

Rate-Limiting Steps in Yeast Protein Translation

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SUMMARY

Deep sequencing now provides detailed snapshots of ribosome occupancy on mRNAs. We leverage these data to parameterize a computational model of translation, keeping track of every ribosome, tRNA, and mRNA molecule in a yeast cell. We determine the parameter regimes in which fast initiation or high codon bias in a transgene increases protein yield and infer the initiation rates of endogenous *Saccharomyces cerevisiae* genes, which vary by several orders of magnitude and correlate with 5' mRNA folding energies. Our model recapitulates the previously reported 5'-to-3' ramp of decreasing ribosome densities, although our analysis shows that this ramp is caused by rapid initiation of short genes rather than slow codons at the start of transcripts. We conclude that protein production in healthy yeast cells is typically limited by the availability of free ribosomes, whereas protein production under periods of stress can sometimes be rescued by reducing initiation or elongation rates.

INTRODUCTION

Protein translation is central to cellular life. Although individual steps in translation such as the formation of the 43S preinitiation complex are known in intricate molecular detail, a global understanding of how these steps combine to set the pace of protein production for individual genes remains elusive (Jackson et al., 2010; Plotkin and Kudla, 2011). Factors such as biased codon usage, gene length, transcript abundance, and initiation rate are all known to modulate protein synthesis (Bulmer, 1991; Cha- mary et al., 2006; Cannarozzi et al., 2010; Tuller et al., 2010a; Shah and Gilchrist, 2011; Plotkin and Kudla, 2011; Gingold and Pilpel, 2011; Chu et al., 2011; Chu and von der Haar, 2012), but how they interact with one another to collectively determine translation rates of all transcripts in a cell is poorly understood. Systematic measurements for some of the most critical rates—such as the gene-specific rates of 5' UTR scanning and start codon recognition—are extremely difficult to perform. As a

result, questions as fundamental as the relative role of initiation versus elongation in setting the pace of protein production are still actively debated (Kudla et al., 2009; Tuller et al., 2010a; Plotkin and Kudla, 2011; Gingold and Pilpel, 2011; Chu et al., 2011; Chu and von der Haar, 2012; Ding et al., 2012). Biotechnical applications that exploit these processes stand to gain from a quantitative understanding of the global principles governing protein production (Gustafsson et al., 2004; Salis et al., 2009; Welch et al., 2009).

Recent advances in synthetic biology allow high-throughput studies on the determinants of protein production (Kudla et al., 2009; Welch et al., 2009; Salis et al., 2009). Sequencing techniques such as ribosomal profiling provide snapshots of the translational machinery in a cell (Ingolia et al., 2009; Reid and Nicchitta, 2012). One way to leverage this new information is to develop a computationally tractable model of translation in a cell, to parameterize it from known measurements, and to use it to infer any unknown parameters of global translation dynamics.

Here, we develop a whole-cell model of protein translation, and we apply it to study translation dynamics in yeast. Our model describes translation dynamics to the single-nucleotide resolution for the entire transcriptome. In combination with ribosomal profiling data, we use our model to infer the initiation rates of all abundant yeast transcripts. We systematically explore how the codon usage, transcript abundance, and initiation rate of a transgene jointly determine protein yield and cellular growth rate. Applied to the endogenous genome, our model reproduces one of the defining features of ribosomal profiling measurements: a decrease in ribosome density with codon position. We evaluate both elongation- and initiation-driven hypotheses for the ramp of 5' ribosome densities. We also describe the factors that influence ribosomal pausing along mRNA molecules, as well as the effects of stress on translation.

RESULTS

Model

We developed a continuous-time, discrete-state Markov model of translation. The model tracks all ribosomes and transfer RNA (tRNA) molecules in a cell—each of which is either freely diffusing or bound to a specific messenger RNA (mRNA) molecule at a specific codon position at any time point (Extended Experimental Procedures). Rates of initiation and elongation

Table 1. Summary of Model Parameters

Parameter	Description	Value or Range of Values	References
R^f	number of ribosomes	2×10^5	(Warner, 1999; von der Haar, 2008)
A^f	number of mRNAs	6×10^4	(Zenklusen et al., 2008)
T^f	number of tRNAs	3.3×10^6	(Waldron and Lacroute, 1975)
T_n	number of types of tRNAs	41	(Chan and Lowe, 2009)
T_j^f	number of tRNAs of type j	$\sim 12,000$ – $190,000$	(Chan and Lowe, 2009)
A_i^f	number of mRNAs of type i	1–1,254	(Ingolia et al., 2009)
p_i	gene-specific initiation probability	$\sim 3.5 \times 10^{-6}$ – 0.115	(Experimental Procedures)
n	number of genes	3,795	(Ingolia et al., 2009)
D_r	diffusion coefficient of ribosomes	3×10^{-13} m ² /s	(Poltz et al., 2003)
D_t	diffusion coefficient of tRNAs	8.42×10^{-11} m ² /s	(Werner, 2011)
C_r	size of ribosome footprint in codons	10	(Ingolia et al., 2009)
s	tRNA competition coefficient	7.78×10^{-4}	(Experimental Procedures)
V	volume of the cell	4.2×10^{-17} m ³	(Siwiak and Zielenkiewicz, 2010)

See also Table S1.

are based on physical parameters that have been experimentally determined in yeast, including the cell volume, the abundances of ribosomes and tRNAs, and their diffusion constants (Tables 1 and S1 available online). Transition rates among states are parameterized in seconds so that the model describes the dynamics of translation in real time, as opposed to using arbitrary discrete time steps. We provide a precise definition of the Markov state space, as well as pseudocode and complete source code in Data S1 and S2 and also Table S2.

Unlike many other models of translation (Gilchrist and Wagner, 2006; Mitarai et al., 2008; Reuveni et al., 2011), which treat each mRNA molecule in isolation and assume an inexhaustible supply of free ribosomes that initiate the message at a constant rate, our model keeps track of every tRNA, mRNA, and ribosome molecule in the cell simultaneously, and so it captures the indirect effects of one gene's translation on another's (Figure 1). In particular, if many ribosomes are engaged in translating the mRNAs of one gene, this reduces the pool of free ribosomes and tRNAs available to translate other genes.

Our model makes a number of simplifying assumptions. Most importantly, our model treats the total number of ribosomes, tRNA molecules, and mRNA molecules in the cell as fixed quantities because the dynamics of their production and decay are typically slower than those of protein translation (García-Martínez et al., 2004; Larson et al., 2011). We specify the total number of ribosomes and tRNA molecules to agree with their experimentally determined values in an exponential-phase yeast cell: 2×10^5 and 3.3×10^6 , respectively (Waldron and Lacroute, 1975; Warner, 1999; von der Haar, 2008; Siwiak and Zielenkiewicz, 2010; Chu and von der Haar, 2012). We infer gene-specific initiation probabilities (Extended Experimental Procedures) so that 85% of ribosomes are bound to mRNAs in equilibrium in agreement with measurements in yeast (Arava et al., 2003; Zenklusen et al., 2008). We further assume that tRNA charging is fast, which is reasonable because 80% of all tRNAs are charged at any given time in exponential-phase cells (Varshney et al., 1991; Jakubowski and Goldman, 1992; Chu et al., 2011).

As a result of these parameters, the equilibrium number of free ribosomes available in the cell is typically smaller than the number of available charged tRNAs of each species. In this regime, we will show that protein production is generally limited by the rate of translation initiation in the sense that increasing the initiation probability of an mRNA molecule will typically increase the rate at which protein is produced, but increasing its codon elongation rates generally will not increase production. The initiation-limited regime agrees with the long-standing view of endogenous protein synthesis (Andersson and Kurland, 1990; Bulmer, 1991; Eyre-Walker and Bulmer, 1993; Lackner et al., 2007; Plotkin and Kudla, 2011), but it contrasts with other models of translation that assume an inexhaustible supply of ribosomes, which are always available for initiation of an mRNA regardless of how many ribosomes are bound to other mRNAs (Mitarai et al., 2008; Reuveni et al., 2011; Tuller et al., 2011).

We implemented our Markov model of translation using the Gillespie algorithm. We simulated 1,500 s of translation and extracted the final 500 s to collect data on translation dynamics in equilibrium (Experimental Procedures). Our implementation requires about 1,300 s of computation time to simulate all initiation and elongation events in a wild-type cell for 1,500 s. In these simulations, at equilibrium, the mean elongation rate is 9.3 aa/s (median = 9.5 aa/s), and the mean distance between consecutive bound ribosomes is 60 codons (median = 34). Both of these quantities agree with empirical measurements in yeast (Arava et al., 2003).

Codon Bias and Transgene Expression

Optimizing a transgene's codon usage to the tRNA content of a cell often improves protein yield (Gustafsson et al., 2004; Welch et al., 2009), but the underlying mechanisms have not been systematically explored. To study this in a quantitative model, we simulated translation of a transgene within the context of a *Saccharomyces cerevisiae* cell containing 3,795 endogenous genes whose transcript levels and gene-specific initiation

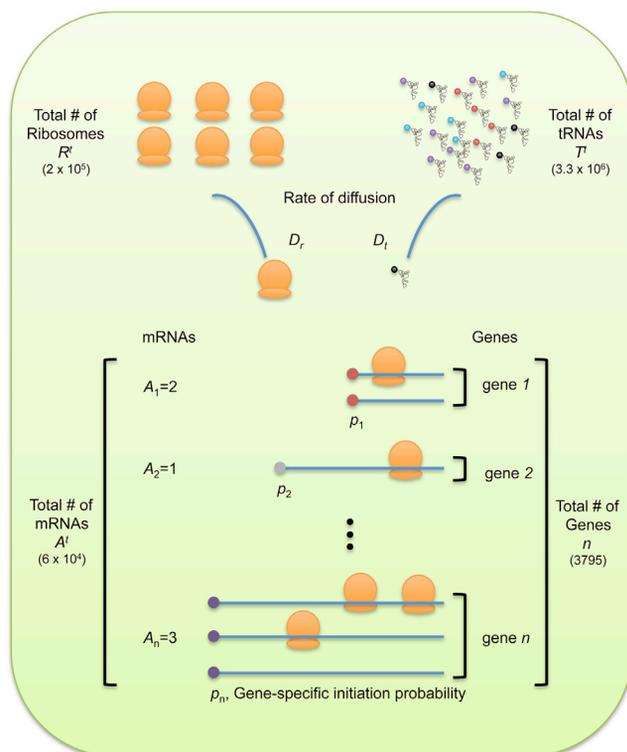


Figure 1. A Computational Model of Protein Translation

The model tracks the status of all ribosomes, tRNAs, and mRNAs in a cell in continuous time. At any time point, each tRNA and ribosome molecule is either diffusing freely in the cell or is bound to a specific mRNA molecule at a specific codon position. Translation initiation occurs when a free ribosome diffuses to an mRNA and subsequently, with an mRNA-dependent probability, scans to its start codon. The rate of elongation of each subsequent codon depends on the abundance of free cognate tRNAs and their diffusion to the bound ribosome. All rates are based on experimentally determined parameters, including the cell volume, numbers of mRNAs, total abundances of ribosomes and tRNAs, and their diffusion constants. A precise definition of the Markov state space, illustrative pseudocode, and the complete source code for simulation are provided in the [Supplemental Information](#). See also [Figure S1](#), [Tables S1](#) and [S2](#), and [Data S1](#) and [S2](#).

probabilities were estimated from ribosomal profiling data (Ingolia et al., 2009) (Experimental Procedures). By varying the codon adaptation index (CAI) (Sharp and Li, 1987) and transcript level of the transgene across many simulations, we delineated the regimes for which increasing codon bias is expected to increase protein yield and by what mechanisms.

Using the green fluorescent protein (GFP) as an example transgene, we found that increasing the CAI of a transgene significantly improves the rate of proteins produced per mRNA molecule only when the transgene mRNA accounts for a substantial proportion of all the mRNA in the transcriptome (Figure 2 and Table S4). For a transgene whose messages account for 50% of the cell's mRNA content, for example, increasing CAI from almost zero to one results in nearly 3.6-fold more proteins produced per transcript per second (Figure 2B, triangles), whereas optimizing CAI in a transgene expressed at only 1% of the transcriptome results in a more modest increase (~50%)

in its rate of protein production (Figure 2B, squares). These results help explain the divergent views of biotechnological studies, which often report large gains in protein production upon optimizing transgene CAI (Gustafsson et al., 2004), and evolutionary studies of endogenous translation, which typically report very small effects of CAI on protein production per message (Bulmer, 1991; Tuller et al., 2010b; Gingold and Pilpel, 2011; Plotkin and Kudla, 2011). The discrepancy arises because transgenes are usually overexpressed and comprise a substantial fraction of all cellular mRNA, whereas endogenous genes are expressed at 1% of the transcriptome or less.

Why does codon bias strongly influence protein yield only when a gene has high mRNA abundance? The reason has to do with the effects of codon bias on the pool of free ribosomes, as seen in Figure 3. At equilibrium, neglecting rare abortion events, the rate of protein production from any given mRNA (i.e., the rate of polypeptide termination) must equal the rate of initiation on that mRNA, which, in turn, depends primarily on the abundance of free ribosomes in the cell. Increasing the CAI of a gene will increase its codon elongation rates and thus decrease the density of ribosomes on each of its mRNAs, but the overall effect on the pool of free ribosomes is small when the gene accounts for a small proportion (<1%) of mRNA in the transcriptome, as virtually all endogenous genes do. As a result, increasing the CAI of a gene at low mRNA abundance is not expected to strongly increase the rate of protein production, as our simulations confirm (Figure 2). By contrast, for a transgene at very high abundance (e.g., 50% of cellular mRNA), a significant fraction of all ribosomes in the cell are bound to its mRNAs. Increasing the CAI of such a gene leads to a significant increase in the pool of free ribosomes (Figure 3) and thus a significant increase in initiation rates and protein production from all mRNAs in the cell, including from the transgene itself.

Our simulations confirm the mechanistic role of free ribosomes in shaping the relationship between codon bias and protein yield. For a transgene at high abundance, such as 50% of the transcriptome, increasing its CAI causes a 3.2-fold increase in the equilibrium number of free ribosomes in the cell (Figure 3B), which accounts for the great majority of the concomitant 3.6-fold increase in its protein production. By contrast, for a transgene expressed at low levels (e.g., 1% of transcriptome), increasing CAI results in only 3% more free ribosomes (Figure 3B), which is not sufficient to explain the concomitant 50% increase in transgene protein production. (Nonetheless, a 3% fitness gain suffices to explain selection for codon bias in highly expressed endogenous genes over evolutionary timescales.) In this case, the gain in transprotein production is explained instead by reduced ribosomal trafficking at the 5' end of transgene mRNAs: about 47% more transgene mRNAs are available to be initiated (that is, they are not bound by a ribosome at their 5' end) when $CAI \approx 1$ compared to $CAI \approx 0$ in our simulations of such a transgene.

In summary, increasing transgene codon bias has a modest effect on translational efficiency, which is limited to the transgene mRNAs themselves and is caused by reduced ribosomal occupancy of their 5' ends, whereas increasing CAI can have a huge effect on protein production globally—which is caused by an increased pool of free ribosomes—when the transgene

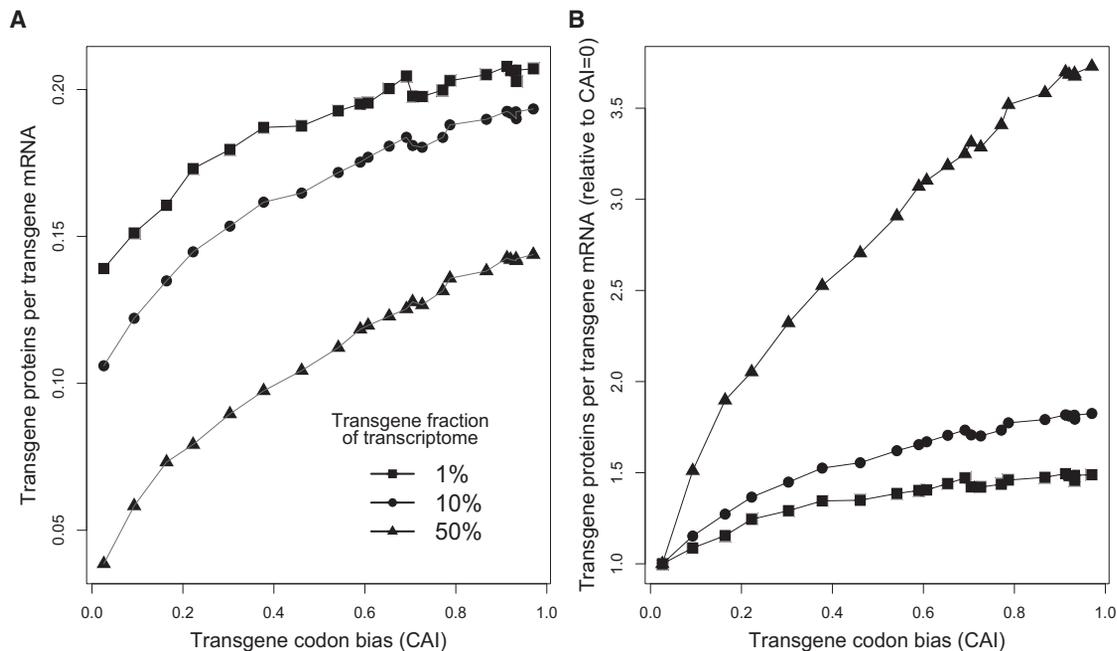


Figure 2. The Effects of Transgene Codon Bias on Protein Production

(A and B) We simulated translation in a wild-type yeast cell with the addition of a transgene. Transgene mRNA levels were set at 1%, 10%, or 50% of all cellular mRNA. We measured the number of transproteins produced per transgene mRNA over 500 s in equilibrium (A). As (A) shows, increasing the codon bias of the transgene generally increases the efficiency of its translation. However, when the transgene is expressed at low levels (e.g., transgene mRNAs constituting 1% of transcriptome), then the gain in translation efficiency achieved by optimizing codon bias is moderate (~50% gain, comparing CAI \approx 1 to CAI \approx 0, squares in [B]). By contrast, when the transgene mRNAs constitute a large fraction of the total transcriptome, then the gain in translation efficiency by optimizing codon bias is far greater (3.6-fold gain, triangles in [B]). See also Tables S3 and S4 and Figure S2.

has very high transcript abundance. These results (Figures 2 and 3) hold whenever protein translation is limited by the pool of ribosomes freely available for initiation, as is the case in healthy yeast cells (Arava et al., 2003; Zenklusen et al., 2008). When a cell is starved for tRNAs or amino acids, by contrast, or when the pool of available ribosomes is artificially inflated, the effects of codon bias on protein yield are due solely to reduced ribosomal interference along translating mRNAs, as discussed below.

Whereas Figures 2 and 3 quantify translation dynamics for a transgene expressed at three different abundances, Table S4 provides analogous results for a full range of transcript abundances. In the simulations described above, we maintained a constant transcriptome size in nucleotides so that an increase in the abundance of transgene mRNA comes at the expense of endogenous transcripts. Nonetheless, we found the same results when transgene mRNAs were simply added to the endogenous transcriptome (Table S4 and Experimental Procedures). Likewise, we found the same qualitative results for three other simulated transgenes with very different sequences and amino acid compositions than GFP (Table S4). Whereas Figure 2 reports the rate of protein production per transgene mRNA molecule, Table S4 reports the corresponding total rate of transprotein production in the cell, which is often the most important consideration in biotechnical applications. Most of the relationships between codon bias and protein yield per message also hold for total protein yield.

Initiation Rate and Transgene Expression

Translation initiation in eukaryotes is a multistep process involving multiple protein complexes. Our model simplifies this process into its two critical components: the arrival of a free ribosome at the 5' end of an mRNA molecule, whose rate is determined by the number of free ribosomes and their diffusion constant, and the probability that such a ribosome then successfully binds and scans to the start site of the mRNA to irreversibly initiate translation. This initiation probability is known to depend strongly on the sequence of the transcript (Andersson and Kurland, 1990; de Smit and van Duin, 1990; Eyre-Walker and Bulmer, 1993; Kudla et al., 2009; Tuller et al., 2010b). In the simulations above, we set the initiation probability of the transgene at the 95th percentile of endogenous initiation probabilities because transgenes are typically optimized for rapid initiation (Salis et al., 2009; Welch et al., 2009). Here, we explore more generally how the probability of transgene initiation, once a ribosome has diffused to a transgene mRNA, influences protein production.

As Figure 4A shows, high codon bias will significantly increase protein yield only when the initiation probability of a transgene exceeds the (abundance-weighted) average initiation probability of the endogenous transcriptome. This is true irrespective of transgene abundance (Table S4), and it makes intuitive sense by considering, once again, the effects of initiation and elongation on the pool of free ribosomes. Increasing a gene's codon bias typically reduces the density of ribosomes along its mRNA

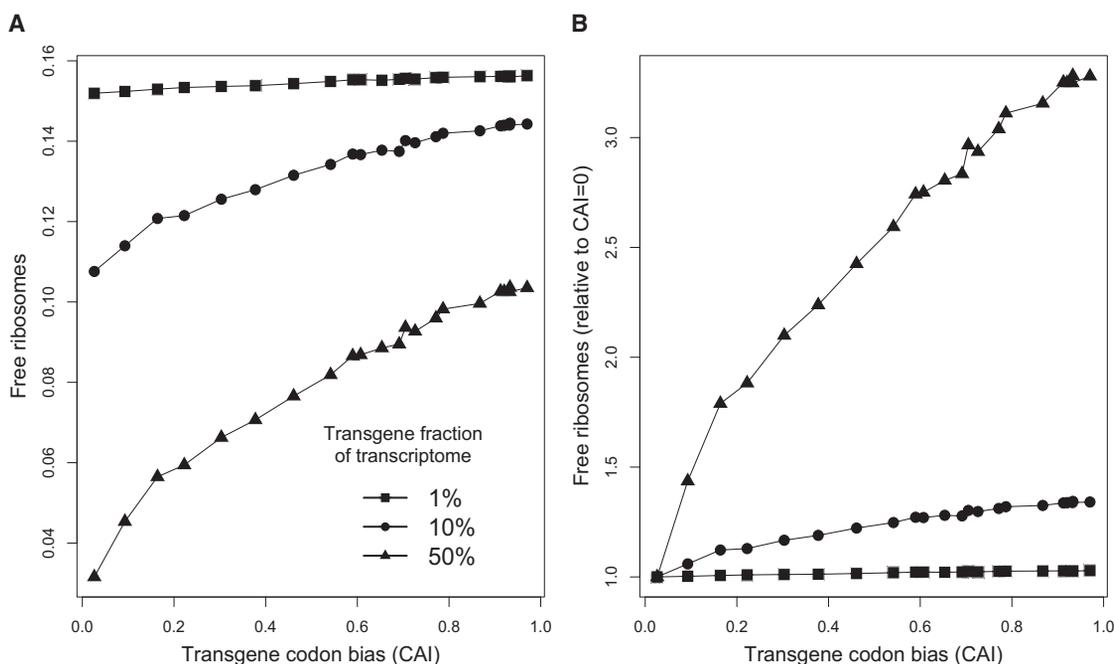


Figure 3. The Effects of Transgene Codon Bias on the Pool of Free Ribosomes

(A and B) As in Figure 2, we simulated translation of a transgene added to a wild-type yeast cell. Transgene mRNA levels were set at 1%, 10%, or 50% of all cellular mRNA. We measured the equilibrium fraction of ribosomes that are free (unbound). Increasing codon bias of the transgene reduces the number of ribosomes bound to its transcripts and thereby increases the pool of free ribosomes (A), especially when the transgene accounts for a large proportion of all cellular mRNA. For example, when transgene mRNAs comprise 50% of the total transcriptome, then optimizing codon bias of the transgene from CAI \approx 0 to CAI \approx 1 causes a 3.2-fold increase in the number of free ribosomes (triangles in [B]), which explains a large proportion of the corresponding gain in transprotein production. See also Table S4.

molecules due to faster elongation. When a highly expressed transgene has high initiation probability, its ribosomal density will be high as well, and so increasing codon bias can substantially replenish the pool of free ribosomes, which, in turn, increases initiation rates and protein yields. However, when a transgene has low initiation probability, regardless of its mRNA abundance, there are relatively few ribosomes bound to its mRNAs, and so increasing codon bias has a limited effect on its ribosomal densities and on the pool of free ribosomes (Figure 4B and Table S4). These results underscore the critical role of rapid initiation in allowing codon bias to modulate transgene protein yields.

Initiation Probabilities of Endogenous Genes

One of the most challenging problems in understanding protein translation remains the estimation of initiation rates for endogenous genes. As described above, translation initiation depends first on the arrival of a free ribosome to an mRNA and then on the ribosome binding and successfully scanning to the transcript's start codon (de Smit and van Duin, 1990). Despite their importance, the initiation probabilities of each transcript are the only parameters in our model that have not been measured empirically. Therefore, we used our model to infer the gene-specific initiation probabilities from ribosomal occupancy data (Ingolia et al., 2009).

To make this inference, we assumed that the cell is in equilibrium, and we derived analytic approximations for the steady-

state density of ribosomes on each mRNA molecule (Extended Experimental Procedures) in terms of the unknown initiation probabilities. These approximations neglect the possibility of ribosomal interference along each message, but they are nonetheless extremely accurate in the parameter regime of a healthy yeast cell ($R > 0.9$; Figures S1A and S1B). We then inverted our equations to infer gene-specific initiation probabilities from observed densities of ribosomes on transcripts. An alternative method of estimating initiation probabilities from profiling data was independently developed by Siwiak and Zielenkiewicz (2010). We validated that our analytical method can indeed reliably infer initiation probabilities when we simulate ribosome profiling data for *S. cerevisiae* genes with known initiation probabilities (Figure S1B). Using this method, we inferred the initiation probabilities for the 3,795 *S. cerevisiae* genes whose ribosomal densities have been reliably measured (Ingolia et al., 2009).

The initiation probabilities we inferred for yeast genes vary by many orders of magnitude. According to these estimates, the average time between initiation events on a given mRNA molecule ranges from 4 s (fifth percentile) to 233 s (95th percentile), with a median value of 40 s. This variation provides the cell considerable range for tuning protein levels by modulating initiation probabilities of genes.

Experiments with individual genes (Hall et al., 1982; Duan et al., 2003) and with large sets of coding sequences (Kudla et al., 2009) suggest that strong 5' mRNA structure reduces the rate of initiation, presumably by obstructing ribosomal-mRNA binding.

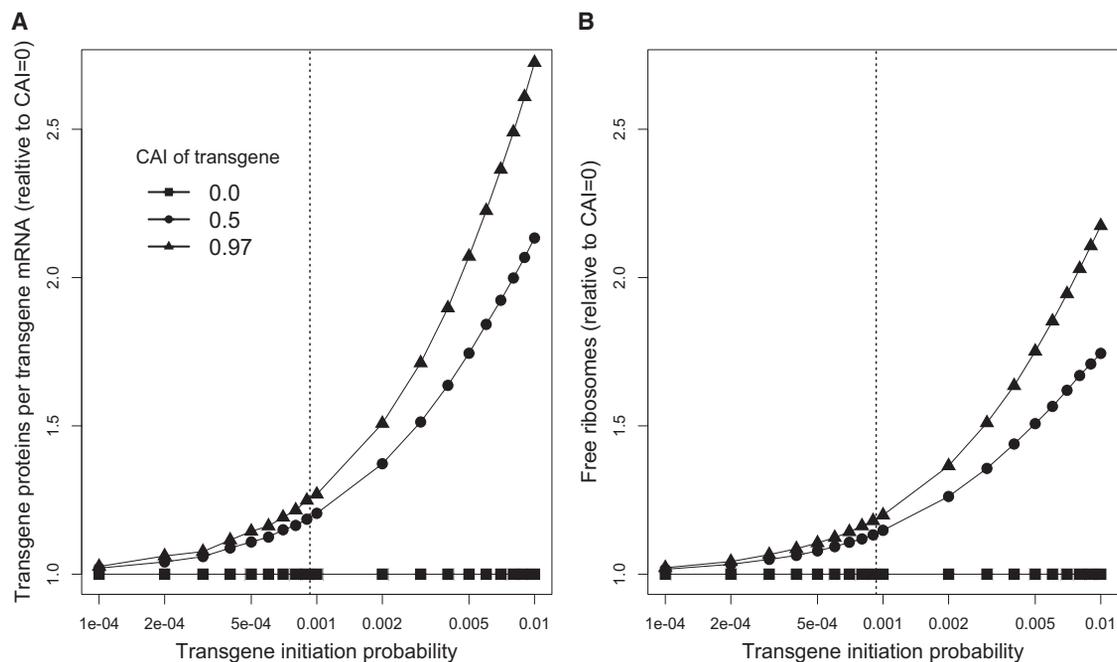


Figure 4. The Effects of Initiation Probabilities on Protein Production and Pool of Free Ribosomes

(A and B) As in Figure 2, we simulated translation of a transgene added to a wild-type yeast cell. Transgene mRNA levels were set at 25% of all cellular mRNA. We measured the number of transgene proteins produced per transgene mRNA (A), as well as the equilibrium fraction of ribosomes that are free (B); both quantities are expressed relative to the case of transgene with CAI \approx 0. The dashed vertical line denotes the average initiation probability of endogenous yeast genes. Increasing codon bias of a transgene significantly increases the rate of protein production only when the transgene's initiation probability exceeds the average initiation probability of endogenous genes. See also Table S4 and Figure S2.

Using a large set of synthetic GFP genes that vary synonymously, we confirmed experimentally that 5' mRNA folding plays a predominant role in determining protein levels in *S. cerevisiae* (Figure S2), which is similar to the role it plays in *Escherichia coli* (Kudla et al., 2009). In light of these experiments, we compared the initiation probabilities we estimated for 3,795 endogenous yeast genes with their predicted 5' mRNA folding energies (nucleotides -4 to $+37$, Experimental Procedures) and found a strong positive correlation (Pearson correlation $R = 0.125$ and $p < 10^{-13}$; Figure 5A). These results suggest that 5' mRNA structure systematically modulates initiation rates across the yeast genome.

Interestingly, we also found a negative correlation between initiation probability and open reading frame (ORF) length ($R = -0.56$ and $p < 10^{-15}$; Figure 5B), even after controlling for mRNA expression level (partial correlation, $R = -0.425$ and $p < 10^{-15}$). This trend suggests that shorter yeast genes have experienced selection for faster initiation, and so it provides a mechanistic explanation for the greater density of ribosomes typically observed on short genes (Arava et al., 2003; Lackner et al., 2007). Note that shorter genes are known to be more densely packed with ribosomes despite the fact that they tend to have significantly higher CAI (t test, $p < 10^{-4}$) and presumably faster elongation. This result again indicates the dominance of initiation, as opposed to elongation, in determining the density of ribosomes on transcripts.

We performed several controls to ensure that our estimates of initiation probabilities are not biased by gene length (Extended

Experimental Procedures). We found no significant differences in the inferred initiation probabilities when artificially doubling the lengths of all transcripts (Kolmogorov-Smirnov, $p > 0.9$). Moreover, we validated that we can reliably infer initiation probabilities from simulated ribosomal profiling data even when gene length and initiation probabilities are positively correlated (Figures S1C and S1D and Extended Experimental Procedures), indicating that the negative correlation observed in the real yeast data is not an artifact of our inference procedure.

Why should short genes experience selection for fast initiation? Short genes are enriched for constitutively expressed housekeeping and ribosomal genes (Hurowitz and Brown, 2003), which must produce protein as rapidly as possible. In addition, housekeeping genes tend to have shorter 5' UTRs and are under weaker posttranscriptional regulation (Hurowitz and Brown, 2003; Lin and Li, 2012). The probability of successful ribosomal binding and scanning on an mRNA may depend on the length of its 5' UTRs; indeed, we find that genes with shorter 5' UTRs exhibit higher inferred initiation probabilities ($p < 10^{-10}$). In addition to de novo initiation, recently terminated ribosomes can reinitiate translation on the same mRNA, a process known as ribosome recycling. The probability of successful reinitiation may depend on an mRNA's 3' UTR length (Tanguay and Gallie, 1996; Gallie, 1998). Consistent with this hypothesis, we find genes with longer 3' UTRs have higher initiation probabilities ($p < 10^{-5}$). However, unlike 5' folding energy, we find no significant correlation between 3' UTR folding energy and the initiation probability of a gene.

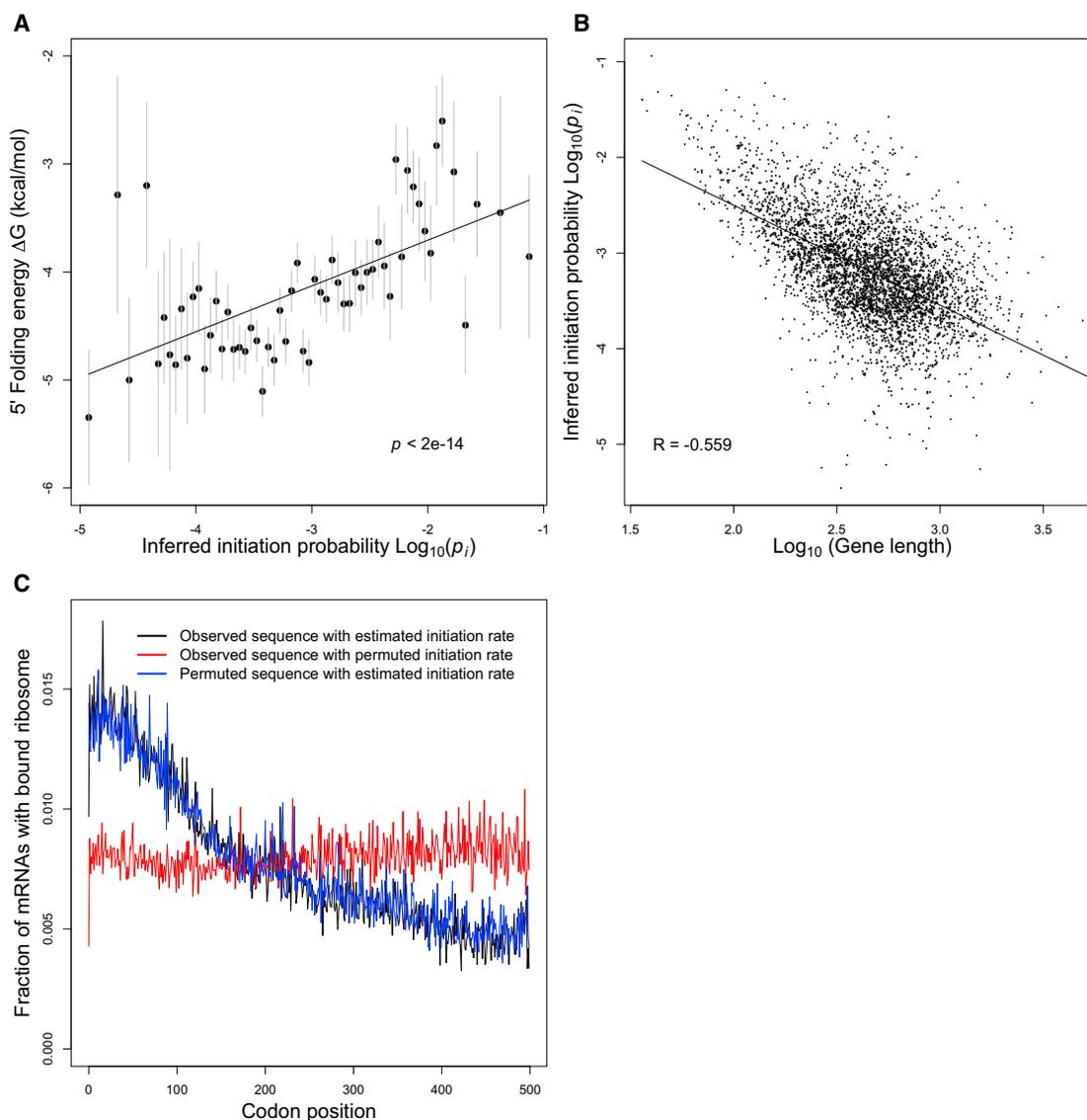


Figure 5. Fast Initiation of Short Genes Causes a 5' Ribosomal Ramp

(A) Yeast genes with weak 5' mRNA structure initiate quickly. We inferred the initiation probabilities of 3,795 endogenous yeast genes from ribosomal profiling data (Ingolia et al., 2009). A gene's initiation probability correlates strongly with the estimated energy of its 5' mRNA structure. The gray bars indicate 1 SD of folding energies of binned genes.

(B) Initiation probabilities of yeast genes also correlate with ORF lengths, suggesting that short genes have experienced selection for faster initiation.

(C) Simulations of translation in a wild-type yeast cell recapitulate the "ramp" of 5' ribosomes observed in empirical ribosomal profiling data (Ingolia et al., 2009). The figure shows the density of ribosomes bound to mRNAs as a function of codon position, averaged across the simulated transcriptome (black). The ramp is preserved in simulations that permute the codons within each gene (blue), but the ramp is disrupted when permuting the initiation probabilities among genes (red). Thus, we infer that the ramp of 5' ribosomes is caused primarily by the trend toward faster initiation in short genes, rather than by the ordering of codons within each gene.

See also Figures S3, S4, and S5.

The "Ramp" of 5' Ribosomes

A defining feature of ribosome profiling data in yeast (Ingolia et al., 2009) and humans (Reid and Nicchitta, 2012) is a striking decrease in ribosome density with codon position, averaged across the transcriptome. This observation has led to the "ramp" hypothesis, which attributes higher ribosome densities to slower codons in the 5' ends of mRNAs (Tuller et al., 2010a;

Reuveni et al., 2011; Tuller et al., 2011). Slow 5' codons are thought to reduce ribosomal interference further down the length of the mRNA, leading to more efficient translation (Tuller et al., 2010a).

Our simulations of translation in a yeast cell recapitulate the empirical observation of declining ribosome density with codon position, averaged across the transcriptome (Figure 5C). The

ability of our model to recapitulate this striking spatial trend is nontrivial because we did not use any position-specific information from the ribosomal profiling data in order to parameterize the model (we used only the average ribosome density per mRNA).

Our computational model allows us to systematically determine which processes are responsible and which ones are dispensable in explaining the 5'-to-3' ramp of decreasing ribosome density. We propose an alternate explanation for this trend: the ramp can be explained by the simple fact that shorter yeast genes tend to have higher initiation probabilities (Figure 5B) and correspondingly higher densities of ribosomes overall (Arava et al., 2003; Lackner et al., 2007). Because short genes are disproportionately weighted in early codon positions as opposed to late codon positions, their elevated ribosome densities will cause an apparent ramp in the transcriptome-wide average ribosome density with codon position.

We used our model to distinguish between our initiation-driven hypothesis and the elongation-driven hypothesis for the ramp of 5' ribosomes (Tuller et al., 2010a, 2011; Reuveni et al., 2011). If the ramp were caused primarily by slow codons near the 5' ends of genes, then the ramp would disappear upon randomizing codon order within each gene, whereas if the ramp were caused primarily by faster initiation rates in shorter genes, then it would disappear upon permuting initiation rates among genes. We found that simulations permuting codon order within genes still exhibit the ramp of 5' ribosome densities (Figure 5C), whereas permuting initiation probabilities among genes removes the ramp (Figure 5C). Both of these results support the initiation-driven and reject the elongation-driven hypothesis for the cause of the 5' ribosome ramp.

Aside from using our simulation model, we can also analyze the raw ribosomal profiling data of Ingolia et al. (2009) to dissect the causes of the apparent 5' ribosome ramp. When we remove all positional information from the profiling data and use only the observed average ribosome density on each mRNA, assuming a uniform density along each mRNA, we still observe a decline in transcriptome-wide average ribosome density with codon position (Figure S3A). In addition, when inspecting the profiling data on a gene-by-gene basis, we find that just as many genes exhibit a trend of increasing ribosome density as exhibit a trend of decreasing ribosome density (Figure S3B and Extended Experimental Procedures). Finally, we have plotted average ribosome density by codon position for genes binned by ORF length, which is analogous to Figure S11 from Ingolia et al. (2009) but with more stringent length bins (Figure S4). These plots show no consistent 5'-to-3' ramp, and many show 3'-to-5' ramps (Figure S4). Taken together, these analyses of the raw profiling data confirm the conclusions drawn from our simulations: the apparent 5' ribosome ramp in yeast is not caused primarily by a higher density of ribosomes near the 5' end of each message but rather by a greater overall density of ribosomes on shorter mRNA molecules due to their faster rates of initiation.

Comparison to Other Models of Translation

Several models of translation, such as the ribosome flow model and other TASEP-based models, have been used to justify the role of codon ordering in determining spatial patterns of ribosomes along mRNAs (Reuveni et al., 2011; Tuller et al., 2011).

Such models of translation consider each mRNA in isolation of all others, assuming a constant rate of initiation. In other words, TASEP models implicitly assume a constant, inexhaustible supply of free ribosomes and free tRNAs in the cell, so that the 5' end of each mRNA is typically saturated with ribosomes (Reuveni et al., 2011), and the speed of elongation then sets the pace of protein production. Such models make sense only if ribosomes are in overabundance in the cell. As a result, the total number of ribosomes bound to mRNAs estimated by such models ($>5 \times 10^5$, Extended Experimental Procedures) exceeds the empirical measurement of the total number of ribosomes in a yeast cell ($1.87 \times 10^5 \pm 5.6 \times 10^4$; von der Haar, 2008) by a factor of 2.5.

When we artificially increase the number of ribosomes and tRNAs in our simulations beyond their empirically measured abundances, we can recapitulate the patterns produced by TASEP models of translation (Figure S5A). In this regime, which we argue is unrealistic, we still observe a decrease in the average ribosome density with codon position, but this ramp is caused by collisions along each mRNA, and it persists regardless of gene-specific initiation probabilities or codon ordering within genes (Figure S5B). Thus, models of translation in both initiation- and elongation-limited regimes produce similar global patterns of ribosomal densities with codon position but for entirely different and contradictory mechanisms. Only the initiation-limited regime is consistent with empirical measurements of ribosome abundances in the yeast cell.

Ribosomal Interference and Codon Usage

Our simulations allow us to estimate the amount of time a ribosome spends waiting for a tRNA at each codon position, called ribosomal pausing, and also the amount of time a ribosome wastes at any position due to interference by an adjacent downstream ribosome that prevents further elongation, called ribosomal stalling. We identified the sequence features of a gene that predispose it to ribosomal pausing or stalling (Experimental Procedures).

Using GFP as an example transgene simulated at 50% mRNA transcriptome abundance, we found that increasing the transgene's codon bias tends to decrease the overall density of ribosomes on its mRNAs, as well as the frequency of ribosomal stalling (Figure 6). For a transgene with high CAI, the probability of finding a ribosome bound at a given codon is negatively correlated with the abundance of corresponding iso-accepting tRNAs (Pearson correlation, $R = -0.802$), but this correlation is much weaker for a transgene with low CAI ($R = 0.042$ and $p > 0.05$). In other words, the waiting time per codon is largely determined by the abundance of corresponding tRNAs for a gene with high CAI. But for a gene with low CAI, ribosome densities are higher overall and so the waiting time at each codon is also influenced by interference with downstream ribosomes and, therefore, is not easily predicted from tRNA abundances. In fact, regardless of CAI, there is a strong correlation between ribosomal stalling at a position and the probability of ribosomal pausing 10 codons downstream ($R = 0.958$ for high CAI and $R = 0.644$ for low CAI). Because the probability of pausing in a high-CAI transgene sequence is correlated with tRNA abundances, it is possible to predict the positions of ribosomal stalling from the transgene

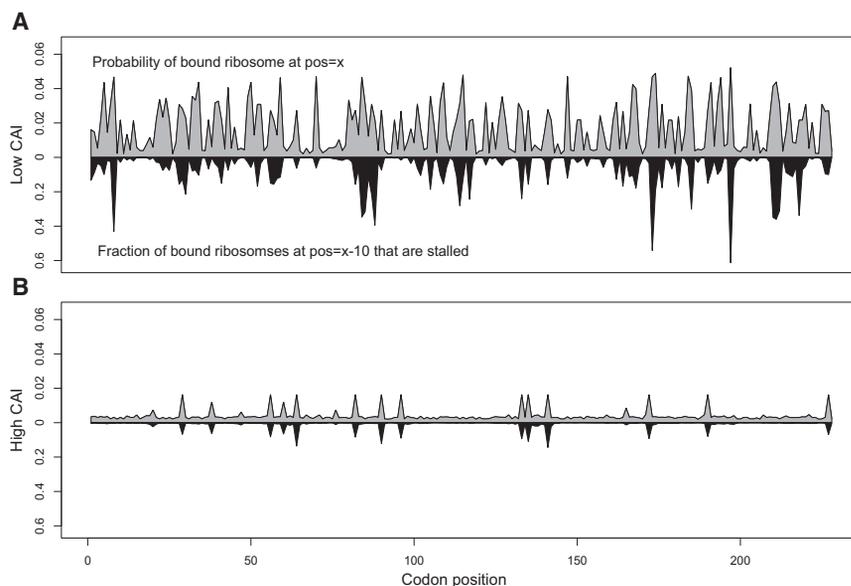


Figure 6. Codon Usage and Ribosomal Pausing and Stalling

(A and B) The influence of codon usage and amino acid sequence on ribosomal pausing and stalling for a simulated transgene with either (A) low or (B) high codon adaptation, expressed at 50% transcript abundance. Gray bars indicate the probability of finding a ribosome bound at a given codon position x , and black bars indicate the probability of finding a ribosome stalled at position $x-10$ (i.e., a ribosome whose further elongation is obstructed by another ribosome). High codon adaptation reduces both ribosome density and ribosome interference. The probability of a ribosome stalling at a position correlates strongly with the probability of a ribosome pausing 10 positions ahead ($R = 0.947$ for high CAI and $R = 0.644$ for low CAI). For a transgene with high CAI, the probability of finding a ribosome bound at a given codon position is strongly anticorrelated with the abundance of iso-accepting tRNAs for that codon ($R = 0.786$), but not for a transgene with low CAI ($R = -0.042$).

sequence alone. Understanding the effects of amino acid and codon usage on pausing and stalling may prove useful in designing transgene sequences to minimize ribosomal interference on its mRNAs.

Protein Translation under Stress

The simulations of translation described above were performed under parameters of optimal cell growth. Translation dynamics likely differ when a cell experiences stress. To investigate how protein production is affected by stress and how a cell might adapt in response, we simulated translation under conditions of amino acid starvation. We modeled starvation of a particular amino acid by reducing the abundance of its (charged) cognate tRNAs by either 2-, 5-, or 10-fold. As expected, we found that the rate of total protein production decreases under stress (Figures 7A and S6A). Furthermore, starvation of different amino acids can have radically different effects on protein production. For example, 10-fold starvation of amino acids Ala, Leu, Glu, Gln, or Ser decreases total protein production by at least 10-fold, whereas an equivalent starvation of Met, Trp, or His reduces protein production by less than 25% (Figure 7A). As expected, the effect of starvation of a particular amino acid is significantly correlated with its abundance encoded in the transcriptome ($p < 0.01$ in all cases).

Our simulations reveal that decreased protein synthesis upon starvation is caused primarily by a decrease in the pool of free ribosomes (Figures S6A and S6B). When tRNAs corresponding to a specific amino acid are in short supply, elongation of their codons becomes rate limiting, as has been predicted theoretically (Elf et al., 2003) and observed experimentally (Welch et al., 2009). As our simulations demonstrate, this effect creates traffic jams that increase the density of ribosomes on all mRNAs and increase the fraction of bound ribosomes that are stalled (Figure S6D). The increased density of bound ribosomes in turn decreases the pool of free tRNAs of all species, as each bound ribosome sequesters one tRNA in its P site. At equilibrium, the

limited pool of free ribosomes and tRNAs reduces the initiation and elongation rates of all transcripts (Figure S6C) and hence retards total protein production.

Eukaryotic cells have evolved mechanisms to cope with stress, which we can analyze mechanistically using our model of translation. During amino acid starvation, eukaryotic cells respond (1) by repressing the production of ribosomal proteins and rRNAs (Moehle and Hinnebusch, 1991) and (2) by phosphorylating eIF2 α by GCN2, which retards the formation of initiation complexes (Krishnamoorthy et al., 2001; Zhang et al., 2002; Hinnebusch and Lorsch, 2012). In order to study these adaptive responses, we simulated the repression of ribosomes by reducing the total number of ribosomes in the cell, and we simulated the phosphorylation of eIF2 α by reducing the initiation probabilities of all genes by a fixed factor. Under mild stress conditions (2- to 5-fold decrease in charged tRNAs), reducing either the ribosome abundance or initiation probabilities was detrimental to protein production (Figure S7 and Table S5). However, when the cell experiences severe amino acid starvation, reducing ribosome abundance or initiation probabilities can partly rescue protein production (Figures 7B and S7 and Table S5). This increase in protein production, albeit not to the levels of the wild-type cell, is quite significant. This counter-intuitive behavior can be explained by the fact that, under severe stress conditions, the cell becomes elongation limited instead of initiation limited. As a result, reducing the initiation rates of genes not only increases the pool of free ribosomes (Table S5) but also the pool of free tRNAs, especially the ones corresponding to the starved amino acid. This leads to an increase in the elongation rate of all genes and, hence, overall protein production.

Stress-induced repression of ribosomes and phosphorylation of eIF2 α have previously been thought to be adaptive because they minimize resource waste. Our simulations indicate that such responses may also have a direct benefit of rescuing protein production and therefore increasing cell growth.

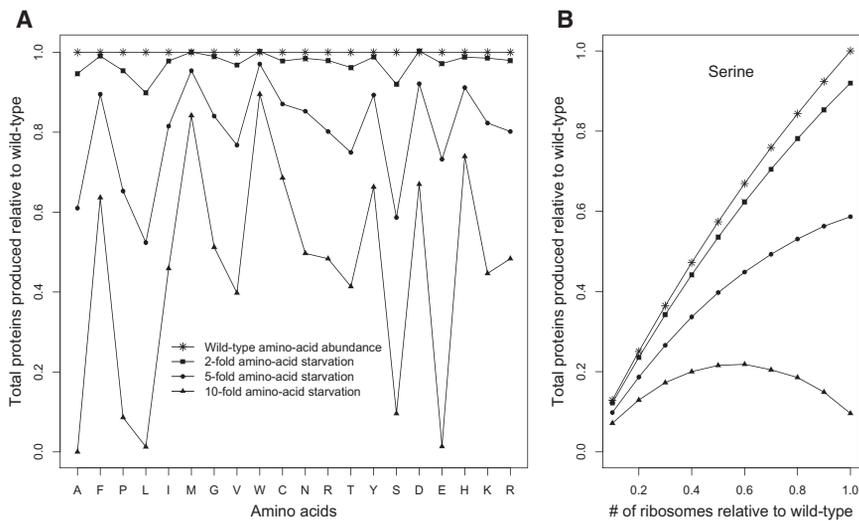


Figure 7. The Effects of Amino Acid Starvation and Cellular Stress Response on Protein Production

(A) For each amino acid, we reduced the relative abundance of its (charged) cognate tRNAs by either 2-, 5-, or 10-fold and then simulated translation in the cell. Although a stronger stress always leads to lower protein production, the strength of this effect varies widely among amino acids.

(B) During starvation, cells often respond by repressing ribosome production, which we modeled by reducing the total number of ribosomes in the cell and resimulating translation. Reducing ribosomes under normal or mild stress conditions always reduces protein production. However, during severe stress, reducing ribosomes by a moderate amount can partly rescue protein production, as in the case of serine shown in (B).

See also Figures S6 and S7 and Table S5.

DISCUSSION

We have used a whole-cell simulation model to study the dynamics of translation. This approach allows us to map the parameter regimes in which high codon adaptation is expected to increase transgene protein yield and by what mechanisms—revealing the critical role of free ribosomes in constraining initiation and protein production. This approach also elucidates the basic determinants of translation dynamics in the endogenous yeast transcriptome, providing estimates of initiation probabilities for all abundant yeast mRNAs. We have found a strong correlation between ORF length and initiation probability, which, we argue, provides a simple explanation for the apparent ramp of 5' ribosome densities observed in ribosomal profiling data.

Whether endogenous protein production is initiation or elongation limited remains actively debated (Gingold and Pilpel, 2011; Plotkin and Kudla, 2011). It cannot easily be determined a priori which process should be limiting because the cellular abundances of some tRNA species are comparable to the abundance of ribosomes. Nonetheless, a long string of early experiments by Andersson and others established the empirical fact that initiation limits production for most endogenous proteins in healthy cells (Andersson and Kurland, 1990; Bulmer, 1991). Our simulations—and especially our results on how slow codons in an abundant mRNA retard protein production by depleting free ribosomes (Figure 2)—confirm and quantify the longstanding initiation-limited view of protein synthesis. Moreover, from an evolutionary perspective, it makes more sense for a cell to err on the side of producing a slight excess of tRNAs as opposed to an excess of ribosomes because ribosomes are much more costly to synthesize than tRNAs. Finally, it is important to note that the TASEP-based models of translation (e.g., Reuveni et al., 2011) cannot, even in principle, be used to assess whether protein production is limited by available ribosomes because such models assume a fixed, inexhaustible supply of free ribosomes. Nor can such models, which treat each mRNA molecule independently, assess how the codon usage of a transgene influ-

ences the pool of free ribosomes in a cell and thus feeds back to alter initiation rates of all transcripts and cell growth.

Although our simulations allow us to quantify translation dynamics in a cell, our model makes many simplifying assumptions, as mentioned previously. For instance, we assume that the total numbers of ribosomes, tRNAs, and mRNAs remain constant, which we have argued is a reasonable approximation based on empirical data (García-Martínez et al., 2004; Larson et al., 2011). Nonetheless, spatial heterogeneities in the distributions of tRNAs, mRNAs, and ribosomes (Reid and Nicchitta, 2012; Qian et al., 2012), which our model neglects, could modulate the effective diffusion constants of those molecules. We have also assumed that, upon elongation, a free tRNA is instantly recharged and available for further translation. Although this assumption is clearly violated in reality, tRNA charging is generally thought not to limit protein production, with about 80% of all tRNAs charged at all times due to strong negative feedback on aminoacyl synthetases (Varshney et al., 1991; Jakubowski and Goldman, 1992; Chu et al., 2011) (but see Brackley et al. [2011] and Qian et al. [2012]). Nonetheless, in conditions of amino acid starvation, the availability of charged tRNAs may become a limiting factor in protein production (Elf et al., 2003; Welch et al., 2009), as reflected by our simulations of translation under stress.

Our model also ignores the details of termination, as well as translation errors. Although missense errors do not affect overall protein yield or the pool of free ribosomes, such errors can reduce the amount of “functional” protein produced or even produce detrimental, misfolded protein products (Drummond and Wilke, 2008, 2009). Systematically predicting which mutations will cause nonfunctional or deleterious protein folds is not feasible, but nonetheless, mistranslation remains a strong force of selection on codon usage over evolutionary timescales (Drummond and Wilke, 2008, 2009). By contrast, premature termination or nonsense errors affect both protein yield and the pool of free ribosomes. Because the probability of a nonsense error at a codon is inversely proportional to the amount of tRNAs available (Gilchrist, 2007; Shah and Gilchrist, 2010), incorporating

nonsense errors into our model would tend to exaggerate the effects of CAI and mRNA abundance on protein yield.

Aside from the systematic processes described above, our model also neglects a host of other sequence-specific features that are known to influence protein production and cellular fitness in specific cases, such as cotranslational requirements for ribosomal pausing (Kimchi-Sarfaty et al., 2007), internal mRNA structures that may retard elongation (Tuller et al., 2010b), synonymous codons required for proper splicing (Chamary et al., 2006), the effects of tRNA isoforms, neighboring codon interactions, and the recently discovered rRNA-mRNA interactions that operate in *E. coli*, but not in yeast (Li et al., 2012). Although each of these effects has been observed in a few empirical cases, it is difficult to predict when they will operate and what consequences they will have in general. Like all models, our model of translation should be particularly useful when it fails to match measurements of protein production for individual transcripts, indicating the action of some factor missing from the model that influences the translation of a particular gene. Nonetheless, these types of highly sequence-specific factors are unlikely to alter the general conclusions we have drawn from our model, such as the predominant role of free ribosomes in setting the overall pace of translation and the role of initiation rates in causing a ramp of 5' ribosome densities.

EXPERIMENTAL PROCEDURES

S. cerevisiae Transcriptome

To define the mRNA transcriptome, we selected the 3,795 genes from *S. cerevisiae* (S288c June 6, 2008 release; Cherry et al., 2012) for which Ingolia et al. (2009) obtained reliable estimates of average ribosomal densities. We fixed the total number of mRNAs to 60,000 (Zenklusen et al., 2008) and sampled mRNAs based on the relative abundances measured by Ingolia et al. (2009), ensuring that each gene had at least one mRNA represented in the transcriptome. mRNA abundances ranged from 1 to 1,254 molecules per gene (Tables 1 and S1). The (mRNA) transcriptome size was then defined as the total number of nucleotides comprised by the 60,000 mRNA molecules.

Generating Transgenes with Various CAI Values

We generated nucleotide sequences of GFP and other transgenes with various different CAI values. To produce a specified CAI value, we calculated relative synonymous codon usage (RSCU) in *S. cerevisiae* from 134 ribosomal genes (Table S3) (Sharp and Li, 1987). We then sampled codons based on RSCU. There are typically many nucleotide sequences with the same, or very similar, CAI values. Thus, for each simulation involving transgenes, we used ten sequences of similar CAI values and equal mRNA abundances to represent the transgene, in order to alleviate noisy, sequence-specific effects.

Calculating 5' Folding Energy

Coding sequences and UTRs for *S. cerevisiae* were downloaded from Ensemble (<http://www.ensemblgenomes.org>). We removed sequences with lengths not equal to a multiple of three, with premature stop codons, or with a continuous string of >3 ambiguous N symbols. We used RNAfold (Hofacker et al., 1994) to estimate the mRNA folding energy from base -4 to 37 for each gene, using default parameters.

Estimating Ribosomal Interference

To identify regions of ribosomal pausing and interference on a transgene sequence, we simulated translation in the cell with a transgene accounting for 50% of the (mRNA) transcriptome. We ran the simulation for 500 s in equilibrium and sampled the state of the system every second. We used the average number of ribosomes bound at each position to quantify the

frequency of ribosomal pausing. To quantify the frequency of ribosomal stalling, we calculated the fraction of bound ribosomes at a position that also have another bound ribosome ten codons (positions) ahead on that mRNA in the same time sample.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, two data files, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.05.049>.

LICENSING INFORMATION

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