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The Sua5 Protein Is Essential for Normal Translational Regulation in Yeast[▽]

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The anticodon stem-loop of tRNAs requires extensive posttranscriptional modifications in order to maintain structure and stabilize the codon-anticodon interaction. These modifications also play a role in accommodating wobble, allowing a limited pool of tRNAs to recognize degenerate codons. Of particular interest is the formation of a threonylcarbamoyl group on adenosine 37 (t^6A_{37}) of tRNAs that recognize ANN codons. Located adjacent and 3′ to the anticodon, t^6A_{37} is a conserved modification that is critical for reading frame maintenance. Recently, the highly conserved YrdC/Sua5 family of proteins was shown to be required for the formation of t^6A_{37}. Sua5 was originally identified in a screen by virtue of its ability to affect expression from an aberrant upstream AUG codon in the *cyc1* transcript. Together, these findings implicate Sua5 in protein translation at the level of codon recognition. Here, we show that Sua5 is critical for normal translation. The loss of *SUA5* causes increased leaky scanning through AUG codons, +1 frameshifting, and nonsense suppression. In addition, the loss of *SUA5* amplifies the 20S RNA virus found in *Saccharomyces cerevisiae*, possibly through an internal ribosome entry site-mediated mechanism. This study reveals a critical role for Sua5 and the t^6A_{37} modification in translational fidelity.

tRNAs require extensive modification in the anticodon and stem-loop domain (ASL) to function in codon recognition and maintenance of reading frame during translation. The anticodon, consisting of nucleosides 34 to 36, needs to base pair correctly with the codon for accurate translation. Modifications added to nucleosides in the tRNA anticodon region help to stabilize this interaction. Positions 34 and 37 in the ASL are two of the most modified nucleosides (8). Posttranscriptional modifications at wobble position 34, aided by modified nucleoside 37, which is most often a purine, permit noncanonical base pairing to occur at the third base pair. Importantly, this allows wobble to occur and thereby expands the decoding ability of tRNAs (3). Modified nucleosides also serve to minimize conformational movements of the ASL and as such, are important for providing structure to this region (2). This increases the affinity of each tRNA for the cognate codon in the decoding site, providing more free energy for the 30S ribosome to undergo a conformational rearrangement needed to close on the correct codon-anticodon complex for accurate decoding (45, 49, 59).

Purine 37 is a highly conserved nucleoside located 3′ adjacent to the anticodon that can undergo extensive modifications which are critical for stabilizing codon-anticodon interactions. Of the myriad modifications that exist at position 37, the presence of an N^6-threonylcarbamoyl group on adenosine 37 (t^6A_{37}) is one of the most ubiquitous and conserved (22). t^6A_{37} is found in most tRNAs decoding ANN (N representing one of the four canonical nucleotides, A, G, C, or U). Its main role is to create a planar hydrophobic structure that stacks above the third base of the anticodon and the first base of the codon, thus stabilizing the weaker A · U base pair (2, 3). The presence of t^6A_{37} in $tRNA^{Arg}_{UCU}$, $tRNA^{Ile}_{GAU}$, and $tRNA^{Lys}_{UUU}$ has been shown to increase ribosome binding and stabilize binding to the codon (41, 65, 69). Together with a modified wobble nucleoside 34, t^6A_{37} structures the ASL of $tRNA^{Lys}_{UUU}$ for optimal binding to the codon at the ribosomal decoding center (45). In addition, a modified purine 37 also maintains an open anticodon loop structure by preventing undesirable intraloop hydrogen bonding between nucleosides 32 and 38 (60). Therefore, t^6A_{37} plays an important role in the accurate decoding of the genome. However, while it is known that the loss of modified purines such as 2-methylthio-N^6-(*cis*-hydroxyisopentenyl) adenosine ($ms^2io^6A_{37}$) and *N*1-methylguanosine (m^1G_{37}) increase +1 translational frameshifting, the absence of t^6A_{37} on the maintenance of translational reading frame in vivo has not been determined (2).

Recently, Sua5 was shown to be required for the formation of t^6A_{37} (15). Bulk tRNA isolated from a *sua5* deletion strain was found to lack the t^6A_{37} modification. This defect was complemented by either wild-type Sua5 or its *Escherichia coli* homolog, YrdC. The absence of t^6A_{37} was correlated with slow growth, suggesting that t^6A_{37} is critical for normal cellular growth. In agreement with its requirement for t^6A_{37} formation, YrdC preferentially binds partially modified $tRNA^{Thr}$ lacking only the threonylcarbamoyl group. Consistent with the requirement of ATP for t^6A_{37} formation (14), this family of proteins has been shown to bind (15) and hydrolyze ATP (1).

Sua5 belongs to the unique Sua5/YrdC/YciO family of proteins and is a highly conserved protein found in all species (15, 61). First discovered in a screen for suppressors of an aberrant upstream start codon (uAUG), a mutation in Sua5 somehow enhanced initiation at a downstream endogenous start codon to partially restore translation of the coding region. This screen

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utilized *CYC1* alleles harboring uAUGs that effectively reduced Cyc1 protein expression (25, 52). *SUA* (*s*uppressor of *u*pstream *A*TG) genes that enhanced Cyc1 protein levels were identified (52). One of these suppressor candidates, *sua5*, partially restored the amount of Cyc1 protein expressed from the mutant transcript (*cyc1-1019*) without altering the transcriptional start site (46, 52). These results implicated Sua5 in the fidelity of start site selection during translation initiation. Recent data shed light on the mechanism and would suggest that the loss of *SUA5* and subsequent absence t^6A_{37} from initiator $tRNA^{Met}$ could adversely affect the efficiency of base pairing with the AUG codon. Failure to base pair correctly with the first favorable AUG that the ribosome encounters could result in dissociation and reinitiation or continued scanning for subsequent downstream AUG codons, thereby accounting for the partial rescue of Cyc1 expression in the *sua5* mutant.

Appropriate modification of the adenosine at position 37 (A37) in the ASL of tRNAs is undoubtedly important for translation. Pathogenic mutations in A37 of human mitochondrial tRNAs have been implicated in a wide range of maternally transmitted diseases (37, 40, 62, 68). For example, human mitochondrial initiator $tRNA^{Met}$ harboring the A4435G (A37 to G37) mutation was shown to contribute to Leber's hereditary optic neuropathy (53). In cells harboring the A4435 mutation, decreased levels of mitochondrial $tRNA^{Met}$ could impair mitochondrial translation (53). Hence, examining how the loss of *SUA5* affects general translation in *Saccharomyces cerevisia* provides an ideal system to elucidate the in vivo effects of tRNAs that lack t^6A_{37}. In this study, we show that the loss of *SUA5* increases translation initiation of upstream open reading frames (uORFs) containing *GCN4* by increasing leaky scanning through the upstream start codons. Considering that the initiator $tRNA^{Met}$ contains t^6A_{37}, it was not surprising that the loss of *SUA5* exerted the greatest adverse effect on translation initiation. However, given that t^6A_{37} is found in other tRNAs with NNU anticodons, we reasoned that *SUA5* depletion would be detrimental to other steps of translation. In addition to defects in translation initiation, we observed that the loss of *SUA5* affected reading frame maintenance and increased +1 frameshift events. Translation termination efficiency was also altered, as demonstrated by an increased read-through of stop codons. Surprisingly, cells that lack *SUA5* also showed a drastic amplification of a persistent yeast 20S RNA virus, and our data suggest that this increase may be mediated through an internal ribosome entry site (IRES)-dependent mode of translation initiation.

MATERIALS AND METHODS

Yeast strains. All yeast strains used in this study were derived from W303-1A or W303-1B (*can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*). Strains used in Fig. 6C and D were derivatives of 5V-H19 (*MAT*a *ade2-1 can1-100 leu2-3,112 ura3-52 SUQ5*; gift from M. D. Ter-Avanesyan). The coding regions of *SUA5* and *GCN2* were disrupted using the one-step PCR-mediated gene disruption method (23, 38). For complementation of the deletion of *SUA5* in Fig. 1, the indicated plasmids, p*yrdC*, p*mIRIP*, and p*sua5-1* (*LEU2*) were introduced into a *sua5*Δ strain complemented with plasmid-borne p316*SUA5* by the plasmid shuffle technique (18). To obtain the plasmid shuffle haploid strain, tetrads were obtained from a heterozygous null diploid containing p316*SUA5*. To create the Tet-off Sua5 strain (described below), pTetr*HASUA5* was introduced by the plasmid shuffle technique.

Plasmids. Unless otherwise indicated, all plasmids constructed were based on the pRS series of plasmids (57). Vectors containing constitutive promoters were previously described (44). p*SUA5* was constructed by cloning a 1.7-kb BamHI-SalI fragment containing the *SUA5* ORF and the endogenous promoter. p*yrdC* was constructed by cloning a 0.6-kb BamHI-SalI fragment containing *yrdC* that was amplified from pmk107 (31) (a gift from M. Rydén-Aulin) into a pRS415TEF vector. p*sua5-1* was constructed by cloning a 1.9-kb BamHI-SalI fragment containing *sua5-1* and the endogenous promoter that was amplified from YMH123 (a gift from M. Hampsey) (46). p*mIRIP* was constructed by subcloning a 0.8-kb EcoRI-SalI fragment from pBD-*GAL4*-mIRIP (29) (a gift from O. Mirochnitchenko) into a pRS424TEF vector. pTetr*HASUA5* was constructed by ligating a 1.3-kb EcoRI (blunted with Klenow)-EagI *HA-SUA5* fragment into pCM184 (a gift from E. A. Craig) previously digested with BamHI (blunted with Klenow) and NotI (4, 20). p*IMT4* was constructed by subcloning a 170-bp XhoI fragment containing *IMT4* from p1780-IMT (5) into a pRS424 vector.

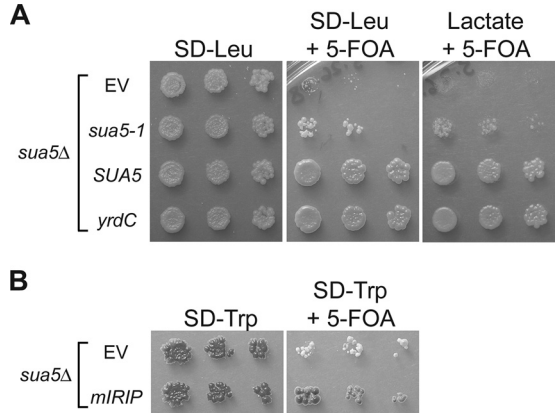

FIG. 1. Growth defects of the *sua5*Δ mutant are complemented by the *E. coli* homolog *yrdC* and mouse homolog *mIRIP*. (A) *sua5*Δ cells containing p316*SUA5* were transformed with pRS315 (EV), p315*sua5-1* (S107F), p315*SUA5*, or p315TEF*yrdC*. Yeast cultures at equal cell densities were serially diluted fivefold and spotted onto the indicated media. 5-Fluoroorotic acid (5-FOA) was added to the media to select for cells that had lost the complementing plasmid p316*SUA5*. (B) *sua5*Δ cells containing p316*SUA5* were transformed with pRS314 (EV) and p424TEF*mIRIP*. Fivefold serial dilutions of the yeast cultures at equal cell densities were spotted onto the indicated media.

Media. Yeast strains were grown in rich medium (1% yeast extract, 2% peptone, 2% dextrose [YPD]) or synthetic dropout (SD) media lacking the indicated components to select for plasmids. Solid media contained 2% agar. For sporulation, diploids were patched on GNA presporulation medium (5% dextrose, 3% nutrient broth, 1% yeast extract, 2% Bacto agar) for 1 day (30°C), repatched onto GNA presporulation medium for a second day (30°C), and then transferred onto minimal sporulation medium (1% KOAc, 2% agar) and grown for ~5 days (25°C) before micromanipulation of asci. For Tet repression of *SUA5*, doxycycline (DOX; Sigma) was added to media at a final concentration of 6 μg/ml. For efficient repression in liquid media, cells were grown overnight in media containing 6 μg/ml DOX.

Northern analyses. Total RNA was isolated as previously described (56). Briefly, total RNA was extracted using three organic extractions: once in hot, acidic phenol-chloroform and twice in cold, acidic phenol-chloroform. The RNA was precipitated overnight with 3 M sodium acetate (pH 5.2) and 100% ethanol at −20°C. The RNA pellet was washed once in 70% ethanol and resuspended in diethylpyrocarbonate-treated water. Equal volumes (16 μg) of RNA were loaded onto a 1% denaturing formaldehyde agarose gel in 1× MOPS (morpholinepropanesulfonic acid) buffer. RNA was transferred onto a nylon membrane (Amersham Hybond-N) by capillary action overnight. The membrane was stained with methylene blue to visualize the 18S and 25S rRNA. The RNA was UV cross-linked onto the membrane. The membrane was prehybridized in Amersham Rapid-hyb buffer at 65°C for 1 hour. Radioactive probes for the Northern analysis were prepared by random primed labeling *lacZ* PCR product (Amersham Rediprime II catalog no. RPN1633). The oligonucleotides used to amplify *lacZ* product were 5′-GCGCCCATCTACACCAAC (forward oligonucleotide) and 5′-GGCTTCATCCACCACATACAG (reverse oligonucleotide). Hybridization was performed at 55°C for 2 hours. The membrane was washed three times: once for 15 min at room temperature, followed by a 45-min wash at 65°C (1×

SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA {pH 7.7}], 0.1% sodium dodecyl sulfate [SDS]), and a final wash for 45 min at 65°C (0.5× SSPE, 0.25% SDS). The membrane was exposed to film for visualization of bands.

Cloning 20S RNA by reverse transcription. The RNA species of interest was excised from 1% nondenaturing agarose gel made with 1× TAE (diluted from 10× TAE [0.4 M Tris, 0.2 M sodium acetate, 0.01 M EDTA, 0.33 M glacial acetic acid; pH 7.4]). The RNA was extracted by the phenol-chloroform method from the gel slice and precipitated. RNA was amplified from the sample by using the Round AB protocol as described previously (64). Briefly, first-strand synthesis was performed using the Stratascript RT enzyme (Stratagene) and primer A (5′-GTTTCCCAGTCACGATAN$_8$-3′). Second-strand synthesis was performed using Sequenase (US Biochemical catalog no. 70775). PCR amplification was performed using specific primer B (5′-GTTTCCCAGTCACGATA-3′) and AccuPrime *Taq* polymerase (Invitrogen). The PCR product was ligated into a pGEM-T Easy vector according to manufacturer's instructions (Promega, catalog no. A1360). Clones were sequenced to identify the insert (Protein and Nucleic Acid Chemistry Laboratories, Washington University School of Medicine).

β-Galactosidase assays. For *GCN4-lacZ* assays, Tet-off Sua5 cells containing the reporter constructs (p180, p227, or p4164) were grown overnight to mid-logarithmic phase in SD-Ura media with or without DOX. To ensure that the addition of DOX did not affect the assay readout, we measured β-galactosidase activity in *sua5*Δ cells complemented with *SUA5* expressed from the endogenous promoter (p*SUA5*). Control experiments confirmed that the addition of DOX did not affect the induction of *GCN4-lacZ* (data not shown). β-Galactosidase reporter activity was assayed by the Galacto-Light chemiluminescent reporter gene assay system (Applied Biosystem). Cells were disrupted in 100 μl of lysis buffer, using glass beads, and the lysate was diluted 10-fold. Seventy microliters of 1× chemiluminescent substrate (Galacton) was added to the diluted lysate and incubated for 1 hour at room temperature. One hundred microliters of light emission accelerator was added, and luminescence was measured on a Sirius single tube luminometer (Berthold Detection Systems) with a delay time of 2.0 s and read time of 5.0 s. For each strain lysate, the specific β-galactosidase activity was calculated from three independent cultures as the number of relative light units per microgram of protein (as determined by Bradford assay [Bio-Rad]). Reporter constructs p180, p226, p227, and p4164 were all generously provided by A. G. Hinnebusch (21, 26, 43).

Dual luciferase assays. For stop codon read-through assays, Tet-off Sua5 cells containing the dual luciferase reporter plasmids expressing either a stop codon or a sense codon (UAG, CAG, UGA, CGA) were grown overnight to mid-logarithmic phase in SD-Ura media with or without DOX. The luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega). Cells were disrupted in 100 μl of passive lysis buffer, using glass beads, and the lysate was diluted 100-fold. Ten microliters of luciferase assay reagent II was added to the diluted lysate, and the firefly luciferase activity was measured with a delay time of 2.0 s and read time of 10.0 s. Ten microliters of the Stop & Glo reagent was added, and the *Renilla* luciferase activity was measured for another 10 s. For each strain lysate, corrected read-through was calculated by expressing the firefly/*Renilla* ratio (stop codon) as a percentage of the firefly/*Renilla* ratio (sense codon). Three independent cultures were assayed in each experiment to obtain the standard deviation. These dual luciferase read-through constructs were generously provided by David Bedwell (32).

***EST3* frameshift assays.** Tet-off Sua5 cells containing either the frameshift or the in-frame construct were grown overnight to mid-logarithmic phase in SD-Leu media with or without DOX. β-Galactosidase reporter activity was assayed by the Galacto-Light chemiluminescent reporter gene assay system (Applied Biosystem) as described above. To measure firefly luciferase activity, 100 μl of luciferase assay buffer (25 mM glycylglycine [pH 7.8], 15 mM potassium phosphate [pH 7.]8, 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, 1 mM dithiothreitol) and 100 μl of 1 mM D-luciferin stock (5 mg D-luciferin [Sigma], 25 mM glycylglycine, 10 mM dithiothreitol) was added to 10 μl of the undiluted lysate. Luminescence was measured with a delay time of 2.0 s and read time of 5.0 s.

***URE2* IRES activity.** Tet-off Sua5 cells containing either the IRES construct (AUG) or the control (CTT) were grown overnight to mid-logarithmic phase in SGal/Raf-Ura media with or without DOX. β-Galactosidase reporter activity was assayed as described above.

Protein analysis. To measure steady-state levels of phosphorylated eIF2α, cells were grown in SD-Trp medium overnight to mid-logarithmic phase. DOX was added to a final concentration of 6 μg/ml to maintain effective Sua5 repression in Tet-off Sua5 cells. Cells were disrupted in lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5 mM MgCl, 10% glycerol), using glass beads. Protein levels were normalized by Bradford assay (Bio-Rad), and samples were loaded onto an SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride membrane (Immobilon) for Western blotting. Membranes were immunoblotted with antibodies recognizing phospho-eIF2α (Cell Signaling Technology catalog no. 9721). Membranes were stripped and immunoblotted with antibodies recognizing total eIF2α (antibody was a kind gift from T. E. Dever).

Polysome profile analyses. Polysomes were prepared and fractionated on a 7% to 47% sucrose gradient as described previously, with some minor modifications (6). Briefly, cells were grown overnight in YPD medium with or without DOX to an optical density at 600 nm of ~0.5, lysed, and loaded onto a 10-ml linear 7% to 47% sucrose gradient. The sucrose gradients were centrifuged at 39,000 rpm for 1.5 h in an SW 41 rotor (Beckman Coulter). Sucrose gradients were fractionated, and the absorbance at 254 nm was monitored using an ISCO density gradient fractionator and a UA-6 detector (Teledyne ISCO). Total protein was precipitated from the fractions by the trichloroacetic acid method. Briefly, 0.5-ml fractions were collected from the sucrose gradients. Trichloroacetic acid was added to the samples at a final concentration of 20%, incubated on ice for 30 min, and centrifuged at 13,000 rpm for 30 min at 4°C using a tabletop centrifuge. The supernatant was removed, and the pellet was washed with ice-cold acetone. The samples were centrifuged again as described above for 5 min. The supernatant was removed, and the pellet was air dried and resuspended in 1× SDS-PAGE loading buffer. To detect p91, protein was loaded onto an SDS-PAGE and subjected to Western blotting. Membranes were immunoblotted with antibodies recognizing p91 (16) (antibody was a kind gift from R. Esteban). Total RNA was isolated from the fractions and subjected to Northern analyses as previously described (6). The oligonucleotide probe specific for the 20S replicon (RW 308) was described previously (47).

RESULTS

The growth defect of the *sua5* deletion strain is complemented by the *Escherichia coli* and mouse homologs of SUA5. The Sua5 protein contains a unique Sua5/YciO/YrdC domain which is widely distributed across species, from *E. coli* to *Homo sapiens* (15, 61). Yeast cells lacking *SUA5* exhibit severe growth defects on both rich and nonfermentable carbon sources, indicating that Sua5 performs a crucial cellular function (46). *SUA5* was previously discovered in a screen for mutations that suppressed the inhibitory effects of an aberrant AUG upstream of the *CYC1* ORF. The loss of *SUA5* was shown to partially restore the levels of Cyc1 protein produced from the mutant transcript (46, 52). The bacterial homolog, YrdC, was previously suggested to be a putative ribosome maturation factor (31). A mutation in *E. coli yrdC* showed a slight defect in the maturation of 16S rRNA, and it was proposed that this mutation affected the ability of 30S subunits to function in translation (30, 31). The *yrdC* mutant also had an increased amount of free 30S and 50S subunits relative to the amount of 70S monosomes. One caveat of these studies was that an additional mutation was required to maintain viability upon loss of *yrdC* (31), and that combination may have produced a synthetic genetic effect. Recent results do not show Sua5 in yeast to play a role in rRNA processing (39). We also did not detect any observable rRNA maturation defects in our *SUA5*-depleted cells (data not shown). To test the functional conservation between the two proteins, we asked whether *E. coli yrdC* could complement the slow-growth phenotype of a *SUA5* deletion strain. We cloned *yrdC* to be expressed from the constitutive yeast *TEF1* promoter. The *yrdC* plasmid was introduced into a *sua5*Δ strain by using the plasmid shuffle technique. The expression of the bacterial *yrdC* gene rescued the growth defects to the same extent as plasmid-borne wild-type *SUA5*, indicating a high degree of functional conservation between the two proteins (Fig. 1A). The *sua5-1* (S107F) mutant allele previously shown to harbor a loss-of-function mutation (46) con-

ferred a weak complementation, while empty vector alone (EV) did not rescue the growth defects of the *sua5*Δ strain (Fig. 1A). The *SUA5* homolog in higher eukaryotes is known as *IRIP* (*i*schemia and *r*eperfusion *i*nducible *p*rotein) (10, 29). The ectopic expression of mouse *IRIP* from the yeast *TEF1* promoter also partially suppressed the slow-growth defect of the *sua5*Δ strain, although the rescue was weaker than that observed with *yrdC* (Fig. 1B). These data highlight the functional conservation of the Sua5/YciO/YrdC protein family.

Loss of *SUA5* inhibits translation initiation and derepresses GCN4 expression. We created a *sua5*Δ strain covered with a plasmid containing hemagglutinin (HA)-tagged *SUA5* under a Tet-repressible promoter (pTetr*HASUA5*) in order to regulate Sua5 expression. The efficient repression of *SUA5* expression by the addition of DOX was verified by Western blot analysis. There was no detectable Sua5 protein by 2 hours after DOX addition (data not shown). Growth of the haploid mutant was fully complemented by *HA-SUA5* expression and suppressed by the addition of the tetracycline analog DOX (Fig. 2A and data not shown). This strain, hereafter referred to as the Tet-off Sua5 strain, was impaired in growth on media containing DOX, recapitulating the slow-growth phenotype of the *sua5*Δ mutant.

Previous data demonstrated that the loss of Sua5 increased the expression of Cyc1 protein from the *cyc1-1019* mRNA harboring an aberrant AUG upstream of the *CYC1* ORF. However, it was unclear if Sua5 was acting at the level of transcription or the level of translation (46, 52). To distinguish between these possibilities, we tested whether Sua5 could affect the translation initiation of Gcn4, a protein under the translational control of four naturally occurring uORFs (uORF1 to -4) (27). The translation of *GCN4* mRNA is derepressed by amino acid starvation through an increase in eIF2α phosphorylation. Increased levels of phosphorylated eIF2α inhibit the formation of ternary complexes (TC). Consequently, ribosomes scan past the uORFs before joining a TC to initiate translation at the main *GCN4* AUG (27). As a result, under conditions where translation initiation is perturbed, such as a reduction in TC levels, *GCN4* expression is increased. To measure translation initiation activity, we used the well-characterized *GCN4-lacZ* reporter (p180) that contains the four uORFs followed by the first 55 codons of the *GCN4* ORF fused in frame to the *lacZ* gene (26, 43). Using this construct, an increase in β-galactosidase activity is indicative of a translation initiation defect. We observed that cells depleted for *SUA5* had β-galactosidase activity threefold higher than that of the wild-type control (Fig. 2B). To test whether the altered *GCN4-lacZ* derepression caused by *SUA5* depletion could be due to an alteration in mRNA levels, we utilized a control *GCN4-lacZ* reporter devoid of all uORFs (p227) (43). The loss of *SUA5* did not cause an appreciable difference in the β-galactosidase activity expressed from this control construct (Fig. 2C). In addition, Northern blots confirmed that a change in *GCN4-lacZ* mRNA levels could not account for the derepression observed with the loss of *SUA5* (Fig. 2D). This suggests that the effect of *SUA5* on the *GCN4* expression is mediated through the translational control via the uORFs and not simply through changes in transcript expression or stability.

The derepression of *GCN4* is dependent on eIF2α phosphorylation by the Gcn2 kinase, which is activated by uncharged

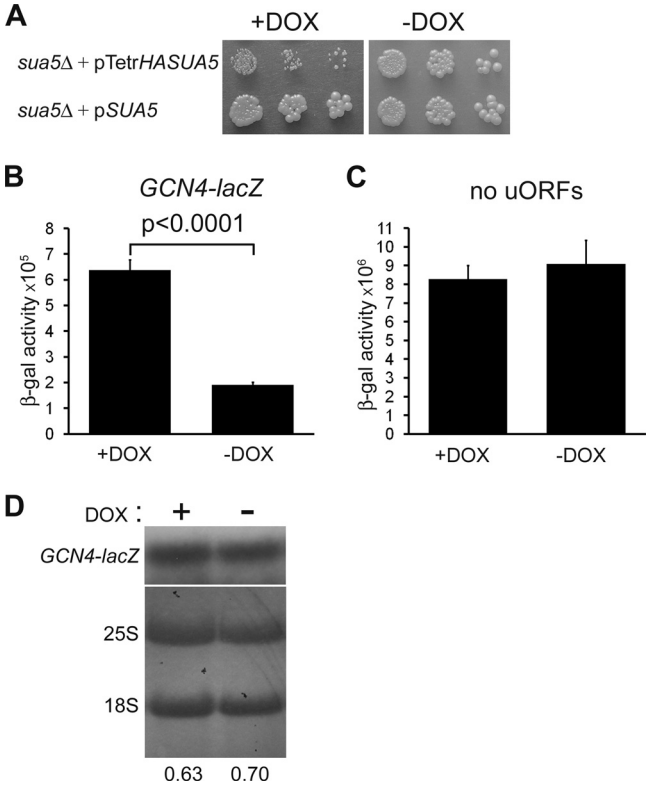

FIG. 2. The loss of *SUA5* derepresses *GCN4* expression at the translational level. (A) Validation of Tet-off Sua5 cells. *sua5*Δ cells containing pTetr*HASUA5* or p314*SUA5* were serially diluted fivefold and spotted onto YPD medium or YPD medium containing 6 μg/ml DOX. (B) The effect of *SUA5* depletion on the expression of a *GCN4-lacZ* construct containing four uORFs (p180) was assayed in Tet-off Sua5 cells grown in the presence or absence of DOX. β-Galactosidase activity was measured in lysates from three independent cultures grown under repressing (nonstarvation) conditions. Specific β-galactosidase activity was calculated based on total protein concentration as determined by Bradford assays. These data are representative of six independent experiments. (C) The effect of *SUA5* depletion on the expression of a *GCN4-lacZ* construct lacking all four uORFs (p227) was measured under the repressing conditions as described for panel A. Error bars represent the standard deviation. The data are representative of three experiments. (D) Northern analyses to measure relative *GCN4-lacZ* transcript levels in Tet-off Sua5 cells grown in the presence or absence of DOX. 25S and 18S rRNA bands on the membrane were visualized by methylene blue staining. *GCN4-lacZ* levels were normalized to 18S rRNA levels to determine the relative ratios indicated below the figure. Band intensities were quantified using Image J software (http://rsb.info.nih.gov/ij).

tRNAs (27). Since Sua5 is required for t^6A_{37} modification in a large subset of tRNAs, it was possible that the absence of this modification affected aminoacylation (50), thus increasing the levels of uncharged tRNAs, and consequently eIF2α phosphorylation. To examine the possibility that the activation of the Gcn2 kinase by uncharged tRNAs was responsible for the translation initiation defect, we asked if a deletion of the Gcn2 kinase could abolish the activation of *GCN4* in *SUA5*-depleted cells. Interestingly, even in the absence of the Gcn2 kinase, *SUA5*-depleted cells had higher *GCN4-lacZ* expression than did wild-type cells (Fig. 3A). Moreover, we found that the *SUA5*-depleted cells had reduced steady-state levels of phos-

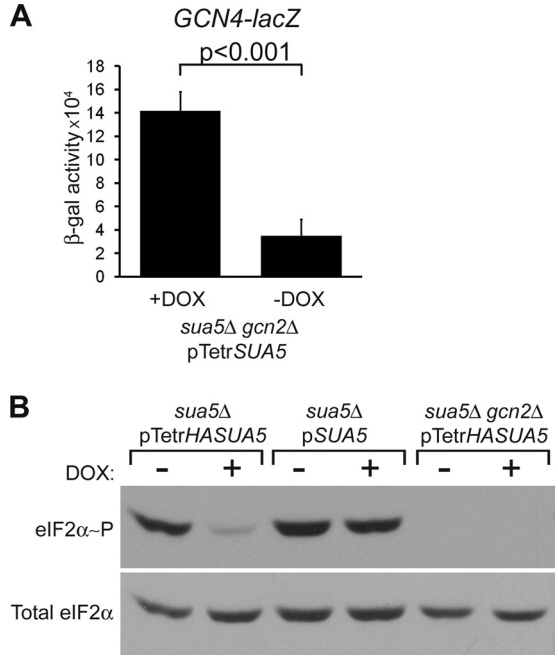

FIG. 3. The Gcn2 kinase is not required for *GCN4-lacZ* translational derepression in *SUA5*-depleted cells. (A) The expression of *GCN4-lacZ* (p180) was assayed in *sua5*Δ *gcn2*Δ cells containing pTetr*HASUA5* grown in the presence or absence of DOX. β-Galactosidase activity was measured in lysates from three independent cultures grown under repressing (nonstarvation) conditions. Specific β-galactosidase activity was calculated based on total protein concentration as determined by Bradford assays. Error bars represent the standard deviation. These data are representative of three experiments. (B) Cell cultures of the indicated genotype were grown in the presence or absence of DOX. Equal quantities of protein were loaded onto an SDS-PAGE and analyzed for levels of phosphorylated eIF2α by Western blot analysis. The membrane was stripped and immunoblotted for total eIF2α.

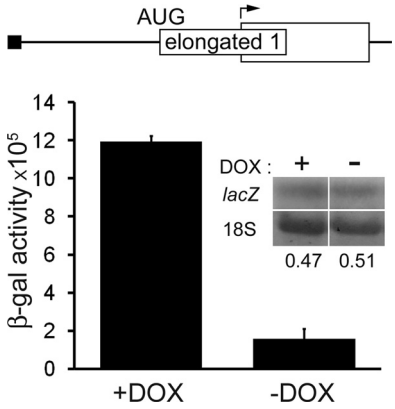

FIG. 4. The loss of *SUA5* increases the frequency of leaky scanning. The construct p4164 contains an elongated uORF1 that overlaps with the beginning of the *GCN4-lacZ* ORF. The AUG of the elongated uORF1 is noted, and the arrow indicates the start codon of the main *GCN4-lacZ* ORF. Translation initiation from the downstream AUG indicated by the arrow would produce β-galactosidase activity. Tet-off Sua5 cells were grown in the presence or absence of DOX. β-Galactosidase activity was measured in lysates from three independent cultures. Specific β-galactosidase activity was calculated based on total protein concentration as determined by Bradford assays. Error bars represent the standard deviation. These data are representative of three experiments ($P < 0.0001$). The inset figure shows the relative levels of *lacZ* expressed from p4164 in Tet-off Sua5 cells grown in the presence or absence of DOX. 18S rRNA bands on the membrane were visualized by methylene blue staining. The *lacZ* levels were normalized to 18S rRNA levels to determine the relative ratios indicated below the figure. Band intensities were quantified using Image J software.

phorylated eIF2α (Fig. 3B). This reduction was not likely due to reduced Gcn2 kinase activity, since we found that *SUA5*-depleted cells still responded to stress appropriately by phosphorylating eIF2α (data not shown). This leaves open the interesting possibility that there could be an increase in the dephosphorylation or turnover of phosphorylated eIF2α in the absence of *SUA5* by mechanisms yet to be discovered. Taken together, these data indicate that the inhibition of translation initiation caused by *SUA5* depletion is not a result of activating the Gcn2 kinase by increasing the pool of uncharged tRNAs.

Loss of *SUA5* increases the frequency of leaky scanning through start codons. There are many reasons that could account for the increased *GCN4-lacZ* expression in *SUA5*-depleted cells (27). However, given the requirement of Sua5 in t^6A_{37} formation, we considered whether the ribosomes might bypass the uAUGs (leaky scanning) due to incorrect codon-anticodon base pairing to initiate more frequently at the AUG of *GCN4* (27). To examine this possibility, we utilized a well-characterized construct that requires leaky scanning of the upstream AUGs for expression (21). This construct (p4164) contains a modified version of uORF1 that overlaps the main *GCN4* ORF in a different reading frame so that ribosomes that initiate at uORF1 will prematurely terminate downstream of the *GCN4* start codon (21). Consequently, any translation of *GCN4-lacZ* in the context of this elongated uORF1 occurs via leaky scanning (21, 35). We measured β-galactosidase levels in the elongated uORF1-*lacZ* construct and found that they had higher *GCN4-lacZ* expression than did the cells expressing *SUA5* (Fig. 4). This supports the hypothesis that the loss of *SUA5* results in an increased frequency of leaky scanning through the uAUG codons to initiate translation at the AUG of the *GCN4-lacZ* ORF. Since the 43S preinitiation complex will have had a TC assembled before scanning the 5′ untranslated region for the AUG of the elongated uORF1, it is unlikely that the first AUG is bypassed due to insufficient levels of TC (12, 51). In support of this rationale, we did not observe a reduction in the leaky scanning in *SUA5*-depleted cells when all four components of the TC (eIF2α, -β, and -γ and Met-$tRNA_i^{Met}$) (17) were overexpressed from a high-copy plasmid (data not shown). Increasing the pool of initiator $tRNA^{Met}$ (*IMT4* expressed from a multicopy vector) also did not reduce the leaky scanning observed in *SUA5*-depleted cells (data not shown). In addition, the slow-growth defect associated with *SUA5* depletion was not rescued by overexpression of the TC or *IMT4* alone (data not shown).

Loss of *SUA5* increases +1 frameshift and read-through of stop codons. The widespread presence of t^6A_{37} in tRNAs with an NNU anticodon led us to examine how the loss of *SUA5* affected other steps in translation. The absence of two other purine modifications, 2-methylthio-N^6-(*cis*-hydroxyisopentenyl) adenosine (ms^2io^6A37) and *N*1-methylguanosine (m^1G37), induce +1

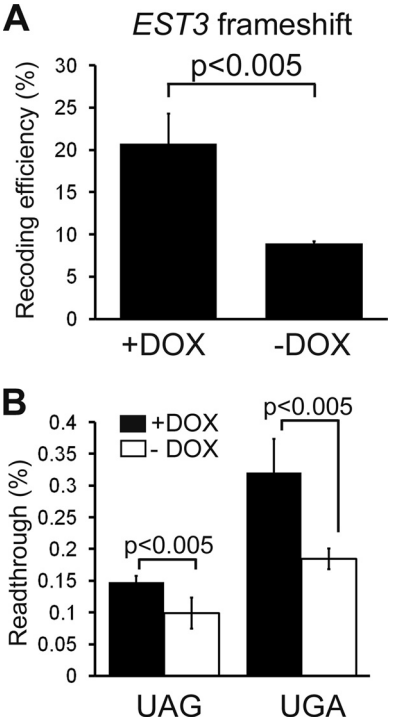

FIG. 5. *SUA5* depletion increases the frequency of +1 frameshifting and read-through of UAG and UGA stop codons. (A) The levels of +1 frameshifting in three independent cultures of the Tet-off Sua5 strain were assayed using a dual reporter construct with the minimal *EST3* frameshifting sequence inserted between the *lacZ* and firefly luciferase ORFs. The control constructs contain an in-frame sequence inserted between the two ORFs. The frameshift efficiency is expressed as a firefly luciferase/β-galactosidase ratio. Recoding efficiency is obtained by expressing the ratio from the frameshift construct as a percentage of the in-frame control. The data presented was collected from three independent experiments. (B) Read-through levels in three independent cultures of the Tet-off Sua5 strain were assayed using the dual luciferase read-through constructs. The dual luciferase constructs contained either a stop codon (UAG, UGA) or a sense codon (CAG, CGA) inserted between the *Renilla* and firefly luciferase ORFs. The amount of read-through in each construct is expressed as a firefly/*Renilla* ratio. Corrected read-through is obtained by expressing the ratio from the stop codon constructs as a percentage of the sense codon controls. The data presented for each read-through construct was collected from at least four independent experiments.

frameshift errors (24). To examine the effect of losing t^6A_{37} on reading frame maintenance, we utilized a +1 frameshifting sequence in *EST3* that uses an AGU codon recognized by a rare tRNA ("hungry codon") (42, 48). The presence of this "hungry codon" frequently causes the ribosome to stall and the tRNA to slip into an overlapping codon (48). Using this *EST3* reporter construct, we found that *SUA5*-depleted cells have a level of +1 frameshifting that is twofold higher than that of the wild-type cells (Fig. 5A).

The efficiency of translational stop codon read-through by natural nonsense suppressor tRNAs is also modulated by base modifications within or 3′ to the anticodon of these tRNAs (7). Hence, we wanted to examine whether the absence of t^6A_{37} affects the ability of tRNAs to suppress stop codons. We used a dual luciferase reporter system (32) which allowed us to monitor stop codon read-through efficiency in a manner that would take into account the alterations in translation initiation in *SUA5*-depleted cells. We observed that *SUA5* depletion resulted in a modest but significant and reproducible increase in the read-through of UAG and UGA codons (1.5-fold and 1.7-fold, respectively) (Fig. 5B). Taken together, these results highlight the role of *SUA5* and t^6A_{37} modification in various aspects of translation.

Loss of *SUA5* upregulates persistent 20S RNA virus. In light of the role of Sua5 in perturbing various phases of translation, we next wanted to ascertain the global translational status in *SUA5*-depleted cells. To accomplish this, we measured the abundance of polysomes by sucrose gradient-velocity sedimentation. While we noted a general decrease in the ribosomal peaks in the polysome profiles of *sua5*Δ cells, consistent with a general translation defect, one striking difference was the presence of a prominent pre-40S peak (Fig. 6A). This unusual peak was diminished when we complemented the *sua5*Δ strain with a plasmid expressing *SUA5* (Fig. 6D). It was interesting that the loss of Sua5 could dramatically upregulate a novel heavy sedimenting species, so we decided to elucidate the contents of this peak. Surprisingly, we found that it contained an abundant RNA species sedimenting between the 18S and 25S rRNA (Fig. 6B, top panel). Subsequent isolation and identification of this RNA species revealed that it belongs to the 20S RNA narnavirus that persistently infects most laboratory strains of *S. cerevisiae* (66). We confirmed that this peak contained the 20S RNA by Northern blot analysis of polysome profile fractions (Fig. 6B, bottom panel). The 20S RNA is a single-stranded RNA molecule that encodes only one polypeptide: the 91-kDa RNA-dependent RNA polymerase, known as p91 (19). Since there was a drastic upregulation of the 20S RNA in cells deleted for *SUA5*, we tested whether there was a corresponding increase in the translation of the encoded protein, p91. By Western blot analysis, we found that *sua5*Δ cells accumulated higher levels of p91 (Fig. 6C) than did the complemented strain (Fig. 6D). The highest levels of p91 were found in the region corresponding to the pre-40S peak. Since p91 and 20S are known to associate (19), the aberrant pre-40S peak likely represents the heavier sedimenting 20S-p91 ribonucleoprotein complexes.

The 20S RNA has a highly structured 5′ end which is likely to be uncapped (55), suggesting that a cap-independent mode of translation initiation is a likely mechanism for viral protein expression. One possible mechanism is via the use of an IRES. The presence of a highly structured IRES in the 5′ untranslated region of a transcript can recruit the 43S ribosome in a cap-independent manner for translation initiation (58). Without knowing what region of the 20S RNA stimulates translation, we pursued this idea by utilizing a *URE2-lacZ* fusion containing a *URE2* IRES element which has been shown to support translation initiation (33, 34, 54). This construct contains a 5′ stem-loop that functions to inhibit cap-dependent scanning of the 40S subunit and a *URE2-lacZ* fusion driven by an IRES. Hence, the production of β-galactosidase results from internal initiation from the IRES (33, 34, 54). Using this reporter system, we found that *SUA5*-depleted cells showed a modest but significant and reproducible 1.5-fold enhancement of *URE2* IRES activity (Fig. 6E). We propose that the loss of translational fidelity in cells depleted for *SUA5* results in a downregulation of global cap-dependent translation. The 20S

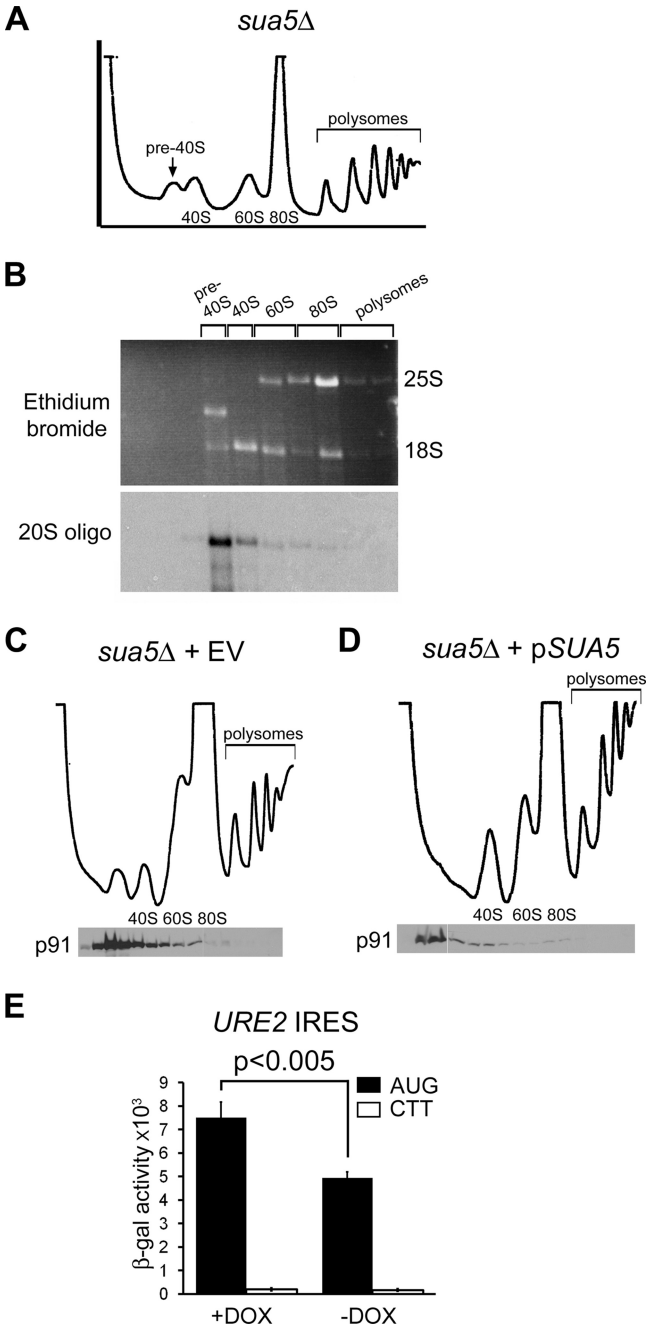

FIG. 6. The loss of *SUA5* results in the accumulation of the 20S:p91 ribonucleoprotein complex. (A) The *sua5*Δ strain was grown in YPD medium to mid-logarithmic phase. Ribosomes and polyribosomes in cell lysates were resolved by velocity sedimentation through 7% to 47% sucrose gradients containing 30 mM $MgCl_2$. The arrow indicates the aberrant pre-40S peak. (B) The pre-40S peak contained an abundant RNA species. Fractions (0.5 ml) were collected from the gradients described for panel A. RNA was precipitated from each fraction, resolved on a formaldehyde-agarose gel, and subjected to Northern analysis. The top panel shows that the RNA species from the pre-40S peak runs between the 18S and 25S rRNA as visualized by ethidium bromide under UV light. The lower panel shows the Northern blot after hybridization with a probe specific for the 20S RNA. (C) Cells deleted for *SUA5* were subjected to polysome profile analyses as described for panel A. Fractions (0.5 ml) were collected from the gradients. Protein was precipitated, resolved on SDS-PAGE, and analyzed for the levels of p91 by Western blot analysis. (D) The same conditions as described for panel C using *sua5*Δ cells complemented with p*SUA5*. (E) The ability to stimulate the *URE2* IRES activity was assayed in three independent Tet-off Sua5 cultures. The control construct contains a CTT mutation in place of the internal AUG. Specific β-galactosidase activity was calculated based on total protein concentration as determined by Bradford assays. Error bars represent the standard deviation. These data are representative of four experiments.

RNA however, may be able to continue translation initiation by means of a potential IRES-like element. Accordingly, the increase in 20S RNA translation could produce more p91, which then binds to the 20S RNA, further stabilizing the ribonucleoprotein complex.

DISCUSSION

One of the most extensively modified nucleosides in the tRNA is purine 37 in the ASL. A few enzymes responsible for these modifications have been identified, and their role in modulating codon recognition during translation has been examined (8). The hypermodified nucleoside t^6A_{37} is uniquely conserved in the tRNAs found across most species (22). Although ample in vitro data point to the essentiality of this modification in accurate codon-anticodon base pairing, there is a lack of in vivo studies describing the effect of its loss, since the enzyme responsible for the t^6A_{37} modification was unknown. The recent discovery of the YrdC/Sua5 family as the putative enzymes required for t^6A_{37} formation has allowed us to determine how the absence of this conserved modification caused by *SUA5* depletion affects translational fidelity.

Our findings provide in vivo evidence that loss of the Sua5 protein exerts a dramatic effect on translation initiation. Considering that Sua5 is required for the formation of t^6A_{37} (15), coupled with the strong in vitro data that t^6A_{37} is crucial for stabilizing codon-anticodon interactions (2, 3), the leaky scanning defect is likely due to reduced fidelity in base pairing between AUG and the hypomodified initiator $tRNA^{Met}$. One possible scenario to explain the increase in leaky scanning is that when the preinitiation complex arrives at a favorable AUG, the lowered affinity of base pairing leads to incorrect binding between the codon and anticodon. As a consequence, the preinitiation complex fails to arrest scanning and continues downstream to base pair with the next favorable AUG. Previously published data are also consistent with this idea, including mutations in the 18S rRNA that affect accurate codon-anticodon base pairing (12). Specifically, mutation of C1400 of the 18S rRNA elicits a leaky scanning phenotype. Moreover, this defect cannot be corrected by an overexpression of TC or initiator $tRNA^{Met}$ alone (12). The failure of *IMT4* overexpression to rescue the leaky scanning observed in *SUA5*-depleted cells suggests that merely increasing the pool of t^6A_{37} hypomodified initiator $tRNA^{Met}$ cannot correct the base-pairing defect. Hence, it is unlikely that the absence of t^6A_{37} simply increases the dissociation rate of the initiator $tRNA^{Met}$ from the preinitiation complexes prior to subunit joining.

Utilizing the well-defined translational regulation of *GCN4* as a readout, we demonstrated that the uORF bypass and increased translation of the main ORF caused by *SUA5* depletion was not acting through the Gcn2 kinase. Hence, the loss of

t^6A_{37} did not simply lead to the accumulation of uncharged tRNAs, which would activate the Gcn2 kinase and derepress translation of *GCN4*. This makes sense given that the t^6A_{37} modification occurs on a myriad of tRNAs, such as isoleucine, threonine, asparagine, lysine, serine, arginine, and methionine, and further highlights its relevance for decoding but not recognition by aminoacyl-tRNA synthetases (3). Moreover, the absence of t^6A_{37} from $tRNA^{Ile}$ diminished ribosome binding efficiency but had no effect on aminoacylation (41). It was also shown that a pathogenic mutation in A37 of human mitochondrial $tRNA^{Ile}$ had only a minimal effect on aminoacylation efficiency (36).

As expected, the loss of Sua5 expression and t^6A_{37} had general consequences on the fidelity of translation. In addition to the defects in translation initiation, our data showed that *SUA5* depletion increased +1 frameshifting. The *EST3* frameshift sequence contains the AGU codon which is decoded by a rare tRNA. In this scenario, the AGU-decoding tRNA also contains the t^6A_{37} (8). Hence, the hypomodified tRNA may be less efficient in base pairing with AGU, further aggravating the ribosome stalling and enhancing +1 slippage of the tRNA into the overlapping codon. We also found an increase in the translational read-through of stop codons in *SUA5*-depleted cells. One likely scenario to explain this effect is that the absence of t^6A_{37} improves the efficiency of natural nonsense suppressors. Most of the cytoplasmic $tRNA^{Gln}$ isolated from mice and tobacco that suppress UAG/UAA contain an unmodified A37 (7). The interaction of the $tRNA^{Gln}$ suppressors with stop codons involves a noncognate base pairing at the third anticodon position, which may be facilitated by the unmodified A37 (7). Furthermore, it was shown that other hypermodifications at A37, such as i^6A (N^6-isopentenyladenosine) and ms^2i^6A (2-methylthio-N^6-isopentenyladenosine), negate noncognate base pairing at the third anticodon (9, 63, 67). The effects of *SUA5* depletion on translational frameshifting and stop codon read-through are modest, but significant and reproducible. Moreover, since t^6A_{37} is critical for stabilizing codon-anticodon base pairing and reading frame maintenance, the loss of the modification could lead to an increase in misincorporation of amino acids via near-cognate tRNAs during translation. Thus, the polypeptides generated could have missense mutations that reduce activity, stability, or expression. Hence, in our enzymatic assays used to measure translational fidelity which will require proper β-galactosidase or luciferase activity, we could in fact be underestimating the defect caused by the loss of *SUA5*, yet the defects were still significant.

The *E. coli* homolog YrdC was first isolated as a mutant protein that suppressed a temperature-sensitive (*ts*) allele of bacterial release factor 1 (RF1) involved in translation termination (30). It was suggested that the reduction in functional ribosomes in the *yrdC* mutant could be slowing down overall translation initiation to balance the slow termination rates in the RF1 mutant, hence suppressing the *ts* phenotype (30). Recent data have provided another mechanism to explain the suppression. The absence of hypermodifications at the wobble base 34 of ASL also suppressed the *ts* RF1 mutant (28). The suggested mechanism of suppression stems from the effect of a hypomodified tRNA occupying the P site preceding the UAG, which serves to induce a frameshift event and a subsequent read-through of the stop codon. This would then alleviate the detrimental effects caused by ribosome stalling at the UAG in the *ts* RF1 mutant (28). In light of the recent finding that YrdC can complement the t^6A_{37} deficiency, it is intriguing to speculate that the absence of hypermodified A37 in *yrdC* mutants could play a similar role in suppressing the *ts* RF1, further supporting the role of Sua5 and YrdC in modulating reading frame maintenance.

In summary, the results presented here provide a link between the loss of the important modification t^6A_{37} and translational defects in vivo. The slow growth of *SUA5*-depleted cells on rich media could be a consequence of increased translational errors, although we cannot rule out the role of Sua5 in modulating telomere lengths (39). However, it is unlikely that the effects on telomere length can fully account for the growth phenotype, since the translationally defective *sua5-1* (S107F) mutant still had wild-type telomere lengths (39). Given the fact that mitochondrial tRNAs also possess t^6A_{37} (13), it is plausible that an impairment in the mitochondrial protein expression could adversely affect growth on nonfermentable carbon sources. Additionally, the high level of conservation of t^6A_{37} in tRNAs across species underscores the utility of using yeast as a model to investigate the role of t^6A_{37} hypomodification in human mitochondrial diseases (11).

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