Identification of the mRNA targets of tRNA-specific regulation using genome-wide simulation of translation

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ABSTRACT

tRNA gene copy number is a primary determinant of tRNA abundance and therefore the rate at which each tRNA delivers amino acids to the ribosome during translation. Low-abundance tRNAs decode rare codons slowly, but it is unclear which genes might be subject to tRNA-mediated regulation of expression. Here, those mRNA targets were identified via global simulation of translation. In-silico mRNA translation rates were compared for each mRNA in both wild-type and a tRNAGln CUG sup70-65 mutant, which exhibits a pseudohyphal growth phenotype and a 75% slower CAG codon translation rate. Of 4900 CAG-containing mRNAs, 300 showed significantly reduced in silico translation rates in a simulated tRNA mutant. Quantitative immunoassay confirmed that the reduced translation rates of sensitive mRNAs were tRNAGln CUG concentration-dependent. Translation simulations showed that reduced tRNAGln CUG concentrations triggered ribosome queues, which dissipated at reduced translation initiation rates. To validate this prediction experimentally, constitutive gcna2 kinase mutants were used to reduce in vivo translation initiation rates. This repaired the relative translational rate defect of target mRNAs in the sup70-65 background, and ameliorated sup70-65 pseudohyphal growth phenotypes. We thus validate global simulation of translation as a new tool to identify mRNA targets of tRNA-specific gene regulation.

INTRODUCTION

Translation of mRNA into protein represents the final stage of the gene expression pathway in which the transcribed mRNA is read by the ribosomal machinery, which translocates along the open reading frame to interpret the encoded peptide sequence. Its complexity can be likened to that of an industrial production line, involving not only the ribosomes and hundreds of ancillary translation factors, but also a population of transfer RNAs, of which there are 3 million in a yeast cell (1). In response to a cognate interaction between the tRNA anticondor and mRNA condon, tRNAs bring amino acids to the actively elongating ribosome at rates of up to 22 amino acids per second (2).

Due to genetic code redundancy, most amino acids are encoded by a family of codons, in turn recognised by more than one tRNA of a given amino acid-accepting type, the so called iso-acceptors. The different tRNAs of each isoacceptor exhibit a particular cellular abundance dictated by that tRNA's gene copy number. In yeast, these vary by as much as 11-fold within a single isoacceptor class of tRNA (3). There is very good evidence that the concentration of tRNAs defines the rate of translation of its cognate condon(s), which can affect overall translational rate, but also protein folding and mRNA secondary structure interactions (4,5). For example, tRNA concentration controls the rate of translation elongation through a run of tandem codons of one type, and regulates the rate of translation of individual condons whose cognate tRNA is in low abundance (6–10). The frequency of ribosomal drop-off is also increased by the translational pause caused by a rare condon (11,12). tRNA concentration also regulates translational +1 frameshifting through control of the length of pause of the elongating ribosome (12–14). Most highly expressed genes, whose transcripts form a large proportion of the transcriptome (ribosomal protein mRNAs, glycolytic mRNAs) utilise condons that are translated by the most abundant tR-
NAs (15). This codon bias probably serves to avoid the detriment to cellular fitness caused by ribosome queuing in response to rare-tRNA induced ribosomal pausing.

Translation is the most resource- and energy-consuming process in the cell, and is therefore highly regulated by a range of protein trans factors in response to environment and nutrient availability (16–22). However, while it is clear that tRNA concentration can regulate the translation rate of individual codons, the extent to which translation of any given mRNA is regulated by tRNA concentration is unknown. Particularly unclear is the regulatory role played by low abundance codons; these are translated by a correspondingly rare tRNAs with known effects on translation pausing. Mathematical modelling of translation has been used to predict that globally, translation is governed principally by ribosome limitation (23–25), presumably ensuring that ribosomes are well-spaced on mRNAs. This can be described as initiation-regulated translation. This reflects the imperative that ribosome queuing incurs a fitness cost, and therefore that most mRNA translation should be initiation-regulated by ribosome availability. Indeed, when ribosome profiling was used to report the effects of deleting some types of yeast tRNA through gene deletion, no significant effects were seen on ribosome pausing, evidence used to argue for a minimal role for tRNA regulation of translation (26). Other studies however make the case that ribosome pausing often reveals pausing at non-optimal codons in yeast (8). Furthermore, a recent study using meticulous measurement of translation velocities on Neurospora mRNAs showed clearly that non-optimal codons significantly slow translation, while abundant codon stretches are rapidly translated (27). Supporting a regulatory role for rare codons, modelling of translation suggests that there are sub-populations of mRNAs whose translation are elongation-regulated (28,29), in which codons translated by low-abundance tRNAs play a regulatory role. These predictions were experimentally validated on artificial mRNAs by controlling the rate of initiation on reporters engineered to contain multiple rare codons (30). However, the extent to which rare codons can exert a regulatory influence on a wide range of natural mRNA sequences, and the requirements for rare codon disposition or configuration to achieve such regulation, is unclear.

A regulatory role for tRNAs in controlling translation is moreover strongly suggested by observations from a range of different organisms. In yeast, a genome-wide tRNA deletion initiative identified a wide range of growth phenotypes and transcriptional stress responses in yeast, particularly associated with deletion of tRNA genes with a low total gene complement (31). Also in yeast, mutant glutamine tRNAs cause the slowed decoding rate of tandem CAG codons (6), and confer a pseudohyphal growth phenotype (32). Streptomyces bldA mutants in the tRNA_UGA_Leu gene cause an inability to form aerial hyphae and produce antibiotics, because translation of mRNAs containing the extremely rare UGA codon is compromised (33,34). In Escherichia coli, mutations in a range of tRNAs specifically compromise phage lambda replication (35) or cause elevated mutagenesis frequencies (36), while in Salmonella, tRNA mutation of tRNA^Arg^_UCU specifically reduce production of fimbriae at the translational level (37). The levels of tRNA charging will also vary in response to amino acid starvation stress, and modelling predicts that in E. coli this may regulate groups of amino acid biosynthetic genes (38). More broadly, tRNA modification, which can affect tRNA stability and translational decoding properties, may be central to regulating particular genes, or groups of genes. Mutations in the yeast Trm9 tRNA modification gene drive altered expression of genes enriched in codons targeted by Trm9-modified tRNAs (39). Similarly, mutations in the eukaryotic Elongator complex, catalysing a uridine tRNA wobble position modification, cause a range of specific phenotypic consequences including telomeric silencing, DNA damage responses, transcriptional elongation and exocytosis (40,41). This is likely to be due to altered translation of sub-sets of genes containing codon targets of the Elongator-modified tRNAs, since all Elongator phenotypes can be complemented by extra copies of key modified tRNAs (41).

Taken together, this body of evidence strongly suggests there is an important role played by the differential concentrations of tRNA species as regulators of the flux of ribosomes along each open reading frame, and thus, the gene-specific translational rate. To identify novel targets of gene expression regulation by low abundance tRNAs, we use global stochastic modelling of translation in Saccharomyces cerevisiae to predict the translational rate of every mRNA. Using this model we simulate the translational rate of each mRNA in a wild-type cell, versus that in a cell in which the concentration of a rare glutamine tRNA has been reduced 4-fold, mimicking the molecular phenotype of the yeast sup70-65 allele of the tRNA^Cln^_CUG (6). The mathematical model predicts several hundred genes that are sensitive to tRNA-specific regulation by this tRNA^Cln^, and we show using a focused experimental investigation that the model predictions are successfully validated. The use of global modelling of cellular translation, combined with experimental validation, reveals that although in global terms cellular translation is remarkably resilient to changes in tRNA concentration, nevertheless there are significant numbers of genes whose translational rate is sensitive to the concentrations of rare tRNAs. We show further that translational sensitivity to the concentration of any rare tRNA is determined not simply by the extent of use of the corresponding codon in an mRNA, but most likely by the configuration of those rare codons within the coding sequence, combined with the relative contents and dispositions of other types of rare codon in that gene. The configuration of rare codons, and their permutation with other rare codons, is thus revealed as an exquisitely sensitive modulator of gene expression.

**MATERIALS AND METHODS**

**Mathematical modelling of translation**

A stochastic model of translation was employed to simulate translation across yeast mRNAs (29). This model, based on the paradigmatic Totally Asymmetric Simple Exclusion Process (TASEP), represents the mRNA as a lattice, where each site of the lattice symbolises a codon (42,43). Ribosomes are then described as particles that hop onto the first
site of the lattice, move along it translating the codons into amino acids, and hop off the lattice at the last site. Particles are considered to have a footprint of 9 codons to represent the actual ribosome width (44). Moreover, they cannot overtake each other, and a particle cannot initiate translation if the first 9 sites of the lattice are not free. Importantly, ribosomes advance through the lattice following a two-state dynamic: (1) recognition of the cognate tRNA with rate $k_i$ proportional to the concentration of that tRNA, and (2) translocation to the next codon with rate $y = 35$ s$^{-1}$, a rate independent of the specific codon (45). Thus our model simulates the stochastic movement of ribosomes along the mRNA, considering the actual ribosome width in terms of codons that they can cover, according to an exclusion process, i.e. two ribosomes cannot occupy the same codon. Importantly, the model also considers the internal biochemical cycle that the ribosome undergoes between each hopping event from one codon to the next. Hence, our model more closely represents the biomechanics of the translation process in comparison to other models (24,46,47) while preserving computational efficiency (each simulation only takes tenths of a second). mRNA-specific translation initiation rates $\alpha$ were derived using an integrated analysis of experimental data using model simulation as described previously (29). The termination rate $\beta$ was considered not limiting and fixed equal to the fastest rate (i.e. $\beta = y$) (48). In establishing the model, a series of simplifying assumptions were made; since there was no expectation that the depletion of a tRNA would affect ribosome biosynthesis, the ribosome concentration was invariant through the simulations. Likewise, tRNA charging by the aminoacyl tRNA synthetases was not expected to be affected, a decision made on the basis of experimental measurements of charging of the glutamine and histidine tRNAs in both wild-type and sup70-65 mutant conditions (6), thus the proportions of tRNAs in the charged condition remained fixed. Likewise the ribosomal translocation rate ($\gamma$), following binding of the cognate tRNA, was invariant in the model.

This model predicts the average occupancy of each codon on the mRNA during translation, as well as the resulting translation rate, i.e. how many proteins per unit time are produced. Simulations were run by using a continuous time Monte-Carlo algorithm based on the Gillespie algorithm (49), coded in C++. Using this model, simulations for each of the 5500 yeast open reading frames (ORFs) were run until steady state was reached, after which then data were collected. Two sets of simulations were performed: (i) mRNA-specific translation initiation rates (designated $\alpha$) were derived using an integrated analysis of experimental data using model simulation as described previously (29). Simulation of translation was carried out using a standard range of codon-specific decoding rates as described (29), or (ii) with the decoding rate of individual tRNAs (e.g. tRNA$^{\text{Gin}}_{\text{CUG}}$) decreased to 25% of their wild-type value to replicate a tRNA depletion condition. The translational rate $J$ of any given mRNA, equivalent to a rate of synthesis of that protein, was recorded during the course of the simulation. Where required, $J$ was recorded following simulation across a range of translation initiation values of $\alpha$. The codon-dependent ribosomal density used to reconstruct the ribosome occupancy profile across the mRNA was extracted by identifying the codon position of the ribosomal A-site.

S. cerevisiae strains and growth conditions Strains MLD17 (MATa/α adel adel his3-11/ his3-1 1 trp1-1/ trp1-1 ura3-52/ ura3-52) and MLD14 (MATα/a sup70-65/sup70-65 adel/ADE1 his3-11/his3-11 leu2-3,112/LEU2 trp1-1/trp1-1 ura3-52/ura3-52) were kindly provided by Prof RA Singer (Dalhousie University, Halifax, Canada) (32). Cells were grown at 30°C on solid or liquid YPD (1% yeast extract, 2% peptone, 2% glucose) (50) or, after transformation, on the appropriate synthetic-defined (SD) or synthetic-complete (SC) selective medium (50). Where required, glucose was substituted with 1% galactose for gene induction.

Plasmids Plasmids expressing HA-tagged open reading frames under the control of GAL1 promoter (FA7/ YII152W, NDLI, STE18, YDL012C, ATG16, RCF1, LCL2, TRP4, ADH1, CDC19, MCM1, OPI1 and PBP2) were purchased from the Thermo Scientific Open Biosystems Yeast ORF Collection (Thermo Fisher Scientific Biosciences GmbH). To construct plasmids pYIL152-CAA, pFA7-CAA and pNDL1-CAA, the respective open reading frames, in which all glutamine CAG codons were substituted with CAA codons, were synthesized by Eurofins Genomics and sub-cloned into CAA vectors (wild-type; plasmid p722, E1522K; p915, E1537G; p914 and M719V-E1522K; p1055 (52)) were kindly provided by Prof G. Pavitt (University of Manchester, UK) and sub-cloned into pRS413 (53) following PCR amplification using primers AGGTCGACCGTATCGA TTGTCGATGAAAGTAGTAA and TAGAAGTGGATGGATGTAGAC and GTAAGA...
TCTCATAGAAGCG (listed 5’-3’). For normalisation of loaded and transferred RNA samples, a specific probe for the yeast *SCRI* mRNA was amplified from genomic DNA using primers TCTTCTCCTCAGCTCAGA and CACC TTGCTGACGCTG (listed 5’-3’). PCR products were radio-labelled by random priming and RNA levels were quantified using a Fuji FLA-3000 phosphoimager.

*Flow cytometry analysis.* Three independent cultures of MLD14 and MLD17 strains (transformed with the p722 wild-type *GCN2* or M719V/E1522K *gen2* allele plasmid p1055 as described above) were grown in 5ml YPD media to mid-log phase. One millitre samples of each culture were harvested, washed and resuspended in sterile phosphate buffered saline. The forward-scatter (FSC) of samples was measured using the blue 488 nm laser in a Becton Dickinson LSR Fortessa Cell Analyser. Typically 20 000 cells were analysed for each sample and the same cytometer settings (photomultiplier tube values, etc) were used throughout the experiment. The resulting data was analysed using FlowJo (version 10). To analyse the data, a population gate was created for the control culture containing the 95% lowest FSC value population. By applying this gate to cultures or strains representing the test population, the percentage of cells with FSC larger than 95% of the wild-type population was determined to define pseudohyphal cell chains.

*Plasmid retention assay.* Cytometry analysis was carried out on cells grown in non-selective YPD medium to optimise chain formation. The extent of plasmid retention was quantified during growth on non-plasmid selective media for the *gen2* allele plasmids that conferred a growth disadvantage. Three independent cultures of yeast strains MLD14 (*sup70-65/sup70-65*) and MLD17 (*SUP70* wild-type; transformed with wild-type p722 or plasmid p1055 expressing the M719V/E1522K *GCN2* allele as described above) were grown to mid-log phase in YPD media. Cells were harvested and plated for single colonies on either complete medium to quantify total colony-forming units, or on selective medium to identify plasmid transformants. The percentage retention of *GCN2* allele plasmids could thus be calculated. Plasmid retention in strain MLD17 was typically 59% for wild-type *GCN2* transformants and 22% for plasmid carrying the constitutively active *gen2*-M719V-E1522K allele.

**RESULTS**

**Cell-wide modelling of translation identifies the targets of tRNA regulation of translation**

There are known examples where a tRNA can regulate the expression of specific sets of genes, e.g. the *Streptomyces bldA* tRNA that controls sporulation and antibiotic production (34). Mutant alleles of the *Saccharomyces cerevisiae* *SUP70* gene encoding tRNA<sub>Gln</sub><sup>CUG</sup> exhibit a slowed translation rate of the cognate CAG codon caused by a 4-fold reduced abundance of tRNA<sub>Gln</sub><sup>CUG</sup> (6) (Figure 1A). This in turn triggers inappropriate nitrogen-starvation responses in N-repelete growth media, including the constitutive formation of pseudohyphal chains of cells in this normally single-celled fungus (32) (Figure 1B). However it is unclear if this reduction in the abundance of an already rare tRNA affects translation rates globally, or if translation of specific sub-sets of mRNAs are particularly prone to alterations in tRNA<sub>Gln</sub><sup>CUG</sup> abundance. If the latter, this group of transcripts must encode a protein(s) required for suppression of the pseudohyphal response in a wild-type cell. The identification challenge is considerable; for example, over 2500 yeast genes have between 1 and 4 CAG codons, and only 600 ORFs are CAG-free (Figure 1C). As a simple consequence of codon bias, highly expressed proteins are CAG-free. However, for CAG-containing mRNAs there is a very weak correlation between the cellular abundance of a protein and its mRNA’s CAG content (Figure 1D). Therefore it is likely that the absolute content of CAG codons is not a determining factor in gene expression, but rather the position of CAG codons in the ORF (56). Slowly translated CAG codons situated early (5’) in an ORF may cause queuing of ribosomes back to the mRNA cap, inhibiting efficient ribosome recruitment. However, there is no correlation between position of the first CAG codon, and protein abundance (Figure 1E). It was therefore not possible to use bioinformatic approaches alone to predict which mRNAs might be specifically targeted due to inefficient decoding by tRNA<sub>Gln</sub><sup>CUG</sup>.

We instead adopted a novel alternative approach to identify the targets of tRNA<sub>Gln</sub><sup>CUG</sup> regulation, and simulated the translation of all 5500 yeast open reading frames (ORF), using a two-state ribosome model of translation (Materials and Methods; Figure 2A). The rate of translation of each ORF was simulated in the first instance using wild-type yeast tRNA concentrations. Simulations were then repeated using the tRNA complement of a *sup70-65* mutant, in which the concentration of tRNA<sub>Gln</sub><sup>CUG</sup> was reduced to 25% of the wild-type value, as determined experimentally (6).

Comparison of the rates of translation of each ORF in wild-type and tRNA mutant backgrounds revealed that although the majority of mRNAs were unaffected by the reduction in CAG-decoding tRNA, nevertheless there were approximately 300 target mRNAs that the model simulation predicted would be as much 2-fold down-regulated (Figure 2B). The translation of these ORFs clearly responds markedly to reductions in the level of the single copy yeast tRNA<sub>Gln</sub><sup>CUG</sup>, identifying these genes as potential targets for regulation by a single-copy tRNA.

**Specific mRNAs are regulated by rare-tRNA abundance**

The model predictions (Figure 2B), generated using our mathematical model of translation, were then validated experimentally. Proteins whose translational expression was predicted to be compromised by the reduction in concentrations of tRNA<sub>Gln</sub><sup>CUG</sup> in the yeast *sup70-65* mutant were quantified in wild-type and *sup70-65* backgrounds.

Accordingly, a range of 8 ORFs was selected whose expression was predicted by the simulation to be sensitive to tRNA<sub>Gln</sub><sup>CUG</sup> levels. One example in this group was *FA7R*, an ORF of 221 codons containing 12 CAG codons. A control group was also selected, comprising ORFs whose reading frames in some cases contained significant numbers of CAG codons, but whose translation was nevertheless predicted
Figure 1. CAG codon usage in the transcriptome. (A) The CAG glutamine codon is recognised by the single copy SUP70 gene, encoding tRNA^Gln\(_{\text{CUG}}\). The sup70-65 allele defines a variant lacking a base-pairing interaction at the base of the anticodon stem. (B) The sup70-65 mutation causes a pseudohyphal growth phenotype. (C) The CAG frequencies were recorded for all S. cerevisiae ORFs. (D) The cellular abundance of yeast proteins (66) was plotted against the binned proportional content of CAG codons relative to ORF length. (E) For each yeast ORF, the position of the first (5′-most) CAG codon was recorded and plotted against the cellular protein abundance.

by the model simulation to be insensitive to levels of the tRNA^Gln\(_{\text{CUG}}\). For example, MCM1 contains 29 CAG codons in an ORF of length 286, but was nevertheless predicted to be unresponsive to tRNA^Gln\(_{\text{CUG}}\). CAG contents for all genes are listed in Supplementary Table S1.

These ORFs, tagged with an HA epitope-protein A fusion, were expressed in both wild-type and sup70-65 mutant yeast under control of the GAL (galactose-regulatable) promoter on a plasmid. The transformants were grown exponentially, then total protein and RNA was isolated for analysis by quantitative Western and Northern blot from three independent biological replicates, normalised for gel loading in each case (Figure 3A). Northern blot phosphoimager data was used to quantitate mRNA levels, which were used to normalise mean protein expression levels of each of the target ORFs. Any effects of alterations in transcription or mRNA stability were thus excluded.

This analysis was conducted both for the test ORF set predicted to be tRNA^Gln\(_{\text{CUG}}\)-sensitive, and for the control tRNA^Gln\(_{\text{CUG}}\)-insensitive gene set (Figure 3B and C respectively). The results clearly show that the expression levels of the tRNA^CUG\(_{\text{CUG}}\)-sensitive ORFs are significantly reduced across the range of genes tested (a mean of 54% relative to the wild-type control; Figure 3B cf model predictions, Supplementary Table S1) while the control set of tRNA^CUG\(_{\text{CUG}}\)-insensitive ORFs, selected because model simulation identified their translational rate as unaffected by levels of the glutamine tRNA, showed a mean expression level in the sup70-65 mutant of 103% relative to wild-type. The measured reductions in protein expression caused by the sup70-65 mu-
Figure 2. Modelling ribosome flux on the total yeast transcriptome identifies the targets of glutamine \textit{tRNA}_{CUG}\textit{CUG} regulation. (A) Mathematical modelling of ribosome flux along each mRNA employed a Totally Asymmetric Simple Exclusion Process (TASEP) in which ribosomal particles of 9-codon width join a lattice representing the mRNA with rate $\alpha$, and translate the lattice, codon by codon using 2-state dynamics. These are defined by cognate tRNA finding rate $k_i$, dictated by tRNA abundance, and translocation rate $\gamma$ for ribosomes charged with the cognate tRNA, not shown in the figure (67). Ribosomes then terminate with rate $\beta$. (B) The TASEP model was used to simulate in turn each of the 5500 yeast mRNAs using published initiation and stepping rates (29). Simulations were performed using wild-type tRNA concentrations, and again using a concentration of \textit{tRNA}_{CUG}\textit{CUG} reduced to 25\% of normal levels to mimic the \textit{sup70-65} mutant as determined experimentally (6). Translation rates for each mRNA under the two conditions are plotted.

tation (Figure 3B) were highly correlated with the original model simulation quantitative predictions of reduced translation (Figure 2 and Supplementary Table S1), with a correlation coefficient $R^2 = 0.63$.

Taken together, the results showed clearly there are genes in yeast that are sensitive to variations in the levels of rare tRNAs, and that in silico simulation of translation can successfully predict the identity of those mRNA sequences. Moreover, the experimental validation of the model predictions for control ORFs demonstrates that alone, the content of a rare codon such as CAG in an ORF is not predictive of its sensitivity to the concentration of its cognate tRNA.

Specific translation defects in the \textit{sup70-65} mutant are \textit{tRNA}_{CUG}\textit{CUG}-dependent

In order to confirm that the compromised translational efficiency measured (Figure 3) was due to the presence of CAG codons in the open reading frame, the mathematical model of translation was first used to predict the effect of replacing all CAG codons with the synonymous CAA glutamine codon. As expected, for the three candidate CAG-sensitive genes chosen, replacement of all CAG codons with CAA

Figure 3. Experimental confirmation of the targets of glutamine \textit{tRNA}_{CUG}\textit{CUG} regulation. (A) Quantitative Western blotting was performed for target proteins (predicted to be sensitive to cellular levels of \textit{tRNA}_{CUG}\textit{CUG}), HA-tagged and expressed in either wild-type or \textit{sup70-65} mutant yeast; three examples are shown. Triplicate biological replicates were assayed for HA-tagged protein expression following gel loading normalisation. RNA expression levels for each HA-tagged construct were quantified using \textit{SCR1} control probe. (B) CAG-regulated mRNAs; HA-tagged protein expression levels were quantified in wild-type (black bars) and \textit{sup70-65} yeast (grey bars) for eight putative targets of \textit{tRNA}_{CUG}\textit{CUG} regulation ($n = 3$, ± standard error of the mean). Protein expression levels were normalised using the levels of the corresponding mRNA expression levels determined by northern blot. (C) Non-CAG-regulated mRNAs; The same process was repeated for a series of control ORFs, whose expression is not predicted to be responsive to \textit{tRNA}_{CUG}\textit{CUG} ($n = 3$, ± standard error of the mean).
rendered the \textit{in silico} translation immune to a simulated reduction of tRNA\textsubscript{CUG}\textsuperscript{Gln} concentrations (Figure 4A).

The model predictions were then confirmed experimentally. Alleles of the YIL152W, FAR7, and NDL1 genes were synthesised in which all CAG codons were replaced by their synonymous CAA counterpart. Expression of these CAA-replacement alleles was assessed using quantitative Western blots, normalised for mRNA concentration as before. The results showed clearly that the expression levels of each of the genes in the \textit{sup70-65} mutants was restored to almost wild-type levels, identifying the presence of the CAG codons as the sole cause of the reduced expression in the \textit{sup70-65} mutant (Figure 4B, compare with Figure 3B).

To further confirm the mechanism via which translational efficiency (translational rate) of these three genes is compromised in the tRNA\textsubscript{CUG}\textsuperscript{Gln} mutant, both the wild-type and the \textit{sup70-65} mutant were separately transformed with a multi-copy plasmid expressing a wild-type copy of the \textit{SUP70} gene, encoding tRNA\textsubscript{CUG}\textsuperscript{Gln}. This is known to complement the \textit{sup70-65} pseudohyphal growth phenotype, and normalise the CAG codon translation rate (32). As predicted, the ectopic tRNA expression in the mutant restored the translational efficiency of both of the two tested mRNAs to wild-type levels (Figure 4C).

The relative rates of translation initiation and elongation govern the sensitivity of mRNA translation to rare-tRNA concentrations

Whereas wild-type yeast grows in single budded cell form, a yeast \textit{sup70-65} mutant forms long, pseudohyphal chains of cells. We show that the reduced tRNA\textsubscript{CUG}\textsuperscript{Gln} concentrations in the \textit{sup70-65} mutant cause reduced expression of a specific sub-set of CAG-containing genes (Figure 3), and we suggest that it is this failure to translate one or more specific mRNAs at wild-type rates that triggers the formation of pseudohyphae. However, how reduced concentrations of tRNA\textsubscript{CUG}\textsuperscript{Gln} inhibit the translational rate of some mRNAs is unclear. Reducing the concentration of a given tRNA stochastically reduces the translation rate of its cognate codon (6–10). Such extended translational pauses can cause ribosomal queuing, which if they extend back to the 5′ end of the mRNA can compromise recruitment of ribosomal subunits to the 5′ mRNA cap, and thus translational efficiency of the mRNA (30). We therefore hypothesised that the reduced translation rate of CAG codons produces ribosome queues which inhibit ribosome recruitment on the YIL152W and FAR7 mRNAs, explaining why they exhibit reduced expression in the \textit{sup70-65} mutant.

If the translation rate is being limited by a rate-limiting step at the elongation stage, forming a ribosomal queue, then significantly reducing the rate of translation initiation will introduce a more rate-limiting step earlier in the translation process, at the point of ribosome joining to the mRNA. This in turn will cause the ribosomal queue to dissipate. Using the mathematical model of translation we confirmed that this is in fact the case by simulating translation of three genes known to be sensitive to tRNA\textsubscript{CUG}\textsuperscript{Gln} concentrations; for each gene the ratio of translation rate in \textit{sup70-65} to that in wild-type tends to a value of 1 as the \textit{in silico} translation...
initiation rate is decreased. This signifies that the sup70-65 translational defect, relative to wild-type, should be masked at low rates of translational initiation (Figure 5A).

The model simulation was then used to quantitate the ribosome density profile across the FAR7 mRNA. In the simulated sup70-65 background, ribosome densities at the S' end of the FAR7 mRNA were significantly raised at high initiation rates (Figure 5B), indicative of ribosome queuing. In contrast, when the in silico initiation rate was reduced, ribosome queues were reduced, and ribosomal density at the earliest, S'-most, codon in the ORF was reduced to wild-type levels in the simulated tRNA_Gln mutant condition. To confirm the queuing behaviour was specific for the predicted tRNA_Gln-sensitive mRNAs such as FAR7, we also simulated translation on the CAG-rich, but tRNA_Gln-insensitive MCM1 mRNA. As expected, at the physiological initiation rate, ribosome densities at the MCM1 S' end were identical in wild-type and sup70-65 simulations (Supplementary Figure S1), explaining why Mcm1 protein expression levels in the sup70-65 mutant were indistinguishable from that in the wild-type (Figure 3C). The model prediction was thus clear; the mutant tRNA_Gln induces the formation of ribosome queues on tRNA_Gln-sensitive mRNAs such as FAR7, queues which should dissipate when the translation initiation rate in the sup70-65 background is reduced, thus masking the sup70-65 mutant phenotypes.

To experimentally validate this model prediction, we employed constitutively-active mutants of the Gcn2 protein kinase (gcn2C) to reduce the global rate of translation initiation via phosphorylation of the essential translation initiation factor eIF2-α (52). We reasoned that reducing the translation initiation rate would make translation initiation, rather than translation elongation, the rate-limiting step in translation, and prevent the formation of ribosome queues at CAG codons that would otherwise extend to the mRNA S' end. This would eliminate the translational disadvantage suffered by these mRNAs in a sup70-65 translation system, relative to their expression level in a wild-type cell. Ameliorating the translational block should in turn diminish the severity of the sup70-65 mutant phenotypes, including pseudohyphal chain formation. We therefore transformed a plasmid bearing a gcn2C allele into either wild-type or sup70-65 yeast, and used cell cytometry and, separately, direct counting of cell chains, to quantify the effect on the pseudohyphal growth phenotype.

Cytometric analysis, using forward scatter as an indicator of cell size, showed clearly that whereas the wild-type population exists as single cells, a significant proportion of a sup70-65 population is composed of cell chains exhibiting large forward scatter (Figure 6A). Crucially, reducing the translation initiation rate through expression of the gcn2C allele in the sup70-65 mutant caused a significant, almost 2-fold shift in the population away from chains and towards single cells (Figure 6A, bar chart). That change may have even been greater had there not been a significant loss of the growth-inhibitory gcn2C expressing plasmid from the transformed cells (Materials and Methods; 22%-59% plasmid retention), caused by the requirement to grow the cells under plasmid non-selective conditions.

Direct microscopic observation of cells allowed quantification of a chain formation index to indicate the extent of pseudohyphal formation in wild-type and sup70-65 strains (6). This revealed that gcn2C expression caused at least 2-fold reductions in chain formation in the sup70-65 mutant (Figure 6B). Thus the cell developmental phenotype caused by reductions in rare tRNA concentration can be significantly reduced through down regulation of the global translation initiation rate.

It was important to verify that a gcn2C-driven reduction in the translation initiation rate can also improve the impaired translation of a sup70-65-sensitive mRNA such as FAR7, as predicted by the in silico translation simulation (Figure 5A). A number of gcn2C constitutive alleles have been identified, exhibiting a range of eIF2-α kinase activities and thus slowed growth phenotypes (52). These were employed to produce a range of translation initiation rates. In separate experiments, three different gcn2C alleles exhibiting low, medium or high constitutive eIF2 kinase activities were transformed into either wild-type or sup70-65 strains, and the translation of FAR7 mRNA monitored, normalised relative to their mRNA levels.

The results revealed that expressing constitutively active Gcn2 protein in the sup70-65 strain increased the translational efficiency of FAR7 mRNA relative to that in wild-type cells (Figure 6C). Moreover, a graded, increasing response of FAR7 mRNA translational efficiency was recorded in the sup70-65 background in response to expressing gcn2C alleles of increasing constitutive activity; E1562K, E1537G or M719V/E1522K. Note that as the constitutive eIF2 phosphorylation activity increases across this series, so the translation initiation rate decreases. We also observed that the measured content of Far7p relative to total cell protein, (i.e. prior to ‘percentage of wild-type’ normalisation, Figure 6C) increased in the mutant background as the initiation rate decreased. Although the translation rate of Far7p in the mutant background is expected to decrease with decreasing initiation rate, it does so at a slower rate than in the wild type background, and also slower than the average protein in the cell. Thus as the initiation rate is reduced, Far7p content as a fraction of total cellular protein increases in the mutant background because the absence of ribosome queues at low initiation rates allows the FAR7 mRNA to more effectively recruit ribosomes. The physiological ratio of translation efficiency of FAR7 mRNA relative to the translation efficiency of the mRNA cellular pool is thus restored. Together, this analysis indicated that reducing translation initiation rates caused an amelioration of the elongation-inhibitory effects of slow codons within an mRNA.

Low abundance tRNAs can act as master regulators of specific subsets of mRNAs

The demonstration that the rare glutamine tRNA_Gln is able to regulate a specific set of mRNA translation events raised the possibility that other tRNAs might exhibit similar regulatory potential to govern the translation rate of specific sub-sets of mRNAs. For example, the abundance of yeast tRNA_Arg is known to be low enough to trigger a ribosomal pause-driven ribosomal frameshift in certain con-
Figure 5. The rate of translation elongation at CAG codons, relative to the rate of translation initiation, governs ribosome queue formation and thus tRNA\textsubscript{Gln}\textsubscript{CUG} sensitivity. (A) TASEP simulation of translation of three tRNA\textsubscript{Gln}\textsubscript{CUG}-sensitive mRNAs was conducted across a range of values of $\alpha$, the translation initiation rate (Figure 2). Simulations were conducted in either a wild-type tRNA background, or a sup70-65 tRNA background, and a ratio of these translational efficiencies plotted against each value of the translation initiation rate. (B) These same TASEP simulations were used to record the codon-specific ribosomal density across the FAR7 ORF to indicate the positions of ribosome queuing. The ribosomal density across the FAR7 was recorded in a wild-type strain (filled circle symbols) and the sup70-65 mutant condition (open triangle symbols), at the physiological initiation rate of 0.3 events/s (dashed lines, blue symbols), and again at a 6-fold slower rate of 0.05 events/s (solid lines). The ribosomal density across codons 1–30 is presented, showing that the ribosomal density at the mRNA 5′ end in the sup70-65 mutant is greater than that in the wild-type at the high initiation rate, but that ribosome queues dissipate at the lower initiation rate, eliminating this density differential at the 5′-most codons. (C) Codons 1–24 of the FAR7 open reading frame, with the positions of the CAG codons indicated (underlined).

Importantly, a similar, simulated reduction in the concentration of abundant tRNAs (in this case defined as those with a gene copy number > 4) produced no effect on translational rate of any mRNA. This indicated that translation is extremely robust to marked changes in the concentrations of abundant tRNAs.

Each of the sets of genes showing sensitivity to the concentration of a given rare tRNA did not overlap with one another, indicating that codon content and configuration render given mRNAs sensitive to particular rare tRNA species. Thus some coding sequences may contain disproportionate numbers of a rare codon of a given type configured in a particular arrangement so as to trigger queue formation, making that mRNA the target for regulation by alterations in the concentration of its cognate tRNA. To test this hypothesis, we examined whether the sensitivity of a given mRNA to reductions in the concentration of tRNA\textsubscript{Gln}\textsubscript{CUG} was inversely correlated with a specific content of CAG codons, combined with the absence of other rare codons (ratio of CAG codons: other rare codons). Indeed
Reductions in the global translation initiation rate ameliorate the mRNA-specific translational inefficiencies caused by reduced tRNA\(^{\text{Gln}}\) concentration. (A) Cell cytometry was used to assay the prevalence of pseudohyphal chains that typify the sup70-65 mutant. Forward scatter measurements indicated the extent of formation of large chains of cells. Pseudohyphal growth was assessed in wild-type and sup70-65 yeast in the presence of either a wild-type ectopic GCN2 allele (dark-shaded frequency plot), or a constitutively-active gen2 allele (light-shaded). Population sizes of large chains and single budded cells were quantified using the cytometry data and plotted in the bar chart. (B) To confirm these observations, the degree of pseudohyphal chains formation in wild-type or sup70-65 cells, transformed with a plasmid expressing either CGN2 or gen2 was assessed by direct microscope observation. Pseudohyphal chains were counted, and a chain formation index used to capture the extent of pseudohyphal growth during growth on complete (YPD) or minimal medium (SC) (6). (C) HA-tagged FAR7 was expressed in wild-type and sup70-65 yeast transformed with ectopically expressed CGN2 or gen2 genes to either maintain, or reduce, global translation initiation rates respectively. Three different gen2 alleles were used with increasing degrees of constitutive eIF2 phosphorylation activity (E1522K < E1573G < M719V, E1522K). FAR7 expression was quantified using Western blotting, normalised for mRNA expression level as in Figure 3 (n = 3, ± standard error of the mean). The Far7p expression level in the mutant sup70-65 was expressed as a percentage of the expression level in a wild-type cell.

**DISCUSSION**

The role of tRNA abundance in controlling the efficiency of mRNA translation has been the subject of much debate, with some research concluding that control of translation initiation, rather than elongation, is by far the most dominant effect on translational efficiency (26,57), or that ribosome limitation is key to controlling translation efficiency (23). The importance of these influences cannot be overstated, nevertheless there is mounting evidence that tRNAs can and do regulate sub-sets of mRNAs (34). mRNAs may therefore fall into two classes, those that are principally initiation-regulated, and those that are elongation-regulated and responsive to tRNA concentration (28–30). The challenge then is to identify which mRNAs are sensitive to the concentration of any given tRNA. Only then will it be possible to understand how dynamic changes in tRNA concentration can control expression of some genes at the translational level.

In this work, we investigated an unusual mutant form of an essential, single gene copy tRNA in yeast that reduces the translation rate of CAG glutamine codons by 75% while still maintaining viability (6,32). This molecular phenotype causes a constitutive pseudohyphal growth phenotype (Figure 1), almost certainly through tRNA-driven changes in gene expression in the sup70-65 mutant. The sup70-65 mutant thus represents a powerful tool to analyse the effects of depleting an essential tRNA species. However, identifying the mRNA translation events sensitive to this tRNA required a new approach. For the first time we used global simulation of translation across all 5500 yeast mRNAs to replicate either wild-type translation, or translation in a sup70-65 mutant background. In this way, we effectively...
Figure 7. Translation is sensitive to changes in concentrations of rare tRNAs. (A) Translation of all 5500 yeast ORFs was simulated using the TASEP model representing a wild-type spectrum of tRNA concentrations, and again for a simulated cell where the concentration of one tRNA type was reduced to 25% of wild-type levels. The wild-type translation rate for each ORF is plotted against the corresponding tRNA-depleted rate. This exercise was repeated for all 42 species of yeast cytoplasmic tRNA. The results for 6 tRNAs are presented, three types whose encoding genes are multicopy (Gene Copy Number GCN > 4; left column), and three single gene-copy tRNAs (GCN = 1; right column). (B) A disproportionately high content of CAG codons within an ORF, relative to other rare codons, together with the configuration of those CAG codons, may drive ORF sensitivity to tRNA Gln CUG concentrations. To test this, for the 90 most sup70-65 sensitive ORFs, the CAG rare codon content, normalised to content of other rare codons, was plotted against the translation efficiency ratio (sup70-65/wild-type) revealing a negative correlation ($R^2 = 0.21$).
which sup70-65 mutant tRNA would block Gln3 dynamics if is a component were needed for Gln3 nuclear relocation. For example, the study found that the concentration of low abundance tRNAs that primarily respond to growth conditions (65). Intriguingly, in Lactococcus, it is the concentration of low abundance tRNAs that primarily responds to growth rate, exactly the sub-population that our study predicts has the greatest regulatory role on mRNA translation. Our work has identified the translation regulatory targets of a key single copy tRNA when that tRNA is significantly depleted through mutation. This prompts the obvious question of how tRNA concentrations might vary physiologically in a wild-type cell to control gene expression at the translational level. The view that concentrations of individual tRNA species are fixed, and determined only by tRNA gene copy number is looking increasingly simplistic. We know that the developmental regulation of the Streptomyces bldA tRNA is not a special case (60), since as long ago as 1994 researchers were reporting differential regulation of the four members of the S. cerevisiae seryl-tRNA family in response to growth rate and carbon source (61). Differential, albeit moderate, regulation of the E. coli tRNAs in response to growth rate has also been reported (62). More recently, tRNA abundance in human was reported to vary across tissue type (63,64), and in Lactococcus sp in response to growth conditions (65). Intriguingly, in Lactococcus, it is the concentration of low abundance tRNAs that primarily responds to growth rate, exactly the sub-population that our study predicts has the greatest regulatory role on mRNA translation (Figure 7).

Our study also reveals the regulatory potential inherent in the selection of rare codon types in each ORF. Simulated depletion of each of the single copy tRNAs in yeast revealed in each case several hundred genes that were translationally down-regulated in response to depletion of a single gene copy tRNA (Figure 7). These gene sets were however largely non-overlapping (Supplementary Table S2). Thus evolutionary selection of particular rare codon types within an ORF, and the potential exclusion of other rare codon types could render an ORF sensitive to one particular rare tRNA species. Indeed, there was some evidence that examples of this might exist; we showed that mRNAs most sensitive to tRNACUG depletion contained a high ratio of CAG codons relative to other non-CAG rare codons (Figure 7B). This could offer a partial explanation for why some CAG-containing mRNAs were sensitive to tRNACUG concentrations, and other ORFs with a high CAG content were insensitive (Figure 3). However, the configuration of those CAG codons is also of paramount importance; many CAG-containing genes are completely unaffected by the sup70-65 tRNA milieu, and we show that in one such insensitive gene, MCM1, containing 25 CAG codons, the ribosomal density at the 5’ end is not significantly altered in the sup70-65 background relative to wild-type, underlining the importance of configuration.

This study has unequivocally clarified our understanding of the regulatory role that can be played by low abundance tRNAs. The translational pausing that results when a ribosome translates a rare tRNA’s cognate codon can cause a bottleneck in translation elongation. If this rate-limiting step is slower than the rate of translation initiation on that mRNA, ribosome queues will then result. Ribosome queues that extend back to the 5’ cap of an mRNA, representing an elongation ‘bottleneck’, will lead to reduced translational efficiency through failure to compete for, and recruit, new ribosomal subunits to the now occluded 5’ cap. We thus show the crucial role in determining translational efficiency played by the balance between initiation and elongation rates. In addition to this fundamental insight into the control of gene expression by tRNAs, our study has also broken new ground through the use of an in silico screen to rapidly probe transcriptome-wide translational rate in a computer model. This approach opens the door for completely new analytical approaches to understanding translational regulation on a system-wide scale in the future.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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