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Characterizing IGR IRES-mediated translation initiation for use in yeast cell-free protein synthesis

Research Paper

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Eukaryotic cell-free protein synthesis (CFPS) systems are limited, in part, by inefficient translation initiation. Here, we report three internal ribosome entry site (IRES) sequences from the *Dicistroviridae* family that are highly active in yeast CFPS. These include the intergenic region (IGR) IRES from cricket paralysis virus (CrPV), plautia stali intestine virus (PSIV) and *Solenopsis invicta* virus 1 (SINV1). Optimization of combined transcription and translation (Tx/Tl) CFPS reactions primed with linear DNA containing the CrPV IGR IRES resulted in batch synthesis yields of 0.92 ± 0.17 μg/mL luciferase. Further template engineering, such as including the first 12 nt of native CrPV gene, increased yields to 2.33 ± 0.11 μg/mL. We next observed that the inclusion of a 50 nt poly(A) to the 3′ end of the IGR IRES-mediated message increased yields an additional 81% to 4.33 ± 0.37 μg/mL, without any effect on mRNA stability or copy number. This was surprising because the CrPV IGR IRES requires no known translation initiation factors. Lastly, we investigated a method to inhibit background expression through competitive inhibition by supplying the reaction with 5′ cap structure analog. This study highlights the crucial role translation initiation plays in yeast CFPS and offers a simple platform to study IRES sequences.

Introduction

Cell-free protein synthesis (CFPS) is a rapidly growing field that complements *in vivo* approaches [1,2]. Advantages of CFPS include direct access to and control of the reaction environment, as well as rapid process and product development pipelines for high-throughput protein expression [1,2]. Yeast is a particularly attractive candidate for eukaryotic CFPS because of its simple methods for cultivating cells and extract preparation and its widespread use as a model organism [3,4]. However, yeast CFPS platforms remain limited by low expression yields [1–5].

In eukaryotic protein expression platforms, translation initiation is often considered the rate-liming step in protein synthesis [6]. One strategy used to successfully improve protein synthesis in eukaryotic CFPS reaction platforms is to leverage internal ribosome entry sites (IRESs) that are strong inducers of translation initiation [7,8]. IRESs are a special class of RNA sequences found in the 5′ untranslated region (UTR) of mRNA that form complex secondary structure used for translation initiation [9,10]. Because they can initiate translation in a cap-independent fashion by binding to the ribosome, IRESs are attractive for combined transcription and translation (Tx/Tl) CFPS reactions [2]. IRES sequences are present in many native organism genes used when the cell switches to cap-independent expression during a stress response [11]. In addition, virus genes use IRES sequences effectively to hijack the translation machinery of the cell and are considerably better at initiating translation compared to their cellular IRES counterparts as a result of evolutionary pressure [9,10].

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Viral IRES can be grouped into four classes based on their secondary structure, translation initiation factor requirement, and whether or not the ribosome locates the AUG start codon through scanning or by directly binding the IRES adjacent to the start codon (Table 1) [10]. Type I and Type II IRES include *Picornavirus* such as poliovirus (PV) and encephalomyocarditis virus (EMCV), respectively, and require several eukaryotic initiation factors (eIF) to initiate translation, but differ with respect to how the ribosome locates the start codon. Type III IRES include *Flavivirus* such as hepatitic C virus (HCV), and only require eIF2 and eIF3 for initiation. Type IV IRES include *Dicistrovirus* intergenic region (IGR) IRES from cricket paralysis virus (CrPV) and plautia stali intestine virus (PSIV), and are unique to the extent that they do not require any initiation factors to initiate translation [12]. Each of these IRES sequences forms complex secondary structure that binds the ribosome and (in most cases) translation initiation factors [13–16].

With respect to eukaryotic CFPS reactions, the yeast [2] and wheat germ [17] platforms typically use the non-IRES cap-independent translation enhancer Ω sequence from the tobacco mosaic virus (TMV) to initiate translation, whereas rabbit reticulocyte lysate (RRL) has optimal expression initiated by the EMCV IRES [8]. In addition, CrPV IGR IRES can be used effectively in CFPS reactions with extract prepared from the insect cell line Sf21, CHO cells and human K562 cells [7,18]. The CrPV IGR IRES is a particularly attractive option for eukaryotic CFPS because no translation factors are required for initiation (Table 1) [7]. Therefore, CrPV IGR IRES initiation bypasses regulatory effects and diffusion limitations of initiation factors. By contrast, the TMV Ω sequence is thought to have overlapping function with the 5′ cap and requires the eIF4F complex and remaining cap-dependent translation initiation factors to initiate translation [19].

The goal of this work was to evaluate IRES-mediated translation in yeast Tx/Tl reactions in an effort to assess their ability to activate highly robust and efficient protein synthesis. However, of the four types of IRES discussed, only IRES from Type IV have been used to activate translation initiation in yeast *in vivo* [20,21]. The commonly used IRES from Types I–III such as PV, EMCV and HCV do not function in *Saccharomyces cerevisiae* [21]. Although the reason for this is not completely resolved, HCV is thought to be inhibited by a small inhibitor RNA sequence that sequesters factors needed for IRES-mediated translation in yeast [22]. Further possibilities include a lack of IRES *trans*-acting factors (ITAFs) in *S. cerevisiae* [23] or differences in ribosomal and initiation factor structure across kingdoms [24].

Because only Type IV IGR IRES sequences have been observed to work in yeast *in vivo*, we focused our efforts on these sequences. We observed that the three IGR IRES sequences tested yielded similar levels of synthesized protein. We specifically optimized reactions with CrPV IGR IRES-mediated translation initiation to improve protein synthesis yields (on a μg/mL basis). Next, we investigated template engineering strategies, such as inclusion of native codons from the CrPV genome at the 5′ end of the translated region of the mRNA and a poly(A) tail at the 3′ end of the mRNA, which led to a nearly fivefold increase in protein expression. Lastly, given the fact we had activated a fully orthogonal cap-independent translation initiation pathway (i.e. without any eIF requirement), we investigated methods to inhibit background expression of endogenous mRNA.

Materials and methods

Template construction

Ω-Mediated linear DNA was amplified as described previously [5]. IRES-mediated linear DNA was amplified via overlap PCR. Gene blocks containing IRES sequences were ordered from Integrated DNA Technologies (IDT, Coralville, IA). Nucleotide (nt) sequences and gene blocks of each IGR IRES evaluated can be found in Supplementary Table 1. A 32 nt sequence was added upstream of the IRES containing a 10 nt GC clamp and spacer followed by the T7 RNA polymerase promoter sequence. The luciferase reporter gene was amplified from either the ATG start codon or the second naturally translated codon (GAA) with the appropriate IRES overlap sequence. Overlap PCR reactions were used to combine and amplify the IRES gene block and reporter gene. Alterations to the final PCR construct were guided by adjusting the flanking sequences of primers used amplify the IRES and reporter gene accordingly. Primer sequences used in this study can be found in Supplementary Table 2.

Crude extract preparation and cell-free protein synthesis

Methods for crude extract preparation from *S. cerevisiae* S288c were identical to the methods described previously [4]. CFPS reactions and autoradiography protocols were identical to the methods described previously [3]. ^{3}H-UTP incorporation assays were identical to the methods described previously [5]. Where described, the reaction was supplied with 1.5 mM G(5′)ppp(5′)G RNA Cap Structure Analog (cap analog) from New England Biolabs (Ipswich, MA).

Results

IGR IRES expression in yeast CFPS

We began our investigation by assessing three types of Type IV IGR IRESs that had been previously used to initiate translation in *S. cerevisiae in vivo*: CrPV, PSIV and *Solenopsis invicta* Virus 1 (SINV1) [20]. Specifically, we carried out 15 μL batch Tx/Tl reactions for

TABLE 1

IRES types and their translation initiation requirements [10,12]

Type	Viral IGR IRES	eIFs required	AUG scanning or direct binding
Type I	Poliovirus (PV)	eIF4G, eIF4A, eIF3 and eIF2	Scanning
Type II	Encephalomyocarditis virus (EMCV)	eIF4G, eIF4A, eIF3 and eIF2	Direct binding
Type III	Hepatitic C virus (HCV)	eIF3 and eIF2	Direct binding
Type IV	Cricket paralysis virus (CrPV)	None	Direct binding

four hours at 21°C and 30°C and monitored the formation of luciferase, which was used as model reporter protein. We evaluated two different temperatures because IGR IRES-mediated translation is sensitive to the secondary structure of the RNA, which is affected by temperature [20]. All three IGR IRES sequences with the canonical ATG start codon of luciferase were able to initiate translation in yeast CFPS reactions (Fig. 1). Furthermore, we found enhanced expression for each of the constructs at 30°C (Fig. 1).

Next, we assayed combined Tx/Tl reactions by initiating translation with the first translated codon from the native IGR IRES genome, instead of the canonical ATG start codon. This was important to test because it has been previously reported that translation is improved by initiating translation with the first translated codon from the parent virus gene [7]. In our experiments, we found similar expression among the different constructs (Fig. 1). Although translation was not specifically improved by altering the first translated codon, our results confirmed that translation is initiating through the cap-independent IRES-specific mechanism because cap-dependent translation requires the canonical ATG start codon [20].

We next sought to improve combined Tx/Tl from linear DNA templates equipped with IGR IRES leader sequences (Supplementary Figure 1). We chose to move forward with the CrPV(GCT) leader sequence because all templates performed equally well and the CrPV IGR IRES is most commonly used [7,20,25]. Specifically, we carried out a series of optimization experiments to explore the effects of temperature, magnesium glutamate, potassium glutamate, EGTA, glycerol, creatine phosphate, putrescine, spermidine and amino acids on batch Tx/Tl reactions. As a result of our comprehensive optimization, putrescine was removed from the reaction, glycerol was lowered from 11% to 4% (v/v), and temperature was set to 27°C (Supplementary Figure 1). The final optimized reaction conditions

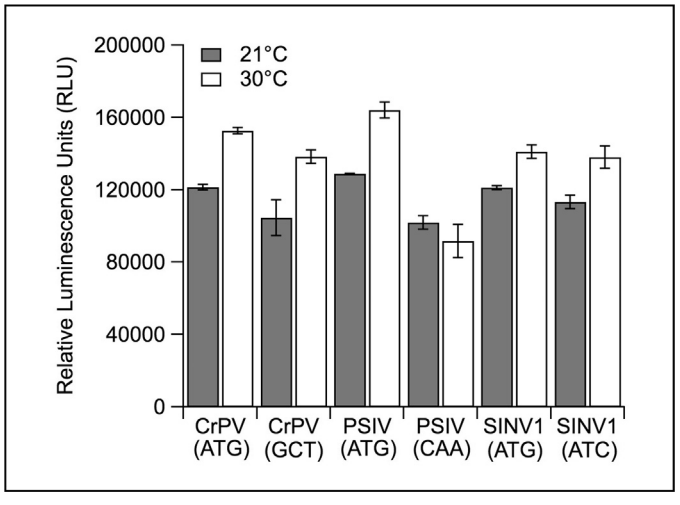

FIGURE 1

IGR IRES expression in yeast CFPS reactions. Fifteen μL batch Tx/Tl reactions were prepared in separate 1.5 mL tubes and incubated for four hours at 21°C (gray column) or 30°C (white column). Active luciferase synthesis is shown at the end of the batch reaction and quantified via relative luminescence units (RLU). Two construct variants were tested for each IGR IRES sequence, including either the canonical ATG sequence as the first translated codon or the first translated codon from the native viral structural gene including GCT for CrPV, CAA for PSIV, and ATC for SINV1. Values display means with error bars representing the standard deviation of three independent experiments.

led to a threefold increase in protein synthesis yields from 0.32 ± 0.02 to 0.92 ± 0.17 μg/mL active luciferase (Supplementary Table 3).

CrPV IGR IRES template considerations

When expressed naturally *in vivo*, the CrPV IGR IRES is located between two genes that are expressed in tandem (Fig. 2a) [20]. Because there is a naturally occurring upstream sequence (US) of the CrPV IGR IRES, we investigated the effect of including 0–40 nt of this sequence in the DNA template (Fig. 2b). We observed that the inclusion of 10–40 nt upstream of the IGR IRES sequence abolished its activity and this strategy was not pursued further.

While the upstream IGR IRES sequence did not enhance translation, we turned our attention to the first 12 nt of the natural structural gene that directly follows the IGR IRES (Fig. 2a). Previously, the first 6–11 nt of the structural gene have been shown to be important for IRES function [26]. Therefore, we generated PCR fragments that contained additional nt present in the CrPV structural gene and added them directly upstream of the luciferase gene. Additional nt were added in triplicate in the form of structural gene codons (SC) in order to preserve the correct translation frame. In Fig. 2c, we observed a 154% increase in luciferase synthesis yield from 0.92 ± 0.17 to 2.33 ± 0.11 μg/mL when including the first 12 nt of the native CrPV structural gene.

Poly(A) tail effects on CrPV IGR IRES-mediated translation

Up to this point, all experiments included only a T7 terminator sequence downstream of the stop codon at the 3′ end of the gene. However, translation of yeast transcripts is known to be strongly dependent on the inclusion of a poly(A) tail [27,28]. Therefore, we compared the optimized CrPV IGR IRES linear DNA template with and without the addition of a 50 nt poly(A) tail, which we have previously shown to be optimal in yeast combined Tx/Tl [2]. The addition of the poly(A) tail resulted in an 81% increase in luciferase synthesis yields up to 4.33 ± 0.37 μg/mL (Fig. 3a). Although no translation initiation factors are required for CrPV IGR IRES-mediated translation initiation (Table 1), we hypothesized that the addition of the poly(A) tail may (i) assist in competing with endogenous mRNA for the translation apparatus (a known issue for yeast CFPS reactions [3,27]), (ii) assist in mRNA stability or (iii) assist in ribosomal recruitment to the mRNA via translation initiation factors, thus allowing more efficient translation initiation.

To test these hypotheses, we first treated the extract with Micrococcal Nuclease (MNase) to remove endogenous mRNA in the lysate. Endogenous mRNA from the cell lysate competes with mRNA encoding the target protein of interest for the translational machinery [3]. If the poly(A) tail's main effect was due to helping the desired template compete with endogenous mRNAs, we expected to observe that protein synthesis from templates with or without the poly(A) tail would be similar in lysates treated with MNase. By contrast, we observed the opposite effect. The benefit in protein synthesis realized with the inclusion of a poly(A) tail was maintained regardless of MNase treatment (Fig. 3a). This result suggested that the benefit observed from the poly(A) tail was not due to endogenous mRNA competition effects.

We next investigated the stability of the mRNA transcripts with or without the presence of the poly(A) tail (Fig. 3b). Strikingly, the mRNA profiles for both templates were identical during a

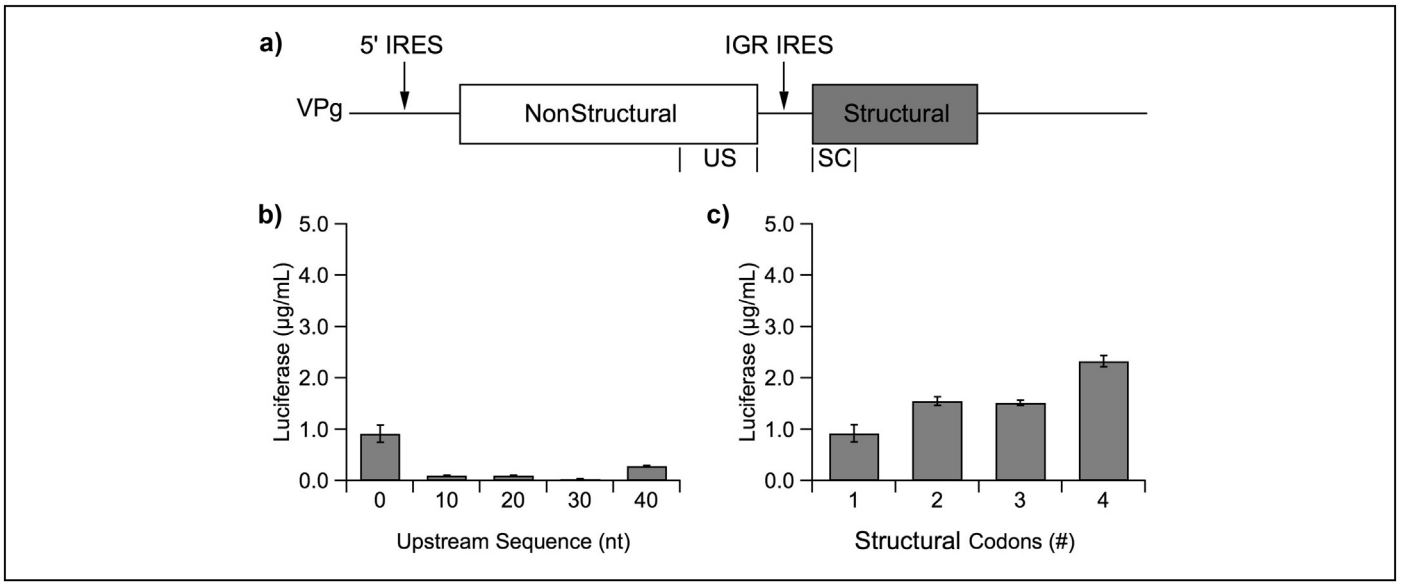

FIGURE 2

CrPV IGR IRES template considerations. **(a)** Representation of CrPV genome (not to scale) [20]. VPg = viral protein genome linked (acts as a primer during RNA synthesis), 5′ IRES = IRES sequence found at the 5′ end of the genome, IGR IRES = intergenic region IRES (used in this study), US = upstream sequence region included in linear DNA template expressed in reactions in **(b)**, and SC = structural codons included in linear DNA templates expressed in reactions in **(c)**. Tx/Tl reactions primed with DNA containing the CrPV IGR IRES sequence with variations to the flanking sequences to the IRES both (b) directly upstream containing 0–40 nt of the native nonstructural gene and (c) downstream as the first 1–4 translated codons (1–12 nt) from the native structural gene. Fifteen µL batch reactions were prepared in separate 1.5 mL tubes and incubated for four hours. Active luciferase synthesis is shown at the end of the batch reaction and quantified via relative luminescence compared to a purified recombinant luciferase standard. Values display means with error bars representing the standard deviation of three independent experiments.

four-hour batch reaction. These data suggest that the improved protein synthesis with the poly(A) tail addition was not a function of mRNA stability. We also compared protein synthesis during the course of the reaction to see if there was a difference in the duration of protein synthesis or the rate of protein synthesis (Fig. 3c). We found the reaction duration was maintained for both constructs, around three hours. However, the rate of protein synthesis differed significantly with a linear synthesis rate between 0.5 and 1.5 hours of 2.06 ± 0.01 µg/mL and 1.01 ± 0.13 µg/mL-hour, with and without the addition of the poly(A) tail, respectively.

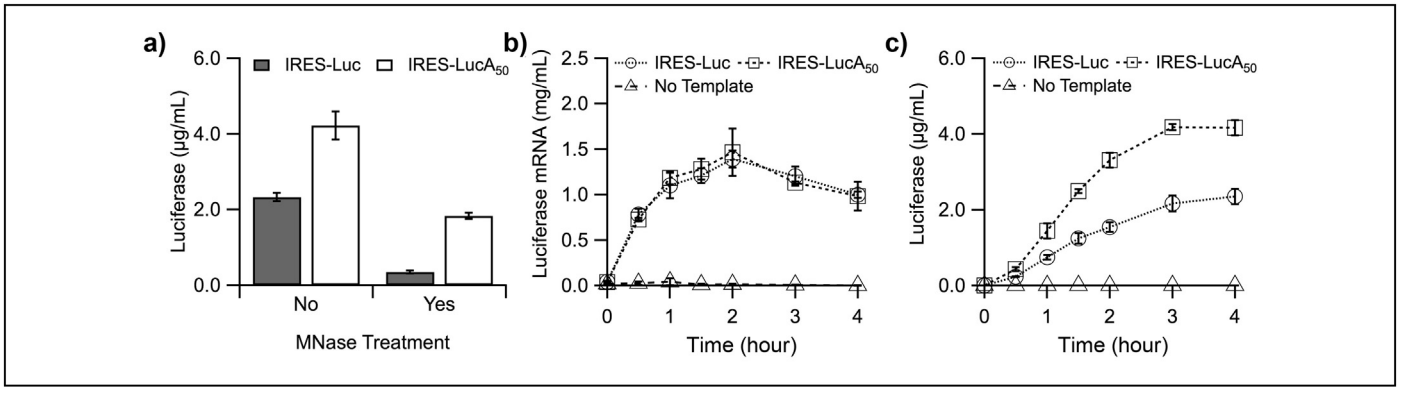

FIGURE 3

Poly(A) tail effects on CrPV IGR IRES-mediated translation in yeast CFPS reactions. **(a)** CrPV IGR IRES-mediated translation of luciferase containing 12 nt from the SC region was compared in batch yeast CFPS reactions with or without the addition of a 50 nt poly(A) tail on the 3′ end of the mRNA. Extract was pretreated with Micrococcal Nuclease (MNase) as described previously [3]. Active luciferase synthesis is shown at the end of the four-hour batch reaction and quantified via relative luminescence compared to a purified recombinant luciferase standard. **(b)** Time course analysis of luciferase mRNA during the course of a batch reaction primed using DNA with and without the addition of the 50 nt poly(A) tail. In each reaction, 15 µL batch reactions were prepared in separate 1.5 mL tubes and incubated for four hours. Luciferase mRNA was quantified via scintillation counter via ^{3}H-UTP incorporation assay [5]. **(c)** Time course analysis of protein synthesis during the course of a batch reaction with and without the addition of the poly(A) tail. CrPV IGR IRES-mediated translation of luciferase (IRES-Luc) with a T7 terminator on the 3′ end of the template is displayed as either a gray column or circle icon. CrPV IGR IRES-mediated translation of luciferase (IRES-LucA$_{50}$) with a 50 nt poly(A) tail on the 3′ end of the template is displayed as either a white column or square icon. No template added to the reaction is displayed as the triangle icon. Values display means with error bars representing the standard deviation of three independent experiments.

Endogenous mRNA translation suppression

Following demonstration of robust and efficient translation from mRNA templates harboring CrPV IGR IRES leader sequences, we next aimed to inhibit background cap-dependent translation. This was because cap-dependent translation of endogenous mRNA accounts for up to 2/3 of the total protein synthesized in yeast CFPS [3]. Endogenous mRNA is present because our yeast CFPS platform avoids the use of MNase treatment in order to improve yields (see Fig. 3, which showcases the deleterious effect of MNase treatment on reaction performance) [3]. It would be preferable to selectively activate expression of only our target protein without also translating endogenous mRNA. The CrPV IGR IRES provides and orthogonal translation initiation pathway that does not depend on the mRNA cap-binding protein eIF4E to initiate translation. Thus, to inhibit background expression, we sought to reduce cap-dependent translation of endogenous mRNA by supplying the CFPS reaction with 1.5 mM G(5′)ppp(5′)G RNA Cap Structure (cap analog). Cap analog is known to strongly inhibit cap-dependent translation at these concentrations through competitive inhibition with eIF4E [27]. The addition of cap analog inhibited Ω-mediated translation by >97% yet improved the expression of CrPV IGR IRES-Luciferase with a poly(A)$_{50}$ tail by 18% to 4.98 ± 0.16 μg/mL (Fig. 4a). There was no obvious effect when CFPS was performed with the CrPV IGR IRES-Luciferase template lacking the poly(A) tail. Previously, it has been shown that the TMV Ω sequence has overlapping function with the 5′ cap and interacts directly with the cap-binding protein eIF4E containing eIF4F complex [19]. However, more recently it has also been shown that the 5′ leader of TMV Ω sequence does not necessarily require eIF4F and can also initiate translation through a ribosome scanning mechanism [29]. Our results suggest that eIF4F was specifically required for the activity of the Ω sequence in the yeast CFPS system used here. There was no obvious effect when CFPS was performed with the CrPV IGR IRES-Luciferase template lacking the poly(A) tail.

To confirm the presence or absence of background translation, we prepared an autoradiogram of CFPS reactions after four hours of incubation supplied with DNA containing the TMV Ω sequence or CrPV IGR IRES (Fig. 4b). We observed that the addition of 1.5 mM cap analog almost completely eliminated Ω-mediated translation and background translation without the addition of a DNA template (Fig. 4b; lanes 1–2 and 7–8). By contrast, the addition of 1.5 mM cap analog had a relatively small impact on background translation with CrPV IGR IRES-mediated translation (Fig. 4b; lanes 3–6).

Discussion

Translation initiation plays a crucial role in protein synthesis, is often considered the rate limiting step, and is tightly regulated *in vivo* [6]. Here, we described and optimized methods to initiate translation in yeast combined Tx/Tl reactions that use IGR IRES sequences. IGR IRES sequences offer several advantages over the previously used TMV Ω sequence, including the removal of the requirement for translation initiation factors. As a consequence, advances made here should remove translation initiation regulatory concerns and reduce diffusion limitations associated with the dilute cell-free environment [11].

When evaluating various *Dicistrovirus* IGR IRES sequences, we found that CrPV, PSIV and SINV1 IGR IRES are all able to initiate translation in yeast CFPS reactions (Fig. 1). The CrPV IGR IRES,

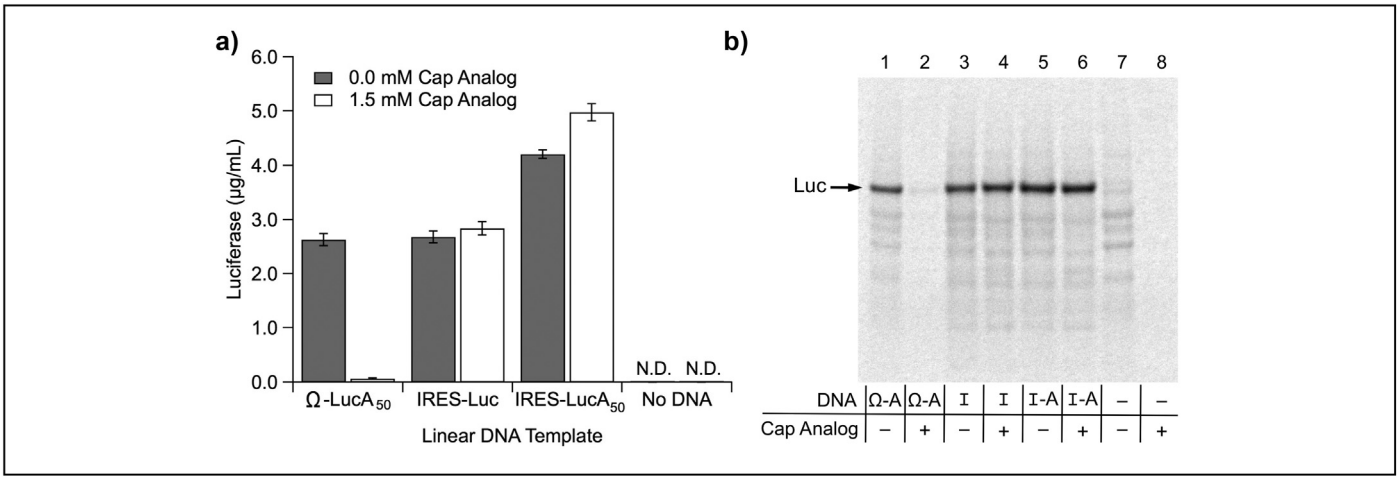

FIGURE 4

Effects of cap analog addition on Ω- and CrPV IGR IRES-mediated translation in yeast CFPS reactions. **(a)** Fifteen μL batch reactions were prepared in separate 1.5 mL tubes and incubated for four hours, with the addition of 0.75 μL ^{35}S-methionine per reaction. The DNA templates used to prime the reaction included either the TMV Ω sequence (Ω-LucA$_{50}$) or the CrPV IGR IRES sequence at the 5′ end with (IRES-LucA$_{50}$) or without (IRES-Luc) a 50 nt poly(A) tail on the 3′ end. Reactions primed in the absence of exogenous DNA are also included. Additionally, the reactions were prepared with (white columns) or without (gray columns) the addition of 1.5 mM G(5′)ppp(5′)G RNA Cap Structure (cap analog). All reactions were run under optimized conditions for IRES expression found in Supplementary Table 3. Values display means with error bars representing the standard deviation of three independent experiments. **(b)** Autoradiograph of ^{35}S-Met incorporation into luciferase following a yeast CFPS reaction primed with or without the aforementioned DNA templates and with or without the addition of 1.5 mM cap analog. 5 μL of sample was loaded onto an NuPAGE 4–12% Bis-Tris Gel following the manufacturers instructions. Lane 1: Ω-LucA$_{50}$ DNA template (Ω-A) with 0 mM cap analog, lane 2: Ω-LucA$_{50}$ DNA template (Ω-A) with 1.5 mM cap analog, lane 3: IRES-Luc DNA template (I) with 0 mM cap analog, lane 4: IRES-Luc DNA template (I) with 1.5 mM cap analog, lane 5: IRES-LucA$_{50}$ DNA template (I-A) with 0 mM cap analog, lane 6: IRES-LucA$_{50}$ DNA template (I-A) with 1.5 mM cap analog, lane 7: no DNA template with 0 mM cap analog, and lane 8: no DNA template with 1.5 mM cap analog. Luciferase (Luc; 60.7 kDa) is the dominant band found in lanes 1 and 3–6. The image was replicated two additional times with similar results.

being the most widely studied [7,20,25], was subsequently optimized for protein synthesis (Fig. 1 and Supplementary Figure 1). We observed that IGR IRES-mediated translation was sensitive to temperature and the reaction environment, probably due to a strong dependence on correct secondary structure necessary to initiation translation [20].

Because the flanking regions to the IGR IRES probably play a significant role in determining its secondary structure and activity, we evaluated 40 nt of the native sequence upstream of the IGR IRES. We observed that addition of USs to the reporter construct abolished protein synthesis activity (Fig. 2b). One possible explanation for this unanticipated result could be the addition of this sequence at the 5′ end of the IGR IRES disrupts of the IRES structure itself. It may be interesting to add the entire gene naturally present to the 5′ region and measure its effects on expression to see whether or not this trend continues. However, this was not accessible by our two-step PCR method and therefore not pursued in this study. Conversely, we evaluated the addition of up to 12 nt (4 codons) of the naturally translated CrPV structural gene to the 5′ end of the luciferase gene and saw a 154% improvement in synthesis yields over the inclusion of only 3 nt (Fig. 2c).

To further engineer the template, we investigated the addition of a 50 nt poly(A) to the 3′ end of the IGR IRES-mediated transcript. Because the CrPV IGR IRES recruits the ribosome directly and does not require initiation factors to initiate translation (Table 1), no poly(A) tail sequence is required. For example, no improvement in expression was seen in mammalian cell-free extracts when using the CrPV IGR IRES combined with a poly(A) tail [30]. By contrast, we surprisingly observed an 81% increase in CFPS yields when using the CrPV IGR IRES with a poly(A) tail as compared to the transcript with no poly(A) tail (Fig. 3a).

While it is clear that the protein synthesis yields increased with the inclusion of the poly(A) tail, the mechanism of action is still unknown. Even though a poly(A) tail is not required for CrPV IRES expression, we hypothesize that the addition of the poly(A) tail may assist in recruiting the ribosome to the mRNA through interaction with PolyA Binding Protein 1 (Pab1). This is an established mechanism for activation of yeast IRESs during a stress response [31], thus allowing more efficient translation initiation. Indeed, the time course analysis of the CFPS reaction displays an enhanced rate of protein synthesis with the addition of the poly(A) tail (Fig. 3c). If translation initiation is the rate limiting step in protein synthesis, this result would reinforce the hypothesis that the poly(A) tail enhances IGR IRES-mediated translation initiation in yeast CFPS reactions. On the basis of the previous work of Sarnow [27,28] and others that have shown yeast use the poly(A) tail to activate endogenous IRESs during stress [31], it is possible that this phenomenon is restricted to yeast-based cell-free protein synthesis reactions. This should not be considered contradictory to other published results, such as what was reported in work with mammalian cell-free systems [30], in particular due to the strong dependence between yeast translation and Pab1 in combination with the dilute cell-free reaction environment in comparison to the native cytoplasm.

By activating IGR IRES-mediated cap-independent translation, we activated an orthogonal translation initiation pathway that is not dependent on the cap-binding protein eIF4E. We thus went on to show that Ω-mediated translation is inhibited when the cap structure analog is supplied to the reaction (Fig. 4). Interestingly, our results are consistent with a previous report that suggests the TMV Ω sequence has overlapping function with the 5′ cap and interacts directly with the cap-binding protein eIF4E containing eIF4F complex [19], yet differ from a recent report suggesting an eIF4F independent mechanism is also possible [29]. It could be that there are multiple mechanisms or that our results are specific to yeast.

Although, the background translation was completely inhibited in the presence of cap analog in reactions primed with DNA containing the Ω sequence, any positive effects on synthesis of the protein of interest mediated by the CrPV IGR IRES were modest (Fig. 4a). Curiously, background translation may not be completely inhibited by the addition of cap analog when the reaction was primed with DNA containing the CrPV IGR IRES (Fig. 4b). This result was unexpected, suggesting additional complexity surrounding translation initiation that is yet unresolved. Looking forward, alternative strategies that could be implemented include pretreating the extract with decapping enzymes or protease such as 2A that cleave the eIF4E biding region from eIF4G [32].

Although yields were significantly improved for IGR IRES-mediated translation in yeast CFPS reactions over the previous state-of-the-art, Ω-mediated translation remains a superior choice using our system with yields exceeding 8 μg/mL luciferase [4]. Interestingly, the CrPV IGR IRES has been used very successfully in CFPS platforms from higher eukaryotes including insect, CHO and K562 cells [7,18]. Because the function of IRES sequences is sensitive to its secondary structure, it is possible that the biochemical interactions between the CrPV IGR IRES sequence and the ribosome is not conserved between higher eukaryotes and yeast. The crystal structure of the CrPV IGR IRES within the ribosome shows that this IRES uniquely sits inside the P-site of the ribosome, mimicking the structure of methionyl-tRNA [16]. This suggests that the evolution of the viral CrPV IGR IRES may be host-organism specific. If so, this provides an opportunity for directed evolution, such as using *in vitro* compartmentalization [33] or ribosome display [34], to significantly improve IRES activity in yeast.

Lastly, by adopting a platform that uses the CrPV IGR IRES to initiate translation, translation initiation regulation concerns, which have been shown to be active in higher eukaryotic CFPS platforms [35–38], are eliminated. We anticipate that this will facilitate more general use of yeast CFPS for systems and synthetic biology applications in years to come. Furthermore, the newly optimized platform offers improvements in sensitivity for CrPV IGR IRES-mediated translation in yeast cell-free translation reactions that can be used for future biochemical studies.

Conflict of interest statement

The authors declare no commercial or financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2014.07.001.

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