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Multicopy tRNA Genes Functionally Suppress Mutations in Yeast eIF-2α Kinase GCN2: Evidence for Separate Pathways Coupling *GCN4* Expression to Uncharged tRNA

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GCN2 is a protein kinase that stimulates translation of *GCN4* mRNA in amino acid-starved cells by phosphorylating the α subunit of translation initiation factor 2 (eIF-2). We isolated multicopy plasmids that overcome the defective derepression of *GCN4* and its target genes caused by the leaky mutation *gcn2-507*. One class of plasmids contained $tRNA^{His}$ genes and conferred efficient suppression only when cells were starved for histidine; these plasmids suppressed a *gcn2* deletion much less efficiently than they suppressed *gcn2-507*. This finding indicates that the reduction in *GCN4* expression caused by *gcn2-507* can be overcome by elevating $tRNA^{His}$ expression under conditions in which the excess tRNA cannot be fully aminoacylated. The second class of suppressor plasmids all carried the same gene encoding a mutant form of $tRNA^{Val}$(AAC) with an A-to-G transition at the 3′ encoded nucleotide, a mutation shown previously to reduce aminoacylation of $tRNA^{Val}$ in vitro. In contrast to the wild-type $tRNA^{His}$ genes, the mutant $tRNA^{Val}$ gene efficiently suppressed a *gcn2* deletion, and this suppression was independent of the phosphorylation site on eIF-2α (Ser-51). Overexpression of the mutant $tRNA^{Val}$ did, however, stimulate *GCN4* expression at the translational level. We propose that the multicopy mutant $tRNA^{Val}$ construct leads to an accumulation of uncharged $tRNA^{Val}$ that derepresses *GCN4* translation through a pathway that does not involve GCN2 or eIF-2α phosphorylation. This GCN2-independent pathway was also stimulated to a lesser extent by the multicopy $tRNA^{His}$ constructs in histidine-deprived cells. Because the mutant $tRNA^{Val}$ exacerbated the slow-growth phenotype associated with eIF-2α hyperphosphorylation by an activated $GCN2^c$ kinase, we suggest that the GCN2-independent derepression mechanism involves down-regulation of eIF-2 activity.

In *Saccharomyces cerevisiae*, starvation for an amino acid, or a defective aminoacyl-tRNA synthetase, leads to increased transcription of more than 30 genes encoding amino acid biosynthetic enzymes in numerous pathways. This regulatory mechanism, known as general amino acid control (reviewed in reference 19), involves the transcriptional activator GCN4, which binds upstream of each amino acid biosynthetic gene subject to the general control. Expression of *GCN4* itself is regulated by amino acid availability at the level of translation initiation. Four short upstream open reading frames (uORFs) in the leader of *GCN4* mRNA prevent translation initiation at the *GCN4* start codon under nonstarvation conditions. In amino acid-starved cells, many ribosomes which have translated the first uORF are able to scan past the remaining three uORFs in the leader (uORFs 2 to 4) and reinitiate further downstream at the *GCN4* coding sequences. The GCN4 protein thus produced activates transcription from its target genes and thereby stimulates amino acid biosynthesis.

The protein kinase GCN2 is required to stimulate (derepress) *GCN4* expression in response to amino acid starvation. GCN2 phosphorylates the α subunit of translation initiation factor 2 (eIF-2) in amino acid-starved yeast cells (9). In mammalian cells, phosphorylation of eIF-2α inhibits a second initiation factor called eIF-2B that catalyzes exchange of bound-GDP for GTP on eIF-2 following each round of initiation (16). Because only the GTP-bound form of eIF-2 can deliver initiator $tRNA^{Met}$ to the ribosome, impairing the activity of eIF-2B leads to a reduction in the amount of active eIF-2 that is available for translation initiation. There is now strong evidence that phosphorylation of eIF-2 stimulates *GCN4* translation by reducing the activity of the yeast equivalent of eIF-2B (4, 8, 9). It has been proposed that the ensuing reduction in the level of active eIF-2 is responsible for allowing ribosomes to scan past uORFs 2 to 4 in the *GCN4* leader without rebinding charged initiator $tRNA^{Met}$, suppressing reinitiation at these sites and allowing utilization of the *GCN4* start site instead (9).

Uncharged tRNA is thought to play an important role in coupling the phosphorylation of eIF-2α by GCN2 to the availability of amino acids in the cell because mutants defective for an aminoacyl-tRNA synthetase show elevated expression of genes under the control of *GCN4* without being starved for the cognate amino acid (25, 28, 35). In addition, strong genetic evidence was obtained that derepression of *GCN4* in mutants containing a defective lysyl-tRNA synthetase is dependent on GCN2 function (25). Because it was also shown directly that GCN2 is required for increased *GCN4* expression in cells starved for histidine (17) or tryptophan (55), it has been assumed that GCN2 can be activated by uncharged $tRNA^{His}$, $tRNA^{Trp}$, or $tRNA^{Lys}$. GCN2 contains a regulatory domain of about 530 amino acids adjacent to the kinase catalytic domain that is homologous to histidyl-tRNA synthetases (HisRSs) from *S. cerevisiae*, humans, and *Escherichia coli* (51). This HisRS-like domain is required for the ability of GCN2 to stimulate *GCN4* expression in vivo but is dispensable for the

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autophosphorylation activity of GCN2 observed in vitro (52). Given that aminoacyl-tRNA synthetases distinguish between charged and uncharged forms of tRNA, we proposed that the HisRS-related region of GCN2 monitors the aminoacylation levels of many different tRNAs and activates the adjacent protein kinase moiety in response to an accumulation of any uncharged tRNA (51). This hypothesis is in accord with the isolation of *GCN2*^{c} mutations mapping in the HisRS-related domain that lead to constitutive activation of GCN2 function without increasing the steady-state level of the protein (38). Interestingly, these mutations alter amino acids in the portion of the HisRS-like domain that corresponds to the highly conserved catalytic core in class II aminoacyl-tRNA synthetases that binds the 3′ end of tRNA (6, 12, 40). At present, however, there is no in vitro evidence that GCN2 kinase function can be activated directly by uncharged tRNA.

To identify additional regulatory factors that interact with the GCN2 protein kinase, we screened a yeast genomic library for genes that in multicopy would suppress the low constitutive *GCN4* expression associated with the partially defective *gcn2-507* allele. In this report, we describe two types of tRNA genes that in multicopy restore derepression of *GCN4* in *gcn2-507* mutants. Our studies strongly suggest that the encoded tRNAs affect *GCN4* expression only under conditions in which they cannot be fully aminoacylated. One component of the suppression mechanism appears to be dependent on the residual protein kinase activity conferred by the *gcn2-507* product and may be attributable to increased activation of the defective kinase by high levels of uncharged tRNA. The remaining component is independent of both GCN2 and the phosphorylation site on eIF-2α (serine 51) but requires the uORFs in *GCN4* mRNA. The latter findings clearly indicate the existence of an additional mechanism for induction of *GCN4* translation in response to uncharged tRNA that does not involve phosphorylation of eIF-2α.

MATERIALS AND METHODS

Identification of suppressor plasmids containing tRNA genes. The procedures used for screening high-copy-number libraries of *S. cerevisiae* genomic fragments for plasmids that could complement the nonderepressible phenotype of *gcn2-507* were described previously by Wek et al. (50) in isolating a truncated allele of the *GLC7* gene. Plasmids that did not contain the *GCN4* or *GCN2* gene, as judged by the absence of restriction fragments diagnostic of the coding regions of these genes, were subjected to blot hybridization analysis to detect the presence of tRNA genes. Plasmid DNA was digested with *Eco*RI, *Bgl*II, or *Hin*dIII, and the restriction fragments were separated by gel electrophoresis, transferred to nitrocellulose filters, and probed with either total yeast tRNA (Boehringer Mannheim) or a mixture of four oligonucleotides containing both strands of the tRNA^{His} coding sequence of *S. cerevisiae* (7). Both probes were labeled at the 5′ end by using T4 polynucleotide kinase (41). All plasmids analyzed in this study contained a genomic fragment that hybridized with total yeast tRNA, and a subset of these fragments hybridized with the tRNA^{His} probes. The latter were characterized further by using oligonucleotides complementary to the tRNA^{His} gene to determine the DNA sequences (42) of the tRNA genes and their flanking regions. From this analysis, we identified three plasmids from the YEp13-based library (32), called p857, p866, and p867. Plasmids p857 and p867 were judged to contain the same tRNA^{His} locus present in plasmids pYG2 and pGN545 described previously (7, 33), as we observed only a few nucleotide differences in the 5′- and 3′-flanking sequences between p867, p857, and pYG2; p866 contains a tRNA^{His} gene that was recently identified in the 3′ region of the *NUP116/NSP116* gene, encoding a protein component of the nuclear pore complex (54, 56). Three additional plasmids containing a tRNA^{His} gene were isolated from a genomic library constructed in YEp24 (3): p1573, p1574, and p1575. Plasmids p1574 and p1575 contain the same tRNA^{His} gene isolated on p857 and p867 described above and on pYG2 (7); p1573 contains the same tRNA^{His} gene that was recently identified downstream of the *NUP100* gene, another member of the nuclear pore complex (54). It was proposed that the close linkage of tRNA^{His} genes and the homologous *NUP116/NSP116* and *NUP100* genes arose by gene duplication (54).

To determine the identities of tRNAs encoded by the suppressor plasmids which failed to hybridize with tRNA^{His} probes, finer mapping of these tRNA genes was carried out by cutting each plasmid DNA with additional restriction enzymes and conducting blot hybridization analysis using labeled total yeast tRNA as the probe as described above. The tRNA genes in plasmids p856 and p860 were localized to an 800-bp *Acc*I restriction fragment, and the tRNA gene present in p868 was mapped to a 1-kb *Acc*I fragment. These *Acc*I fragments were subcloned into plasmid pUC19 (57), and the complete DNA sequences of the inserts were determined. All three inserts were found to contain the same mutant tRNA^{Val} gene with a single transition from A to G at position 74 of the tRNA sequence (reading 5′ to 3′) compared with the sequence of wild-type tRNA^{Val}(AAC). This mutant tRNA^{Val} gene was identified previously downstream of the *HEM15* coding sequence (14, 24). The inserts of the other plasmids containing non-tRNA^{His} genes (p862 from the YEp13 library and p1576, p1577, and p1578 from the YEp24 library), were shown to contain the same mutant tRNA^{Val} (AAC) gene by DNA sequence analysis using oligonucleotide primers complementary to the tRNA^{Val} gene sequence.

Plasmid constructions. The tRNA^{His} gene in pGN545 was inserted into the high-copy-number plasmids YEp13 and YEp24 by a two-step cloning procedure. First, a 420-bp *Eco*RV-*Pvu*II fragment was removed from pGN545 and inserted into the *Sma*I site of pUC19, yielding plasmid p3-2. The insert was oriented such that the *Pvu*II junction was adjacent to the *Bam*HI site. The tRNA^{His} gene was removed from p3-2 by using the flanking *Bam*HI and *Asp* 718 sites, the *Asp* 718 site was end filled with the Klenow fragment of DNA polymerase I, and the resulting fragment was inserted into the *Bam*HI site of YEp13, yielding p897. The orientation of the insert in p897 places the 5′ end of the tRNA^{His} gene adjacent to the 2μm sequence. Alternatively, the *Bam*HI-*Asp* 718 fragment from p3-2 was inserted into the *Bam*HI site of YEp24, resulting in p896. The orientation of the insert in this plasmid places the 5′ end of the tRNA^{His} gene adjacent to the *URA3* gene.

A 500-bp *Bam*HI fragment containing the mutant tRNA^{Val} gene was isolated from p856 and inserted into the *Bam*HI site of YEp24, resulting in plasmid p1362. Note that the *Bam*HI site downstream of the tRNA^{Val} gene is not present in the *HEM15* sequence (14, 24); presumably, this site came about during construction of the genomic library, which involved the insertion of restriction fragments from a partial *Sau*3AI digestion of genomic DNA into the *Bam*HI site of YEp13. The orientation of the insert in p1362 puts the 3′ end of the tRNA^{Val} gene adjacent to the *URA3* gene. Plasmid p1361 was constructed by inserting the same *Bam*HI fragment into the *Bam*HI site of low-copy-number plasmid pRS316 (43), such that the 5′ end of the tRNA^{Val} gene was adjacent to the *Spe*I site in the multiple-cloning sequence of the vector. To generate a wild-type version of the tRNA^{Val} gene in a high-copy-number

TABLE 1. Genotypes of yeast strains used in this study

Strain	Genotype	Source or reference
H1149	*MATα gcn2::LEU2 ino1 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	51
H1260	*MATa gcn2::LEU2 ils1-1 leu2-3 leu2-112*	This study
H1402	*MATα ino1 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	15
H1438	*MATα gcn2-507 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	50
H1450	*MATa gcn2-507 gcn3-101 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ* p457[*GCN3 URA3*]	50
H1470	*MATα gcn2-507 gcn3::URA3 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	50
H1472	*MATα gcn2::LEU2 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	50
H1473	*MATα gcn2::URA3 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	50
H1486	*MATα GCN2 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	50
H1576	*MATa gcn2-507 ils1-1 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1613	*MATα GCN2^c-E532K,E1522K ino1 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	38
H1870	*MATa gcn2::LEU2 ils1-1 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1894	*MATα gcn2Δ ura3-52 leu2-3 leu2-112 trp1-Δ63*	T. E. Dever
H1934	*MATα gcn2-507 sui2Δ his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ* p1097[*SUI2 LEU2*]	This study
H1935	*MATα gcn2-507 sui2Δ his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ* p1098[*SUI2-S51A LEU2*]	This study
H1936	*MATα gcn2-507 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1937	*MATα gcn2::LEU2 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1938	*MATα GCN2 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1939	*MATα gcn2-507 sui2Δ ura3-52 leu2-3 leu2-112 HIS4-lacZ* p1097[*SUI2 LEU2*]	This study
H1940	*MATα gcn2-507 sui2Δ ura3-52 leu2-3 leu2-112 HIS4-lacZ* p1098[*SUI2-S51A LEU2*]	This study
H1941	*MATα ino1 ura3-52 leu2-3 leu2-112 rho^0 HIS4-lacZ*	38
H1953 (Y64)	*MATα gcn2-507 sui2Δ ura3-52 leu2-3 leu2-112 ino1 HIS4-lacZ* p1097[*SUI2 LEU2*]	50
H1954 (Y65)	*MATα gcn2-507 sui2Δ ura3-52 leu2-3 leu2-112 ino1 HIS4-lacZ* p1098[*SUI2-S51A LEU2*]	50
H1955 (Y66)	*MATα gcn2-K559V sui2Δ ura3-52 leu2-3 leu2-112 ino1 HIS4-lacZ* p1097[*SUI2 LEU2*]	50
L780	*MATa ils1-1*	G. R. Fink

plasmid, the nucleotide at position 74 in the tRNA coding sequence was changed from a G to an A residue by site-directed mutagenesis (27, 47), and a 500-bp *Bam*HI fragment containing the wild-type tRNA^{Val} gene was inserted into YEp24, resulting in plasmid p1308.

A 5-kb *Bam*HI fragment from pFM20 (29) containing the wild-type *ILS1* gene was cloned into the *Bam*HI site of the vector pRS316, generating plasmid p1301. Plasmids containing tRNA^{Ile}(UAU) in low or high copy number were constructed as follows. First, a 500-bp *Eco*RI-*Nco*I fragment containing the tRNA^{Ile} gene present upstream of the *GCD6* gene (2) was isolated from plasmid pJB6, the *Nco*I site was end filled with the Klenow fragment of DNA polymerase I, and the fragment was inserted between the *Sma*I and *Eco*RI sites of the low-copy-number plasmid pRS316 to generate p1302. To construct a high-copy-number plasmid containing the tRNA^{Ile} gene, a 500-bp *Bam*HI-*Sal*I fragment containing the gene was isolated from p1302 and inserted between the *Bam*HI and *Sal*I sites of the high-copy-number plasmid YEp24, yielding plasmid p1304.

pAH7 is a nonreplicating derivative of YIp5 (45) bearing a 11-kb *Bam*HI fragment containing the *HIS1* gene (20) inserted at the *Bam*HI site. Plasmid p180 is a derivative of the low-copy-number *URA3* plasmid YCp50 (36) containing a *GCN4-lacZ* translational fusion with all four uORFs in the mRNA leader intact (18). Plasmid p227, derived from p180, contains base substitutions in the ATG start codons of all four uORFs, leaving only the *GCN4* AUG intact (31). Plasmid p585 is a derivative of YCp50 containing the *GCN2* gene (52). pAH15 is a derivative of the high-copy-number *LEU2* vector YEp13 containing *GCN2* (21). Plasmids pGN163 and pGN172 contain the *HTS1* gene on the high-copy-number vector YEp24 or the single-copy vector YCp50, respectively (33). Plasmid pGN545 (33) is a derivative of YEp24 containing the same tRNA^{His} gene present on plasmid pYG2 (7).

Yeast strains. All yeast strains used in this study are listed in Table 1. Strains H1934 and H1935 were constructed by a plasmid-shuffling technique described by Dever et al. (9). Strains H1936, H1937, H1938, H1939, and H1940 are isogenic *HIS1* derivatives of strains H1438, H1472, H1486, H1934, and H1935, respectively, that were constructed by transforming each strain to Ura$^+$ His$^+$ with plasmid pAH7. Ura$^-$ His$^+$ segregants of these transformants which had lost the *URA3* plasmid marker were selected by their resistance to 5-fluoro-orotic acid (1). Strain H1576 was constructed by tetrad analysis of a cross between *ils1-1 gcn2::LEU2* strain H1260 and *gcn2-507* strain H1438, being identified as a Tsm$^-$ (*ils1-1*) and 3′-aminotriazole-sensitive (3-AT^s) Leu$^-$ (*gcn2-507*) ascospore clone. The *ils1-1* allele was introduced into strain H1260 by crossing strain L780 by strain H1149 and backcrossing a Tsm$^-$ 3-AT^s Leu$^+$ ascospore with H1149. Strain H1870 was constructed from H1576 by a one-step gene replacement of *gcn2-507* with the *gcn2::LEU2* allele carried on plasmid p500, as described previously (52).

Assay of *HIS4-lacZ* and *GCN4-lacZ* fusions. Assays were conducted on cell extracts prepared from cultures grown in SD medium containing only the required supplements as described previously (26). For repressing conditions, saturated cultures were diluted 1:50 and harvested in mid-logarithmic phase after 6 h of growth. For derepressing conditions, cultures were grown for 2 h under repressing conditions and then for 6 h after the addition of 3-AT to 10 mM, 5-methyltryptophan (5-MT) to 2 mM, or sulfometuron methyl (SM) to 0.5 μg/ml. Values shown are the averages from two to five independently derived transformants. β-Galactosidase activities are expressed in units, defined as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

Analysis of RNA levels. Transformants were grown under repressing and derepressing conditions as described above, using 3-AT to produce histidine starvation. Total RNA was extracted and subjected to formaldehyde-agarose gel electrophoresis and RNA blot hybridization analysis using radiolabeled DNA probes, all as described previously (21). *GCN4*

and *GCN4-lacZ* mRNAs were both probed with a 300-bp *Bgl*II-*Xho*I fragment isolated from plasmid p285 (30), *PYK1* mRNA was probed with a *Hin*dIII fragment isolated from plasmid pFR2 (22), $tRNA^{Val}$ was probed with a 500-bp *Bam*HI fragment isolated from plasmid p1361, and $tRNA^{His}$ was probed with an oligonucleotide 41 residues in length containing roughly the 3′ half of the coding sequence for $tRNA^{His}$.

Isoelectric focusing PAGE. Strains were grown for 6 h in SD medium or, in the case of valine starvation, SD supplemented with 1 mM leucine and 0.5 mM isoleucine. For starvation conditions, cultures were supplemented with 3-AT at 10 mM or SM at 2 μg/ml (in the presence of 1 mM leucine and 0.5 mM isoleucine) 1 h prior to harvesting. Preparation of total protein extracts, vertical slab gel isoelectric focusing polyacrylamide gel electrophoresis (PAGE), and detection of eIF-2α by immunoblot analysis using antiserum prepared against a TrpE–eIF-2α fusion protein (5) were carried out as described previously (9) except that antigen-antibody complexes were detected by using an enhanced chemiluminescence system (Amersham) as instructed by the vendor.

RESULTS

Multiple copies of $tRNA^{His}$ genes restore derepression of histidine biosynthetic genes in a *gcn2-507* mutant. We isolated genes from two different high-copy-number plasmid libraries of *S. cerevisiae* genomic sequences that overcome the inability of *gcn2-507* mutants to derepress histidine biosynthetic genes under conditions of histidine starvation. The *gcn2-507* mutation is a two-codon insertion (Glu-Leu) at amino acid position 1177 in the domain that is related in sequence to HisRS (51). It is a leaky mutation, permitting low-level growth on medium containing 3-AT, an inhibitor of the *HIS3* product, at a concentration that completely inhibits the growth of strains deleted for *GCN2*. The genomic library constructed in plasmid YEp13 was introduced into the *gcn2-507* strain H1450, and transformants showing increased resistance to 3-AT were isolated. Plasmids recovered from these transformants that conferred 3-AT resistance when reintroduced into the *gcn2-507* strain were selected for further analysis.

The suppressor plasmids were digested with several restriction endonucleases, and the results of this analysis showed that several chromosomal loci were represented among the first 11 plasmids that we examined. Two of the eleven plasmids were found to contain restriction maps diagnostic of *GCN2* (39, 51) or *GCN4* (17) and were not analyzed further. The isolation of a plasmid containing *GCN4* was not surprising because it is known that overexpression of *GCN4* partially bypasses the requirement for GCN2 in the derepression of *HIS* genes subject to the general control (19). A third plasmid was found to contain a truncated allele of the *GLC7* gene, encoding a type I protein phosphatase, and the mechanism of suppression of *gcn2-507* by this plasmid was described previously (50).

In view of our hypothesis that uncharged tRNA is an activator of GCN2 kinase function, we considered the possibility that some of the remaining suppressor plasmids contained $tRNA^{His}$ genes. According to this hypothesis, overexpression of $tRNA^{His}$ would produce high levels of uncharged tRNA in histidine-starved cells that would lead to greater activation of the *gcn2-507* kinase than could occur with a wild-type complement of $tRNA^{His}$. This increased activation would compensate for the reduced kinase function associated with the *gcn2-507* product. To determine whether the remaining suppressor plasmids encoded $tRNA^{His}$, we conducted DNA blot hybridization analysis on restriction digests prepared from these plasmids, using radiolabeled total yeast tRNA as the probe. The results showed that all eight plasmids contained genomic inserts that hybridized with the labeled tRNA probe (data not shown). We next determined whether these same fragments would hybridize with probes made from synthetic oligonucleotides corresponding to *S. cerevisiae* $tRNA^{His}$ (see Materials and Methods). The results of this experiment indicated that only three of the eight suppressor plasmids hybridized with the $tRNA^{His}$-specific probes (data not shown), implying that the remaining plasmids encoded one or more nonhistidine tRNAs.

The presence of a $tRNA^{His}$ gene on the three suppressor plasmids that hybridized with $tRNA^{His}$-specific probes was confirmed by DNA sequence analysis. The sequencing results indicated that two of the three suppressor plasmids derived from the same chromosomal locus and contained a $tRNA^{His}$ gene that had been isolated previously on a plasmid named pYG2 (7); the third suppressor in this group encoded the same $tRNA^{His}$ molecule but had flanking sequences which correspond to a $tRNA^{His}$ gene located downstream of the *NUP116/NSP116* locus (54, 56). In subsequent screening of a second high-copy-number plasmid library, three suppressor plasmids with unique restriction patterns were also found to contain $tRNA^{His}$ genes (see Materials and Methods). Two of the latter plasmids contained part of the same $tRNA^{His}$ locus isolated previously on plasmid pYG2 (7); the third derives from a $tRNA^{His}$ locus present downstream of the *NUP100* gene (54). Taken together, we isolated six plasmids containing $tRNA^{His}$ genes originating from three distinct chromosomal loci.

The fact that three different chromosomal loci isolated as high-copy-number suppressors of *gcn2-507* contained wild-type $tRNA^{His}$ genes strongly suggested that the suppressor activity was conferred by this gene. To obtain additional evidence for this conclusion, we examined whether a small fragment containing the $tRNA^{His}$ gene was sufficient to suppress the *gcn2-507* phenotype. A 420-bp fragment containing the $tRNA^{His}$ gene from plasmid pGN545 isolated previously (33) was inserted into two different high-copy-number plasmids, and the resulting constructs (p896 and p897) were tested for suppression of *gcn2-507* in strain H1438.

As shown in Fig. 1, H1438 transformed with vector alone is sensitive to 3-AT and unable to grow on medium lacking histidine. The histidine requirement of H1438 is attributable to the leaky *his1-29* allele that provides insufficient enzyme activity for histidine biosynthesis when GCN4-mediated derepression of *HIS1* transcription is impaired by a *gcn2* mutation. From previous studies (50), we know that greater derepression of *GCN4* translation is required for 3-AT resistance than for histidine prototrophy in a *his1-29 gcn2-507* strain. The high-copy-number construct p896 bearing the subcloned $tRNA^{His}$ gene conferred a His^+ phenotype and resistance to 3-AT in strain H1438 indistinguishable from that conferred by the parent high-copy-number plasmid pGN545 or another multicopy genomic clone containing $tRNA^{His}$ (Fig. 1; compare results with p896, p1574, pGN545, and the vector pRS316 for the *gcn2-507* strain). We also verified that cells containing a high-copy-number plasmid bearing the $tRNA^{His}$ gene express elevated levels of $tRNA^{His}$ by blot hybridization analysis of total RNA using radiolabeled $tRNA^{His}$ sequences as the hybridization probe (Fig. 2). These results are consistent with the idea that overproduction of $tRNA^{His}$ restores derepression of *HIS* genes in histidine-starved cells containing the *gcn2-507* kinase.

Multiple copies of a mutant $tRNA^{Val}$ gene suppress the derepression defect in *gcn2* mutants. We next sought to determine the identity of the nonhistidine tRNA genes con-

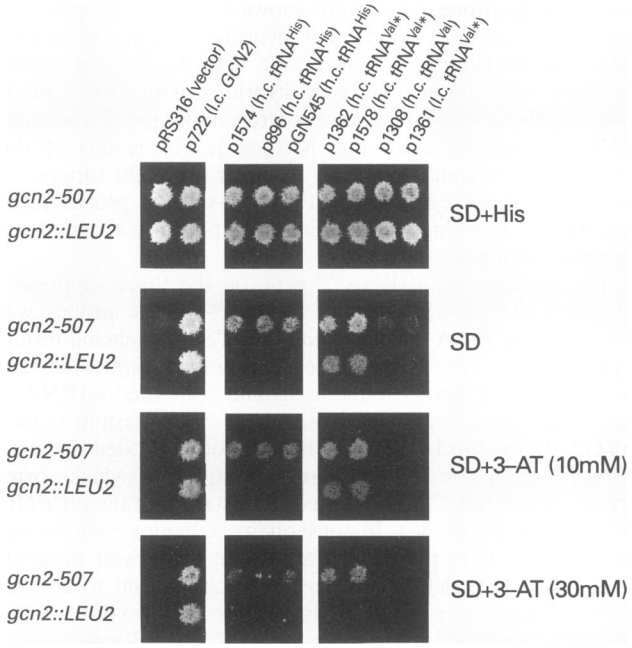

FIG. 1. High-copy-number plasmids containing wild-type tRNA^{His} or the mutant tRNA^{Val*} gene increase expression of histidine biosynthetic genes in *gcn2-507* mutants. Isogenic strains H1438 (*his1-29 gcn2-507*) and H1472 (*his1-29 gcn2::LEU2*) transformed with the indicated plasmids were replica plated to SD medium containing histidine (SD+His), SD lacking histidine (SD), or SD lacking histidine and supplemented with either 10 mM 3-AT [SD+3-AT (10 mM)] or 30 mM 3-AT [SD+3-AT (30 mM)] and incubated for 3 days at 30°C. p722 is a low-copy-number (l.c.) plasmid containing *GCN2*; p1574 and pGN545 are high-copy-number (h.c.) genomic clones containing the identical tRNA^{His} gene; p896 is a high-copy-number subclone containing the tRNA^{His} gene from pGN545; p1578 is a high-copy-number genomic clone containing the mutant tRNA^{Val*} gene; p1362 is a high-copy-number subclone containing the tRNA^{Val*} gene; p1308 is a high-copy-number subclone containing the wild-type tRNA^{Val} gene; p1361 is a low-copy-number subclone containing the mutant tRNA^{Val*} gene.

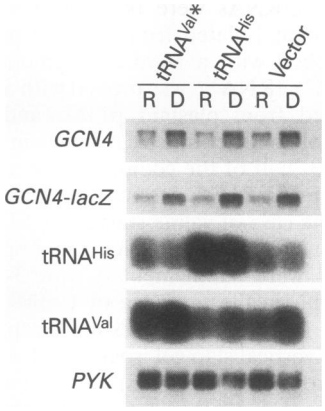

FIG. 2. High-copy-number constructs containing tRNA^{His} or tRNA^{Val*} genes lead to overexpression of the encoded tRNAs but do not affect levels of *GCN4* mRNA. Strain H1894 was cotransformed with the low-copy-number plasmid p180 containing a *GCN4-lacZ* fusion and either p856 (high-copy-number tRNA^{Val*}), p857 (high-copy-number tRNA^{His}), or vector alone (YEp13). Transformants were grown on SD medium with minimal supplements (repressing conditions [R]) or on SD medium supplemented with 3-AT to produce histidine starvation (derepressing conditions [DR]). Total RNA was extracted and subjected to formaldehyde-agarose gel electrophoresis and RNA blot hybridization analysis using radiolabeled probes specific for the indicated transcripts.

tained on the five remaining suppressor plasmids. Using blot hybridization analysis with total tRNA as the probe, we mapped these tRNA genes to relatively small restriction fragments. DNA sequence analysis of these fragments revealed that all five originated from a single chromosomal locus that was sequenced previously (14, 24), encoding a tRNA that differs by a single nucleotide from tRNA^{Val} with anticodon AAC. The mutant tRNA^{Val} sequence contains G instead of A at position 73, the 3′-terminal nucleotide encoded in the DNA. To investigate whether this mutant tRNA^{Val} (henceforth designated tRNA^{Val*}) had suppressor activity, a 500-bp fragment containing the altered gene was subcloned into high-copy-number and low-copy-number plasmids (yielding p1362 and p1361, respectively). In addition, we used site-directed mutagenesis to convert the tRNA^{Val*} coding sequence in the high-copy-number construct p1362 to the wild-type sequence of this tRNA^{Val} gene, yielding p1308. All of these constructs were tested for suppression of the derepression defect in the *gcn2-507* strain H1438 as described above for the tRNA^{His} suppressors. Construct p1362 bearing the subcloned tRNA^{Val*} gene suppressed *gcn2-507* to the same extent as did the corresponding high-copy-number tRNA^{Val*} constructs isolated from the genomic libraries, such as p1578 (Fig. 1). These tRNA^{Val*} constructs were more efficient suppressors than were the multicopy tRNA^{His} plasmids, restoring growth of the *gcn2-507* strain even at high concentrations of 3-AT. In contrast, the low-copy-number construct p1361 bearing the tRNA^{Val*} gene and the high-copy-number construct p1308 containing the wild-type tRNA^{Val} gene had little or no suppressor activity. Using RNA blot hybridization analysis, we verified that the high copy-number plasmids containing the tRNA^{Val*} gene lead to elevated levels of this tRNA^{Val} isoacceptor (Fig. 2). These results support the idea that overexpression of the mutant tRNA^{Val*} is responsible for derepression of histidine biosynthesis in *gcn2-507* cells.

The position altered in the mutant tRNA^{Val*} often plays an important role in the recognition of tRNA by its cognate aminoacyl-tRNA synthetase and is known as the discriminator base (6, 12, 40). All known tRNA^{Val} species from prokaryotic and eukaryotic organisms contain an A nucleotide at position 73 (44), and an A-to-G mutation at this position prevented aminoacylation both of a model yeast tRNA^{Val}(AAC) substrate by yeast valyl-tRNA synthetase (13) and of wild-type *E. coli* tRNA^{Val}(UAC) by the *E. coli* enzyme (46). From these findings, we presume that the altered tRNA^{Val} encoded by our suppressor plasmids cannot be aminoacylated efficiently in vivo. Accordingly, the ability of the mutant tRNA^{Val*} gene or the wild-type tRNA^{His} gene to function in multicopy as a suppressor of *gcn2-507* is consistent with the idea that the general control response can be restored in *gcn2-507* cells by increasing the level of an uncharged tRNA. This could be achieved either by overexpression of wild-type tRNA^{His} under conditions of histidine starvation, in which case the excess tRNA^{His} would not be fully charged, or by overexpression of the mutant tRNA^{Val*} that would not be charged irrespective of valine availability.

Evidence that overexpression of mutant tRNA^{Val*} elicits a derepression independent of GCN2. All of the suppressor plasmids that we isolated from the genomic libraries yielded less suppression of a *gcn2* deletion than of *gcn2-507*. Thus, in

TABLE 2. High-copy-number plasmids containing the wild-type $tRNA^{His}$ or mutant $tRNA^{Val*}$ gene restore derepression of *HIS4-lacZ* expression in *gcn2-507* mutants

Gene[a]	Plasmid	β-Galactosidase activity (U)[b]			
		H1936 (*gcn2-507*)		H1937 (*gcn2::LEU2*)	
		R	DR	R	DR
None	YEp24	120	130	120	130
GCN2	p585	150	700	200	750
$tRNA^{His}$ (h.c.)	p1574	130	440	140	160
$tRNA^{Val*}$ (h.c.)	p1362	360	660	410	480
$tRNA^{Val*}$ (l.c.)	p1361	100	110	94	80
$tRNA^{Val}$ (h.c.)	p1308	150	110	160	130

[a] h.c., high copy number; l.c., low copy number.

[b] Measured in extracts of yeast strains H1936 and H1937 bearing the indicated plasmids, grown under repressing (R; nonstarvation) or derepressing (DR; histidine starvation imposed by 3-AT) conditions as described in Materials and Methods. The results shown are averages of assays conducted on two to five independent transformants, and the individual measurements deviated from the average values shown here by 30% or less.

the *gcn2::LEU2* strain H1472, the suppressor plasmids encoding $tRNA^{His}$ conferred no growth either on medium lacking histidine or on 3-AT medium (Fig. 1). The suppressor plasmids encoding $tRNA^{Val*}$ differed from those containing the $tRNA^{His}$ gene in conferring histidine prototrophy and weak growth on 3-AT medium in H1472. (It is noteworthy that the plasmids containing the subcloned $tRNA^{His}$ or $tRNA^{Val*}$ genes resembled the corresponding parental plasmids in conferring less suppression of the *gcn2* deletion versus *gcn2-507*, providing further evidence that the tRNA genes on these plasmids are responsible for the suppressor phenotype.) The ability of the high-copy-number $tRNA^{Val*}$ constructs to give significant suppression of the *gcn2* deletion implies the existence of a GCN2-independent derepression response to uncharged tRNA.

To quantitate the degree of suppression conferred by the tRNA genes in the presence of the *gcn2-507* and *gcn2::LEU2* alleles, we assayed a *HIS4-lacZ* fusion present in a pair of strains isogenic with those described in Fig. 1 after transformation with various suppressor plasmids. Derepression of *HIS4* transcription under starvation conditions is mediated by the GCN4 protein and is thus dependent on stimulation of *GCN4* translation by GCN2 (19). As shown in Table 2, transformants of both the *gcn2-507* and the *gcn2::LEU2* strains containing plasmid-borne wild-type *GCN2* exhibited four- to fivefold derepression of *HIS4-lacZ* expression in response to histidine limitation imposed by 3-AT. In contrast, transformants of these strains bearing vector alone showed no derepression of *HIS4-lacZ* expression under the same starvation conditions. The presence of the high-copy-number plasmid p1574 encoding $tRNA^{His}$ in the *gcn2-507* strain increased *HIS4-lacZ* expression under starvation conditions by a factor of 3 to 4 but had no significant effect under nonstarvation conditions. In addition, the multicopy plasmid encoding $tRNA^{His}$ led to only a small increase in *HIS4-lacZ* expression in the *gcn2::LEU2* strain, consistent with the growth tests on 3-AT plates shown in Fig. 1. Thus, most of the *HIS4* derepression associated with high-copy-number $tRNA^{His}$ genes is dependent on the *gcn2-507* product.

The high-copy-number plasmid p1362 bearing $tRNA^{Val*}$ led to greater *HIS4-lacZ* expression under starvation conditions than was seen with the multicopy plasmid encoding $tRNA^{His}$ in the *gcn2-507* strain and also conferred significant derepression in the *gcn2::LEU2* strain (Table 2). In fact, a large portion of the *HIS4* derepression conferred by $tRNA^{Val*}$ appears to be independent of *GCN2*, in that we observed a nearly fourfold increase in *HIS4-lacZ* expression in the *gcn2::LEU2* strain transformed with the high-copy-number $tRNA^{Val*}$ gene compared with vector alone. In addition, *HIS4-lacZ* expression under starvation conditions was only 1.5-fold higher in *gcn2-507* transformants than in *gcn2::LEU2* transformants bearing p1362 (660 versus 480 U). Another noteworthy difference between the $tRNA^{His}$ and $tRNA^{Val*}$ suppressors is that the latter increased *HIS4-lacZ* expression on minimal medium in the absence of amino acid starvation. This stimulatory effect appears to be completely independent of *GCN2*, occurring essentially to the same degree in the *gcn2-507* and *gcn2Δ* strains under repressing conditions. In agreement with the growth tests in Fig. 1, the derepression of *HIS4-lacZ* expression conferred by the $tRNA^{Val*}$ suppressor was abolished either by reducing the copy number of the plasmid, as shown with the low-copy-number construct p1361, or by restoring the wild-type $tRNA^{Val}$ sequence in the multicopy construct p1308. Transformants bearing the latter constructs showed *HIS4-lacZ* expression comparable to that of the corresponding transformants containing vector alone (Table 2).

It is interesting that histidine starvation elicited a derepression response in the *gcn2-507* mutant bearing the multicopy $tRNA^{Val*}$ construct p1362 but not when this strain was transformed with vector alone. To explain this observation, we suggest that $tRNA^{Val*}$ and uncharged $tRNA^{His}$ have additive effects in derepressing *HIS4* expression. One way to explain such additivity would be to suggest that stimulation of the *gcn2-507* kinase activity by histidine starvation in cells containing wild-type amounts of $tRNA^{His}$ results in a degree of eIF-2 phosphorylation and inhibition of eIF-2 function that is insufficient to derepress *GCN4* translation. In contrast, overexpression of $tRNA^{Val*}$ would further impair eIF-2 function (either by increasing eIF-2 phosphorylation or by an unknown GCN2-independent mechanism) and exceed a threshold level of inhibition required to derepress *GCN4* translation. Other instances of additive effects of different uncharged tRNAs in the derepression response were encountered and will be described below.

Evidence that suppression of *gcn2-507* by the multicopy tRNA genes involves an accumulation of uncharged tRNA. The results presented thus far can be explained by proposing that derepression of *GCN4* can be restored in the *gcn2-507* mutant by overexpression of wild-type $tRNA^{His}$ under histidine starvation conditions in which the excess $tRNA^{His}$ cannot be charged or by overexpression of the $tRNA^{Val*}$ that cannot be aminoacylated. As shown in Table 2, the high-copy-number plasmid p1308 containing wild-type $tRNA^{Val}$ had no derepressing effect on *HIS4* expression regardless of the availability of histidine, implying that under these conditions, the excess $tRNA^{Val}$ produced from p1308 is being aminoacylated by valyl-tRNA synthetase. This would explain why we failed to isolate other wild-type tRNA genes as suppressors of *gcn2-507* under conditions of histidine limitation. If this interpretation is correct, the high-copy-number $tRNA^{His}$ constructs should suppress *gcn2-507* less efficiently in cells starved for an amino acid besides histidine. This prediction was borne out by the results of the experiment shown in Table 3, in which the abilities of a multicopy $tRNA^{His}$ construct to suppress the derepression defect in *gcn2-507* were compared under conditions of histidine, tryptophan, or leucine-isoleucine-valine starvation. As shown in Table 3, plasmid p1574 bearing the $tRNA^{His}$ gene conferred about threefold derepression of *HIS4-lacZ* expression versus vector alone in the *gcn2-507* strain when

TABLE 3. Effects of a multicopy $tRNA^{His}$ gene on *HIS4-lacZ* expression in *gcn2* mutants in response to starvation for different amino acids

Gene[a]	Plasmid	β-Galactosidase activity (U)[b]							
		H1936 (*gcn2-507*)				H1937 (*gcn2::LEU2*)			
		R	3-AT	5-MT	SM	R	3-AT	5-MT	SM
None	YEp24	120	170	130	360	120	130	140	110
GCN2 (l.c.)	p585	150	700	560	920	200	750	540	910
$tRNA^{His}$ (h.c.)	p1574	130	440	160	530	140	160	130	120

[a] l.c., low copy number; h.c., high copy number.
[b] Measured in extracts of yeast strains H1936 and H1937 bearing the indicated plasmids, grown under repressing (R; nonstarvation) or derepressing conditions produced by histidine starvation imposed by 3-AT, tryptophan starvation imposed by 5-MT, or leucine, isoleucine, and valine starvation caused by SM, all as described in Materials and Methods. The results shown are averages of assays conducted on two to five independent transformants, and the individual measurements deviated from the average values shown here by 30% or less.

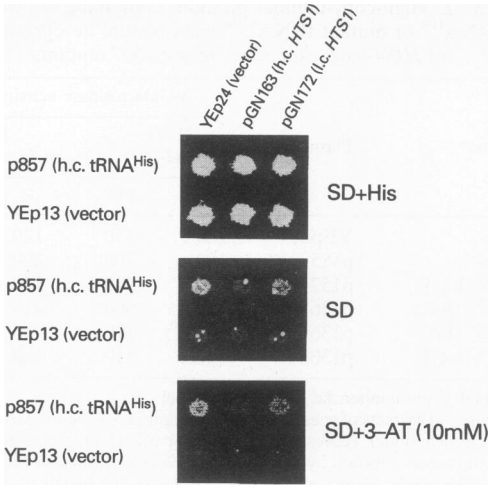

FIG. 3. Suppression of *gcn2-507* by a multicopy $tRNA^{His}$ gene is reduced by increasing the dosage of the *HTS1* gene, encoding HisRS. Strain H1438 (*his1-29 gcn2-507*) was doubly transformed either with a high-copy-number (h.c.) plasmid containing $tRNA^{His}$ (p857) or with vector alone (YEp13) and with either a high-copy-number plasmid containing *HTS1* (pGN163), a low-copy-number (l.c.) plasmid containing *HTS1* (pGN172), or vector alone (YEp24). The transformants were replica plated to SD medium containing histidine (SD+His), SD lacking histidine (SD), or SD lacking histidine and supplemented with 10 mM 3-AT [SD+3-AT (10 mM)] and incubated for 3 days at 30°C.

cells were starved for histidine (440 versus 170 U). By contrast, little or no derepression was conferred by the $tRNA^{His}$ construct versus vector alone in response to starvation for tryptophan (160 versus 130 U), and only a 50% increase in the level of derepression occurred in cells starved for leucine, isoleucine, and valine in the presence versus the absence of the $tRNA^{His}$ construct (530 versus 360 U) (Table 3). Analysis of *HIS4-lacZ* expression in transformants of the *gcn2::LEU2* strain bearing wild-type *GCN2* or vector alone confirmed that each of the three analogs elicited a derepression response that was completely dependent on GCN2.

The data in Table 3 indicate that starvation for leucine, isoleucine, and valine with SM elicited much greater derepression of *HIS4-lacZ* expression in the *gcn2-507* transformants bearing vector alone (from 120 to 360 U) or the *GCN2*-containing plasmid (from 150 to 922 U) than was seen when these strains were starved for histidine or tryptophan. These findings suggest that SM was more effective than the other two analogs in activating both GCN2 and the *gcn2-507* product, presumably because it imposes starvation for multiple amino acids. As just noted, introduction of the multicopy $tRNA^{His}$ construct into the *gcn2-507* mutant led to a 50% increase in *HIS4-lacZ* expression in the presence of SM (from 360 to 530 U) but had no significant effect under nonstarvation conditions. To explain this finding, we suggest that the excess $tRNA^{His}$ is not fully charged by histidyl-tRNA synthetase, even in the absence of 3-AT treatment, but this amount of uncharged $tRNA^{His}$ is insufficient to cause derepression of *GCN4* expression. In contrast, the uncharged $tRNA^{Leu}$, $tRNA^{Val}$, and $tRNA^{Ile}$ produced in response to SM-induced starvation can activate the *gcn2-507* product and bring about phosphorylation of eIF-2 at a level sufficient to derepress *GCN4* translation. In the latter situation, addition of even a small amount of uncharged $tRNA^{His}$ can lead to further derepression either through greater activation of the *gcn2-507* product or by an alternative mechanism for down-regulating eIF-2.

A second prediction of our model is that overexpression of the HisRS should reduce the amount of uncharged $tRNA^{His}$ present in transformants containing a multicopy $tRNA^{His}$ construct under histidine starvation conditions and thereby reduce the suppression of *gcn2-507*. To test this prediction, we introduced the *HTS1* gene encoding HisRS on either high-copy-number or low-copy-number plasmids into transformants of *gcn2-507 his1-29* strain H1438 bearing a high-copy-number $tRNA^{His}$ construct or vector alone and analyzed the 3-AT sensitivity of the doubly transformed strains. As shown in Fig. 3, all transformants containing vector alone had the expected His^- phenotype and sensitivity to 3-AT characteristic of *gcn2-507 his1-29* strains, in the presence or absence of plasmid-borne *HTS1*. Strains containing the $tRNA^{His}$ construct and either no plasmid-borne *HTS1* or low-copy-number *HTS1* were His^+ and resistant to 3-AT, although the latter were less 3-AT resistant than the former. In addition, strains containing both the $tRNA^{His}$ construct and high-copy-number *HTS1* were 3-AT sensitive and His^-. These findings suggest that decreasing the amount of uncharged $tRNA^{His}$ by overexpression of the corresponding HisRS reduces derepression of histidine biosynthetic genes, supporting the idea that uncharged $tRNA^{His}$ is responsible for suppression of *gcn2-507* in strains bearing the multicopy $tRNA^{His}$ plasmid.

A third prediction of our model is that increasing the concentration of uncharged tRNA by a mutation in an aminoacyl-tRNA synthetase should mimic the suppressing effect of a mutant tRNA that cannot be aminoacylated. To test this possibility, we analyzed yeast strains containing the *ils1-1* allele, encoding a mutant form of isoleucyl-tRNA synthetase. This mutation leads to slow growth at 30°C and inviability at 36°C, presumably due to insufficient charging of $tRNA^{Ile}$ at these temperatures (28). We tested *ils1-1 gcn2 his1-29* mutant strains at 30°C for suppression of the His^- and $3\text{-}AT^s$ phenotypes of the *gcn2-507* or *gcn2::LEU2* mutations after transforming them with a plasmid bearing wild-type *ILS1* or with different plasmids containing a wild-type $tRNA^{Ile}$ gene. When transformed with vector alone, the *his1-29 gcn2-507 ils1-1* strain was His^+ $3\text{-}AT^s$, whereas the corresponding *his1-29 gcn2::LEU2 ils1-1* transformant was His^- $3\text{-}AT^s$ (Fig. 4). These phenotypes indicate that partial derepression of *HIS* genes was occurring in the *his1-29 gcn2-507 ils1-1* mutant and was dependent on the *gcn2-507* product. Introduction of the plasmid bearing *ILS1*, which should restore a wild-type level of $tRNA^{Ile}$ charging, led to a His^- phenotype in the *his1-29*

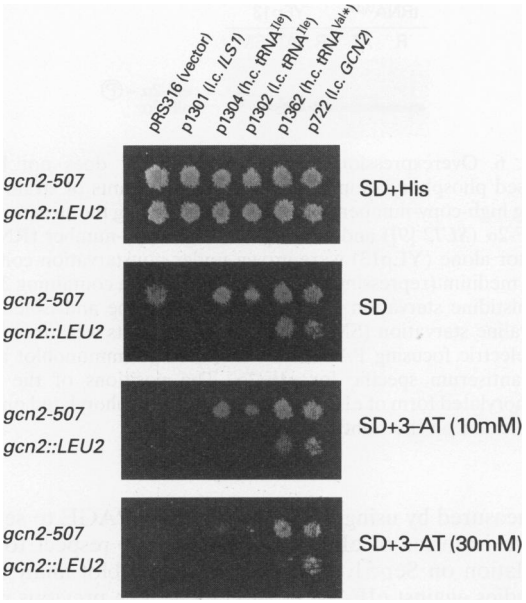

FIG. 4. Suppression of *gcn2-507* by the *ils1-1* mutation and multiple copies of the tRNA^{Ile} gene. Isogenic strains H1576 (*ils1-1 gcn2-507 his1-29*) and H1870 (*ils1-1 gcn2::LEU2 his1-29*) were transformed with the indicated plasmids and replica plated to SD medium containing histidine (SD+His), SD lacking histidine (SD), or SD lacking histidine and supplemented with either 10 mM 3-AT [SD+3-AT (10 mM)] or 30 mM 3-AT [SD+3-AT (30 mM)] and incubated for 3 days at 30°C. p1301 is a low-copy-number (l.c.) plasmid containing the *ILS1* gene; p1304 is a high-copy-number (h.c.) plasmid containing a tRNA^{Ile} (UAU) gene; p1302 is a low-copy-number plasmid containing same the tRNA^{Ile} gene; p1362 is a high-copy-number plasmid containing the tRNA^{Val*} gene; p722 is a low-copy-number plasmid containing the wild-type *GCN2* gene.

gcn2-507 ils1-1 strain. Moreover, introduction of low- or high-copy-number plasmids containing the tRNA^{Ile} gene led to detectable 3-AT resistance in the *his1-29 gcn2-507 ils1-1* strain (Fig. 4).

To explain these results, we suggest that reduced charging of the wild-type complement of tRNA^{His} under histidine starvation conditions in the *gcn2-507 ILS1 his1-29* transformant does not activate the *gcn2-507* product sufficiently to derepress *GCN4*. However, when this deficit in tRNA^{His} charging is combined with reduced charging of tRNA^{Ile} in the *gcn2-507 ils1-1 his1-29* mutant, eIF-2 function is diminished sufficiently to derepress *GCN4* and its target genes in the histidine pathway. When the amount of uncharged tRNA^{Ile} is increased to even higher levels by overexpression of tRNA^{Ile} in the *ils1-1* mutant, the resulting derepression of *GCN4* and *HIS* genes is great enough to confer 3-AT resistance as well as histidine prototrophy. An important feature of this hypothesis is its ability to explain how increasing the level of uncharged tRNA can stimulate growth under starvation conditions by derepressing *GCN4* expression instead of retarding it further by decreasing the rate of translation elongation.

To determine whether the accumulation of uncharged tRNA^{Ile} in the *ils1-1* mutant led to derepression of *HIS4* expression by activating the *gcn2-507* product, we assayed *HIS4-lacZ* expression in isogenic *ils1-1 gcn2-507* and *ils1-1 gcn2::LEU2* strains transformed with the *ILS1* construct p1301 or vector alone (Table 4). When the strains were grown at 30°C, a semipermissive temperature for *ils1-1* mutants (28), we found that *HIS4-lacZ* expression was about threefold higher in

TABLE 4. Effects of the *ils1-1* mutation on *HIS4-lacZ* expression in *gcn2* mutants grown under semipermissive conditions

Gene	Plasmid	β-Galactosidase activity (U)[a]	
		H1576 (*gcn2-507*)	H1870 (*gcn2::LEU2*)
None	YEp24	320	180
ILS1	p1301	97	80

[a] Measured in extracts of yeast strains H1576 and H1870 bearing the indicated plasmids, grown under semipermissive conditions for the *ils1-1* mutation (30°C), all as described in Materials and Methods. The results shown are averages of assays conducted on five independent transformants, and the individual measurements deviated from the average values shown here by 30% or less.

the *gcn2-507 ils1-1* strain transformed with vector alone versus the *ILS1* construct (320 versus 97 U), confirming the derepressing effect of uncharged tRNA^{Ile} on *HIS4* expression. *HIS4-lacZ* expression was significantly lower in the *gcn2::LEU2 ils1-1* strain transformed with vector compared with the corresponding *gcn2-507 ils1* transformant (320 versus 180 U), indicating that the residual kinase activity of the *gcn2-507* product contributed to the derepression elicited by uncharged tRNA^{Ile}. However, it was evident that the *ils1-1* charging defect also led to significant derepression in the *gcn2::LEU2* strain. These findings suggest that the ability to derepress histidine biosynthetic genes conferred by the *ils1-1* mutation in the *gcn2-507* strain involves contributions from both GCN2-dependent and GCN2-independent derepression mechanisms that each respond to uncharged tRNA.

Derepression of *HIS4* by mutant tRNA^{Val*} is independent of eIF-2α phosphorylation on Ser-51. The fact that the tRNA^{Val*} construct led to a substantial increase in *HIS* gene expression in the *gcn2* deletion strain (Fig. 1 and Table 2) strongly suggests that a large component of the derepression response elicited by this mutant tRNA does not involve phosphorylation of the α subunit of eIF-2 on Ser-51, as GCN2 is the only known yeast kinase that catalyzes this reaction (9). To rule out the possibility that Ser-51 was being phosphorylated by an unknown eIF-2α kinase in response to overexpression of tRNA^{Val*}, we compared suppression of the *gcn2-507* mutation by tRNA^{Val*} in isogenic strains expressing either wild-type eIF-2α (encoded by *SUI2*) or a mutant form of the protein in which Ser-51 is replaced by alanine (encoded by *SUI2-S51A*) (9). This alanine substitution was shown previously to abolish phosphorylation of eIF-2α by GCN2 and the GCN2-dependent activation of *GCN4* translation in amino acid-starved cells (9). As shown in Table 5, when both strains were transformed with a plasmid containing *GCN2*, derepression of *HIS4-lacZ* in response to histidine starvation was restored in the transformant expressing wild-type eIF-2α but not in the strain expressing eIF-2α-S51A, in accord with our previous findings (9). In contrast, the derepression conferred by the high-copy-number tRNA^{Val*} construct was only about 30% lower in the *gcn2-507 SUI2-S51A* transformant than in the corresponding *gcn2-507 SUI2* strain (360 versus 540 U). As expected, the low-copy-number tRNA^{Val*} construct and the high-copy-number wild-type tRNA^{Val} constructs gave results very similar to those for the vector alone. These results support the idea that overexpression of the mutant tRNA^{Val*} derepresses the general control system by a mechanism independent of phosphorylation of eIF-2α on Ser-51.

The derepression response elicited by multicopy tRNA^{Val*} in the absence of Ser-51 on eIF-2α was demonstrated in a different set of strains, using growth on 3-AT medium as the indicator of *HIS* gene expression. As shown in Fig. 5, the

TABLE 5. Importance of the GCN2 phosphorylation site in eIF-2α at Ser-51 on the ability of the multicopy tRNA^{Val*} gene to restore derepression of *HIS4-lacZ* expression in *gcn2-507* strains

Gene[a]	Plasmid	β-Galactosidase activity (U)[b]			
		H1939 (*SUI2*)		H1940 (*SUI2-S51A*)	
		R	DR	R	DR
None	YEp24	160	150	150	110
tRNA^{Val*} (h.c.)	p1362	340	540	350	360
tRNA^{Val*} (l.c.)	p1361	96	110	89	110
tRNA^{Val} (h.c.)	p1308	140	110	110	110
GCN2	p585	130	520	93	87

[a] h.c., high copy number; l.c., low copy number.
[b] Measured in extracts of yeast strains H1939 and H1940 bearing the indicated plasmids, grown under repressing (R; nonstarvation) or derepressing (DR; histidine starvation imposed by 3-AT) conditions as described in Materials and Methods. The results shown are averages of assays conducted on two to five independent transformants, and the individual measurements deviated from the average values shown here by 30% or less.

high-copy-number tRNA^{Val*} construct suppressed the 3-AT^s phenotype of *gcn2-507* in strains expressing either wild-type eIF-2α (*SUI2*) or eIF-2α-S51A (*SUI2-S51A*) or containing the *gcn2-K559V* allele that encodes a catalytically inactive kinase (51). Note that in this strain background, the high-copy-number tRNA^{His} construct also elicited a derepression response in the absence of *GCN2* function and Ser-51 on eIF-2α (Fig. 5). Measurements of *HIS4-lacZ* expression in these strains confirmed the existence of GCN2-independent derepression conferred by the multicopy tRNA^{His} constructs (data not shown).

Given that the major component of the derepression of *HIS4* conferred by overexpression of tRNA^{Val*} occurs independently of *GCN2* (Table 2) and Ser-51 on eIF-2α (Table 5), we reasoned that the presence of this mutant tRNA should not lead to a significant accumulation of phosphorylated eIF-2α. To test this prediction, we measured the level of phosphorylated eIF-2α present in *GCN2* strains containing either high-copy-number tRNA^{Val*} or vector alone, under starvation and nonstarvation conditions. The level of eIF-2α phosphorylation

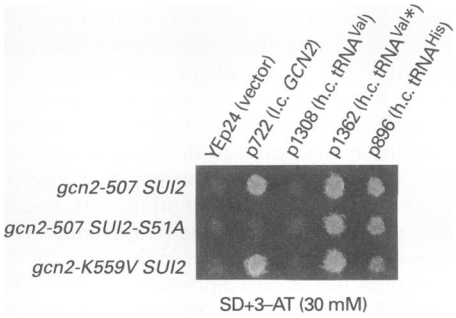

FIG. 5. Derepression of histidine biosynthetic genes by multicopy tRNA^{Val*} in the absence of GCN2 and Ser-51 of eIF-2α. Isogenic strains H1953 (*gcn2-507 SUI2*), H1954 (*gcn2-507 SUI2-S51A*), and H1955 (*gcn2-K559V SUI2*), containing a catalytically inactive GCN2 protein, were all transformed with the indicated plasmids, replica plated to SD medium containing 30 mM 3-AT, and incubated for 3 days at 30°C. p722 is a low-copy-number (l.c.) plasmid containing the wild-type *GCN2* gene; p1308 is a high-copy-number (h.c.) plasmid containing the wild-type tRNA^{Val} gene; p1362 is a high-copy-number plasmid containing the tRNA^{Val*} gene; p896 is a high-copy-number plasmid containing the tRNA^{His} gene.

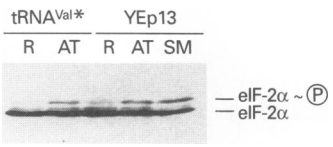

FIG. 6. Overexpression of mutant tRNA^{Val*} does not lead to increased phosphorylation of eIF-2α. Transformants of strain H1938 bearing high-copy-number plasmid p925 containing the structural gene for eIF-2α (*SUI2* [9]) and either p856 (high-copy-number tRNA^{Val*}) or vector alone (YEp13) were grown under nonstarvation conditions on SD medium (repressing [R]) or on SD medium containing 3-AT to elicit histidine starvation (AT) or SM plus leucine and isoleucine to elicit valine starvation (SM). Total protein extracts were fractionated by isoelectric focusing PAGE and subjected to immunoblot analysis using antiserum specific for eIF-2α. The positions of the basally phosphorylated form of eIF-2α and the form phosphorylated on Ser-51 are indicated on the right.

was measured by using isoelectric focusing PAGE to separate the two isoforms of eIF-2α that differ with respect to phosphorylation on Ser-51, followed by immunoblot analysis with antibodies against eIF-2α. In agreement with previous results, starvation of the strain bearing vector alone with 3-AT led to an increase in the proportion of eIF-2α that is phosphorylated (Fig. 6). Similar results were obtained under conditions of valine starvation produced by addition of SM in the presence of isoleucine and leucine. In contrast, we observed little or no increase in phosphorylated eIF-2α in the *GCN2* transformant bearing the high-copy-number tRNA^{Val*} construct relative to the strain containing vector alone under nonstarvation conditions or in the presence of 3-AT (Fig. 6). (Recall that overexpression of tRNA^{Val*} elicits derepression of *HIS4* under nonstarvation conditions; Table 2.) These results indicate that elevated levels of the mutant tRNA^{Val*} do not stimulate eIF-2α phosphorylation by GCN2, even though starvation for valine (which should generate wild-type uncharged tRNA^{Val}) clearly does.

Derepression of *GCN4* by the mutant tRNA^{Val*} occurs at the translational level. In view of the fact that derepression of *HIS4* by multicopy tRNA^{Val*} was largely independent of eIF-2α phosphorylation, it was important to determine whether it could be attributed to increased synthesis of GCN4 protein. To address this question, we measured the expression of a plasmid-borne *GCN4-lacZ* fusion containing all four uORFs and another fusion lacking the uORFs in a strain that was deleted for *GCN2* and transformed with high-copy-number constructs encoding tRNA^{Val*}, tRNA^{His}, or vector alone. As shown in Table 6, the tRNA^{Val*} construct increased expression of the *GCN4-lacZ* fusion on p180 containing all four uORFs, whereas the tRNA^{His} construct had no effect on the expression of this fusion. These results are consistent with the idea that the *GCN2*-independent derepression of *HIS* genes conferred by high-copy-number tRNA^{Val*} involves increased expression of *GCN4*. In addition, they provide another indication that the derepression conferred by the multicopy tRNA^{His} constructs, at least in certain strains, is largely dependent on *GCN2* function. The derepression of *GCN4-lacZ* expression conferred by the tRNA^{Val*} construct in the *gcn2*Δ strain was about half as efficient as that observed in the isogenic *GCN2* strain deprived of histidine (Table 6). This result is in accord with the partial derepression of *HIS4-lacZ* produced by tRNA^{Val*} in *gcn2*Δ cells (Table 1).

We carried out two experiments to evaluate whether the increase in *GCN4* expression elicited by the high-copy-number tRNA^{Val*} construct occurred at the translational level. First,

TABLE 6. Derepression of *GCN4-lacZ* expression by the high-copy-number $tRNA^{Val*}$ gene in a *gcn2Δ* strain requires uORFs in *GCN4* mRNA

Gene[a]	Plasmid	β-Galactosidase activity (U)[b]			
		p180		p227	
		R	DR	R	DR
None	YEp13	5	6	1,300	1,600
$tRNA^{Val*}$ (h.c.)	p856	20	30	990	1,200
$tRNA^{His}$ (h.c.)	p857	5	6	1,100	1,100

[a] h.c., high copy number.

[b] Measured in extracts of yeast strain H1894 doubly transformed with p180 or p227 harboring *GCN4-lacZ* fusions containing all four uORFs or no uORFs, respectively, in the *GCN4* mRNA leader and the *LEU2* plasmids p856, containing high-copy-number $tRNA^{Val*}$, p857, containing high-copy-number $tRNA^{His}$, or vector alone. Assays were carried out on extracts of cells grown under repressing (R; nonstarvation) or derepressing (DR; histidine starvation imposed by 3-AT) conditions. The results shown are averages of assays of two to five independent transformants, and the individual measurements deviated from the average values shown here by 30% or less. In separate experiments on strains isogenic to H1894, we found that introduction of wild-type *GCN2* on a single-copy plasmid led to *GCN4-lacZ* expression from p180 under derepressing conditions at a level approximately twofold higher than that given by the $tRNA^{Val*}$ construct (49).

we determined whether the increase in *GCN4-lacZ* expression produced by this construct was dependent on the uORFs in the *GCN4* leader. In agreement with previous findings (31), the *GCN4-lacZ* fusion on p227 lacking uORFs exhibited high constitutive expression in the presence or absence of amino acid starvation in the *gcn2Δ* mutant, as the uORFs are required for translational control of *GCN4*. The presence of the multicopy $tRNA^{Val*}$ construct did not increase *GCN4-lacZ* expression from the p227 construct (Table 6). Second, we determined the effect of overexpressing multicopy $tRNA^{Val*}$ on the steady-state levels of *GCN4* and *GCN4-lacZ* mRNAs. As shown in Fig. 2, we observed no increase in the level of *GCN4* and *GCN4-lacZ* mRNAs in strains bearing the high-copy-number $tRNA^{Val*}$ (or $tRNA^{His}$) constructs relative to vector alone. These results suggest that the derepression of *GCN4* conferred by the $tRNA^{Val*}$ construct in a *gcn2Δ* strain occurs at the translational level and is mediated by the uORFs, even though it is independent of phosphorylation of eIF-2α by GCN2.

Although the increased translation of *GCN4* mRNA elicited by the $tRNA^{Val*}$ construct occurred independently of eIF-2α phosphorylation, it was possible that it involved negative regulation of eIF-2 by some other means. We reasoned that if this hypothesis was correct, overexpression of mutant $tRNA^{Val*}$ should exacerbate the growth defect associated with hyperphosphorylation of eIF-2α by genetically activated forms of GCN2. Strains expressing such alleles exhibit slow growth due to an inhibition of general translation initiation caused by hyperphosphorylation of eIF-2α (9, 38) and the attendant down-regulation of the eIF-2 recycling factor, eIF-2B (8, 49). Thus, the active form of eIF-2 is rate limiting for general translation in *GCN2*^{c} strains. As shown in Fig. 7, a strain containing the *GCN2*^{c}*-E532K,E1522K* allele grew more slowly when it was transformed with a high-copy-number $tRNA^{Val*}$ construct than when it was transformed with either vector alone or the multicopy plasmid bearing wild-type $tRNA^{Val}$. In contrast, the $tRNA^{Val*}$ construct had no effect on the rate of colony formation in a *GCN2* strain (data not shown). Importantly, it also did not exacerbate the growth defect of a ρ^0 strain (H1941) that is respiratory deficient (data not shown), nor did it exacerbate that associated with certain *GCN4* alleles containing mutations in the transcriptional activation domain that

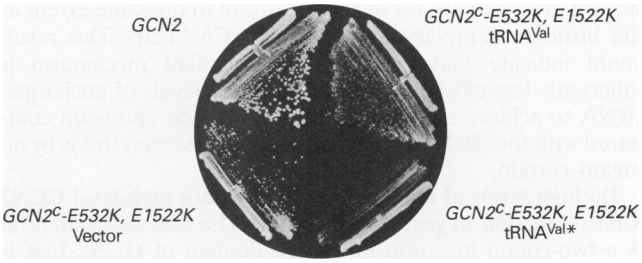

FIG. 7. Overexpression of mutant $tRNA^{Val*}$ exacerbates the slow-growth phenotype of an activated allele of *GCN2*. Strain H1613 containing the *GCN2*^{c}*-E522K,E1532K* allele was transformed with high-copy-number plasmids p1362 (containing $tRNA^{Val*}$), p1308 (containing $tRNA^{Val}$), or vector alone (YEp24). Isolated colonies of each transformant, as well as a YEp24 transformant of wild-type strain H1402, were grown on SD plates at 30°C for 3 days, and the growth rate of transformants was compared with that of the isogenic wild-type strain H1402.

lead to slow growth when overexpressed (10a). Three such *GCN4* alleles were introduced into strain H1894 along with either the multicopy $tRNA^{Val*}$ construct p856 or vector YEp13. For each *GCN4* construct, transformants bearing p856 grew indistinguishably from those containing YEp13 (data not shown). Thus, overexpression of $tRNA^{Val*}$ specifically exacerbated the growth defect associated with a hyperactivated GCN2 kinase. These findings suggest that overproduction of $tRNA^{Val*}$ leads to a reduction in eIF-2 function that is insufficient to inhibit cell growth unless combined with an impairment of eIF-2 function caused by a different mechanism, such as hyperphosphorylation of eIF-2α.

DISCUSSION

To identify regulatory factors that interact with the eIF-2α kinase GCN2, we isolated genes from multicopy plasmid genomic libraries that overcome the derepression defect of the leaky *gcn2-507* allele. By focusing on those plasmids that showed substantially less suppression of a *gcn2* deletion compared with *gcn2-507*, we hoped to obtain genes that would restore the ability of the *gcn2-507*-encoded kinase to phosphorylate eIF-2α in amino acid-starved cells. In this report, we described the properties of mutant and wild-type tRNA genes that were isolated as suppressors of *gcn2-507*. Several lines of evidence indicate that increasing the amount of wild-type $tRNA^{His}$ under conditions in which the excess tRNA cannot be efficiently aminoacylated, or overexpressing a mutant $tRNA^{Val}$ that is defective for aminoacylation, leads to increased translation of *GCN4* mRNA. Most of the derepression response conferred by wild-type $tRNA^{His}$ genes was dependent on the residual kinase function of the *gcn2-507* product. In contrast, derepression elicited by the mutant $tRNA^{Val*}$ was largely independent of GCN2 and the phosphorylation site on eIF-2α. In some strains, a significant GCN2-independent derepression response was also produced by overexpression of wild-type $tRNA^{His}$ under histidine starvation conditions and therefore is not unique to the mutant $tRNA^{Val}$. Similarly, our results indicate that accumulation of uncharged $tRNA^{Ile}$ in a mutant containing an attenuated isoleucyl-tRNA synthetase leads to a significant general control response in a strain lacking GCN2. These latter results identify a second mechanism for stimulating *GCN4* translation that is responsive to multiple species of uncharged tRNA but does not involve antagonism of eIF-2B by phosphorylation of eIF-2α. The mutant $tRNA^{Val*}$ construct

did not derepress *GCN4* in a *gcn2Δ* strain to the same extent as did histidine deprivation of isogenic *GCN2* cells. This result could indicate that the GCN2-independent mechanism is inherently less efficient or requires higher levels of uncharged tRNA to achieve the same level of *GCN4* derepression compared with the GCN2-mediated pathway; however, this is by no means certain.

Do high levels of uncharged tRNA restore high-level GCN2 kinase function in *gcn2-507* mutants? The *gcn2-507* mutation is a two-codon insertion in a large domain of GCN2 that is similar in sequence to HisRSs. This domain is required in vivo for GCN2-mediated derepression of *GCN4* translation in response to amino acid starvation (51) and is thought to have a regulatory function in detecting uncharged tRNAs and activating the adjacent protein kinase domain of GCN2. This idea is supported by the isolation of dominant mutations that activate GCN2 under nonstarvation conditions and map in the portion of GCN2 HisRS-related sequences that are most conserved among class II aminoacyl-tRNA synthetases (38). When otherwise wild-type *gcn2-507* strains were starved for histidine with 3-AT, we did not observe a significant general control response; however, partial derepression was restored in these strains by overexpressing wild-type $tRNA^{His}$ (Table 2). The fact that the $tRNA^{His}$ constructs had a much smaller derepressing effect on a *gcn2Δ* strain could be interpreted to indicate that increasing the amount of uncharged $tRNA^{His}$ beyond the level that can be achieved with a wild-type complement of $tRNA^{His}$ genes permits enhanced activation of *gcn2-507* kinase function. For example, if the *gcn2-507* mutation decreases the binding affinity for uncharged $tRNA^{His}$, this defect could be overcome by increasing the concentration of uncharged $tRNA^{His}$.

This mechanism could also explain the following situations in which combining different types of uncharged tRNA restored derepression of *HIS* gene expression in *gcn2-507* cells. First, histidine starvation did not elicit significant derepression in an otherwise wild-type *gcn2-507* mutant, but we consistently observed increased *HIS4-lacZ* expression when transformants of this strain bearing the multi-copy $tRNA^{Val*}$ construct were starved for histidine (Table 2). Second, the ability of a *his1-29 gcn2-507* mutant to derepress *HIS* genes and grow on medium lacking histidine was restored by impairing aminoacylation of $tRNA^{Ile}$ by the *ils1-1* mutation (Fig. 4). Third, the presence of the multicopy $tRNA^{His}$ construct did not elicit derepression in a *gcn2-507* mutant under nonstarvation conditions but did so when the cells were starved for leucine, isoleucine, and valine with SM (Table 3). These observations could be accounted for by proposing that the level of uncharged $tRNA^{His}$ produced in each situation did not activate the *gcn2-507* product sufficiently to achieve the minimum level of eIF-2α phosphorylation needed to derepress *GCN4* translation. The critical level of uncharged tRNA and activation of GCN2 kinase function would be attained, however, when the uncharged $tRNA^{His}$ was combined either with the mutant $tRNA^{Val*}$ (which presumably cannot be charged) or with the uncharged form of wild-type $tRNA^{Ile}$, $tRNA^{Val}$, or $tRNA^{Leu}$. The idea that phosphorylated eIF-2α must reach a critical level before it can affect *GCN4* translation is in accord with our previous observations that a small fraction of eIF-2α is phosphorylated by GCN2 under conditions in which *GCN4* translation is fully repressed and that amino acid starvation simply increases the proportion of eIF-2α that is phosphorylated (9).

While the observations discussed above are consistent with the idea that high levels of uncharged tRNA restored derepression of *GCN4* by activating *gcn2-507* kinase function, there is an alternative explanation for these results. Because excess amounts of each tRNA that we examined elicited a significant derepression response in *gcn2Δ* strains, the derepression observed in *gcn2-507* strains could represent the additive effects of constitutive eIF-2α phosphorylation by the *gcn2-507* kinase and a GCN2-independent pathway that down-regulates eIF-2 without phosphorylation of the α subunit. The idea that two independent mechanisms combine to reduce eIF-2 activity below a critical level seems quite plausible for $tRNA^{Val*}$ because of its marked effect on *GCN4* expression in the absence of *GCN2* or Ser-51 of eIF-2α (Tables 2, 5, and 6) and the absence of increased eIF-2α phosphorylation in *GCN2* strains overexpressing this mutant tRNA (Fig. 6). This explanation must also be considered for $tRNA^{His}$, however, because we have been unable to observe increased eIF-2α phosphorylation in *gcn2-507* or *GCN2* strains bearing a high-copy-number $tRNA^{His}$ construct versus vector alone (data not shown). The fact that wild-type $tRNA^{His}$ genes showed a much greater dependence on *gcn2-507* for derepressing *GCN4* than did the $tRNA^{Val*}$ gene (Table 2) might simply reflect higher levels of uncharged tRNA produced by mutant $tRNA^{Val*}$ genes versus the wild-type $tRNA^{His}$ genes under our histidine starvation conditions. Consequently, the $tRNA^{Val*}$ gene would be less dependent on the eIF-2α phosphorylation catalyzed by *gcn2-507* for reducing eIF-2 activity below the critical level needed to derepress *GCN4* translation. Because suppression of *gcn2-507* by the $tRNA^{His}$ constructs is relatively inefficient and is at least partially attributable to the GCN2-independent pathway, we do not regard our inability to observe increased eIF-2α phosphorylation in *gcn2-507* cells bearing the $tRNA^{His}$ construct as an unequivocal indication that the *gcn2-507* kinase cannot be hyperactivated by overproduction of uncharged $tRNA^{His}$.

A mechanism for stimulating *GCN4* translation by uncharged tRNA that is independent of eIF-2α phosphorylation. Because the GCN2-independent derepression of *GCN4* elicited by $tRNA^{Val*}$ required the uORFs, we believe that it occurs at the translational level and involves a reduction in ribosomal reinitiation at uORFs 2-4. The fact that $tRNA^{Val*}$ constructs elicited derepression in the absence of Ser-51 on eIF-2α rules out the possibility that a second eIF-2α kinase phosphorylates Ser-51 and is activated by high levels of uncharged tRNA. However, the fact that overexpressing the mutant $tRNA^{Val*}$ exacerbated the growth defect conferred by a $GCN2^c$ allele is indicative of reduced levels of the active form of eIF-2. This finding suggests that the GCN2-independent pathway accessed by $tRNA^{Val*}$ derepresses *GCN4* translation by decreasing eIF-2 function. This could involve phosphorylation of the β or γ subunit of eIF-2, or of one of the subunits of eIF-2B itself, for which there is some evidence from animal systems (10, 53). Another possibility is that some other initiation factor that interacts with eIF-2 and is required for reinitiation on *GCN4* mRNA is down-regulated by the GCN2-independent derepression mechanism in the presence of uncharged tRNA.

The GCN2-independent response to $tRNA^{Val*}$ may be related to the transient derepression of *GCN4* translation that occurs following a nutritional shift-down from amino acid-rich to minimal medium, as the latter is also independent of GCN2 (48). It is noteworthy that formation of 43S preinitiation complexes decreases in such a nutritional shift-down (48), as eIF-2 function is required for the formation of this initiation intermediate (16), and our results suggest that overexpression of the mutant $tRNA^{Val*}$ leads to a reduction in eIF-2 activity (Fig. 7). Krupitza and Thireos (23) reported derepression of *GCN4* translation in a yeast cell-free system that was triggered by relatively high levels of uncharged tRNA and was depen-

dent on the uORFs but did not require GCN2. Perhaps a nutritional shift-down leads transiently to high levels of uncharged tRNA during the time it takes to mobilize intracellular pools of amino acids for aminoacylation (48), mimicking the addition of uncharged tRNA to the cell-free system. Recent results indicate that the cyclic AMP-dependent protein kinase (cADPK) is involved in the transient increase in *GCN4* translation following a nutritional shiftdown but not for the long-term starvation response that depends on GCN2. Moreover, it was found that constitutive activation of cADPK leads to partial derepression of *GCN4* translation on nutrient-rich medium (11). It will be interesting to determine whether the derepressing effect of the multicopy tRNA genes observed in *gcn2*Δ strains requires activation of cADPK and to investigate whether these tRNAs utilize the same signalling mechanism employed during a nutritional shift-down.

There are some puzzling aspects of our results which may indicate that fundamental differences exist between the GCN2-dependent and GCN2-independent systems in the recognition of uncharged tRNA. The GCN2-dependent system seems to provide the sole response to amino acid starvation imposed by the inhibitory analogs 3-AT, 5-MT, and SM. We saw no evidence for derepression in response to these analogs mediated by the GCN2-independent mechanism in *gcn2*Δ strains (Table 3). This is noteworthy because these strains are unable to grow in the presence of the analogs, presumably because of insufficient aminoacylation of tRNAs for the limiting amino acids. The levels of uncharged tRNA produced under these starvation conditions elicit substantial eIF-2α phosphorylation by GCN2 (Fig. 6). On the other hand, overexpression of the mutant $tRNA^{Val*}$ under nonstarvation conditions produced a sizable derepression response that was completely independent of GCN2 and did not lead to detectable eIF-2α phosphorylation (Fig. 6). It is surprising that overexpression of the mutant $tRNA^{Val*}$ does not appear to activate GCN2 kinase function.

One way to account for these observations would be to propose that GCN2 is not directly activated by uncharged tRNA. This possibility is at odds with results indicating that GCN2 mediates the derepression response elicited by mutations in the lysyl- and isoleucyl-tRNA synthetases (25) (Table 4). It would also be necessary to explain why GCN2 contains a HisRS-like regulatory domain (52) and is found associated with translating ribosomes (37), characteristics which we accounted for previously by postulating that GCN2 detects uncharged tRNA at the ribosome during the process of translation (37). A second explanation for our results would be that the mutant $tRNA^{Val*}$ is not recognized by GCN2. Even though valine starvation activates eIF-2α phosphorylation by GCN2 (Fig. 6), it is possible that this response is mediated by other $tRNA^{Val}$ isoacceptors. A third possibility is that GCN2 does not have access to the large pool of mutant $tRNA^{Val*}$. This could occur, for example, if GCN2 can be activated only when bound to translating ribosomes and if tRNAs are channeled between ribosomes and aminoacyl-tRNA synthetases in the manner proposed for mammalian cells (34). According to the channeling model, aminoacyl-tRNA is directly transferred from the synthetase to elongation factor 1α to the ribosome without dissociation into the cell fluid. If the excess uncharged tRNA produced in strains containing the high-copy-number $tRNA^{Val*}$ was excluded from this synthetase/EF-1α/ribosome cycle, it would be unable to stimulate GCN2. This exclusion might occur because the mutation in $tRNA^{Val*}$ weakens its interaction with valyl-tRNA synthetase. It is more difficult to explain how wild-type $tRNA^{His}$ could be excluded from the postulated synthetase/EF-1α/ribosome cycle; although we noted that the GCN2-independent pathway was stimulated much less effectively by the uncharged $tRNA^{His}$ than by the mutant $tRNA^{Val*}$. Perhaps an excess of uncharged wild-type $tRNA^{His}$ decreases the maturation of one or more tRNA species, leading to an accumulation of unchargable tRNA similar to $tRNA^{Val*}$. In the future, we hope to learn how the mutant $tRNA^{Val*}$ is recognized by the GCN2-independent pathway and why it fails to activate GCN2 as efficiently as occurs in valine-starved cells.

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