Increased expression of the fluorescent reporter protein ymNeonGreen in *Saccharomyces cerevisiae* by reducing RNA secondary structure near the start codon.

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ABSTRACT

Expression of a new fluorescent reporter protein called mNeonGreen, that is not based on the jellyfish green fluorescent protein (GFP) sequence, shows increased brightness and folding speed compared to enhanced GFP. However, in *in vivo* brightness of mNeonGreen and its yeast-optimized variant ymNeonGreen in *S. cerevisiae* is lower than expected, limiting the use of this high quantum yield, fast-folding reporter in budding yeast. This study shows that secondary RNA structure near the start codon in the ymNeonGreen ORF inhibits expression in *S. cerevisiae*. Removing secondary structure, without altering the ymNeonGreen protein sequence, led to a 2 and 4-fold increase in fluorescence when expressed in *S. cerevisiae* and *E. coli*, respectively. In *S. cerevisiae*, increased fluorescence was seen with strong and weak promoters and led to higher transcript levels suggesting greater transcript stability and improved expression in the absence of stable secondary RNA structure near the start codon.

1. Introduction

Fluorescent reporter proteins such as the green fluorescent protein (GFP), originally identified from jellyfish, *Aequorea victoria*, have been improved upon over the last two decades making them very useful as markers for gene expression [1,2]. Among the properties improved are, generating a single emission wavelength, increased brightness, shifted emission wavelength, increased speed of folding, codon adaptation for improved expression, and monomerization (Table 1).

While GFP based on *A. victoria* has been vastly improved, relatively low overall brightness and slow maturation time still limit its usefulness as a reporter for low abundance proteins or fast-changing gene expression patterns. Additional fluorescent proteins (FPs) have been isolated and characterized in efforts to overcome these issues. One of these newer FPs, LanYFP, is a tetrameric yellow fluorescent protein from *Branchistoma lanceolatum* that is 4-fold brighter than enhanced GFP (EGFP). mNeonGreen, a monomerized GFP based on LanYFP, is 2.7-fold brighter than EGFP with an estimated maturation time of less than 10 min [7]. Consistent with this report, mNeonGreen protein maturation time measured in *Drosophila melanogaster* was about 7 min [10]. Faster folding of this fluorescent protein is expected to increase the temporal resolution of fast-changing gene expression systems. mNeonGreen has also been shown to express well in mammalian cells and localize correctly when expressed as C- or N-terminal fusions. Expression of mNeonGreen in *Caenorhabditis elegans* showed 3–5 fold more fluorescence than GFP optimized for expression in *C. elegans* [11] and a codon-optimized form of mNeonGreen based on human codon usage was 1.4 fold brighter in mammalian cells than the non-optimized mNeonGreen [12]. mNeonGreen expression was also characterized in budding yeast, *Saccharomyces cerevisiae*, against 26 other fluorescent proteins. Among GFPs, mNeonGreen was identified as the brightest when normalized to an equimolar control FP [8]. However, overall expression of mNeonGreen in *S. cerevisiae* was low, although the codon-optimized mNeonGreen (ymNeonGreen WT) was able to increase fluorescence by ~25% [8]. Additional studies of the ymNeonGreen WT variant also showed *in vivo* brightness to be much lower than expected based on its *in vitro* properties [13] and *in vivo* fluorescence when expressed in additional cell lines and microorganisms. A possible

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Fluorescent reporter property definitions: Excitation peak (λex), emission wavelength (λem), extinction coefficient (ε), quantum yield (QY). Brightness values represent the product of QY and ε.

Table 1
Properties of fluorescent reporter proteins.

| Reporter    | λex (nm) | λem (nm) | ε (mM⁻¹·cm⁻¹) | QY | Brightness | Association State | Maturation (min) | Reference |
|-------------|----------|----------|----------------|----|------------|-------------------|------------------|-----------|
| avGFP       | 488      | 507      | 55.9           | 0.60 | 33.6       | Weak dimer        | 25               | [3]       |
| EGFP        | 488      | 507      | 55.9           | 0.60 | 33.6       | monomer           | 25               | [4]       |
| mEGFP       | 501      | 511      | 89.4           | 0.39 | 34.9       | Weak dimer        | 25               | [5]       |
| GFPmut3     | 501      | 511      | 89.4           | 0.39 | 34.9       | Weak dimer        | 25               | [4]       |
| yEGFP       | 501      | 511      | 89.4           | 0.39 | 34.9       | Weak dimer        | 25               | [4]       |
| LanYFP      | 513      | 524      | 150            | 0.95 | 142.5      | Tetramer          | ND               | [7]       |
| mNeonGreen  | 506      | 517      | 116            | 0.80 | 92.8       | Monomer           | <10              | [7]       |
| ymNeonGreen | 506      | 517      | 116            | 0.80 | 92.8       | Monomer           | <10              | [8]       |

Table 2
Plasmids used in this study.

| Plasmid | Description | Source |
|---------|-------------|--------|
| pRS414  | pBluescript II SK +, TRP1, CEN6, ARSH4 | [23]   |
| pRH164  | pRS414 + P lacT7 - MCS - T lacT7 | [24]   |
| pRH908  | pRS414 + P lacT7 - ymNeonGreen WT - T lacT7 | This work |
| pRH922  | pRS414 + P lacT7 - yEGFP3 - T lacT7 | This work |
| pRH927  | pRS414 + P lac – ymNeonGreen WT - T lacT7 | This work |
| pRH929  | pRS414 + P lac – ymNeonGreen WT - T lacT7 | This work |
| pRH930  | pRS414 + P lac – ymNeonGreen WT - T lacT7 | This work |
| pRH932  | pRS414 + P CYCI - ymNeonGreen WT - T lacT7 | This work |
| pRH933  | pRS414 + P CYCI - ymNeonGreen WT - T lacT7 | This work |

a The HXT7 promoter (P lacT7) used in this work refers to the truncated, constitutive promoter, containing 390 nucleotides upstream of the HXT7 ORF [25]. RM designation refers to a ymNeonGreen variant with the same amino acid sequence as wild-type ymNeonGreen, but RNA modified (RM) to reduce mRNA structure from nucleotide 1 to 50 of the open reading frame.

b Media preparation, cell growth, transformation, and statistical analyses were performed as previously described [22]. All plasmids and microorganisms used in this study are listed in Tables 2 and 3, respectively.

2. Materials and methods

2.1. Strains, media, statistics, and general methods

Media preparation, cell growth, transformation, and statistical analyses were performed as previously described [22]. All plasmids and microorganisms used in this study are listed in Tables 2 and 3, respectively.

2.2. Plasmid construction

Plasmids pRH908, pRH922, pRH927, pRH929, pRH930, pRH932, and pRH933 were constructed using pRH164 as the parent. Plasmid pRH164 was digested with BsaHII and the NEBuilder HiFi DNA Assembly kit (NEB; Ipswich, MA, USA) was used to join DNA fragments to the vector. Yeast recombinational repair was used to repair the digested vector. Yeast recombinational repair was used to repair the digestion plasmid using the co-transformed DNA fragment as a template. Two hundred nucleotides of homologous sequence on the ends of the DNA fragment were correct prior to transformation into cells for fluorescence analysis. All plasmids were sequence-verified to confirm the inserted DNA fragments were correct prior to transformation into cells for fluorescence analysis.

Table 3
Microorganisms used in this study.

| Strain | Source | Genotype (description) |
|--------|--------|------------------------|
| YRH1879 | NEB | E. coli fhuA2 [pagI-lacZ] U169 phoA glnV44 b80O(lacZ) M15 gyrB96 recA1 relA1 mfdA1 thi-1 hsdR17 |
| YRH1900 | CEN | S. cerevisiae MATa ura3–52 trp1–289 leu2–3,112 his3Δ1 |
| YRH1902 | CEN | pRH929 (pRS414 + P lacT7 – ymNeonGreen WT – T lacT7) |
| YRH1903 | CEN | pRH933 (pRS414 + P CYCI – ymNeonGreen WT – T lacT7) |
| YRH1906 | YRH1879 | This work |
| YRH1908 | CEN | pRH922 (pRS414 + P lacT7 – yEGFP3 – T lacT7) |
| YRH1909 | YRH1907 | This work |
| YRH1910 | CEN | pRH927 (pRS414 + P lacT7 – ymNEONGreen WT – T lacT7) |
| YRH1911 | CEN | pRH930 (pRS414 + P lac – ymNEONGreen WT – T lacT7) |
| YRH1912 | CEN | pRH929 (pRS414 + P CYCI – ymNeonGreen WT – T lacT7) |
| YRH1913 | CEN | pRH933 (pRS414 + P CYCI – ymNeonGreen WT – T lacT7) |

C. elegans used in this study are listed in Tables 2 and 3, respectively.

Table 4
In vivo fluorescence analysis of reporter proteins.

| Reporter    | λex (nm) | λem (nm) | ε (mM⁻¹·cm⁻¹) | QY | Brightness | Association State | Maturation (min) | Reference |
|-------------|----------|----------|----------------|----|------------|-------------------|------------------|-----------|
| avGFP       | 488      | 507      | 55.9           | 0.60 | 33.6       | Weak dimer        | 25               | [3]       |
| EGFP        | 488      | 507      | 55.9           | 0.60 | 33.6       | monomer           | 25               | [4]       |
| mEGFP       | 501      | 511      | 89.4           | 0.39 | 34.9       | Weak dimer        | 25               | [5]       |
| GFPmut3     | 501      | 511      | 89.4           | 0.39 | 34.9       | Weak dimer        | 25               | [4]       |
| yEGFP       | 501      | 511      | 89.4           | 0.39 | 34.9       | Weak dimer        | 25               | [4]       |
| LanYFP      | 513      | 524      | 150            | 0.95 | 142.5      | Tetramer          | ND               | [7]       |
| mNeonGreen  | 506      | 517      | 116            | 0.80 | 92.8       | Monomer           | <10              | [7]       |
| ymNeonGreen | 506      | 517      | 116            | 0.80 | 92.8       | Monomer           | <10              | [8]       |

Fluorescent reporter property definitions: Excitation peak (λex), emission wavelength (λem), extinction coefficient (ε), quantum yield (QY). Brightness values represent the product of QY and ε.
2.3. Codon bias and RNA folding

The codon adaptive index (CAI) calculator ([https://www.genscript.com/tools/rare-codon-analysis](https://www.genscript.com/tools/rare-codon-analysis)) was used to determine the CAI for each ymNeonGreen variant in *E. coli* vs. *S. cerevisiae*. CAI calculations were performed using the first 20 codons, of which 9 codons were changed to reduce secondary structure. RNA structure images and statistics were calculated using default settings for the mfold web server [27], [http://www.unafold.org/mfold/applications/rna-folding-form-v2.php](http://www.unafold.org/mfold/applications/rna-folding-form-v2.php). To be consistent with calculated folding energies reported in several previous studies of secondary RNA structure in *S. cerevisiae*, the default temperature of 37 °C was used to calculate maximum folding stability (ΔG, Gibbs free energy change for folding, kcal/mol). Lower ΔG values indicate increased stability of the secondary structure. Where multiple RNA structures were possible, free energies reported are for the most stable structure.

2.4. ymNeonGreen fluorescence detection

Yeast strains were diluted in 3 mL of SC-trp with 20 g/L glucose to an optical density (OD600) of 0.01. All cultures were grown overnight in triplicate at 30 °C in a rotating drum at 80 rpm to an OD600 of 1. A 20 µL sample of each culture was transferred to single well of a black, clear-bottom, 96 well microplate (Corning; Corning, NY, USA) and 180 µL of fresh media was added. ymNeonGreen fluorescence and optical density were measured using a Synergy HTX Multi-Mode microplate reader (BioTek; Winsooki, Vermont, USA). Fluorescence was measured using a 485/20 nm filter for excitation and a 580/20 nm filter for emission. Measurements were read from the top with a read height of 1 mm and gain set at 50. Fluorescence data were background-corrected and normalized to background-corrected OD600 (FLU/OD). Cells from remaining culture were collected for RT-PCR by centrifugation for 5 min at 5000 rpm and resuspended in 0.5 mL of RNALater™ (Ambion; Austin, TX, USA).

Bacterial strains were grown overnight at 37 °C in a shaking incubator in 1 mL of LB medium plus ampicillin (LBAmp). The overnight culture was diluted into 200 µL of fresh 37 °C LBamp medium to an optical density (OD900) of 0.05. Quadruplicate 200 µL cultures were incubated at 37 °C in a black, clear-bottom, 96 well microplate for 4 h at 150 rpm. Fluorescence was measured as described above.

2.5. Quantitative RT-PCR

Cells were grown and collected by centrifugation as described above. Yeast cells were resuspended in 0.5 mL of RNALater™ and incubated at room temperature for 1 hour. Cells were collected again by centrifugation, supernatant removed, and stored at −80 °C. Total RNA from triplicate cultures was prepared using an RNAsiy mini spin column (Qiagen; Hilden, Germany) and DNase treated with TURBO DNA-free (Ambion; Austin, TX, USA). Total RNA concentration was measured using a spectrophotometer (Nanodrop ND-1000, Thermo Scientific; Waltham, MA, USA) and diluted in water to 10 ng/µL. Three technical replicate RT-PCR reactions were performed from each of three biological replicates using a Rotor-Gene real-time PCR cycler (Qiagen), a QuantiTNova SYBR® green RT-PCR kit (Qiagen), and 20 ng of total RNA. Primers used for ymNeonGreen mRNA measurement were (NG-F, 5′-TCGCTAAACC-TATGGCCTGCT-3′) and (NG-R, 5′-GAGCCCTTTGCACTTCTT-3′). Primers used for ACT1 mRNA measurement were (ACT1-F, 5′-GCTTCTACGCGTTTCCATCGA-3′) and (ACT1-R, 5′-GGGACACACTGATTTCTACAAA-3′). Primer efficiencies were determined using a serial, 2-fold dilution of RNA and each primer set was determined to be 100% efficient. ymNeonGreen measurements were normalized to ACT1 mRNA using the 2−ΔΔCt method [28].

3. Results and discussion

3.1. ymNeonGreenRM variant with reduced secondary RNA structure

Natural 5′ untranslated regions (UTRs) from *Saccharomyces cerevisiae* genes have evolved to be rich in adenine nucleotides and weakly-folded with an average ΔG of −4.3 kcal/mol [29–32]. Several studies in *S. cerevisiae* show that stable secondary structure in the 5′ UTR, and specifically near the start codon, is detrimental to protein expression, mainly due to inhibition of translation [20,21,33–35]. In trying to understand why mNeonGreen and the codon optimized variant ymNeonGreenWT expressed poorly in *S. cerevisiae* compared to its expression in other organisms, we looked for secondary RNA structure near the start codon. Yeast promoters used in previous studies (PPEK[1]), as well as the two yeast promoters used in this study (PACT7 and P_CYC2), had secondary structure present at nucleotides −50 to −1 with folding energies of −5.10, −5.20, and −5.20 kcal/mol, respectively (Supplemental Fig. 1.). These ΔG values are in the natural range of secondary structure stability for yeast promoters and are not likely to result in poor translation. Both mNeonGreen and ymNeonGreenWT form hairpin RNA structures with folding energies of −15.10 and −14.50 kcal/mol, respectively (Fig. 1.). Protein translation in *S. cerevisiae* is sensitive to the presence of secondary structure and has been shown to be inhibited by RNA structures with folding free energies of approximately −10 kcal/mol [18–21]. To determine if the secondary structure in the ymNeonGreenWT mRNA was inhibiting expression, we changed the nucleotide sequence to reduce the secondary structure to −4.40 kcal/mol, which is comparable to natural *S. cerevisiae* 5′ UTRs (Fig. 1.).

In changing the nucleotide sequence, we kept the protein sequence identical to that of ymNeonGreenWT so differences in fluorescence could not be attributed to changes in quantum yield or protein stability. Eleven nucleotides, corresponding to 9 codons, were changed to make the RNA-modified (RM) variant, ymNeonGreenRM. Most of the nucleotide changes were in the wobble base of the codon (Fig. 2). Approximately 63% of the mRNA sequence downstream of the start codon (nucleotides +1 to 51) of the ymNeonGreenWT ORF is part of secondary structure with a ΔG = −14.50 kcal/mol. For the ymNeonGreenRM variant, only 27% of the downstream sequence is double-stranded. Additionally, the secondary structure (ΔG = −4.40 kcal/mol) is split between two separate small RNA hairpins that do not include the start codon, instead of one large RNA hairpin including part of the start codon for ymNeonGreenWT (Fig. 1.).

3.2. Expression of ymNeonGreen variants in *Escherichia coli*

Translation in prokaryotes initiates internally, with the ribosome binding site encompassing a 30-nucleotide window around the start codon. Translation initiation is inhibited by the presence of stable secondary RNA structure in this region ([15], and reviewed in [36]). Most of the mRNA sequence in the ribosome binding site is involved in secondary structure for ymNeonGreenWT. To determine if this structure inhibits expression in *E. coli*, both ymNeonGreen variants were placed immediately downstream of the lac promoter that is present in the pRS series of shuttle vectors [23]. *E. coli* cells containing both ymNeonGreen variants showed fluorescence indicating that translation could proceed in the presence of secondary structure in the WT variant. Removal of the secondary structure resulted in a 3.8-fold increase in fluorescence (Table 4), indicating that the secondary RNA structure was negatively impacting expression in a bacterial expression system. The change in fluorescence with respect to folding energy observed (−0.4-fold change per kcal/mol difference) with ymNeonGreenRM expression is the same magnitude seen in a previous study investigating the effects of secondary mRNA structure on GFP expression in *E. coli* [15].

When changing codons to reduce RNA structure, effort was also made to substitute frequently used codons based on the *S. cerevisiae* codon usage table. As frequently used codons for the same amino acid
were used for substitution, codon bias for the RNA structure modified ymNeonGreenRM variant was not significantly different (Table 5). CAI values calculated using the first 20 codons decreased slightly, while CAI values for the entire ORF did not change (not shown). Thus, increased fluorescence is not likely due to changes in codon bias.

### 3.3. Expression of ymNeonGreen variants in S. cerevisiae

Unlike translation initiation in prokaryotes, initiation for eukaryotes begins with the 40S ribosomal subunit binding at the 5' 7mGppN cap and scanning the mRNA 5' to 3' until the start codon is reached [36]. Secondary structure in the mRNA can inhibit translation either by blocking ribosome entry at the cap, or by impeding movement of the ribosome [16]. To evaluate the effect of RNA secondary structure near the start codon of ymNeonGreenWT on expression, we measured the fluorescence from both variants in S. cerevisiae. Two different promoters were used to compare the effect of differing upstream 5’ UTR sequences. The promoters also differed in strength, to determine if RNA structure in the...
ymNeonGreen variants was more, or less, detrimental to expression when using weak promoters. The ymNeonGreen\textsubscript{FA} variant showed increased fluorescence from both a strong (\(P\textsubscript{HXT7}\)) and a weak (\(P\textsubscript{CYC1}\)) promoter compared to ymNeonGreen\textsubscript{WT} (Fig. 3). This result suggests that the strong secondary RNA structure localized 3’ of the start codon in the wild-type variant (see Fig. 1B) is the dominant structure that forms in vivo and is likely to result in reduced expression regardless of promoter used.

For comparison, fluorescence was also measured for \(S.\) cerevisiae cells expressing yEGFP3 [6], a traditional \(A.\) victoria-based enhanced GFP construct (pRH22), from the HXT7 promoter. Cells expressing yEGFP3 had fluorescence levels of 9445 ± 941 FLU/OD. This level was identical to ymNeonGreen\textsubscript{WT} fluorescence and is consistent with previous reports that ymNeonGreen\textsubscript{WT} expression in yeast does not match the increased brightness predicted based on in vitro analysis and its expression level reported in multiple other organisms [8,13]. Our results suggest that poor performance of ymNeonGreen\textsubscript{WT} in yeast is attributable to the stable secondary RNA structure near the start codon.

The first seven amino acids of ymNeonGreen are identical to the leader sequence used in yEGFP3 (MVSKEGEE), raising the possibility that yEGFP3 expression may also be limited by secondary RNA structure. The same region downstream of the yEGFP3 start codon (nucleotides 1–50) has a folding stability of −10.90 kcal/mol. The secondary structure however is split between two smaller RNA hairpins and does not include the yEGFP3 start codon (Supplemental Fig. 2). The larger of the two RNA hairpins (−7.20 kcal/mol) begins seven nucleotides 3’ of the start codon. Secondary RNA structure has been shown to inhibit translation more efficiently when close to the start codon [21]. Previous studies using the CYC1 promoter showed that a −7.6 kcal/mol RNA hairpin immediately 5’ of the CYC1 start codon could reduce expression 20-fold [18]. Given the stability and proximity of the RNA hairpin to the start codon of yEGFP3 (Supplemental Fig. 2), it may be possible to further improve yEGFP3 expression in \(S.\) cerevisiae by reducing the stability of this hairpin. As the focus of this study was on ymNeonGreen expression, we chose not to pursue these changes with yEGFP3.

### 3.4. Changes in mRNA level

Two recent genome-wide studies in \(S.\) cerevisiae showed significant positive correlation (\(R = 0.95\) and \(R = 0.93\), respectively) between steady state mRNA and protein levels [37,38]. Also, when analyzed at genome-scale, increased stability of secondary structure in the 5’-UTR has been shown to affect mRNA degradation, with strongly-folded 5’-UTR structures correlating with shorter mRNA half-life [39]. The interconnection of translation and mRNA degradation has been well-studied in \(S.\) cerevisiae (reviewed in [40]), and the decreased mRNA half-life is suggested to result from reduced translation [33,35,39,41–44]. Poor translation leads to increased mRNA deadenylation and decapping, resulting in less mRNA, most likely due to competition between the translation initiation complex and mRNA degradation machinery [35,42,45]. Based on this hypothesis, removing strong secondary structure in the 5’ UTR leads to increased translation and decreased deadenylation and decapping, resulting in higher mRNA levels. Thus, we would expect to see an increase in mRNA level if translation was enhanced for the ymNeonGreen\textsubscript{RM} variant. Quantitative RT-PCR analysis of the WT and RM variants, each expressed from two different promoters, was performed to compare ymNeonGreen mRNA levels. As expected, mRNA levels were lower in general for the weaker \(P\textsubscript{CYC1}\) promoter. An increase in mRNA level was observed for ymNeonGreen\textsubscript{RM} compared to the ymNeonGreen\textsubscript{WT} for both promoters (Fig. 4).

Increased mRNA levels for the RM variant could result from either increased transcription of the mRNA or increased stability of the mRNA. mRNA degradation rates were not measured in this study, raising the possibility that transcription rates were higher. However, two different promoters showed an increase in mRNA level for the RM variant. Since the changes to the transcript were in the open reading frame and not in the promoter, we believe it is unlikely that the RM variant increased

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![Fig. 3](image_url)  
**Fig. 3.** Expression of ymNeonGreen variants in \(S.\) cerevisiae. ymNeonGreen expression in reported in fluorescence units per OD (FLU/OD). Fluorescence of cells expressing each ymNeonGreen variant was corrected for background fluorescence and normalized to culture density (OD). Data shown are the average expression for three biological replicates. Error bars represent the standard deviation.

![Fig. 4](image_url)  
**Fig. 4.** Quantitative RT-PCR analysis for ymNeonGreen variants. Primers for ymNeonGreen amplified a region near the 3’ end of the ORF and were the same for both variants. mRNA concentrations for ymNeonGreen variants were normalized to ACT1 mRNA levels using the \(2^{\Delta\DeltaCT}\) method and are shown relative to the strain expressing ymNeonGreen\textsubscript{WT} from the HXT7 promoter. Data shown represents the average mRNA level for three biological replicates. Error bars represent the standard deviation.
transcription from both promoters. A more likely scenario is that the changes made to the RNA structure increased mRNA stability leading to its increased abundance. As numerous studies show that translation and mRNA stability are intrinsically linked in S. cerevisiae (reviewed in [40]), we believe the increase in mRNA level observed is the result of this interplay between mRNA degradation and translation.

3.5. Comparison of strong and weak promoters

Both strong (P\textsubscript{HXT7}) and weak (P\textsubscript{CYC1}) promoters were used in this study to determine if secondary RNA structure affected the promoters differently. With both promoters, ymNeonGreen fluorescence was increased by the removal of secondary RNA structure (see Fig. 3). Fluorescence increased 2-fold when the HXT7 promoter was used, compared to 2.3-fold when the CYC1 promoter was used suggesting that secondary RNA structure may be more detrimental when a weak promoter is used. This result could stem from less frequent translation initiation from the weak promoter and/or decreased ribosome density. Both events would allow more time for formation of inhibitory secondary RNA structures from weak promoters.

It is also possible that translation capacity reaches a maximum for strong promoters and additional mRNA, while leading to increased protein levels, does not lead to more protein per mRNA. In two recent studies that show a genome-wide correlation between mRNA and protein level [37,38], expression appeared to saturate for genes with the highest mRNA levels. Saturation of expression for high abundance mRNAs was also observed in several studies investigating the effect of promoter:terminator combinations [46,47]. These studies showed that the effect of expression-enhancing terminator elements where greatest when using lower expression promoters. Conversely, the effect was minimized significantly for the highest expressing variants. Another study looking at the correlation between protein and mRNA abundance in yeast showed that protein/mRNA ratios were highly variable for low abundance mRNAs, but in general decreased with increased mRNA abundance [48]. We also measured protein/mRNA for our study by calculating the relative fluorescence normalized to relative mRNA concentration (FLU/mRNA, Fig. 5). FLU/mRNA values did not change between the two ymNeonGreen variants when using the stronger HXT7 promoter. The HXT7 promoter used in this study is a highly active, constitutive promoter, that was shown to be over 8-fold more active than several other glycolytic promoters [25]. We did see an increase in mRNA level for the RM variant but did not see a corresponding increase in fluorescence per mRNA. These data are consistent with saturation of expression for abundant mRNAs that has been observed in previous studies. In contrast, when using the weaker CYC1 promoter, we observed that removing secondary RNA structure increased fluorescence per mRNA by 40%. The increase in fluorescence per mRNA from the CYC1 promoter suggests that weak promoters are more susceptible than strong promoters to inhibition of expression by secondary RNA structure. While reducing RNA structure near the start codon was shown to increase expression from both promoters used in this study, additional investigation of mRNA decay rates and ribosome loading densities for the ymNeonGreen variants will provide further understanding of how expression was increased.

4. Conclusions

In summary, this study shows that ymNeonGreen\textsubscript{WT} expression in the budding yeast S. cerevisiae is limited by stable secondary RNA structure (\(\Delta G = -14.50\) kcal/mol) immediately downstream of the start codon. Eliminating the RNA hairpin, while keeping the ymNeonGreen protein sequence the same, increased fluorescence of ymNeonGreen\textsubscript{WT} in both S. cerevisiae and in E. coli approximately 2-fold and 4-fold, respectively. The increase in fluorescence was seen for both weak and strong promoters in S. cerevisiae, further suggesting that changes made to remove RNA secondary structure immediately downstream of the ymNeonGreen\textsubscript{WT} start codon were responsible for the increase observed. Based on these results, we anticipate increased expression of ymNeonGreen\textsubscript{RM} to be seen regardless of S. cerevisiae promoter used. These results also highlight the importance of secondary RNA structure not only in the 5′-UTR, but also immediately 3′ of the start codon, when designing genes and reporter constructs for expression in S. cerevisiae.

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Author contributions

R.E.H. conceived the research, conducted experiments, and prepared the manuscript. J.A.M. and N.N.N contributed to experiment design, data analysis, and assisted in manuscript preparation. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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