cytoplasmic tRNAs. Nucleic Acids Res. **25:**4493–4499.
8. **Becker, H. F., Y. Motorin, M. Sissler, C. Florentz, and H. Grosjean.** 1997. Major identity determinants for enzymatic formation of ribothymidine and pseudouridine in the Tψ-loop of yeast tRNAs. J. Mol. Biol. **274:**505–518.
9. **Bertrand, E., F. Houser-Scott, A. Kendall, R. H. Singer, and D. R. Engelke.** 1998. Nucleolar localization of early tRNA processing. Genes Dev. **12:**2463–2468.
10. **Calvo, O., R. Cuesta, J. Anderson, N. Gutierrez, M. T. Garcia-Barrio, A. G. Hinnebusch, and M. Tamame.** 1999. GCD14p, a repressor of *GCN4* trans-

lation, cooperates with Gcd10p and Lhp1p in the maturation of initiator methionyl-tRNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19:**4167–4181.
11. **Chamberlain, J. R., Y. Lee, W. S. Lane, and D. R. Engelke.** 1998. Purification and characterization of the nuclear RNase P holoenzyme complex reveals extensive subunit overlap with RNase MRP. Genes Dev. **12:**1678–1690.
12. **Cortese, R., H. O. Kammen, S. J. Spengler, and B. N. Ames.** 1974. Biosynthesis of pseudouridine in transfer ribonucleic acid. J. Biol. Chem. **249:** 1103–1108.
13. **Cuesta, R., A. G. Hinnebusch, and M. Tamame.** 1998. Identification of *GCD14* and *GCD15*, novel genes required for translational repression of *GCN4* mRNA in *Saccharomyces cerevisiae*. Genetics **148:**1007–1020.
13a. **Dever, T. E., L. Feng, R. C. Wek, A. M. Cigan, T. D. Donahue, and A. G. Hinnebusch.** 1992. Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. Cell **68:**585–596.
14. **Dever, T. E., W. Yang, S. Åström, A. S. Byström, and A. G. Hinnebusch.** 1995. Modulation of $tRNA_i^{Met}$, eIF-2 and eIF-2B expression shows that *GCN4* translation is inversely coupled to the level of eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complexes. Mol. Cell. Biol. **15:**6351–6363.
15. **Diamond, A. M., I. S. Choi, P. F. Crain, T. Hashizume, S. C. Pomerantz, R. Cruz, C. J. Steer, K. E. Hill, R. F. Burk, J. A. McCloskey, and D. L. Hatfield.** 1993. Dietary selenium affects methylation of the wobble nucleoside in the anticodon of selenocysteine $tRNA^{[Ser]Sec*}$. J. Biol. Chem. **268:**14215–14223.
16. **Engelberg, D., C. Klein, H. Martinetto, K. Struhl, and M. Karin.** 1994. The UV response involving the ras signaling pathway and AP-1 transcription factors is conserved between yeast and mammals. Cell **77:**381–390.
17. **Frugier, M., C. Florentz, and R. Giege.** 1992. Anticodon-independent aminoacylation of an RNA minihelix with valine. Proc. Natl. Acad. Sci. USA **89:** 3990–3994.
18. **Garcia-Barrio, M. T., T. Naranda, R. Cuesta, A. G. Hinnebusch, J. W. B. Hershey, and M. Tamame.** 1995. GCD10, a translational repressor of *GCN4*, is the RNA-binding subunit of eukaryotic translation initiation factor-3. Genes Dev. **9:**1781–1796.
19. **Gehrke, C. W., and K. C. Kuo.** 1990. Ribonucleoside analysis by reversed-phase high-performance liquid chromatography, p. A3–A71. *In* C. W. Gehrke and K. C. Kuo (ed.), Chromatography and modification of nucleosides. Elsevier, Amsterdam, The Netherlands.
20. **Gietz, R. D., and A. Sugino.** 1988. New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:**527–534.
21. **Gu, X., M. Yu, K. M. Ivanetich, and D. V. Santi.** 1998. Molecular recognition of tRNA by tRNA pseudouridine 55 synthase. Biochemistry **37:**339–343.
22. **Harashima, S., and A. G. Hinnebusch.** 1986. Multiple *GCD* genes required for repression of *GCN4*, a transcriptional activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**3990–3998.
23. **Hellmuth, K., D. M. Lau, F. R. Bischoff, M. Kunzler, E. Hurt, and G. Simos.** 1998. Yeast Los1p has properties of an exportin-like nucleocytoplasmic transport factor for tRNA. Mol. Cell. Biol. **18:**6374–6386.
24. **Hinnebusch, A. G.** 1994. The eIF-2α kinases: regulators of protein synthesis in starvation and stress. Semin. Cell Biol. **5:**417–426.
25. **Hinnebusch, A. G.** 1996. Translational control of *GCN4*: gene-specific regulation by phosphorylation of eIF2, p. 199–244. *In* J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), Translational control. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Hinnebusch, A. G.** 1997. Translational regulation of yeast GCN4: a window on factors that control initiator-tRNA binding to the ribosome. J. Biol. Chem. **272:**21661–21664.
27. **Hinnebusch, A. G., and G. R. Fink.** 1983. Positive regulation in the general amino acid control of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **80:**5374–5378.
28. **Hopper, A. K., L. D. Schultz, and R. A. Shapiro.** 1980. Processing of intervening sequences: a new yeast mutant which fails to excise intervening sequences from precursor tRNAs. Cell **19:**741–751.
29. **Hurt, D. J., S. S. Wang, Y. H. Lin, and A. K. Hopper.** 1987. Cloning and characterization of *LOS1*, a *Saccharomyces cerevisiae* gene that affects tRNA splicing. Mol. Cell. Biol. **7:**1208–1216.
30. **Iida, H., H. Nakamura, T. Ono, M. S. Okumura, and Y. Anraku.** 1994. *MID1*, a novel *Saccharomyces cerevisiae* gene encoding a plasma membrane protein, is required for Ca^{2+} influx and mating. Mol. Cell. Biol. **14:**8259–8271.
31. **Kelmers, A. D., and D. E. Heatherly.** 1971. Columns for rapid chromatographic separation of small amounts of tracer-labeled transfer ribonucleic acids. Anal. Biochem. **44:**486–495.
32. **Koonin, E. V.** 1996. Pseudouridine synthases: four families of enzymes containing a putative uridine-binding motif also conserved in dUTPases and dCTP deaminases. Nucleic Acids Res. **24:**2411–2415.
33. **Kutay, U., G. Lipowsky, E. Izaurraide, F. R. Bischoff, P. Schwartzmaier, E. Hartmann, and D. Gorlich.** 1998. Identification of a tRNA-specific nuclear export receptor. Mol. Cell. **1:**359–369.
34. **Lanker, S., J. L. Bushman, A. G. Hinnebusch, H. Trachsel, and P. P. Mueller.** 1992. Autoregulation of the yeast lysyl-tRNA synthetase gene *GCD5/KRS1* by translational and transcriptional control mechanisms. Cell **70:**647–657.
35. **Lee, Y., D. W. Kindelberger, J. Y. Lee, S. McClennen, J. Chamberlain, and D. R. Engelke.** 1997. Nuclear pre-tRNA terminal structure and RNase P recognition. RNA **3:**175–185.
36. **Li, X., and P. M. Burgers.** 1994. Molecular cloning and expression of the *Saccharomyces cerevisiae RFC3* gene, an essential component of replication factor C. Proc. Natl. Acad. Sci. USA **91:**868–872.
37. **Lucchini, G., A. G. Hinnebusch, C. Chen, and G. R. Fink.** 1984. Positive regulatory interactions of the *HIS4* gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:**1326–1333.
38. **Lund, E., and J. E. Dahlberg.** 1998. Proofreading and aminoacylation of tRNAs before export from the nucleus. Science **282:**2082–2085.
39. **Lygerou, Z., P. Mitchell, E. Petfalski, B. Seraphin, and D. Tollervey.** 1994. The POP1 gene encodes protein component common to the RNase MRP and RNase P ribonucleoproteins. Genes Dev. **8:**1423–1433.
40. **Marton, M. J., C. R. Vazquez de Aldana, H. Qiu, K. Chakraburtty, and A. G. Hinnebusch.** 1997. Evidence that GCN1 and GCN20, translational regulators of *GCN4*, function on elongating ribosomes in activation of the eIF2α kinase GCN2. Mol. Cell. Biol. **17:**4474–4489.
41. **Marton, M. J., D. Crouch, and A. G. Hinnebusch.** 1993. GCN1, a translational activator of *GCN4* in *S. cerevisiae*, is required for phosphorylation of eukaryotic translation initiation factor 2 by protein kinase GCN2. Mol. Cell. Biol. **13:**3541–3556.
42. **Messenguy, F., and J. Delforge.** 1976. Role of transfer ribonucleic acids in the regulation of several biosyntheses in *Saccharomyces cerevisiae*. Eur. J. Biochem. **67:**335–339.
43. **Niederberger, P., M. Aebi, and R. Huetter.** 1983. Influence of the general control of amino acid biosynthesis on cell growth and cell viability in *Saccharomyces cerevisiae*. J. Gen. Microbiol. **129:**2571–2583.
44. **O'Connor, J. P., and C. L. Peebles.** 1991. In vivo pre-tRNA processing in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**425–439.
45. **Samuelsson, T.** 1991. Interactions of transfer RNA pseudouridine synthases with RNAs substituted with fluorouracil. Nucleic Acids Res. **19:**6139–6144.
46. **Samuelsson, T., and M. Olsson.** 1990. Transfer RNA pseudouridine synthases in *Saccharomyces cerevisiae*. J. Biol. Chem. **265:**8782–8787.
47. **Sarkar, S., and A. K. Hopper.** 1998. tRNA nuclear export in *Saccharomyces cerevisiae*: in situ hybridization analysis. Mol. Biol. Cell **9:**3041–3055.
47a. **Sarkar, S., A. K. Azad, and A. K. Hopper.** 1999. Nuclear tRNA aminoacylation and its role in nuclear export of endogenous tRNAs in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **96:**14366–14371.
48. **Schmitt, M. E., and D. A. Clayton.** 1992. Yeast site-specific ribonucleoprotein endoribonuclease MRP contains an RNA component homologous to mammalian RNAse MRP RNA and essential for cell viability. Genes Dev. **6:** 1975–1985.
49. **Sprinzl, M., T. Hartmann, F. Meissner, J. Moll, and T. Vorderwulbecke.** 1987. Compilation of tRNA sequences and sequences of tRNA genes. Nucleic Acids Res. **15:**r53–r188.
50. **Tamura, K., H. Himeno, H. Asahara, T. Hasegawa, and M. Shimizu.** 1991. Identity determinants of *E. coli* $tRNA^{Val}$. Biochem. Biophys. Res. Commun. **177:**619–623.
51. **Tavernarakis, N., D. Alexandraki, P. Liodis, D. Tzamarias, and G. Thireos.** 1996. Gene overexpression reveals alternative mechanisms that induce *GCN4* mRNA translation. Gene **179:**271–277.
52. **Varshney, U., C. P. Lee, and U. L. RajBhandary.** 1991. Direct analysis of aminoacylation levels of tRNA as *in vivo*. J. Biol. Chem. **266:**24712–24718.
53. **Vazquez de Aldana, C. R., M. J. Marton, and A. G. Hinnebusch.** 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.
54. **Vazquez de Aldana, C. R., R. C. Wek, P. San Segundo, A. G. Truesdell, and A. G. Hinnebusch.** 1994. Multicopy tRNA genes functionally suppress mutations in yeast eIF-2α kinase GCN2: evidence for separate pathways coupling *GCN4* expression to uncharged tRNA. Mol. Cell. Biol. **14:**7920–7932.
55. **Wek, R. C., B. M. Jackson, and A. G. Hinnebusch.** 1989. Juxtaposition of domains homologous to protein kinases and histidyl-tRNA synthetases in GCN2 protein suggests a mechanism for coupling *GCN4* expression to amino acid availability. Proc. Natl. Acad. Sci. USA **86:**4579–4583.
56. **Wek, R. C., M. Ramirez, B. M. Jackson, and A. G. Hinnebusch.** 1990. Identification of positive-acting domains in GCN2 protein kinase required for translational activation of *GCN4* expression. Mol. Cell. Biol. **10:**2820–2831.
57. **Wek, S. A., S. Zhu, and R. C. Wek.** 1995. The histidyl-tRNA synthetase-related sequence in the eIF-2α protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. Mol. Cell. Biol. **15:**4497–4506.
58. **Zhu, S., A. Y. Sobolev, and R. C. Wek.** 1996. Histidyl-tRNA synthetase-related sequences in GCN2 protein kinase regulate *in vitro* phosphorylation of eIF-2. J. Biol. Chem. **271:**24989–24994.