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Yeast telomere maintenance is globally controlled by programmed ribosomal frameshifting and the nonsense-mediated mRNA decay pathway

Vivek M. Advani, Ashton T. Belew and Jonathan D. Dinman*

Department of Cell Biology and Molecular Genetics; University of Maryland; College Park, MD USA

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Abbreviations: PRF, programmed ribosomal frameshifting; NMD, Nonsense-Mediated mRNA Decay

We have previously shown that ~10% of all eukaryotic mRNAs contain potential programmed -1 ribosomal frameshifting (-1 PRF) signals and that some function as mRNA destabilizing elements through the Nonsense-Mediated mRNA Decay (NMD) pathway by directing translating ribosomes to premature termination codons. Here, the connection between -1 PRF, NMD and telomere end maintenance are explored. Functional -1 PRF signals were identified in the mRNAs encoding two components of yeast telomerase, EST1 and EST2, and in mRNAs encoding proteins involved in recruiting telomerase to chromosome ends, STN1 and CDC13. All of these elements responded to mutants and drugs previously known to stimulate or inhibit -1 PRF, further supporting the hypothesis that they promote -1 PRF through the canonical mechanism. All affected the steady-state abundance of a reporter mRNA and the wide range of -1 PRF efficiencies promoted by these elements enabled the determination of an inverse logarithmic relationship between -1 PRF efficiency and mRNA accumulation. Steady-state abundances of the endogenous EST1, EST2, STN1 and CDC13 mRNAs were similarly inversely proportional to changes in -1 PRF efficiency promoted by mutants and drugs, supporting the hypothesis that expression of these genes is post-transcriptionally controlled by -1 PRF under native conditions. Overexpression of EST2 by ablation of -1 PRF signals or inhibition of NMD promoted formation of shorter telomeres and accumulation of large budded cells at the G2/M boundary. A model is presented describing how limitation and maintenance of correct stoichiometries of telomerase components by -1 PRF is used to maintain yeast telomere length.

Introduction

Programmed ribosomal frameshift (PRF) signals are *cis*-acting elements located inside of open reading frames in mRNAs that are able to stochastically redirect translating ribosomes to shift into alternative reading frames. In the typical viral context, these elements allow the translational apparatus to bypass a 0-frame encoded in-frame stop codon and continue synthesis of a C-terminally extended fusion protein. The use of programmed -1 ribosomal frameshifting (-1 PRF) by a wide variety of RNA viruses has enabled definition of some broad rules for at least one class of these elements.[1] A typical -1 PRF promoting mRNA sequence motif contains three elements: a heptameric "slippery site" where the translational shift in reading frame actually takes place; a short spacer sequence of usually less than 12 nucleotides; and a downstream stimulatory structure (usually an mRNA pseudoknot). In eukaryotic RNA viruses, the slippery site has the heptameric motif *N NNW WWH*, where the incoming reading frame is indicated.[2] Current models posit that aminoacyl- and peptidyl-tRNAs are positioned on this sequence while the ribosome pauses at the downstream secondary structure, which is thought to provide an energetic barrier to elongating ribosomes, positioning them at the slippery site.[3-7] The nature of the slippery sequence enables re-pairing of the non-wobble bases of both the aa- and peptidyl-tRNAs with the -1 frame codons. mRNA pseudoknots are the most common stimulatory structures but stem-loops and other structures are also capable of stimulating -1 PRF.[8,9]

There are a growing number of examples of functional PRF signals in expressed eukaryotic genes.[10-16] The existence of these PRF signals in a wide variety of viral and prokaryotic genomes suggests an ancient and possibly universal mechanism for controlling the expression of actively translated mRNAs. The past few years have seen the publication of several reports describing *in silico* identification of "recoding signals" using a wide variety of computational approaches.[16-22] While many different bioinformatics methodologies have been used, these tend to fall into two general and complementary strategies. The first, based on the observation that viral frameshifting events direct ribosomes into new ORFs, is to first find out-of-frame ORFs followed

*Correspondence to: Jonathan D. Dinman; Email: dinman@umd.edu
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by the identification of sequences that may function as PRF signals. The strength of this approach is that it can identify new classes of *cis*-acting signals capable of directing efficient PRF; its weakness is that it cannot identify new functional outcomes of frameshifting. The second strategy involves searching for mRNA motifs known to promote efficient PRF. While this approach cannot identify new classes of frameshift signals, it can enable an expansion of our understanding of functional uses for PRF. Using this strategy and a package of bioinformatics and statistical tools, we previously demonstrated that approximately 10% of genes in all eukaryotic genome contain high confidence -1 PRF signals.[16,17,23] Interestingly, like many *cis*-acting elements, -1 PRF signals appear to rapidly evolve, and wet lab studies demonstrated that 9 (out of 9 selected) high confidence putative -1 PRF signals from a variety of *S. cerevisiae* genes promoted efficient recoding in vivo.[24] A searchable database of predicted -1 PRF signals in these genomes is available in the Predicted Ribosomal Frameshift Database (PRFdB) at http://prfdb.umd.edu/.

One key finding of our research is that the outcome and function of -1 PRF differs significantly between viruses and eukaryotic genomic frameshifting. While viral -1 PRF events direct ribosomes into new ORFs, resulting in synthesis of C-terminally extended proteins, the vast majority of -1 PRF events on eukaryotic cellular mRNAs are predicted to direct elongating ribosomes to premature termination signals, suggesting that -1 PRF is used to control mRNA abundance and stability through the nonsense-mediated mRNA decay (NMD) pathway. This hypothesis was supported by the demonstration that a well characterized viral -1 PRF signal can act as a *cis*-acting, NMD-dependent mRNA destabilizing element.[25] A more recent study revealed that functional -1 PRF signals derived from naturally occurring yeast mRNAs can promote rapid degradation of a reporter mRNA by -1 PRF through NMD and, to a lesser extent, the no-go decay (NGD) pathway.[24] That work began to explore the biological significance of -1 PRF by using the yeast EST2 mRNA, which encodes the catalytic subunit of telomerase. There, we showed that the EST2 mRNA is destabilized by -1 PRF, that ablation of all of its predicted -1 PRF signals resulted in its stabilization, and that global increases or decreases in -1 PRF resulted in decreased or increased steady-state abundance EST2 mRNA respectively.[24]

During the course of that study, additional putative -1 PRF signals were computationally identified in the EST2 mRNA and in the mRNAs encoding other components and regulators of telomerase: Est1p, Stn1p, and Cdc13p. Here, we have empirically characterized the computationally predicted -1 PRF signals in these mRNAs, demonstrating that each contains at least one -1 PRF signal that is operational. That the same underlying molecular mechanism is operational is suggested by experiments showing that mutated slippery sites ablate -1 PRF, and by the demonstration that all are responsive to mutants and drugs that were previously shown to alter rates of -1 PRF as directed by a virus derived signal. In general, the ability to function as mRNA destabilizing elements correlated with the extent to which any particular element promoted -1 PRF, and in these cases, ablation of NMD resulted in partial increased accumulation of a reporter mRNA. Importantly, altering rates of EST2 mRNA -1 PRF, either by ablation of EST2 slippery sites, through the activity of extragenic mutants, or by the use of -1 PRF altering drugs mimicked the effects on NMD ablation of yeast telomere length. These findings suggest that -1 PRF is utilized to control the abundance of telomerase components, and thus telomere length in yeast cells.

Results

The mRNAs encoding at least 4 proteins involved in yeast telomere maintenance contain operational -1 PRF signals. A search for -1 PRF signals in the yeast EST2 mRNA revealed 10 slippery sites in the correct reading frames, and further examination of the computationally predicted structures and statistical analyses suggested that 5 of these had the potential to encode operational -1 PRF signals (**Table 1** and **Fig. S1**). Note that, while the signal beginning at position 72 appeared to lack significant mRNA structure, the presence of a "double slippery-site" A AAU UUA AAA made it an attractive candidate nonetheless. Similarly, searches for -1 PRF signals in the mRNAs encoding other proteins involved in telomere maintenance revealed three candidates in EST1, two in STN1 and one in CDC13. Synthetic oligonucleotides containing these sequences were used to clone the predicted -1 PRF signals into standard yeast high copy dual-luciferase reporter plasmids.[26] Note that in order for frameshifted ribosomes to bypass -1 frame termination codons present in the native sequences, either 1 base was deleted from, or 2 bases were added to the spacer regions immediately 3' of the slippery sites, thus directing shifted ribosomes back into the original reading frame and enabling synthesis of the downstream firefly luciferase reporter. While these modifications may change actual rates of -1 PRF, the resulting plasmids nonetheless remain useful for determining whether or not the sequences in question are fundamentally capable of promoting efficient rates of -1 PRF. Employing a cutoff of 1% i.e., approximately 20-fold above non-programmed frameshifting,[26] the EST2 mRNA was found to harbor three functional -1 PRF signals, EST1 contains two, and STN1 and CDC13 each have one (**Table 1**). To ascertain whether -1 PRF promoted by these sequences adhered to canonical simultaneous slippage mechanism,[27] the slippery sites were silently mutagenized so that they would disrupt the slippery sequences while still encoding the same peptide sequences. While none of these slippery site mutations completely abrogated -1 PRF, they all reduced rates of frameshifting 3- to 6-fold. The residual -1 PRF promoting activities can be accounted for by the fact that the constraints imposed by silent mutagenesis retained considerable potentials for non-wobble base-pairing between shifted tRNAs and the -1 frame codons.

The -1 PRF signals in the EST1, EST2, STN1 and CDC13 mRNAs respond to cellular mutations and drugs that globally affect -1 PRF. We have previously characterized a large number of yeast mutants that globally alter rates of -1 PRF.[28] To independently test whether these sequences promote -1 PRF by the

canonical mechanism, rates of -1 PRF were assayed in isogenic cells expressing alleles of *RPL3* or *CBF5* that had previously been shown to stimulate or inhibit -1 PRF as directed by the L-A virus derived sequence.[29-31] As shown in **Figure 1A**, rates of -1 PRF directed by all of the sequences were stimulated in cells expressing the *rpl3-mak8* allele while it was uniformly inhibited in cells expressing the *rpl3-R247A* allele of *RPL3*. Similarly, the *cbf5-D95A* allele of *CBF5* enhanced rates of -1 PRF directed by all of the sequences in question (**Fig. 1A**). Previous studies have demonstrated that rates of -1 PRF can also be affected by the peptidyltransferase inhibitor anisomycin.[32] Consistent with the hypothesis that these are canonical -1 PRF signals, anisomycin uniformly inhibited -1 PRF directed by all of the sequences, and the extent of -1 PRF inhibition was dose-dependent (**Fig. 1B**). In sum, these data support the hypothesis that these sequences all promote -1 PRF through the canonical mechanism.

mRNA accumulation is inversely correlated with the strength of individual -1 PRF signals. Analysis of the predicted outcomes of -1 PRF events suggests that > 95% of the genomic -1 PRF signals should direct translating ribosomes to premature termination codons suggesting that -1 PRF could be used to post-transcriptionally regulate gene expression through the nonsense-mediated mRNA decay (NMD) pathway.[16,23] In a series of proof-of-principle experiments using the well-defined -1 PRF signal from the yeast dsRNA L-A virus we demonstrated that: 1) this signal can function as an mRNA destabilizing element; 2) mRNA destabilization requires the presence of a functional -1 PRF signal; 3) mRNA destabilization requires an intact NMD pathway, and; 4) mRNA half-life is inversely proportional to the frequency of -1 PRF (i.e., the greater the rate of -1 PRF, the greater the frequency of ribosomes being directed to a nonsense codon and vice versa).[25] These principles were subsequently shown to apply to -1 PRF signals identified in 4 endogenous yeast mRNAs.[24] To test whether the -1 PRF signals examined in the current study were also capable of functioning as NMD-dependent mRNA degradation signals, they were cloned into a yeast *PGK1*-reporter and their effects on PGK1 mRNA steady abundances were assayed in isogenic *UPF1* and *upf1*Δ cells as previously described.[24] The presence of an in-frame PTC reduced PGK1 reporter mRNA abundance to 6.0% ± 0.3% of the readthrough control levels, and this was 14.8 ± 0.6-fold more abundant in an isogenic *upf1*Δ strain. The strong EST2_1653 -1 PRF signal also significantly decreased the PGK1 reporter mRNA steady-state abundance (0.04 ± 0.1-fold of readthrough levels), and was increased 21.8 ± 2.8-fold in the *upf1*Δ strain background. As rates of -1 PRF decreased, the effects of these elements on mRNA abundance also decreased. The STN1_1203 and EST2_1215 -1 PRF signals (~24% and ~11% -1 PRF efficiency respectively) resulted in ~60% and ~40% reductions of PGK1 mRNA reporter abundance in wild-type cells (0.32 ± 0.01 and 0.49 ± 0.03-fold readthrough), and were increased 3.22 ± 0.01 and 2.44 ± 0.03 ~fold in *upf1*Δ cells. The EST1_1272 element (~6% -1 PRF) promoted an ~40% decrease in reporter mRNA abundance (0.61 ± 0.02-fold readthrough), which was increased 1.42 ± 0.06-fold in *upf1*Δ cells, and the CDC13 -1 PRF signal (~4% -1 PRF) promoted an ~20% reduction in PGK1 reporter mRNA abundance (0.81 ± 0.13-fold readthrough), while deletion of *UPF1* resulted in a 1.19 ± 0.02-fold increase in the accumulation of this mRNA. Finally, as -1 PRF rates approached 1% EST1_1920, the reductions in PGK1 reporter mRNA abundance were in the range of only 10% (0.91 ± 0.02-fold readthrough), and NMD did not appear to be a significant factor (0.94 ± 0.11). Graphic analyses

Table 1. Most predicted -1 PRF signals in ORFs involved in yeast telomere maintenance promote efficient -1 PRF in yeast cells

[a]ORF	[b]Position	[c]Slippery site Silent slip site Mut (ssM)	[d]% -1 PRF efficiency % -1 PRF ssM
L-A virus gag/pol	1969	G GGU UUA ND	6.5% ± 0.62
EST1	1203	U UUU UUU ND	0.10 ± 0.08
	1272	A AAA AAC G AAG AAC	6.33 ± 0.71 1.29 ± 0.18
	1920	A AAA AAA G AAG AAC	1.73 ± 0.03 0.83 ± 0.15
EST2	72/75	A AAU UUA AAA ND	0.58 ± 0.04
	1215	C CCU UUU U CCA UUC	10.65 ± 0.1.0 1.44 ± 0.14
	1326	A AAA AAA G AAG AAC	1.40 ± 0.04 0.66 ± 0.03
	1653	A AAA AAU G AAG AAC	67.15 ± 2.86 13.12 ± 1.79
	1995	U UUA AAA ND	0.54 ± 0.04
STN1	885	U UUU UUU ND	0.04 ± 0.07
	1203	A AAA AAU G AAG AAC	24.36 ± 1.19 3.23 ± 0.16
CDC13	1272	U UUU UUC C UCU UUC	4.20 ± 0.04 2.94 ± 0.03

[a] Yeast gene. [b]Position in the ORF of the first nucleotide of the predicted -1 PRF signal. Note that 72/75 represents a tandem slippery site beginning at nt 72 of the EST2 ORF. [c]Slippery site sequence of the predicted -1 PRF signal. Below these are shown silent protein coding slippery site mutants (ssM). ND indicates slippery site mutants that were not assayed because the wild-type sequence was determined to not promote efficient -1 PRF. [d]Predicted frameshift signals were cloned in dual luciferase reporters, and -1 PRF efficiencies were monitored in yeast cells. Upper lines show mean and standard deviations of % -1 PRF promoted by wild-type sequences. Lower lines show this data for the ssM constructs.

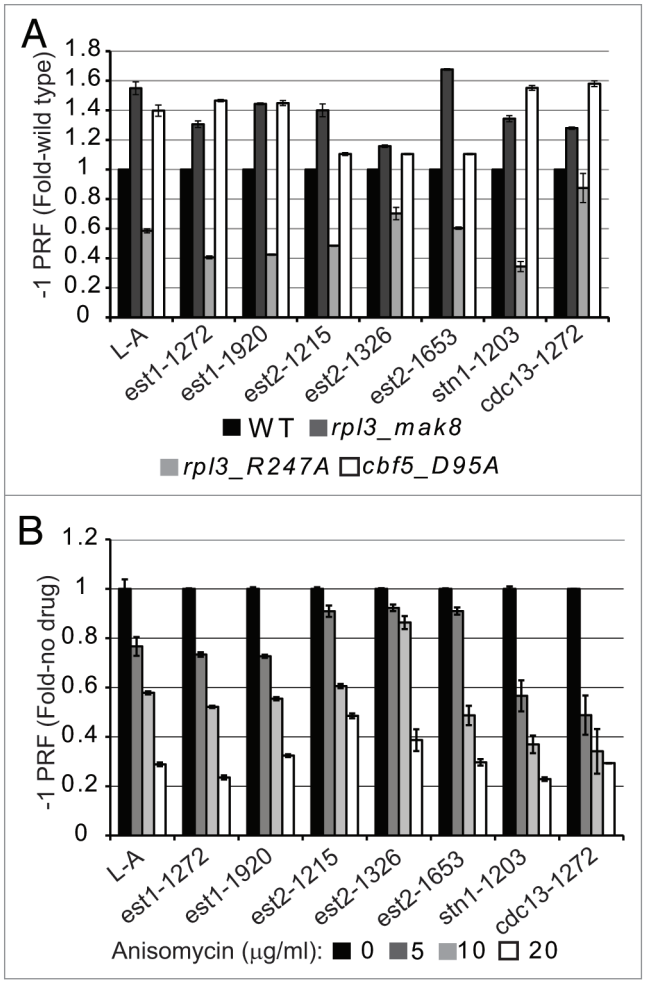

Figure 1. -1 PRF signals in the yeast EST1, EST2, STN1 and CDC13 mRNAs respond to cellular mutants (panel **A**) and a drug (panel **B**) that were previously found to promote changes in -1 PRF promoted by viral -1 PRF signals. -1 PRF was monitored using dual luciferase reporters[26] in isogenic cells expressing *RPL3*, *rpl3-mak8* or *rpl3-R247A* as the sole forms of ribosomal protein L3,[50,51] or the *CBF5* or *cbf5-D95A* allele of the yeast homolog of Dyskerin.[31] All assays were performed enough times to generate meaningful statistical data.[52] Error bars represent standard error. Note that the following the gene name denotes the specific mRNA and first nucleotide of the -1 PRF signal. This nomenclature is used throughout.

of these data reveal exponential relationships between -1 PRF efficiency and mRNA abundance (**Fig. 2A**) which is mirrored by increased abundance of the reporter upon ablation of NMD (**Fig. 2B**).

Steady-state abundance of the cellular EST1, EST2, STN1 and CDC13 mRNAs is inversely affected by changes global in -1 PRF. Consistent with the hypothesis that the presence of operational -1 PRF signals renders these mRNAs substrates for NMD, the EST1, EST2 and STN1 mRNAs have been previously shown to be stabilized upon ablation of NMD.[33,34] Here, these findings were recapitulated by applying qRT PCR methods to total mRNAs isolated from isogenic wild-type and *upf1*Δ *upf2*Δ *upf3*Δ cells (**Fig. 3A**). Given the ability of -1 PRF signals to affect the abundance of the PGK1 reporter mRNA, we tested the hypothesis that global changes in -1 PRF rates would similarly affect the steady-state abundance of the endogenus mRNAs. qRT-PCR analysis of total RNAs isolated from isogenic cells expressing *rpl3-mak8*, *rpl3-R247A*, or *cbf5-D95A* mutants revealed an inverse correlation between changes in -1 PRF and the steady-state abundances of the EST1, EST2, STN1 and CDC13 mRNAs (**Fig. 3B**). Specifically, the steady-state abundances of these mRNAs were decreased when -1 PRF was globally increased (*rpl3-mak8* and *cbf5-D95A*), and increased in *rpl3-R247A*, where -1 PRF is globally inhibited. The steady-state abundances of these mRNAs were also assayed in the presence of increasing concentrations of anisomycin. Similarly, their steady-state abundances were increased in a dose dependent manner, i.e., as -1 PRF rates decreased, the mRNA abundances increased (**Fig. 3C**).

Alteration of -1 PRF in the EST2 mRNA results in telomeres of intermediate length and accumulation of large, multiply budded cells. One would expect that overexpression of telomerase would lead to longer telomeres. However, previous studies made the intriguing finding that telomeres are very short in NMD-deficient cells, and that overexpression of *EST2*, *STN1* or *CDC13* resulted in telomeres of intermediate length.[33,35-37] To test if this is linked to -1 PRF and mRNA abundance, isogenic *est2*Δ, *upf2*Δ and *est2*Δ/*upf2*Δ cells were transformed with low copy (*CEN6*) vectors expressing full-length *EST2* (pEST2), or one containing silent protein coding slippery site mutants (ssM). The mRNA produced from this mutant was previously shown to be ~8.5-fold more abundant than that transcribed from pEST2.[24] Genomic DNAs were harvested, digested with PstI, and a Southern blot was probed for yeast telomeric sequences as previously described.[33] Consistent with previous reports, complementation with the wild-type *EST2* clone restored long telomeres (L) in *est2*Δ cells, and ablation of NMD uniformly resulted in short telomeres (S) (**Fig. 4A**). Importantly, expression of pEST2ssM as the sole source of Est2p promoted intermediate length telomeres (indicated by "I"), recapitulating the *EST2*, *STN1* and *CDC13* overexpression observations. These findings establish a linkage between -1 PRF and telomere length homeostasis through NMD in yeast.

Short telomeres create a "crisis" for dividing cells, triggering a series of signals that result in a delay in mitosis, specifically arrest at the G2/M boundary, as cells attempt to restore their telomeres before committing to division.[38] Cells arrested at this "checkpoint" can continue to grow, increasing in volume, and eventually they can bypass mitosis, producing multiply budded yeast cells. In unpublished observations, we had noted that NMD-deficient cells tend to be unusually large and accumulate with large daughter buds suggestive of a G2/M mitotic delay. In combination with the results presented above, this led to the hypothesis that NMD-dependent defects in telomerase expression might affect the cell cycle. If true, then overexpression of Est2p by the pEST2ssM construct should confer a similar phenotype. This is indeed what we observed: *est2*Δ cells expressing pEST2ssM tended to be somewhat larger than isogenic cells expressing pEST2 and also displayed the large daughter bud

phenotype. This phenotype was exaggerated in *est2*Δ *upf1*Δ cells expressing either pEST2 or pEST2ssM (**Fig. 4B**). These findings suggest that -1 PRF may play an important role in controlling the cell cycle through telomerase expression.

-1 PRF signals as classic, rapidly evolving *cis*-acting elements. A prior analysis of -1 PRF signals from *BUB3*, *EST2*, *SPR6* and *TBF1* orthologs from the closely related yeast species *S. paradoxus*, *S. mikatae*, *S. bayanus*, *S. castellii, S. kudriavzevii* and *S. kluyveri* suggested that while no specific -1 PRF signals appeared to be conserved among these orthologs, the presence of high probability predicted -1 PRF signals in the orthologs of all of these genes (although not in every species) suggested that the mechanism itself may be conserved in these families of mRNAs, an observation that was supported by the finding that no -1 PRF signals were found in any of the orthologs of six genes that were found to lack predicted -1 PRF signals in *S. cerevisiae* (*PGK1, HHT1, TEF2, MIC14, CMD1 and GRX1*).[24] To address whether -1 PRF may be a conserved mechanism in yeast telomere maintenance, the orthologous genes for *EST1*, *STN1*, and *CDC13* were queried. These data are presented in **Table S6**.

One potential -1 PRF signal was found in the EST1 orthologs from *S. paradoxus*, *S. bayanus*, and *S. mikatae*, but the EST1 mRNAs from *S. castelli*, *S. kluyveri* and *S. kudriavzevii* lacked predicted -1 PRF signals. Potential -1 PRF signals were found in the STN1 orthologs from *S. paradoxus*, *S. bayanus*, *S. castelli* and *S. kluyveri* but not in *S. mikatae* or *S. kudriavzevii*. Similarly, orthologous Cdc13 mRNAs from *S. paradoxus*, *S. castelli, S. kluyveri* and *S. mikatae* were found to harbor potential -1 PRF signals, but those from *S. bayanus* and *S. kudriavzevii* did not. The potential evolutionary significance these observations are discussed below.

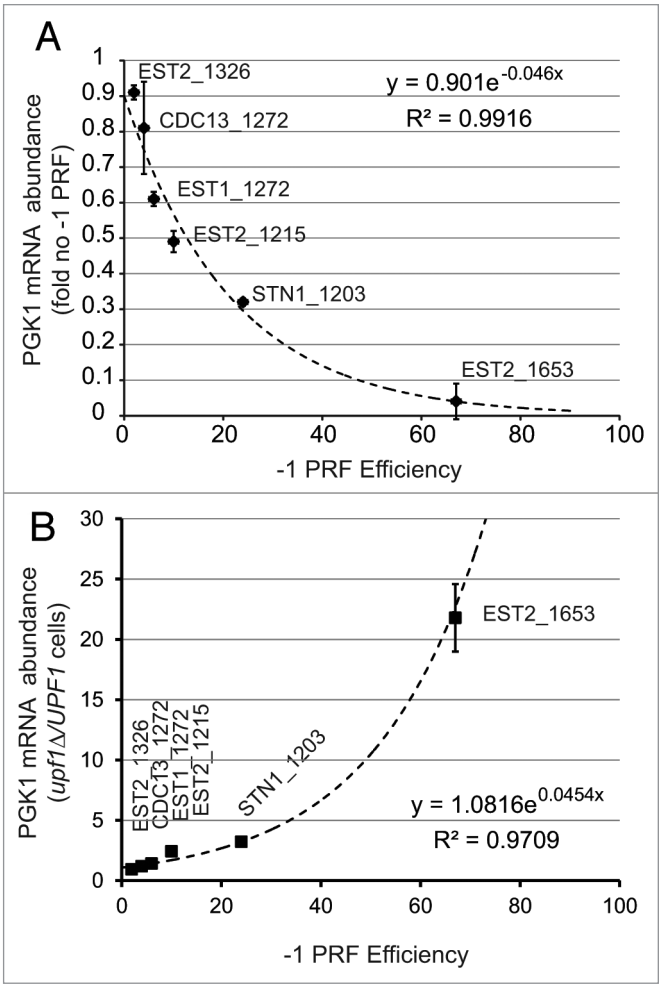

Figure 2. Delineation mathematical relationships between -1 PRF and mRNA abundance in wild-type (Panel **A**), and NMD-deficient cells (Panel **B**). The -1 PRF signals from the yeast EST1, EST2, STN1 and CDC13 mRNAs were cloned into yeast PGK1 reporters in their native orientations so that frameshift events would direct elongating ribosomes to premature termination codons (PTC). A PGK1 vector without inserted sequences was used as a control. These reporters were transformed and expressed in isogenic wild-type (**A**), or *upf1*Δ cells (**B**), and mRNA steady-state abundances were determined by qRT-PCR using the endogenous yeast G3PD mRNA as an internal standard. (**A**) Steady-state abundances of -1 PRF signal containing PGK1 reporter mRNAs/PGK1 control mRNA (i.e., no -1 PRF) in wild-type cells, fit to a logarithmic function. (**B**) Steady-state abundances of -1 PRF signal containing PGK1 reporter mRNAs in isogenic *upf1*Δ vs. *UPF1* cells fit to a logarithmic function. Error bars denote standard deviation.

Discussion

It is now clear that the canonical -1 PRF signals consisting of a heptameric slippery site closely followed by an mRNA pseudoknot that were first characterized in RNA viruses are also widely utilized in eukaryotic cellular mRNAs. Furthermore, it is also well established that, by shifting translating ribosomes to premature termination codons, these elements have mRNA destabilizing activities through the NMD pathway. Here, we have investigated the potential biological relevance of such frameshifting by identifying and characterizing -1 PRF signals in mRNAs encoding yeast proteins involved in telomere maintenance. The active -1 PRF signals in the mRNAs encoding Est1p, Est2p, Stn1p and Cdc13p function to decrease mRNA abundance through NMD. We note that while the EST1, EST2 and STN1 mRNAs were previously found to accumulate in NMD deficient cells, the CDC13 mRNA did not.[33,34,39] Those reports employed microarray technology, while this study used qRT-PCR. As microarrays have substantially lower dynamic range than qRT-PCR, one possibility is that the qRT-PCR method was better able to detect small changes in CDC13 mRNA abundances due to ablation of NMD or changes in frameshifting rates changes that reflect the relatively low levels of -1 PRF activity promoted by the sole element in the CDC13 mRNA. We suggest that these changes were too small to detect using microarrays, although the insensitivity of the CDC13 mRNA to NMD was also confirmed by northern blot.[33] Problematically, the STN1 mRNA has been reported to be an indirect substrate for NMD.[39] The data presented here however showing that this sequence promotes high levels of -1 PRF, that introduction of this -1 PRF signal into the PGK1 reporter results in a very strong decrease in the steady-state abundance of the reporter mRNA (**Fig. 2A**), that the abundance

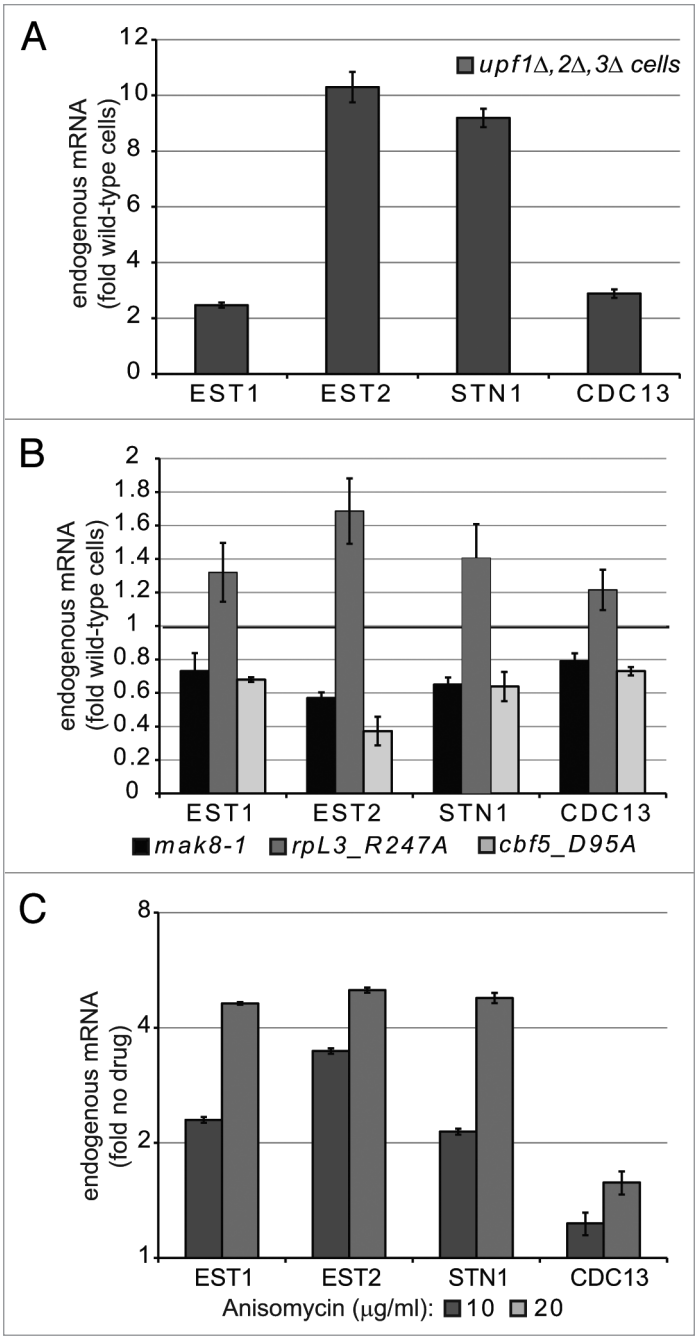

Figure 3. Steady-state abundances of native EST1, EST2, STN1 and CDC13 mRNAs in mutant and drug treated cells. qRT PCR was used to determine steady-state abundances of the native mRNAs using the G3PD mRNA as an internal standard. (**A**) mRNA abundances were monitored in isogenic *UPF1* and *upf1*Δ *upf2*Δ *upf3*Δ cells. (**B**) mRNA abundances in isogenic wild-type and *rpl3-mak8-1*, *rpl3-R247A* (compared with wild-type *RPL3*), and *cbf5-D95A* (compared with *CBF5*) cells. (**C**) mRNA abundances were monitored in wild-type cells treated with the indicated concentrations of anisomycin. Error bars denote standard error.

of this reporter increases ~4-fold when NMD is inactivated (**Fig. 2B**) and that ablation of NMD and inhibition of -1 PRF by anisomycin resulted in increased steady-state abundance of the endogenous mRNA (**Fig. 3**) are more consistent with the hypothesis that this is indeed an mRNA instability element that functions directly through NMD. More in depth studies of this element may resolve this apparent inconsistency.

Interestingly, the wide range of -1 PRF efficiencies promoted by the seven different -1 PRF signals enabled a mathematical analysis of the relationship between -1 PRF and mRNA steady-state abundances (**Fig. 2**). The inverse-exponential correlation between -1 PRF and mRNA abundance suggests that there are limits to changes in -1 PRF above or below which biological effects may be seen. In addition, a phylogenetic analysis revealed that -1 PRF signals in general, but not any specific -1 PRF signals, appear to be utilized in these telomere-associated mRNAs in the yeasts. This is consistent with our previous analyses of -1 PRF signals in other yeast genes, further supporting the hypothesis that rapid rates of mutation in *cis*-acting regulatory elements drives speciation.[24]

How chromosome ends are stably maintained is one of the central questions of modern biology.[40] Telomeres are thought exist in a range of states, from fully capped full-length, to uncapped and short (see model, **Figure 5**, adapted from models proposed in[38,41]). As telomeres age, they progressively shorten, and at some point reach an intermediate, uncapped status. In yeast, telomere shorting promotes recruitment of the MRX+Tel1p and CST complexes (where C = Cdc13p, S = Stn1p, T = Ten1p), inducing checkpoint arrest at the G2/M boundary. Phosphorylation of Cdc13p by Tel1p enables recruitment of telomerase through Est1p, stimulating telomere repair, and releasing cells from checkpoint arrest. Failure to recruit telomerase leads to further telomere shortening, where they eventually resemble double-stranded breaks (DSB). These short telomeres recruit the DSB repair machinery, resulting in strong checkpoint arrest. Eventually, these short telomeres are maintained by this machinery, resulting in telomere end joining, and bypass of checkpoint arrest. The net effect is to "immortalize" telomeres, resulting in longer lifespans, but at the cost of genome integrity.

Previous studies have shown that telomerase is limited in yeast, presumably as a means to ensure its recruitment to uncapped telomeres.[42,43] Importantly, defective expression of individual components of yeast telomerase or telomerase recruiting proteins affects telomere length. For example, overexpression of *TLC1* resulted in very short telomeres,[44] while overexpression of *STN1*, *CDC13*,[35] or *EST2*[44] all promoted the intermediate telomere length phenotype. Furthermore, ablation of NMD resulted in short telomeres,[33,35,36] suggesting that the NMD pathway is epistatic to the activities of individual protein components of the telomere repair machinery. Here, we have demonstrated the presence of operational -1 PRF signals in the EST1, EST2, STN1 and CDC13 mRNAs. One result of -1 PRF events on these mRNAs is to direct elongating ribosomes to premature termination codons, consistent with the fact that they are substrates for NMD (**Fig. 3A**). We have shown that these -1 PRF signals function as mRNA destabilizing elements in an NMD-dependent manner, and that this follows an inverse logarithmic relationship (**Fig. 2A**). Consistent with these observations, the steady-state abundances

of the native EST1, EST2, STN1 and CDC13 mRNAs are inversely correlated with changes in -1 PRF (**Figs. 3B and C**). Intriguingly, the mRNA encoding the third protein component of yeast telomerase, Est3p, harbors a +1 PRF signal[11] and is stabilized in NMD-deficient cells.[33-35] We propose that -1 PRF plays a role in maintaining the correct stoichiometric ratios of telomerase components critical for telomerase recruitment. Overexpression of any one of these components, e.g., by ablation of -1 PRF in the EST2 mRNA, has dominant-negative effects on telomerase recruitment, resulting in accumulation of intermediate length telomeres (see pEST2ssM expressed in *est2*Δ cells, **Figure 4A**). Furthermore, we suggest that global overexpression of all of these components by inactivation of NMD further increases the rate of telomere shortening, hastening the formation of DSB-like, i.e., short telomeres. This model also accounts for the observation that that ablation of -1 PRF in the EST2 mRNA, or ablation of NMD resulted in accumulation of large budded cells, a hallmark of checkpoint arrest at the G2/M boundary (**Fig. 4B**).

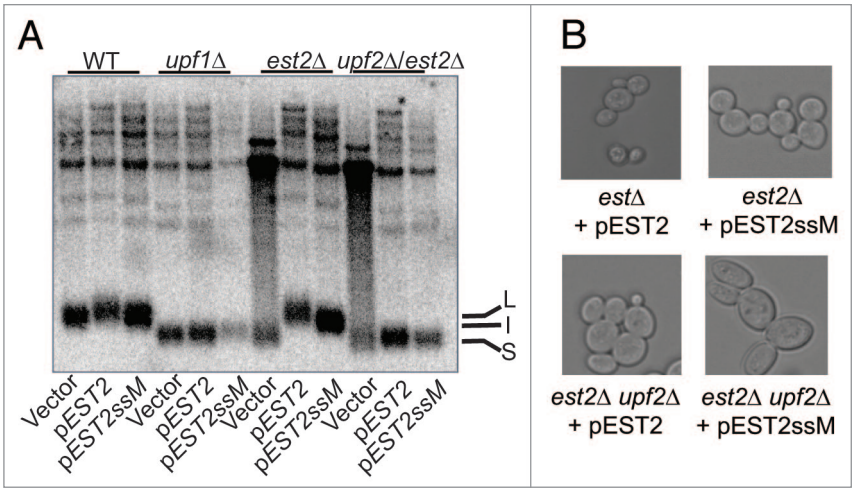

Figure 4. Ablation of -1 PRF in EST2, or of NMD affects telomere length and promotes G2/M cell cycle arrest. (**A**) Southern blot of PstI digested DNAs isolated from isogenic wild-type, *upf1*Δ, *est2*Δ, or *upf1*Δ *est2*Δ cells were transformed with either an empty *CEN6* low copy vector (vector), the same vector expressing the wild-type *EST2* gene (p*EST2*), or one in which the slippery sites of all 5 tested -1 PRF signals had been silently mutated (see **Table 1** and 24). The blot was probed using DNA oligonucleotedes complementary to telomeric repeat sequences as described.[33] Letters along the right hand side indicate (L)ong, (I)ntermediate, and (S)hort telomeres respectively. (**B**) Phase contrast microscopy (100x) of cells used in panel **A** harvested from logarithmically growing cultures.

We are aware that these studies pose more questions than they answer. In particular, we hope that this report inspires deeper research into the impact of -1 PRF in yeast telomere maintenance by laboratories better equipped for such inquiry. More broadly, our studies suggest that -1 PRF is a fundamental molecular mechanism that is used to fine tune a myriad of physiological processes. The big questions in the field that need to be addressed in the future include: 1) which mRNAs are actively engaged in -1 PRF in which cell types? 2) Where along these mRNAs are ribosomes actively shifting? 3) Are there thresholds above or below which changes in -1 PRF have meaningful biological effects? And 4) how and in response to what stimuli can -1 PRF be regulated? The answers to these questions will have broad impact over the next decade.

Materials and Methods

Strains, genetic manipulations, and media. *Escherichia coli DH5*α was used to amplify plasmid DNA. Transformations of *E. coli* were performed as described previously using the calcium chloride method.[45] Yeast cells were transformed using the alkali cation method.[46] Yeast strains used in this study are shown in **Table S1**. Yeast were grown on YPAD and synthetic complete media (H-).[47]

Plasmids and assays of -1 PRF and mRNA steady-state abundance. *URA3*-based high copy dal luciferase and mRNA stability plasmids were previously described.[16] Oligonucleotide primers were purchased from IDT (Coralville, IA) (**Tables S3–S5**). Computationally identified putative -1 PRF signals shown in **Fig. S1** were amplified from yeast genomic DNA by PCR using oligonucleotide primers which terminated in a SalI restriction site at the 5' and BamHI at the 3'. The zero-frame dual-luciferase reporter plasmid (pJD375) along with the -1 PRF signal containing dsDNA fragments were digested using these restriction enzymes and ligated together to generate endogenous -1 PRF signal containing dual-luciferase vectors. The wobble bases of slippery heptamers were mutagenized to synonymous codons in dual luciferase reporters by oligonucleotide site-directed mutagenesis using the QuickChange® Lightning Site-Directed Mutagenesis Kit (Agilent). *PGK1* reporter constructs for individual -1 PRFs were made as previously described.[24] Read through *PGK1* plasmid pJD753 and premature termination codon vector (pJD828) were used as controls for *PGK1* reporter steady-state assays. Oligonucleotide primers were chosen to terminate in KpnI restriction sites and amplify 41 and 30 bases of *Renilla* and firefly luciferase derived sequences respectively. The resulting amplicons were cloned into the KpnI site 492 bases into the *PGK1* open reading frame of the unmodified *PGK1* containing vector (pJD741). A premature termination codon vector (pJD828) was generated by cutting the readthrough (pJD753) with BamHI and backfilling with Klenow fragment. Plasmids expressing full length EST 2 and the EST2ssM mutant were previously described.[24] Assays to determine rates of -1 PRF and mRNA steady-state abundances were performed as previously described.[24] Plasmids used in this study are listed in **Table S2**.

Southern Blot analyses of *est2* mutants. Full length *EST2* expression vectors (pJD641), *EST2* mutant vectors (pJD796), and null plasmids (pJD315) were transformed into WT(JD1281), *EST2* deletion(JD1287), *UPF2*(JD1288) and *EST2/UPF2*(JD1276) deletion strains. Southern analyses were performed as described previously.[36,48] Genomic DNA was extracted from mid-logarithmic cell cultures and digested with PstI in 10 μg aliquots. The

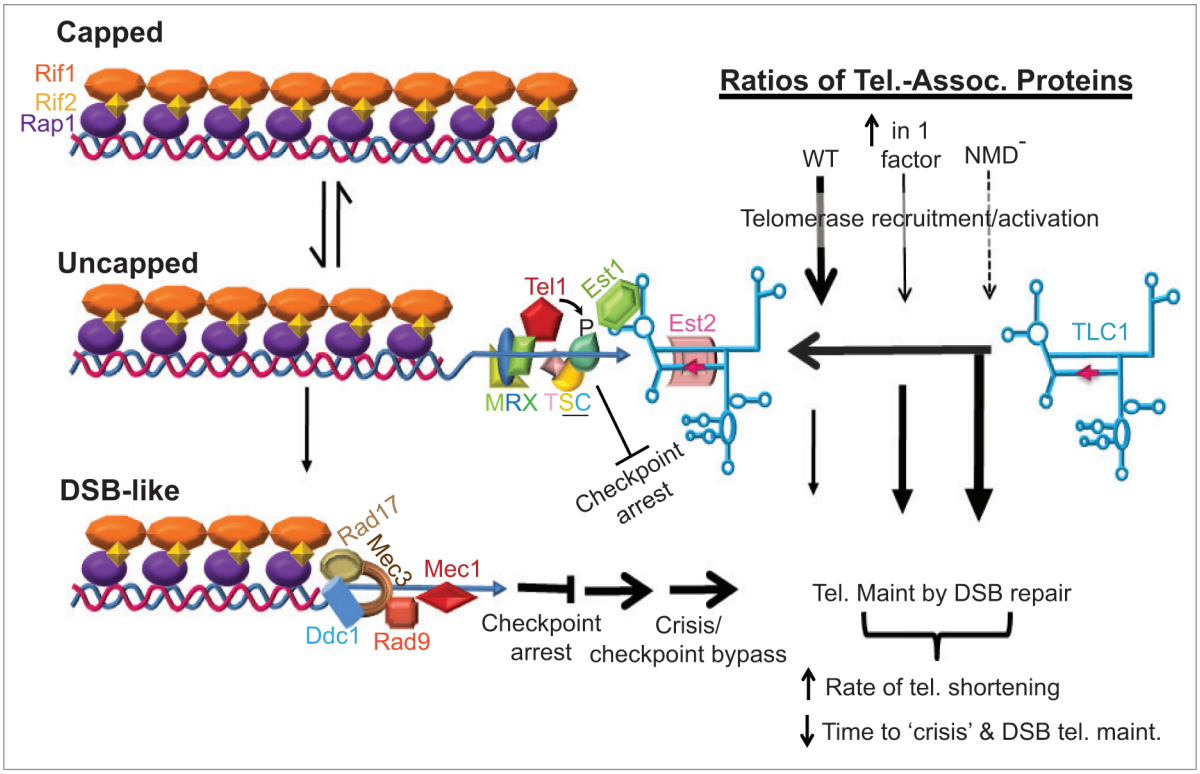

Figure 5. Model: telomerase recruitment to uncapped telomeres is controlled by the relative stoichiometries of telomerase components. Yeast telomeres exist in a range of states, from fully capped and full length to uncapped and short. As telomeres age, they progressively shorten, and at some point reach an intermediate, uncapped status, recruiting the MRX+Tel1p complex. Tel1p phosphorylates Cdc13p (part of the Cdc13, Stn1, Ten1 comples shown as TSC), promoting telomerase recruitment through Est1p, and inducing checkpoint arrest at the G2/M boundary. Telomerase recruitment stimulates telomere repair (up arrow on left), and releases cells from checkpoint arrest. Failure to recruit telomerase leads to further telomere shortening (down arrow on right), where they eventually resemble double-stranded breaks (DSB-like). These short telomeres also promote checkpoint arrest, but since they cannot recruit telomerase they enter crisis. Recruitment of the DSB repair machinery promotes chromosome end joining in some cells, where homologous recombination is used to amplify telomere sequences enabling checkpoint bypass. We propose that maintaining the correct stoichiometric ratios of telomerase components is critical for telomerase recruitment and telomere length homeostasis. Alteration in the expression of one of these factors, e.g., overexpression of Est2p by ablation of -1 PRF, weakly inhibits telomerase recruitment resulting in accumulation of shorter, intermediate length telomeres and accumulation of cells arrested at G2/M. Overexpression of all of these factors by inactivation of NMD has strong dominant-negative effects on telomerase recruitment, increasing the proportion of cells with short telomeres with similarly strong cell cycle effects.

resulting fragments were separated on a 1% agarose gel and transferred to a Hybond-N-membranes (Amersham) and hybridized to a probe derived from yeast telomeric DNA. This probe was prepared from pBC6 (a gift from the Berman lab) by PCR amplifying the telomeric region using M13 oligos. The resulting linear amplicon was used as template to create $\alpha[^{32}P]$ internally labeled telomeric TG repeat sequence using the "Decalabel random priming kit" from Stratagene. Signals were detected using a GeneStorm phosphoimager (Bio-Rad) and quantified using QuantifyOne (Bio-Rad).

Microscopic methods. Yeast cells were visualized using a Zeiss Aziphot microscope at 100x magnification.

Phylogenetic analyses. The *EST1*, *STN1* and *CDC13* orthologs from the genomes of *S. paradoxus*, *S. mikatae*, *S. bayanus*, *S. castellii*, *S. kudriavzevii* and *S. kluyveri* were extracted from the Yeast Gene Order Browser (http://wolfe.gen.tcd.ie/ygob/).[49] Orthologs were identified for all genes and nucleotide sequences were analyzed for the presence of potential -1 PRF signals as previously described.[16,23] Results are compiled in **Table S6**.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:
www.landesbioscience.com/journals/translation/article/24418

References

1. Dinman JD. Mechanisms and implications of programmed translational frameshifting. Wiley Interdiscip Rev RNA 2012; 3:661-73; PMID:22715123; http://dx.doi.org/10.1002/wrna.1126.
2. Harger JW, Meskauskas A, Dinman JD. An "integrated model" of programmed ribosomal frameshifting. Trends Biochem Sci 2002; 27:448-54; PMID:12217519; http://dx.doi.org/10.1016/S0968-0004(02)02149-7.
3. Kontos H, Napthine S, Brierley I. Ribosomal pausing at a frameshifter RNA pseudoknot is sensitive to reading phase but shows little correlation with frameshift efficiency. Mol Cell Biol 2001; 21:8657-70; PMID:11713298; http://dx.doi.org/10.1128/MCB.21.24.8657-8670.2001.
4. Lopinski JD, Dinman JD, Bruenn JA. Kinetics of ribosomal pausing during programmed -1 translational frameshifting. Mol Cell Biol 2000; 20:1095-103; PMID:10648594; http://dx.doi.org/10.1128/MCB.20.4.1095-1103.2000.
5. Plant EP, Jacobs KLM, Harger JW, Meskauskas A, Jacobs JL, Baxter JL, et al. The 9-A solution: how mRNA pseudoknots promote efficient programmed -1 ribosomal frameshifting. RNA 2003; 9:168-74; PMID:12554858; http://dx.doi.org/10.1261/rna.2132503.
6. Plant EP, Dinman JD. Torsional restraint: a new twist on frameshifting pseudoknots. Nucleic Acids Res 2005; 33:1825-33; PMID:15800212; http://dx.doi.org/10.1093/nar/gki329.
7. Liao PY, Choi YS, Dinman JD, Lee KH. The many paths to frameshifting: kinetic modelling and analysis of the effects of different elongation steps on programmed -1 ribosomal frameshifting. Nucleic Acids Res 2011; 39:300-12; PMID:20823091; http://dx.doi.org/10.1093/nar/gkq761.
8. Baril M, Dulude D, Steinberg SV, Brakier-Gingras L. The frameshift stimulatory signal of human immunodeficiency virus type 1 group O is a pseudoknot. J Mol Biol 2003; 331:571-83; PMID:12899829; http://dx.doi.org/10.1016/S0022-2836(03)00784-8.
9. Kollmus H, Hentze MW, Hauser H. Regulated ribosomal frameshifting by an RNA-protein interaction. RNA 1996; 2:316-23; PMID:8634912.
10. Matsufuji S, Matsufuji T, Wills NM, Gesteland RF, Atkins JF. Reading two bases twice: mammalian antizyme frameshifting in yeast. EMBO J 1996; 15:1360-70; PMID:8635469.
11. Morris DK, Lundblad V. Programmed translational frameshifting in a gene required for yeast telomere replication. Curr Biol 1997; 7:969-76; PMID:9382847; http://dx.doi.org/10.1016/S0960-9822(06)00416-7.
12. Morris DR, Geballe AP. Upstream open reading frames as regulators of mRNA translation. Mol Cell Biol 2000; 20:8635-42; PMID:11073965; http://dx.doi.org/10.1128/MCB.20.23.8635-8642.2000.
13. Shigemoto K, Brennan J, Walls E, Watson CJ, Stott D, Rigby PW, et al. Identification and characterisation of a developmentally regulated mammalian gene that utilises -1 programmed ribosomal frameshifting. Nucleic Acids Res 2001; 29:4079-88; PMID:11574691.
14. Manktelow E, Shigemoto K, Brierley I. Characterization of the frameshift signal of Edr, a mammalian example of programmed -1 ribosomal frameshifting. Nucleic Acids Res 2005; 33:1553-63; PMID:15767280; http://dx.doi.org/10.1093/nar/gki299.
15. Wills NM, Moore B, Hammer A, Gesteland RF, Atkins JF. A functional -1 ribosomal frameshift signal in the human paraneoplastic Ma3 gene. J Biol Chem 2006; 281:7082-8; PMID:16407312; http://dx.doi.org/10.1074/jbc.M511629200.
16. Jacobs JL, Belew AT, Rakauskaite R, Dinman JD. Identification of functional, endogenous programmed -1 ribosomal frameshift signals in the genome of Saccharomyces cerevisiae. Nucleic Acids Res 2007; 35:165-74; PMID:17158156; http://dx.doi.org/10.1093/nar/gkl1033.
17. Hammell AB, Taylor RC, Peltz SW, Dinman JD. Identification of putative programmed -1 ribosomal frameshift signals in large DNA databases. Genome Res 1999; 9:417-27; PMID:10330121.
18. Shah AA, Giddings MC, Parvaz JB, Gesteland RF, Atkins JF, Ivanov IP. Computational identification of putative programmed translational frameshift sites. Bioinformatics 2002; 18:1046-53; PMID:12176827; http://dx.doi.org/10.1093/bioinformatics/18.8.1046.
19. Gao X, Havecker ER, Baranov PV, Atkins JF, Voytas DF. Translational recoding signals between gag and pol in diverse LTR retrotransposons. RNA 2003; 9:1422-30; PMID:14623998; http://dx.doi.org/10.1261/rna.5105503.
20. Gurvich OL, Baranov PV, Zhou J, Hammer AW, Gesteland RF, Atkins JF. Sequences that direct significant levels of frameshifting are frequent in coding regions of Escherichia coli. EMBO J 2003; 22:5941-50; PMID:14592990; http://dx.doi.org/10.1093/emboj/cdg561.
21. Moon S, Byun Y, Kim HJ, Jeong S, Han K. Predicting genes expressed via -1 and +1 frameshifts. Nucleic Acids Res 2004; 32:4884-92; PMID:15371551; http://dx.doi.org/10.1093/nar/gkh829.
22. Namy O, Rousset JP, Napthine S, Brierley I. Reprogrammed genetic decoding in cellular gene expression. Mol Cell 2004; 13:157-68; PMID:14759362; http://dx.doi.org/10.1016/S1097-2765(04)00031-0.
23. Belew AT, Hepler NL, Jacobs JL, Dinman JD. PRFdb: a database of computationally predicted eukaryotic programmed -1 ribosomal frameshift signals. BMC Genomics 2008; 9:339; PMID:18637175; http://dx.doi.org/10.1186/1471-2164-9-339.
24. Belew AT, Advani VM, Dinman JD. Endogenous ribosomal frameshift signals operate as mRNA destabilizing elements through at least two molecular pathways in yeast. Nucleic Acids Res 2011; 39:2799-808; PMID:21109528; http://dx.doi.org/10.1093/nar/gkq1220.
25. Plant EP, Wang P, Jacobs JL, Dinman JD. A programmed -1 ribosomal frameshift signal can function as a cis-acting mRNA destabilizing element. Nucleic Acids Res 2004; 32:784-90; PMID:14762205; http://dx.doi.org/10.1093/nar/gkh256.
26. Harger JW, Dinman JD. An *in vivo* dual-luciferase assay system for studying translational recoding in the yeast *Saccharomyces cerevisiae.* RNA 2003; 9:1019-24; PMID:12869712; http://dx.doi.org/10.1261/rna.5930803.
27. Jacks T, Madhani HD, Masiarz FR, Varmus HE. Signals for ribosomal frameshifting in the Rous sarcoma virus gag-pol region. Cell 1988; 55:447-58; PMID:2846182; http://dx.doi.org/10.1016/0092-8674(88)90031-1.
28. Dinman JD, O'Connor M. Mutants that affect recoding. In: Atkins JF, Gesteland RF, editors. Recoding: expansion of decoding rules enriches gene expression. New York, Dordrecht, Heidelberg, London: Springer, 2010:321-344.
29. Meskauskas A, Harger JW, Jacobs KLM, Dinman JD. Decreased peptidyltransferase activity correlates with increased programmed -1 ribosomal frameshifting and viral maintenance defects in the yeast *Saccharomyces cerevisiae.* RNA 2003; 9:982-92; PMID:12869709; http://dx.doi.org/10.1261/rna.2165803.
30. Meskauskas A, Dinman JD. A molecular clamp ensures allosteric coordination of peptidyltransfer and ligand binding to the ribosomal A-site. Nucleic Acids Res 2010; 38:7800-13; PMID:20660012; http://dx.doi.org/10.1093/nar/gkq641.
31. Jack K, Bellodi C, Landry DM, Niederer RO, Meskauskas A, Musalgaonkar S, et al. rRNA pseudouridylation defects affect ribosomal ligand binding and translational fidelity from yeast to human cells. Mol Cell 2011; 44:660-6; PMID:22099312; http://dx.doi.org/10.1016/j.molcel.2011.09.017.
32. Dinman JD, Ruiz-Echevarria MJ, Czaplinski K, Peltz SW. Peptidyl-transferase inhibitors have antiviral properties by altering programmed -1 ribosomal frameshifting efficiencies: development of model systems. Proc Natl Acad Sci U S A 1997; 94:6606-11; PMID:9192612; http://dx.doi.org/10.1073/pnas.94.13.6606.
33. Dahlseid JN, Lew-Smith J, Lelivelt MJ, Enomoto S, Ford A, Desruisseaux M, et al. mRNAs encoding telomerase components and regulators are controlled by UPF genes in Saccharomyces cerevisiae. Eukaryot Cell 2003; 2:134-42; PMID:12582130; http://dx.doi.org/10.1128/EC.2.1.134-142.2003.
34. He F, Li X, Spatrick P, Casillo R, Dong S, Jacobson A. Genome-wide analysis of mRNAs regulated by the nonsense-mediated and 5' to 3' mRNA decay pathways in yeast. Mol Cell 2003; 12:1439-52; PMID:14690598; http://dx.doi.org/10.1016/S1097-2765(03)00446-5.
35. Enomoto S, Glowczewski L, Lew-Smith J, Berman JG. Telomere cap components influence the rate of senescence in telomerase-deficient yeast cells. Mol Cell Biol 2004; 24:837-45; PMID:14701754; http://dx.doi.org/10.1128/MCB.24.2.837-845.2004.
36. Lew JE, Enomoto S, Berman J. Telomere length regulation and telomeric chromatin require the nonsense-mediated mRNA decay pathway. Mol Cell Biol 1998; 18:6121-30; PMID:9742129.
37. Teo SH, Jackson SP. Telomerase subunit overexpression suppresses telomere-specific checkpoint activation in the yeast yku80 mutant. EMBO Rep 2001; 2:197-202; PMID:11266360; http://dx.doi.org/10.1093/embo-reports/kve038.
38. Lydall D. Hiding at the ends of yeast chromosomes: telomeres, nucleases and checkpoint pathways. J Cell Sci 2003; 116:4057-65; PMID:12972499; http://dx.doi.org/10.1242/jcs.00765.
39. Guan Q, Zheng W, Tang S, Liu X, Zinkel RA, Tsui KW, et al. Impact of nonsense-mediated mRNA decay on the global expression profile of budding yeast. PLoS Genet 2006; 2:e203; PMID:17166056; http://dx.doi.org/10.1371/journal.pgen.0020203.
40. Zakian VA. Telomeres: the beginnings and ends of eukaryotic chromosomes. Exp Cell Res 2012; 318:1456-60; PMID:22391099; http://dx.doi.org/10.1016/j.yexcr.2012.02.015.
41. Bianchi A, Shore D. How telomerase reaches its end: mechanism of telomerase regulation by the telomeric complex. Mol Cell 2008; 31:153-65; PMID:18657499; http://dx.doi.org/10.1016/j.molcel.2008.06.013.
42. Mozdy AD, Cech TR. Low abundance of telomerase in yeast: implications for telomerase haploinsufficiency. RNA 2006; 12:1721-37; PMID:16894218; http://dx.doi.org/10.1261/rna.134706.
43. Teixeira MT, Arneric M, Sperisen P, Lingner J. Telomere length homeostasis is achieved via a switch between telomerase-extendible and -nonextendible states. Cell 2004; 117:323-35; PMID:15109493; http://dx.doi.org/10.1016/S0092-8674(04)00334-4.
44. Liao XH, Zhang ML, Yang CP, Xu LX, Zhou JQ. Characterization of recombinant Saccharomyces cerevisiae telomerase core enzyme purified from yeast. Biochem J 2005; 390:169-76; PMID:15813705; http://dx.doi.org/10.1042/BJ20050208.
45. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual. 2 ed. Cold Spring Harbor, NY: Cold Spring Harbor Press, 1989.
46. Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. J Bacteriol 1983; 153:163-8; PMID:6336730.

47. Wickner RB, Leibowitz MJ. Two chromosomal genes required for killing expression in killer strains of Saccharomyces cerevisiae. Genetics 1976; 82:429-42; PMID:773743.
48. Banerjee S, Myung K. Increased genome instability and telomere length in the elg1-deficient Saccharomyces cerevisiae mutant are regulated by S-phase checkpoints. Eukaryot Cell 2004; 3:1557-66; PMID:15590829; http://dx.doi.org/10.1128/EC.3.6.1557-1566.2004.
49. Byrne KP, Wolfe KH. The Yeast Gene Order Browser: combining curated homology and syntenic context reveals gene fate in polyploid species. Genome Res 2005; 15:1456-61; PMID:16169922; http://dx.doi.org/10.1101/gr.3672305.
50. Meskauskas A, Dinman JD. Ribosomal protein L3: gatekeeper to the A site. Mol Cell 2007; 25:877-88; PMID:17386264; http://dx.doi.org/10.1016/j.molcel.2007.02.015.
51. Meskauskas A, Dinman JD. Ribosomal protein L3 functions as a 'rocker switch' to aid in coordinating of large subunit-associated functions in eukaryotes and Archaea. Nucleic Acids Res 2008; 36:6175-86; PMID:18832371; http://dx.doi.org/10.1093/nar/gkn642.
52. Jacobs JL, Dinman JD. Systematic analysis of bicistronic reporter assay data. Nucleic Acids Res 2004; 32:e160-70; PMID:15561995; http://dx.doi.org/10.1093/nar/gnh157.