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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 (t6A37) of tRNAs that recognize ANN codons. Located adjacent to 3’ to the anticodon, t6A37 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 t6A37. Sua5 was originally identified in a screen by virtue of its ability to affect expression from an aberrant upstream AUG codon in the cycl1 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 t6A37 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 N6-threonylcarbamoyl group on adenosine 37 (t6A37) is one of the most ubiquitous and conserved (22). t6A37 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 t6A37 in tRNAArg, tRNAIle, and tRNALeu, has been shown to increase ribosome binding and stabilize binding to the codon (41, 65, 69). Together with a modified wobble nucleoside 34, t6A37 structures the ASL of tRNAArg 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, t6A37 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-N6-(cis-hydroxysopentenyl) adenosine (m2sio6A37) and N1-methylguanosine (m1G37) increase +1 translational frameshifting, the absence of t6A37 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 t6A37 (15). Bulk tRNA isolated from a sua5 deletion strain was found to lack the t6A37 modification. This defect was complemented by either wild-type Sua5 or its Escherichia coli homolog, YrdC. The absence of t6A37 was correlated with slow growth, suggesting that t6A37 is critical for normal cellular growth. In agreement with its requirement for t6A37 formation, YrdC preferentially binds partially modified tRNAArg lacking only the threonylcarbamoyl group. Consistent with the requirement of ATP for t6A37 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
utilized CYC1 alleles harboring uAUGs that effectively reduced Cyc1 protein expression (25, 52). SUA (suppressor of upstream ATG) 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 (cycll-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 t6A37 from initiator tRNA\textsubscript{M}t 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\textsubscript{M}t 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 tRNAMet 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 t6A37. 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\textsubscript{M}t contains t6A37, it was not surprising that the loss of SUA5 exerted the greatest adverse effect on translation initiation. However, given that t6A37 is found in other tRNAs with NNU anticodons, we reasoned that Sua5 deletion 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 trpl-1 ura3-1 ade2-1). Strains used in Fig. 6C and D were derivatives of SV-H19 (MAT\textsuperscript{a} ade2-1 can1-100 leu2-3,112 ura3-352 SUQ5; gift from D. M. 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, pynlC, pnnlRIP, and pnsa5-1 (LEU2) were introduced into a sua5\textsuperscript{Δ} strain complemented with plasmid-borne p316SU45 by the plasmid shuffle technique (18). To obtain the plasmid shuffle haploid strain, tetrads were obtained from a heterozygous null diploid containing p316SU45. To create the Tet-off Sua5 strain (described below), pTetHasu5 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). pSU45 was constructed by cloning a 1.7-kb BamHI-Sall fragment containing the SUA5 ORF and the endogenous promoter, pynlC, was constructed by cloning a 0.6-kb BamHI-Sall fragment containing yrdC that was amplified from pmk107 (31) (a gift from M. Rydén-Aulin) into a pRS415TEF vector. pna5-1 was constructed by cloning a 1.9-kb BamHI-Sall fragment containing sua5-1 and the endogenous promoter that was amplified from YM4123 (a gift from M. Hampsey) (46). pmlRIP was constructed by subcloning a 0.8-kb EcoRI-Sall fragment from pBD-QAL4-mIRIP (29) (a gift from O. Mirochnitchenko) into a pRS424TEF vector. pTetHasu5 was constructed by ligating a 1.3-kb EcoRI (blunted with Klenow)-EagI HA-SU45 fragment into pCM184 (a gift from E. A. Craig) previously digested with BamHI (blunted with Klenow) and NotI (A, 20). pMT4 was constructed by subcloning a 170-bp XhoI fragment containing IMT from pJ780-IMT (5) into a pRS424 vector.

**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 sporulation medium (5% dextrose, 3% nutrient broth, 1% yeast extract, 2% Bacto agar) for 1 day (30°C), repatched onto GNA sporulation 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 UV cross-linked to nylon membranes (Amersham). The membranes were 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 h. Radioactive probes for the Northern analyses were prepared by random primed labeling lacZ PCR product (Amer-sham Rediprime II catalog no. RPN1633). The oligonucleotides used to amplify lacZ product were 5'-GGGCCCATCTACACCAAC (forward oligonucleotide) and 5'-GGCTTCATCCACCACATACAG (reverse oligonucleotide). Hybridization was performed at 55°C for 2 h. 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 2OS 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′-GTTTCCCCAGTCAGCAGATA-G-3′). Second-strand synthesis was performed using Sequenase (US Biochemical catalog no. 70775). PCR amplification was performed using specific primer B (5′-GTTTCCCCAGTCAGCAGATA-G-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 (pB10, 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 sua5Δ cells complemented with SUA5 expressed from the endogenous promoter (pSU145). 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 10.0 s. Ten microliters of the Stop & Glo assay buffer 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 1.0 s. For each strain lysate, corrected read-through was calculated by expressing the firefly/luciferase activity in sua5Δ cells complemented with SUA5 expressed from the endogenous promoter as a percentage of the firefly/luciferase activity in the wild-type strain. The luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega).

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 Cycl1 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 sua5Δ 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 sua5Δ strain (Fig. 1A). The SUA5 homolog in higher eukaryotes is known as IRIP (ischemia and reperfusion inducible protein) (10, 29). The ectopic expression of mouse IRIP from the yeast TEF1 promoter also partially suppressed the slow-growth defect of the sua5Δ 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 sua5Δ strain covered with a plasmid containing hemagglutinin (HA)-tagged SUA5 under a Tet-repressible promoter (pTettrHASUA5) 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 sua5Δ mutant.

Previous data demonstrated that the loss of Sua5 increased the expression of Cyc1 protein from the cycl1-1019 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 tRNAs (27). Since Sua5 is required for t^3^A37 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-
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 sua5Δ genotype cells containing pTettHASUA5 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.

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 t6A37 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 with 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^Met^17) were overexpressed from a high-copy plasmid (data not shown). Increasing the pool of initiator tRNA^Met^17 (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 deletion 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 t6A37 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-hydroxysopentenyl) adenosine (m^7io^6A37) and N1-methylguanosine (m^1G37), induce +1
frameshift errors (24). To examine the effect of losing t₆A₃₇ 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 t₆A₃₇ 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 t₆A₃₇ 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 sua5Δ 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 sua5Δ 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 sua5Δ 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

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**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. Recording 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.
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 t6A37 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 t6A37 modification was unknown. The recent discovery of the YrdC/Sua5 family as the putative enzymes required for t6A37 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 t6A37 (15), coupled with the strong in vitro data that t6A37 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 tRNAMet. 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 tRNAMet alone (12). The failure of IMT4 overexpression to rescue the leaky scanning observed in SUA5-depleted cells suggests that merely increasing the pool of t6A37 hypomodified initiator tRNAMet cannot correct the base-pairing defect. Hence, it is unlikely that the absence of t6A37 simply increases the dissociation rate of the initiator tRNAMet 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"A37 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"A37 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"A37 from tRNA^{Glu} diminished ribosome binding efficiency but had no effect on aminocoylation (41). It was also shown that a pathogenic mutation in A37 of human mitochondrial tRNA^{Glu} had only a minimal effect on aminocoylation efficiency (36).

As expected, the loss of Sua5 expression and t"A37 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"A37 (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"A37 improves the efficiency of natural nonsense suppressors. Most of the cytoplasmic tRNA^{Glu} isolated from mice and tobacco that suppress UAG/UAU contain an unmodified A37 (7). The interaction of the tRNA^{Glu} 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 t"A (N"-isopentenyladenosine) and ms"A (2-methylthio-N"-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"A37 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 translation 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"A37 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"A37 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-t1 (S107F) mutant still had wild-type telomere lengths (39). Given the fact that mitochondrial tRNAs also possess t"A37, 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"A37 in tRNAs across species underscores the utility of using yeast as a model to investigate the role of t"A37 hypomodification in human mitochondrial diseases (11).

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