

Interplay between GCN2 and GCN4 expression, translation elongation factor 1 mutations and translational fidelity in yeast

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ABSTRACT

Genetic screens in *Saccharomyces cerevisiae* have identified the roles of ribosome components, tRNAs and translation factors in translational fidelity. These screens rely on the suppression of altered start codons, nonsense codons or frameshift mutations in genes involved in amino acid or nucleotide metabolism. Many of these genes are regulated by the General Amino Acid Control (GAAC) pathway. Upon amino acid starvation, the kinase GCN2 induces the GAAC cascade via increased translation of the transcriptional activator GCN4 controlled by upstream open reading frames (uORFs). Overexpression of the *GCN2* or *GCN4* genes enhances the sensitivity of translation fidelity assays that utilize genes regulated by GCN4, such as the suppression of a +1 insertion by *S.cerevisiae* translation elongation factor 1A (eEF1A) mutants. Paromomycin and the prion [PSI⁺], which reduce translational fidelity, do not increase GCN4 expression to induce the suppression phenotype and in fact reduce derepression. eEF1A mutations that reduce translation, however, reduce expression of GCN4 under non-starvation conditions. These eEF1A mutants also reduce HIS4 mRNA expression. Taken together, this system improves *in vivo* strategies for the analysis of translational fidelity and further provides new information on the interplay among translation fidelity, altered elongation and translational control via uORFs.

INTRODUCTION

Protein synthesis plays a key role in the efficient and accurate gene expression program required in all cells. The ribosome and soluble protein factors are essential for all steps of protein synthesis. Using the yeast *Saccharomyces cerevisiae*, many protein synthesis factors were first identified as a consequence of their effects on the accuracy of the translation process. These include the translation initiation factor eIF2 subunits identified in a screen for mutations that affect start site selection (1) as well as the termination factor eRF3, which is responsible for the [PSI⁺] omnipotent nonsense suppression phenotype (2).

Many screens for the identification of factors that alter translational fidelity rely on the suppression of altered start codons, nonsense codons or frameshift mutations in genes involved in amino acid or nucleotide metabolism [reviewed in (3)]. Several of the targets of the GCN4 transcription factor have alleles utilized to assess effects in fidelity, including *LYS2*, *TRP1*, *LEU2* and *HIS4* (4). These mRNAs are typically expressed at low levels, thus the identification of mutations that suppress these alleles can be difficult. In the case of the well-characterized [PSI⁺] prion of yeast, the ability to increase the sensitivity for *in vivo* analysis of the presence of this element, and the resulting nonsense suppression phenotype, is facilitated by combining an allele of the *ADE2* gene with an UAA nonsense mutation (*ade2.1*) and a specific UAA suppressor tRNA encoded by *SUQ5* (5).

One possibility is to increase the sensitivity of a wider range of screens for altered fidelity by using the *GCN2* and *GCN4* genes. Both genes are components of the General Amino Acid Control (GAAC) system that increases the expression, indirectly and directly, of a large array of genes belonging to metabolic pathways [reviewed in (6)]. The eukaryotic

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translation initiation factor 2 α (eIF2 α) kinase GCN2 is present in all eukaryotes, and is stimulated by serum or amino acid starvation. The ultimate signal for amino acid starvation is uncharged tRNA that binds to the histidyl-tRNA synthetase (HisRS)-like domain in GCN2 (7). Phosphorylation of the α -subunit of eIF2 is a key mechanism for adjusting the rate of protein synthesis in response to starvation or stress. Phosphorylation of eIF2 α converts eIF2 to a competitive inhibitor of the guanine nucleotide exchange factor eIF2B leading to low levels of ternary complex (TC) [reviewed in (6)].

Through phosphorylation of eIF2 α , GCN2 plays a dual role in down-regulating general translation and specifically up-regulating translation of the transcriptional activator GCN4. The latter is accomplished by four short upstream open reading frames (uORFs) in the GCN4 mRNA (8). Under amino acid-replete conditions, ribosomes translate uORF1, resume scanning and reacquire a TC before reaching uORF2, uORF3 or uORF4. Following translation of these latter uORFs, the majority of ribosomes disassociate from the mRNA so that only a small fraction of ribosomes reach the GCN4 ORF, resulting in low GCN4 translation. Phosphorylation of eIF2 α by GCN2 in amino acid starved cells lowers the level of TC. Consequently, after the translation of uORF1, ribosomes re-acquire the TC after they have scanned through uORF2, uORF3 and uORF4 and instead reinitiate at the GCN4 ORF leading to high GCN4 protein levels. Increased GCN4 protein levels subsequently stimulate transcription of GCN4 target genes, including those encoding numerous amino acid biosynthetic enzymes from 12 different pathways [reviewed in (4)].

In this paper we demonstrate the utility of overexpression of *GCN2* or *GCN4* to enhance the sensitivity of assays for altered translational fidelity in the yeast *S.cerevisiae*. Overexpression of either protein allows enhanced growth on selective media and thus increased the assay sensitivity for translation elongation factor 1A (eEF1A) mutants that specifically suppress a +1 insertion. Since these mutations affect translational fidelity, and GCN4 expression is controlled at the translational level by uORFs, we determined the effect of reduced fidelity on GCN4 expression. These results demonstrate a novel approach to improve *in vivo* strategies for the analysis of translational fidelity as well as the impact of altered fidelity on translational control of GCN4 expression.

MATERIALS AND METHODS

Strains and media

Escherichia coli DH5 α cells were used for plasmid preparation. *S.cerevisiae* strains used in these studies are listed in Table 1. Standard yeast genetic methods were employed (9,10). Yeast cells were grown in either YEPD (1% Bacto yeast extract, 2% peptone and 2% dextrose) or defined synthetic complete media (C or C-) supplemented with 2% dextrose as a carbon source. Yeast cells were transformed by the lithium acetate method (11).

Growth assays

Streaks of yeast cells expressing empty vector (pRS316) or plasmids expressing *GCN2* (p722), *GCN2^c* constitutive mutant alleles E803V (p1253), M788V (p912) or M788V-E1591K (p1055) (12), or *GCN4* (p164), *GCN4* with uORF1 (p235), or *GCN4* with no uORFs (p238) (13) were maintained on C-Ura media and assayed for growth via increased expression of the *his4-713* gene product on C-Ura-His media following 7 days of growth at 30°C. Solid media testing of sensitivity to paromomycin and cycloheximide in C-Ura media was performed via a lawn assay as described previously (14). Growth inhibition was monitored as the zone of inhibition of growth from the filter disc (in mm) repeated at least in triplicate. Liquid growth assays of wild-type strains in the presence of paromomycin or cycloheximide and for [*PSI+*] strains were performed using mid-log phase cells grown in YEPD or complete media and were diluted to an A_{600} of 0.1 or 0.2 and were incubated with a range of concentrations of the various compounds at 30°C in 96-well microtiter plates. All experiments were performed in triplicate and were repeated at least three times.

Assays of GCN4-lacZ reporter expression

Strains were transformed with the *GCN4 lacZ* reporter plasmid [p180 (8)] and were maintained on C-Ura media. Cells were grown in C-Ura-Val-Ile medium for 2 days to saturation and were diluted to an A_{600} of 0.8. The sulfonylurea herbicide sulfometuron methyl (SM), an inhibitor of leucine, isoleucine and valine biosynthesis (15), was added to a final concentration of 2 mg/ml and the cells were grown for 6 h to induce starvation (16). To assess the effect of paromomycin or cycloheximide on reporter expression, the indicated drug was added to cells at the same time as the addition of SM. Extracts were

Table 1. *S.cerevisiae* strains

Strain	Genotype	Reference
MC214	<i>MATα ura3-52 trp1-Δ101 lys2-20 leu2-3,112 met2-1 his4-713 tef1::LEU2 tef2-Δ pTEF2 TRP1</i>	(20)
TKY225	<i>MATα ura3-52 trp1-Δ101 lys2-20 leu2-3,112 met2-1 his4-713 tef1::LEU2 tef2-Δ ptef2-17 TRP1 D156N</i>	(19)
TKY226	<i>MATα ura3-52 trp1-Δ101 lys2-20 leu2-3,112 met2-1 his4-713 tef1::LEU2 tef2-Δ ptef2-19 TRP1 N153T</i>	(19)
TKY229	<i>MATα ura3-52 trp1-Δ101 lys2-20 leu2-3,112 met2-1 his4-713 tef1::LEU2 tef2-Δ ptef218 TRP1 D156E N153T</i>	(19)
TKY252	<i>MATα ura3-52 trp1-Δ101 lys2-20 leu2-3,112 met2-1 his4-713 tef1::LEU2 tef2-Δ pTEF2-4 TRP1 E122K</i>	(24)
TKY235	<i>MATα ura3-52 trp1-Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 pTEF5 URA3</i>	(14)
TKY243	<i>MATα ura3-52 trp1-Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 ptef5-7 LEU2 K120R S121Δ I122Δ</i>	(14)
TKY588	<i>MATα ura3-52 trp1-Δ101 lys2-20 leu2-3,112 met2-1 his4-713 tef1::LEU2 tef2-Δ pTEF2-1 TRP1 E286K</i>	(22)
TKY597	<i>MATα ura3-52 trp1-7 lys2 leu2-3,112 met2-1 his4-713 yef3::LEU2 pYEF3 TRP1</i>	(24)
TKY599	<i>MATα ura3-52 trp1-7 lys2 leu2-3,112 met2-1 his4-713 yef3::LEU2 pyef3 TRP1 F650S</i>	(24)
628-3Aa ψ +	<i>MATα ura3-52 kar1.1 ade2.1 SUQ5 trp1Δ63 leu2-Δ[<i>PSI+</i>]</i>	(5)
628-3Aa ψ -	<i>MATα ura3-52 kar1.1 ade2.1 SUQ5 trp1Δ63 leu2-Δ[<i>psi-</i>]</i>	(5)

prepared and β -gal activity was monitored as described previously (8). In all cases identical cultures were grown at the same A_{600} and for the same time in the absence of SM to determine non-starvation levels of expression. All experiments were performed in a minimum of triplicate.

Northern blot analysis

MC214 or TKY252 expressing empty vector (pRS316), the E803V *GCN2^c* constitutive mutant allele (p1253) or *GCN4* with no uORFs (p238) (13) were grown in C-Ura media at an A_{600} of 0.6–1.0, harvested by centrifugation and total RNA was isolated as described previously (17). RNA (20 μ g) was separated on a 1.4% formaldehyde agarose gel, transferred onto Hybond⁺ membranes overnight by capillary action and crosslinked to the membrane with a Spectrolinker XL-1000 (Spectronics). Hybridization and detection were performed as per the ExpressHyb protocol (Clontech). ³²P-labeled probes for yeast *HIS4* and *ACT1* mRNAs were prepared using the Rad Prime Labeling System kit (Invitrogen). Radioactive signals were detected using a Phosphorimager Typhoon 9410 (Molecular Dynamics) and quantified with the Image-Quant program (Molecular Dynamics).

RESULTS

Expression of an activated form of the eIF2 α kinase GCN2 increases the readout of a reporter allele of reduced translational fidelity in eEF1A mutants

Genetic screens for reduced fidelity typically rely on readout of metabolic reporter genes. However, the levels of the reporter mRNA and the protein produced during translation can be below the level required for growth in the absence of the product of these mRNAs. While suppressor tRNAs are one mechanism to enhance sensitivity, we hypothesized that expression of genes under the control of the GAAC pathway could be increased by elevating the levels of GCN genes. The effects of GCN2 expression on a series of eEF1A mutations were assessed via growth on media that reports altered translational fidelity. All yeast strains used in our studies contain the *his4-713* and *met2-1* alleles, where growth on the C-His or C-Met media requires the suppression of a +1 frameshift mutation. They also contain the *lys2-20* mutation, where growth on C-Lys media indicates the suppression of a nonsense mutation. Three eEF1A mutants with point mutations at N153T (TKY226), D156E N153T (TKY229) and D156N (TKY225) alter the NKXD GTP-binding consensus element (18). Although these mutants show modest effects on nonsense suppression using a sensitive *lacZ* reporter (19), they do not allow growth on C-media lacking His, Met or Lys and thus cannot suppress *his4-713*, *met2-1* or *lys2-20* mutations, respectively. The E122K (TKY252) and E286K (TKY588) mutations in eEF1A were isolated in a screen for dominant suppressors of the *met2-1* allele (20). While both mutations allow growth on C-Met when present as the only form of the protein, neither allows growth on C-Lys (21,22). Thus, eEF1A mutations that were found in a screen for dominant reduced translation fidelity or that target GTP hydrolysis did not show satisfactory results in phenotypic assays scoring for His prototrophy using the +1 frameshift mutations *his4-713*.

We reasoned that this could be due to the fact that the reduced translation fidelity associated with the studied alleles was not strong enough to yield enough functional *HIS4* and *MET2* encoded proteins to allow growth on medium lacking His or Met. Thus, increased mRNA levels of these reporter genes should allow the production of sufficient amounts of functional proteins to render cells prototrophic for these amino acids. Since the transcription of *HIS4*, and to a lesser extent *MET2*, is under the control of GAAC, increased GCN2 and the resultant increased GCN4 levels should render cells with a weak reduction in translation fidelity His⁺ and potentially increase Met⁺ growth.

To induce increased cellular GCN2 and thus GCN4 levels a wild-type *GCN2* gene or a constitutively active *GCN2^c* (E803V) allele on a *URA3 CEN*-based plasmid (12) was transformed into a wild-type strain [MC214 (20)] or a strain expressing one of the mutations in eEF1A. Cells were grown on C-Ura medium to identify any changes in growth. Neither the wild-type nor the five mutant strains showed alterations of growth on C-Ura medium at 30°C with an empty vector control or in the presence of *GCN2* or *GCN2^c* (E803V) (Figure 1A, C-Ura). Analysis of growth in C-Ura-His media that monitors translational fidelity defects indicated that some mutants exhibit enhanced growth in the presence of *GCN2* or *GCN2^c* (E803V). Strains containing eEF1A mutations, E122K or E286K, showed significantly improved growth on C-Ura-His media when an extra copy of *GCN2* or *GCN2^c* (E803V) was present (Figure 1A), indicating increased expression of the *his4-713* gene product. This could occur either through increased suppression of the +1 frameshift signal or through an increase in the level of the *his4-713* mRNA. The three other mutants in the NKXD GTP-binding motif showed no growth on C-Ura-His with *GCN2* or *GCN2^c* (E803V) present, consistent with their lack of a frameshift suppression phenotype (data not shown). There was no noticeable increase in growth on C-Ura-Met media, which monitors a +1 frameshift event at

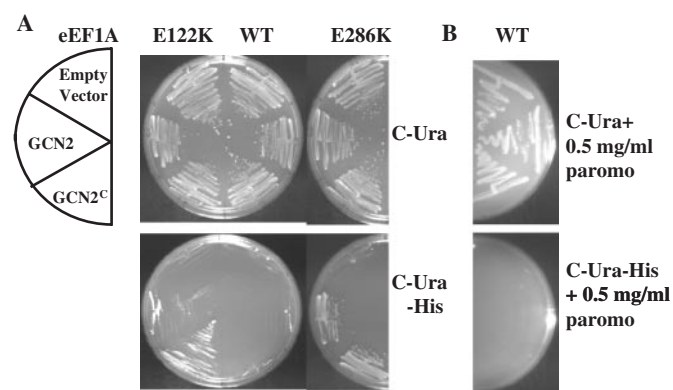


Figure 1. Excess GCN2 activity allows growth of select eEF1A mutant strains on C-Ura-His media, indicating increased expression of the *his4-713* gene product. (A) Either an empty vector (pRS316) or a plasmid expressing wild-type *GCN2* or the constitutively active *GCN2^c* E803V allele was transformed in yeast expressing wild-type eEF1A or the E122K and E286K eEF1A mutants. Cells were grown on either C-Ura (upper panel) or C-Ura-His (lower panel) media for 3 days at 30°C. Growth in the absence of His indicates expression of the product of the *his4-713* +1 insertion allele. (B) Growth was monitored for yeast expressing wild-type eEF1A and the same plasmids as in (A) with the addition of 0.5 mg/ml paromomycin (parom).

met2-1, but this gene is not as responsive as *HIS4* to the GCN4 transcription factor (23). These observations indicate that the fidelity reporter system is probably more sensitive due to increased mRNA levels rather than an increased +1 frameshift. There was no growth on C-Lys that monitors nonsense suppression of *lys2-20* (data not shown). *LYS2* is a target for GCN4, however, the E122K and E286K eEF1A mutations were selected specifically for the suppression of a +1 insertion and the mutations in the NKXD element have been shown previously to have extremely low levels of suppression of nonsense codons. Allele-specific suppression is common, and has been observed previously for these eEF1A alleles as described above (20,21).

To determine if other *GCN2^c* alleles also cause this effect, cells expressing wild-type, N153T, E122K or E286K forms of eEF1A were transformed with plasmids bearing *GCN2^c* (M788V) or *GCN2^c* (M788V-E1591K), which are stronger constitutive alleles (12). Growth was monitored on C-Ura and C-Ura-His media and essentially no growth was seen on C-Ura-His media with the empty vector. *GCN2^c* (M788V) and *GCN2^c* (M788V-E1591K) expressing plasmids resulted in enhanced growth on C-Ura-His media specifically in strains bearing the E122K or E286K forms of eEF1A (data not shown). The N153T mutants strain showed no growth in these conditions, similar to the results obtained with the *GCN2^c* E803V allele.

We wished to determine if this effect was due to altered fidelity and was not a consequence of reduced translation owing to the eEF1A mutants altering the regulation of the uORFs of GCN4. The effect of *GCN2* or *GCN2^c* (E803V) was assessed in strains bearing the *his4-713* allele and mutations in other translation elongation factors that conferred similar growth defects and reduced total protein synthesis to the E122K and E286K eEF1A mutations but do not increase ribosomal frameshifting or nonsense suppression. Strains with the F650S mutation in eEF3 (TKY599) (24) or the K120R S121Δ I122Δ mutations in eEF1B α (TKY243) (14) and the isogenic wild-type strains failed to show any growth on C-His or C-Ura-His media in the presence of a plasmid containing *GCN2* or *GCN2^c* (E803V) (data not shown). Thus, the growth effect is specific to a subset of eEF1A mutant strains that suppress a +1 insertion in the *his4-713* allele.

Excess *GCN2* or *GCN2^c* (E803V) increases paromomycin sensitivity in strains expressing the wild-type but not E122K form of eEF1A

The finding that in strains harboring specific eEF1A mutations the overexpression of *GCN2*, and to a greater extent *GCN2^c* (E803V), results in increased growth on C-Ura-His could indicate increased +1 slippage to produce the *HIS4* encoded protein. Prior work has indicated that increased or decreased sensitivity to the aminoglycoside antibiotic paromomycin correlates with reduced translational fidelity (25,26). Paromomycin induces altered fidelity by affecting aminoacyl-tRNA binding to the A-site of the ribosome (27). Accordingly, if the reduced translation fidelity of eEF1A mutant strains is not exacerbated by excess *GCN2* or *GCN2^c* (E803V), these strains should not be expected to show increased sensitivity to paromomycin as compared with strains harboring native *GCN2*. To assess this possibility, strains expressing

Table 2. The addition of *GCN2* or *GCN2^c* E803V results in increased sensitivity to paromomycin of wild-type but not an eEF1A mutant strain

Strain	Paromomycin (500 mg/ml)	Cycloheximide (1 mM)
WT (empty)	1.5 mm ^a	6.5
WT (<i>GCN2</i>)	2	10
WT (<i>GCN2^c</i> E803V)	3	11
E122K (empty)	2	10
E122K (<i>GCN2</i>)	1.5	11
E122K (<i>GCN2^c</i> E803V)	1.75	11
E286K (empty)	9	11
E286K (<i>GCN2</i>)	7.5	12
E286K (<i>GCN2^c</i> E803V)	5.5	15

^aRadius of the halo of inhibition of growth surrounding a filter disc with 10 μ l of the indicated concentration of drug.

wild-type eEF1A or the E122K and E286K mutant forms in the presence of excess *GCN2* or *GCN2^c* (E803V) were assayed for paromomycin sensitivity as indicated by the inhibition of growth of a lawn of cells around a disc saturated with 500 mg/ml paromomycin and 1 mM cycloheximide. A wild-type strain showed a slight increase in paromomycin sensitivity with excess *GCN2*. The sensitivity was increased with *GCN2^c* (E803V; Table 2). Strains expressing the E122K and E286K mutants of eEF1A are more sensitive to paromomycin than the wild-type strain (21). An E122K mutant strain showed no response to the effects of *GCN2* or *GCN2^c* (E803V) on paromomycin sensitivity. *GCN2*, and to a larger extent *GCN2^c* (E803V), suppressed the paromomycin sensitivity of the E286K mutant strain. Thus, while increasing *GCN2* activity slightly increases paromomycin sensitivity in a wild-type cell, it has little effect in the E122K mutant and actually decreases paromomycin sensitivity in the E286K strain. Cycloheximide sensitivity was assessed in comparison to a drug that also inhibits translation elongation but is not linked to altered translational fidelity. *GCN2* or *GCN2^c* (E803V) expression increased cycloheximide sensitivity for a wild-type or E286K eEF1A strain. Thus, while subtle differences are apparent in the interaction between *GCN2* levels and drug sensitivity, these do not correlate with the His⁺ phenotype.

Exposure to paromomycin does not increase *GCN2* or *GCN2^c* induced growth on C-Ura-His

Since some eEF1A mutants reduce fidelity and show enhanced growth on C-Ura-His in the presence of the *his4-713* mutation with increased *GCN2*, we assessed if the growth was a result of a general reduction in fidelity. Cells with the *his4-713* mutation were treated in liquid culture with concentrations of paromomycin that have been shown previously to cause reduced translational fidelity without affecting cell growth in *S.cerevisiae* (28). Otherwise wild-type cells (MC214) containing the empty vector pRS316, *GCN2* or *GCN2^c* (E803V) were grown on C-Ura or C-Ura-His media supplemented with paromomycin at concentrations of 0.01, 0.1, 0.2 and 0.5 mg/ml. None of these concentrations inhibited the growth of the yeast on C-Ura, or allowed growth on C-Ura-His, with or without *GCN2* or *GCN2^c* (E803V) (Figure 1B and data not shown). Thus, the general reduction in translational fidelity elicited

with paromomycin does not suppress the His⁻ growth phenotype of *his4-713* cells even in the presence of elevated GCN2 function. This is consistent with the interpretation that the growth effects seen on C-His with the eEF1A mutations are not due to reduced fidelity affecting expression independent of the frameshift specific effects of these mutants.

Conditions that reduce fidelity in yeast do not increase GCN4 expression

Since GCN4 expression is under translational control via four uORFs (6), we sought to understand the interplay between translational fidelity and the GCN4 regulation system using well-characterized mechanisms to alter translational fidelity. In order to specifically assay if altered fidelity affects GCN4 expression, cells were treated with paromomycin at increasing concentrations from 0.2 to 1.0 mg/ml and the expression of GCN4 was determined using a *GCN4-lacZ* reporter construct (8). Isoleucine/valine/leucine starvation was induced by growth in C-Ura-Val-Ile to exponential phase followed by the

addition of SM for 6 h. Mutants with defects in inducing translation of GCN4 were unable to increase the expression of GCN4-regulated genes and, therefore, were unable to overcome isoleucine/valine/leucine starvation (15). Paromomycin treatment produced essentially no effect on GCN4-lacZ expression under non-starvation conditions (Table 3). Under starvation conditions without drug, GCN4-lacZ expression was elevated as expected, however, the addition of paromomycin reduced GCN4-lacZ expression. The paromomycin concentration inversely correlated with the amount of GCN4-lacZ derepression (Figure 2A), with the highest paromomycin concentration inhibiting GCN4-lacZ derepression. This effect was not due to paromomycin altering cell growth (Figure 2B). The doubling times showed no significant change at the two lowest levels of treatment 0.2 mM (163.3 ± 14.4 min) and 0.5 mM (169.3 ± 1.2 min) paromomycin, compared with the absence of drug (147.3 ± 5.7 min). A slight increase in doubling time was observed at 1.0 mM paromomycin (196 ± 1.7 min). Thus, altered translation fidelity does not lead to increased GCN4 expression.

Table 3. Translation elongation inhibitors, paromomycin and cycloheximide, do not increase lacZ-GCN4 expression and differentially affect derepression

Paromomycin (mg/ml)	GCN4-LacZ – SM	GCN4-LacZ + SM	Cycloheximide (ng/ml)	GCN4-LacZ – SM	GCN4-LacZ + SM
0	115.5 ± 6.4 ^a	216.5 ± 30.5	0	79.5 ± 13.4	125.6 ± 8.5
0.2	127.6 ± 7.2	165.5 ± 6.8	0.1	84.0 ± 1.3	150.1 ± 9.2
0.5	109.9 ± 9.1	150.8 ± 16.2	1	87.1 ± 12.0	141.2 ± 8.3
1.0	96.6 ± 6.7	101.0 ± 3.2	10	25.9 ± 2.1	82.0 ± 2.5

^aβ-Galactosidase activity (*lacZ* units).

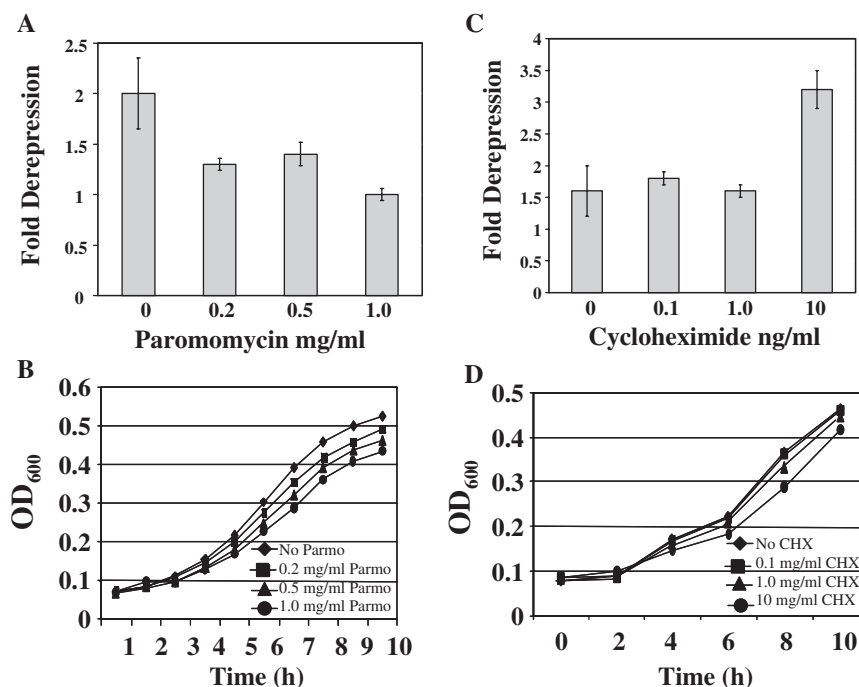


Figure 2. The error inducing aminoglycoside paromomycin does not lead to increased derepression of GCN4-lacZ. (A) Expression of GCN4 was determined using a lacZ-based reported construct (p180) and the fold derepression determined as the ratio of expression under non-starvation (C-Ura-Val-Ile) or starvation (C-Ura-Val-Ile + 2 mg/ml SM for 6 h) conditions at concentrations of paromomycin ranging from 0 to 1.0 mg/ml. (B) Cells were grown in mid-log phase in C-Ura medium with 0 (diamonds), 0.2 (squares), 0.5 (triangles) or 1.0 (circles) mg/ml paromomycin (paromo) in 96-well plates with constant shaking at 30°C and monitored for the OD₆₀₀. Expression of GCN4-lacZ (C) and growth rates (D) were determined as in (A) and (B), respectively, with 0 (diamonds), 0.1 (squares), 1.0 (triangles) or 10.0 (circles) 10 mg/ml cycloheximide (CHX).

To specifically analyze the inhibition of translation without altering fidelity, cells were treated with 0–1 ng/ml cycloheximide, concentrations were chosen based on prior work (29). Treatment with cycloheximide, a drug that inhibits the peptidyl transferase activity of the ribosome, at 0.1 or 1 ng/ml resulted in no effect on GCN4 expression under non-starvation conditions (Table 3) or on the level of derepression (Figure 2C). These same concentrations showed no effect on the growth of the cells (Figure 2D). When cells were treated with high concentrations of drug (10 ng/ml), GCN4 expression was inhibited more under non-starvation than under starvation conditions (Table 3), giving an apparent increase in derepression (Figure 2C).

In order to alter translational fidelity without inhibiting translation elongation, the effect of the element *[PSI+]*, a prion form of the translation termination factor eRF3, was assessed. The presence of *[PSI+]* results in increased nonsense suppression [reviewed in (30)]. Utilizing isogenic *[psi-]* and *[PSI+]* strains (5), no effect of *[PSI+]* was seen on GCN4 expression under non-starvation conditions (Table 4). The derepression of GCN4 under starvation conditions was reduced in the presence of *[PSI+]* (Figure 3A). This was not due to different growth characteristics of *[psi-]* and *[PSI+]* strains (Figure 3B). Thus, in strains with reduced fidelity due to either the presence of *[PSI+]* or paromomycin treatment, the

Table 4. The presence of *[PSI+]*, which reduces translational fidelity, reduces GCN4-LacZ derepression

Strain	GCN4-LacZ – SM	GCN4-LacZ + SM
WT <i>[psi-]</i>	29.2 ± 2.9 ^a	202.0 ± 13.3
WT <i>[PSI+]</i>	33.7 ± 5.6	123.2 ± 12.5

^aβ-Galactosidase activity (*lacZ* units).

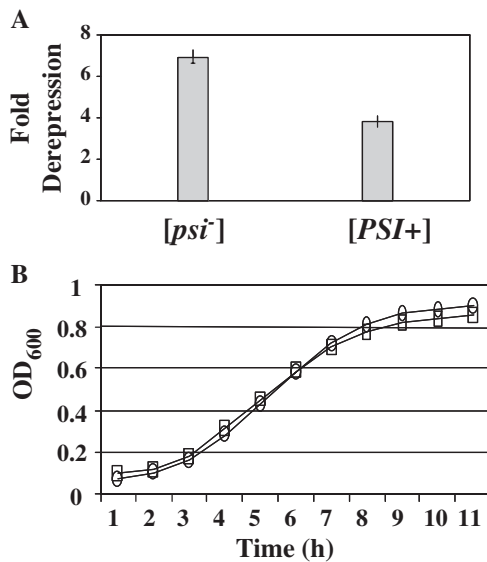


Figure 3. Reduced derepression of GCN4-LacZ is seen in the presence of increased nonsense suppression by *[PSI+]*. (A) Expression of GCN4 was determined using a *lacZ*-based reporter construct (p180) and the fold derepression determined as described in Figure 2 in otherwise isogenic strains without *[psi-]* or with *[PSI+]*. (B) *[psi-]* (open circle) or *[PSI+]* (open squares) cells were grown in mid-log phase in Complete medium in 96-well plates with constant shaking at 30°C and growth monitored by the OD₆₀₀.

derepression of GCN4 is reduced. This indicates that GCN4 expression and derepression are sensitive to conditions that alter translational fidelity.

eEF1A mutants show allele-specific effects on GCN4 expression

To understand the starting levels of GCN4 expression that are the baseline for the GCN2 effect, we directly assessed the effect of mutant forms of eEF1A on GCN4 expression using the *lacZ*-based reporter assays. The assay was performed in strains expressing mutant forms of eEF1A that either fail to show increased growth on C-Ura-His media in the presence of excess GCN2 activity (N153T, D156E N153T and D156N) or the two mutants with enhanced growth under these conditions (E122K and E286K). Previous work had demonstrated that strains bearing N153T, D156E N153T and D156N show essentially wild-type rates of total protein synthesis (19), while strains with either E122K or E286K show a 50% reduction in total translation (24). Expression of the GCN4 reporter under non-starvation conditions was essentially the same for wild-type and D156N mutants strains and was slightly reduced for N153T and N153T D156E mutant strains (Table 5). Expression was dramatically reduced for E122K and E286K mutants to 30% or less of the wild-type level. When compared with expression under starvation conditions, the fold derepression was slightly increased for strains expressing the N153T, D156E N153T and D156N mutations but dramatically increased for expressing the E122K (7.1-fold) and E286K (12.9-fold) mutations to levels 3.7–6.8 times the derepression observed in strains expressing the wild-type eEF1A (Figure 4).

Table 5. Expression of GCN4-LacZ is reduced under non-starvation conditions in E122K and E286K eEF1A mutant strains

eEF1A	GCN4-LacZ activity – SM	GCN4-LacZ activity + SM
WT	63.5 ± 6.0 ^a	124.1 ± 6.5
D156N	70.3 ± 2.2	211.7 ± 29.6
N153T	39.9 ± 2.8	106.6 ± 11.7
N153T D156E	41.5 ± 2.5	165.9 ± 8.1
E122K	18 ± 3.4	128.9 ± 6.3
E286K	14 ± 2.1	128.9 ± 8.8

^aβ-Galactosidase activity (*lacZ* units).

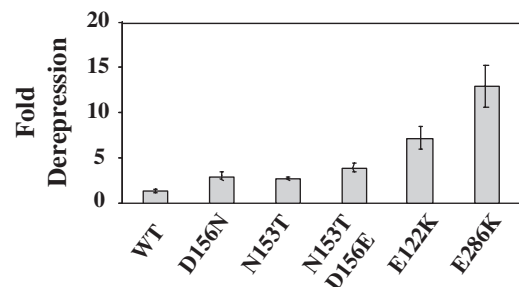


Figure 4. Starvation induces enhanced GCN4-LacZ derepression in strains containing E122K or E286K eEF1A mutants. Expression of GCN4 was determined using a *lacZ*-based reporter construct (p180) and the fold derepression determined as in Figure 2 for strains expressing wild-type (MC214), N153T (TKY226), D156E N153T (TKY229), D156N (TKY225), E122K (TKY252) and E286K (TKY588) forms of eEF1A (19–22).

The level of expression under starvation conditions increased in all cases. Comparison of the absolute expression levels between strains with wild-type, N153T, E122K and E286K mutants, however, were all within experimental error (Table 5). Thus, while the fold derepression was dramatically increased, the total level of GCN4 expression under starvation conditions was the same. Thus, it does not appear that the increased growth of *his4-713* mutant in C-Ura-His was due to an excessive increase in GCN4 expression caused by select eEF1A mutations under starvation or normal conditions but instead the growth effects are caused by the interplay of excess GCN2 activity and the presence of select alleles of eEF1A that suppress a +1 frameshift mutation.

Increased GCN4 is sufficient for increased growth of strains bearing a *his4-713* mutation and specific eEF1A mutations that suppress the +1 insertion

Since the results support the model that GCN4 expression is increased by GCN2 levels and thus *his4-713* mRNA levels, we bypassed the GCN2-mediated induction by utilizing a series of GCN4 expressing plasmids with modifications in the uORFs that regulate GCN4 expression (13). The wild-type *GCN4* expression plasmid has all four uORFs, and its expression is subject to *GCN2* regulation. The *GCN4* construct containing only uORF1 has released some of the repression of GCN4 expression, and the *GCN4* lacking all uORFs is expressed constitutively, leading to constitutive transcription of GCN4-regulated genes including *his4-713*. As shown in Figure 5, a strain expressing the N153T mutant or wild-type eEF1A shows no growth on C-Ura-His with any *GCN4* plasmid. Both E122K and E286K mutant strains, however, show growth with the uORF1 and no ORF *GCN4* constructs. This shows that the eEF1A specificity is the same as seen with elevated GCN2 expression or activity. The transformation efficiency of the *GCN4* overexpression constructs and stable growth of the transformed strains is significantly lower than the *GCN2*

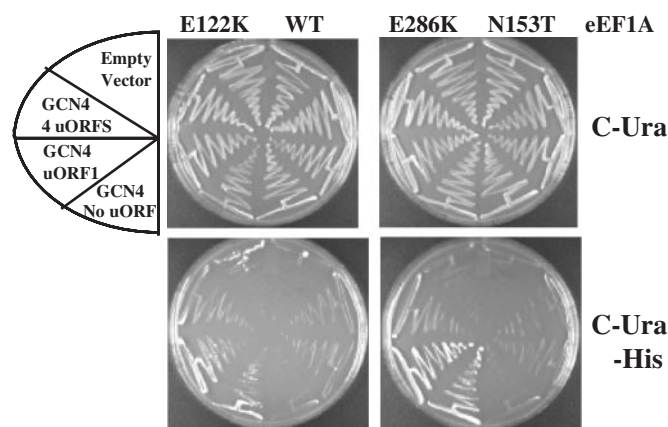


Figure 5. Overexpression of GCN4 promotes eEF1A allele-specific growth on C-Ura-His. Either an empty vector (pRS316) or a plasmid expressing wild-type GCN4, GCN4 with only uORF1 and GCN4 with no uORFs were transformed in yeast expressing wild-type eEF1A or the E122K, N153T and E286K eEF1A mutants. Cells were grown on either C-Ura (upper panels) or C-Ura-His (lower panels) for 3 days at 30°C.

plasmids, indicating these may be less optimal for use in screening applications.

The E122K eEF1A mutant shows reduced expression of the *HIS4* mRNA

The levels of growth on C-Ura medium and the difference in levels of GCN4 activation in wild-type versus the E122K eEF1A mutant support a model where the E122K mutant has reduced levels of GCN4 expression relative to wild-type and that consequently this strain is more sensitive to increased GCN4 activity. To confirm these results in increased *HIS4* expression, a northern blot was performed on wild-type and E122K eEF1A strains expressing a vector alone, a plasmid expressing a constitutively active *GCN2^c* E803V, GCN4 with only uORF1 or GCN4 with no uORFs. As shown in a representative blot in Figure 6A, *HIS4* mRNA levels are higher in the wild-type strain than in the E122K mutant with the empty vector and are grown in C-Ura medium. The addition of *GCN2^c* E803V or GCN4 overactive or overexpressing plasmids increased the expression of *HIS4* mRNA in the E122K mutant strain. Thus, the eEF1A mutant is more strongly repressed for GCN4 activity, and thus responds to elevated GCN2 and GCN4 expression and results in enhanced GCN4 induction under starvation conditions as shown in Figure 4.

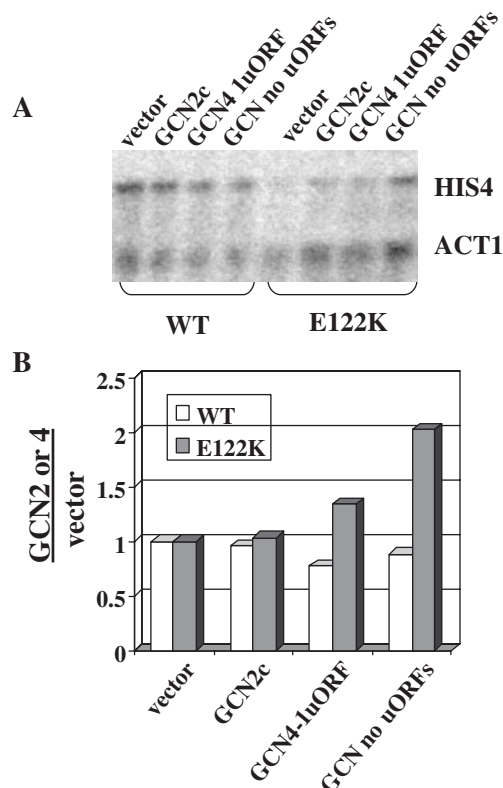


Figure 6. Overexpression of GCN4 promotes higher *HIS4* expression in an eEF1A E122K mutant strain. (A) Either an empty vector (pRS316) or a plasmid expressing a constitutively active *GCN2^c* E803V, GCN4 with only uORF1 or GCN4 with no uORFs. Cells were grown in C-Ura, RNA extracted and *HIS4* and *ACT1* mRNAs detected. (B) *HIS4* levels were normalized to *ACT1* levels per sample and were then expressed as the ratio of that sample to the same strain expressing an empty vector. The average is shown.

DISCUSSION

In our studies we report an improved procedure that allows enhanced *in vivo* scoring of translation fidelity phenotypes. The assay takes advantage of the GAAC system and the fact that many reporter genes used to score for translation fidelity are upregulated by GAAC. Increased GCN2 activity leads to increased expression of *his4-713* due to increased GCN4 translation. Increased *his4-713* mRNA levels allow factors that impair protein synthesis and thus translate sufficient amounts of functional *his4-713* encoded protein to allow cells to grow in the absence of histidine. A plasmid construct harboring the *GCN4* gene with all four uORFs is subject to translational repression and fails to induce growth of the eEF1A E122K or E286K mutations on C-Ura-His media. Removing uORF2, uORF3 and uORF4 leads to increased GCN4 protein levels and growth on C-Ura-His, and further removal of all uORFs resulted in the highest growth. Thus, increasing GCN4 levels gives the same result as increasing GCN2 levels or activity. This supports the model that constitutively increased *his4-713* mRNA levels results in increased sensitivity of the translation fidelity assay (Figures 1 and 5). Since GCN4 should be derepressed on C-His medium, this system must hyper-induce the level of GCN4 to enhance the assay.

The ability to enhance sensitivity of assays of frameshift or nonsense suppression more generally, without requiring a specific nonsense-containing allele/suppressor tRNA pairs such as is commonly used for the analysis of [PSI⁺], has implications in enhanced isolation and characterization of mutations that alter translational fidelity. The use of GCN2 or GCN4 expression has advantages over current approaches. It is not specific to a single stop codon or +1 or -1 frameshift signal-like suppressor tRNA mutations. Thus, in a single strain with multiple alleles it may be possible to simultaneously enhance the sensitivity of suppression of multiple target genes responsive to GCN4 expression. This system is also preferable to the overexpression of a specific reporter mRNA, such as that encoded by *his4-713*, since expression with a constitutive promoter may lead to a very high level of expression that will give a positive growth phenotype. Further, it is possible to vary the sensitivity by using GCN2^c alleles of varying severity. This system is easy to move between strain and with the enhanced sensitivity may allow for the comparison of mutants with intermediate effects on fidelity.

Since the GCN2/GCN4 system is regulated at the level of translation, the effects of reduced translation or fidelity on GCN4 expression were determined. Paromomycin was utilized to induce A-site errors related to aminoacyl-tRNA binding and presentation, and the [PSI⁺] form of eRF3 was utilized to specifically affect nonsense suppression. Both conditions reduced GCN4 expression under starvation conditions. This could be due to increased readthrough of the uORF stop codons, elongating the uORF sequences to inhibit initiation at the GCN4 AUG.

We directly studied the GCN4 expression in strains harboring various eEF1A alleles that reduce fidelity by using the *GCN4-lacZ* reporter gene. The eEF1A E122K and E286K mutations reduced the expression of GCN4 under non-starvation conditions while not affecting GCN4 expression levels under isoleucine/valine/leucine starvation, thereby accounting for the significantly increased level of GCN4

derepression. This might be due to the slowed elongation, as this effect correlates with those eEF1A mutants that show reduced total translation and elongation *in vivo* (24). eEF1A mutations do not, however, affect the expression of GCN4 under amino acid starvation conditions. This effect is further seen by northern blot analysis of the mRNA levels of the GCN4 target gene *HIS4*. The E122K mutant shows an increase in *HIS4* expression in the presence of overactive or overexpressed GCN2 or GCN4, consistent with the enhanced growth on C-Ura-His medium. The E122K mutant also shows much lower expression of *HIS4* with the empty vector compared with the isogenic wild-type eEF1A strain. This correlates with the greater derepression seen for GCN4 under SM starvation and supports the observation that the eEF1A mutant strains show reduced GCN4 expression.

The eEF1A E122K and E286K mutant strains or treatment of yeast with high levels of cycloheximide partially suppress the level of GCN4 expression. Since these are independent mechanisms that reduce translation, it is possible that the reduced elongation rates at uORF2, uORF3 or uORF4 could slow down scanning in the GCN4 leader. The model of GCN4 regulation proposed by Grant *et al.* (13) indicates that slowing of elongation at uORF4 could lead to ribosomes accumulating upstream of the uORF4. Thus, reduced elongation could favor re-initiation at uORF2, uORF3 or uORF4 when TC levels are high and the ribosomes have to longer bind TC. However, under starvation conditions when TC levels are lower, the ribosomes queued in the GNC4 leader would diminish, thus restoring the regulation to normal levels as we observe. Thus, the regulation of uORFs is subject to altered efficiency with altered translation elongation rates or fidelity.

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