RESEARCH ARTICLE

Stop codon readthrough contexts influence reporter expression differentially depending on the presence of an IRES [version 1; peer review: 2 not approved]

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Abstract

Background: Previously we reported the discovery of stop codon readthrough in *AMD1* mRNA followed by ribosome stalling at the end of a conserved Open Reading Frame (*AMD1 tail*). To explain the severe suppression of reporters fused to *AMD1 tail* we proposed a mechanism invoking ribosome queueing. To test this hypothesis, we placed the reporter stop codon in the context of readthrough permissive sequences in a dual reporter vector with downstream reporter expression governed by EMCV IRES. In accordance with our hypothesis, we observed a striking disproportional reduction of upstream reporter activity in response to increased readthrough levels.

Methods: Here we employ dual luciferase assay and western blotting to explore the effects of *AMD1 tail* and control sequences on reporter expression in dual and monocistronic reporter vectors.

Results: With the dual reporter system, the disproportionate reduction of upstream reporter activity is not specific to *AMD1 tail* and occurs as long as the readthrough stop codon context is present at the end of the reporter's ORF. The decreased reporter activity that appears to be induced by the readthrough sequence occurs only in reporters containing EMCV IRES. Monocistronic reporters with the same readthrough context sequence exhibit only a modest reduction in reporter activity. Furthermore, in monocistronic vectors, the disproportionate reduction of reporter levels greatly increased when *AMD1 tail* was translated as a result of readthrough. Such readthrough-mediated reduction was not observed when *AMD1 tail* was substituted with unrelated sequences in agreement with our original hypothesis.

Conclusions: While our findings provide little new information

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regarding the functional role of *AMD1 tail*, they raise caution for the use of viral IRES elements in expression vectors for studying mechanisms of mRNA translation. These findings may also be pertinent to the natural properties of readthrough permissive sequences and of IRES elements, though these require a separate investigation.

Keywords

Translation control, AMD1, stop codon readthrough, IRES, OPRL1, ribosome stalling

Any reports and responses or comments on the article can be found at the end of the article.

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Introduction

Recently we discovered that a proportion of ribosomes translating the *AMD1* mRNA read through its annotated stop codon and continue translating before stalling at the end of a 125-codons-long conserved open reading frame (ORF), referred to as *AMD1 tail* (Yordanova *et al.*, 2018). Ribosome stalling at the end of *AMD1 tail* and its dependence on stop codon readthrough (RT) has been also confirmed in a more recent study (Wangen & Green, 2020). During our initial investigation of this phenomenon we found that fusing the product of *AMD1 tail* translation, i.e. *AMD1* extension, to the C-terminus of reporters leads to nearly complete disappearance of reporter activity (Yordanova *et al.*, 2018). After ruling out extracellular targeting or a protein destabilisation effect of the *AMD1* extension, we proposed a mechanism where ribosome stalling/queuing at the end of *AMD1 tail* results in inhibition of the upstream main ORF translation. A prediction of this mechanism is that increasing the readthrough efficiency at the main ORF stop codon should accelerate the *AMD1 tail* inhibitory effect by enhancing queue formation.

To test this prediction, we employed RT promoting sequences of varying efficiency from *LDHB*, *AQP4* and *OPRL1* genes (Loughran *et al.*, 2014) to titrate ribosomes translating the *AMD1 tail* (Yordanova *et al.*, 2018). By increasing the RT efficiency at a reporter's stop codon to 2.5, 6 and 17% with *LDHB*, *AQP4* and *OPRL1* contexts, respectively, we saw a disproportionately large drop of reporter levels, i.e. beyond what would be expected due to protein degradation if *AMD1* extension had a destabilization effect as proposed for other products of 3'UTRs translation (Arribere *et al.*, 2016). These experiments were performed with a bicistronic vector wherein the termination codon of the Renilla luciferase (Rluc) reporter was placed in varying RT promoting contexts upstream of *AMD1 tail*. A firefly luciferase (Fluc) reporter was expressed via an EMCV IRES (Chamond *et al.*, 2014) to monitor RNA levels.

We saw the potential of using this experimental approach of readthrough enabled ribosome titration to further explore the dynamics of *AMD1 tail* translation and that of other test sequences.

These subsequent experiments revealed unexpected effects of readthrough contexts on reporter expression observed in an EMCV IRES containing vector but not in a vector without the EMCV IRES which we describe here.

Methods

Cloning

Oligonucleotides were synthesized by IDT, Belgium. *AMD1 tail*, *ODC1* PEST and *ACTB* sequences were obtained as gBlocks from IDT. gBlock and primer sequences including those that introduce *OPRL1*, *AQP4* and *LDHB* stop codon context sequences are provided in Extended data File 1 (Yordanova *et al.*, 2020b). The amplicons were generated by standard one or multiple PCR reactions using Phusion High Fidelity DNA Polymerase (NEB) according to the manufacturer instructions. p2luc (Grentzmann *et al.*, 1998) was modified such that the

second luciferase reporter (Fluc) is expressed under the control of the EMCV IRES. Due to the presence of an XbaI restriction site in *AMD1 tail*, the first 65 nts of *AMD1 tail* were omitted for cloning in the monocistronic vector. All constructs were transformed by 90 sec heat shock at 42°C in *E. coli* strain DH5- α and were verified by Sanger sequencing at Eurofins Genomics.

Tissue culture and cell treatment

Human Embryonic Kidney 293A cells (ATCC) were maintained as monolayer cultures, grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS, 1mM L-glutamine and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂. For the dual luciferase assay 4.5×10⁶ HEK293A cells were plated on 10 cm tissue culture dishes. After 24 h the cells were detached with trypsin, suspended in fresh media and transfected in four replicates with Lipofectamine 2000 reagent (Invitrogen), using the 1-day protocol in which suspended cells are added directly to the DNA complexes in 96-well plates. For each transfection, the following was added to each well: 25 ng plasmid DNA and 0.2 μ l lipofectamine 2000 in 25 μ l OptiMem (Gibco). 2×10⁴ cells in 50 μ l DMEM, were added to the transfecting DNA complexes in each well. Transfected cells were incubated at 37°C in 5% CO₂ for 21 h and assayed using the dual luciferase assay. Data shown on the figures was obtained from three independent transfections each with four technical replicates.

Dual luciferase assay

Fluc and Rluc assay buffers were prepared as described in (Dyer *et al.*, 2000). Relative light units were measured on a Veritas Microplate Luminometer fitted with two injectors (Turner Biosystems). Cells transfected in 96 well plate were washed once with 1× PBS and then lysed in 15 μ l of 1× passive lysis buffer (PLB; Promega). Light emission was measured following injection of 50 μ l of each luciferase substrate buffer. Raw data for the dual luciferase assays are available as Underlying data (Yordanova *et al.*, 2020a).

Protein isolation and western blot analysis

Transfections for Western blotting analysis of constructs for Figure 1 were performed in 6 well plates scaled-up from the method described for 96 well plate transfections above. The following was added to each well: 1 μ g plasmid DNA, 7 μ l lipofectamine 2000 in 1 ml OptiMem. A total of 1×10⁶ cells in 3 ml DMEM, were added to the transfecting DNA complexes in each well. Transfected cells were incubated at 37°C in 5% CO₂ for 36 h for Western blotting. Cells were washed with 1× PBS and lysed in 1× PLB (Passive Lysis Buffer, Promega). Luciferase activities in the lysates were measured with the dual luciferase assay. Proteins were separated by 4–12% polyacrylamide gel electrophoresis on pre-made BoltTM 4–12% BisTris Plus gels (Thermo Fisher), transferred onto nitrocellulose membranes (Protran) and incubated with primary Rabbit Anti-Renilla Luciferase Polyclonal Antibody (1000x dilution) (MBL International) (RRID: AB_1520866) in 5% fat-free milk in PBST (1% Tween-20) overnight at 4°C. Incubation with IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody (10,000x dilution) (Abcam, ab216772) was for 0.5 h at room temperature.

Figure 1. *OPRL1* readthrough context mediated inhibition of Rluc levels in EMCV IRES vector. (A) Schematic of the *AMD1*, *ACTB* and *ODC1 PEST* constructs where Rluc stop codon context is varied and Fluc expression is governed by the EMCV IRES. (B) Normalized (Rluc/Fluc) activities. (C) Absolute Rluc values. (D) Absolute Fluc values. (E) Upper panel, Anti-Rluc immunoblots of protein lysates from HEK293A cells transfected with the indicated constructs; termination products are indicated with a black arrowhead, readthrough products (seen only in *ACTB* constructs) are indicated with a red arrowhead; lower panel, normalised (Rluc/Fluc) activities from the protein lysates. See Methods for box plot elements.

Statistical analysis

Box plots were generated with a web tool [BoxPlotR](#). Box plots elements: center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. $n = 12$ sample points. 2-tailed, paired samples t-test was performed in excel (version 2007) on samples as indicated.

Results and discussion

Inhibitory effect of *OPRL1* stop codon context in EMCV IRES vector

To further explore *AMD1 tail* translation in the bicistronic RT constructs we designed and tested two unrelated sequences using the dual luciferase reporter system from our previous work ([Yordanova et al., 2018](#)) ([Figure 1A](#)). The *AMD1 tail* was replaced either with a fragment of *ACTB* coding sequence of equivalent length (381 nt) or with that of mouse ornithine decarboxylase 1 (*ODC1*) C-terminal PEST encoding region (492 nt). *ACTB* was selected to represent a neutral sequence optimized for efficient translation that is not expected to affect the reporter levels. *ODC1* PEST codes for a degradation signal ([Loetscher et al., 1991](#)) and was selected to control for the effects that a degron could have on the reporter when placed downstream of an RT context. In agreement with our previous work, high-level readthrough of *AMD1 tail* resulted in a significant drop in reporter levels in a construct where *AMD1 tail* is placed downstream of the Rluc stop codon in the *OPRL1* RT context ([Figure 1B](#), compare UGA with *OPRL1*). We have attributed this effect to the inhibition of translation by ribosome stalling in *AMD1 tail* ([Yordanova et al., 2018](#)). However, when we replaced *AMD1 tail* with the neutral *ACTB* (no decrease in reporter expected) or the *ODC1* PEST (<20% decrease expected) sequences, they exhibited similar inhibitory behaviour ([Figure 1B](#), compare UGA and *OPRL1*).

One possible explanation for the observed inhibition of reporter levels is that it results from the addition of *OPRL1* context sequence at the end of the reporter's ORF. Both 5' and 3' nucleotides of *OPRL1* context contribute to RT efficiency. To explore the role of the 5' *OPRL1* context, we tested constructs wherein ribosome access to the sequence beyond the stop codon is prevented. For this we substituted UGA in the *OPRL1* context with two UAA codons ([Figure 1B](#), *OPRL1* UAA). In addition, we tested constructs wherein *OPRL1* 5' signal was truncated to only its two last codons instead of six ([Figure 1B](#), tr*OPRL1*). We have recently determined that just two codons 5' of the *OPRL1* stop signal are sufficient for maximal readthrough (Loughran, personal communication).

The termination products of *OPRL1* and *OPRL1* UAA constructs have the exact same amino acid composition, with both having the six *OPRL1* derived amino acids at the C-terminus. For the *OPRL1* UAA construct the reporter levels are indicative of the availability/activity of the termination product only, while for the UGA construct both the readthrough product and the termination product contribute to the reporter levels. *OPRL1* UAA constructs exhibit very similar reporter levels as the *OPRL1* constructs ([Figure 1B](#)) suggesting that the observed

reduction was due to the occurrence of the six *OPRL1* codons just upstream of the stop codon and did not depend on downstream translation. Nonetheless, it should be noted that there is a small difference in the reporter levels between *OPRL1* and *OPRL1* UAA which is most significant for *AMD1* ($p=10^{-15}$, t-test), less significant for *ODC1* PEST ($p=10^{-8}$, t-test), and even less significant for *ACTB* ($p=10^{-4}$, t-test). This could be either due to reduced stability of the readthrough proteins in which case any *AMD1 tail* destabilisation effect must exceed that of *ODC1* PEST degron or else it could be due to *AMD1 tail* translation having an inhibitory effect on the reporter's translation.

Shortening the 5' sequence of *OPRL1* context by deleting four of the six codons largely recovered reporter levels with all three test sequences ([Figure 1B](#), tr*OPRL1*) supporting the idea that these four codons contribute to the observed effect. Like with the full 5' RT context in *OPRL1*, the truncated form in tr*OPRL1* constructs exhibited similar reporter levels with all three test sequences. These findings would appear to argue against an *AMD1 tail* specific inhibitory effect in the RT constructs that we reported in our original work ([Yordanova et al., 2018](#)).

To investigate if *OPRL1* 5' context was interfering with reporter's activity or whether it affected the protein levels, we performed western blotting which showed that the amount of detectable reporter protein was significantly reduced in the presence of the *OPRL1* context for all three test sequences ([Figure 1E](#), compare UGA and tr*OPRL1* vs *OPRL1* and *OPRL1* UAA). This indicates that in these reporters the *OPRL1* 5' context does not simply interfere with Rluc activity. As expected, RT product was detected only with *ACTB* constructs.

The reduction of Rluc levels observed in the presence of *OPRL1* context could be due to a protein destabilising effect of the *OPRL1* derived peptide at the reporter's C-terminus. However, western blotting revealed that the amount of detectable reporter protein was significantly reduced in both the termination and the RT product (as seen for *ACTB* constructs, [Figure 1E](#)), which argues against a C-terminal degron activity. In addition, it has been shown recently that the Venezuelan equine encephalitis virus (VEEV) RT stop codon context promotes ribosome stalling and it has been proposed that such stalling could be a general feature of RT promoting sequences ([Lashkevich et al., 2020](#)). If so, the observed reduction of reporters containing *OPRL1* context could be attributed to slow peptide release and/or reporter mRNA degradation upon activation of ribosome quality control (RQC) pathways ([Ikeuchi et al., 2018](#); [Joazeiro, 2019](#)).

Variation of EMCV IRES governed Fluc expression levels

While Rluc activities normalized over Fluc values report a very similar picture for all three sequences tested ([Figure 1B](#)), this is not the case for the absolute values of these reporters. Addition of the last six codons of *OPRL1* to UAA reporters greatly reduced Rluc levels for *ACTB* and *ODC1* PEST but not for *AMD1* ([Figure 1C](#), *OPRL1* UAA). The most likely explanation for this is that any reduction in Rluc for the *AMD1* reporters was masked by the increased stability of its corresponding mRNA as can be judged from its Fluc activity

(Figure 1D). With the exception of *AMD1* extensions, stop codon contexts that supported efficient termination (as in UGA and *OPRL1* UAA) had three-fold lower Fluc levels as compared to those promoting readthrough (*OPRL1* and *trOPRL1*) which might be expected due to mRNA decay pathways such as Nonsense Mediated Decay (NMD) or No Go Decay (NGD) (Shoemaker & Green, 2012) sensing reduced translation. In case of *AMD1*, enabling readthrough with *OPRL1* context did not lead to further stabilization of mRNA (Figure 1D, *OPRL1* vs *OPRL1* UAA). The relatively high Fluc expression levels observed when *AMD1* tail is placed specifically downstream of *OPRL1* UAA context is intriguing and will need to be investigated further as it may shed light onto the properties of *AMD1* tail and its function in the regulation of *AMD1* expression.

Upstream reporter reduction depends on the RT promoting context and its efficiency

To determine whether these observations are specific to the *OPRL1* stop codon context we next tested the other two RT promoting contexts from our previous study (Yordanova *et al.*, 2018), *AQP4* and *LDHB*. *AMD1*, *ACTB* and *ODC1* PEST constructs where the Rluc stop codon was substituted with RT contexts from *LDHB*, *AQP4* and *OPRL1* of 2.5, 6.5 and

17% RT efficiency respectively (Loughran *et al.*, 2017) and the corresponding controls where the RT UGA codon was substituted with two UAA codons were tested alongside (Figure 2A). Like in our original *AMD1* study, increasing RT efficiency with *LDHB*, *AQP4* and *OPRL1* resulted in reduction of reporter levels (Figure 2B). The drop in reporter levels was disproportionately larger than what is expected to result from degradation of the RT product. However, as in the case of *OPRL1* RT context, constructs with *LDHB* and *AQP4* contexts exhibited the same trend when *AMD1* tail was substituted with *ACTB* and *ODC1* PEST (Figure 2C). With all three RT promoting contexts, a certain degree of recovery of reporter levels was observed upon substitution of UGA with UAAUAA. These results suggest that the observed reporter reduction depends on both, the RT context upstream of the stop codon as well as the readthrough efficiency but not on the extension sequence downstream of the stop codon.

Uncoupling of *OPRL1* RT context and *AMD1* tail translation effects on reporter levels in a monocistronic vector

The results described so far argue that the changes in Rluc reporter levels align with RT efficiency independently of translation downstream of the test sequence. These findings

Figure 2. Effect of *LDHB*, *AQP4* and *OPRL1* RT contexts on Rluc levels in EMCV IRES vector. (A) Schematic of the *AMD1*, *ACTB* and *ODC1* PEST constructs where Rluc stop codon context is varied and Fluc is expressed by EMCV IRES. (B–D) Normalized (Rluc/Fluc) activities were calculated for *AMD1*, *ACTB* and *ODC1* PEST constructs as a percentage of the corresponding UGA construct. Red dashed lines indicate expected reporter levels in case that RT products are degraded. See Methods for box plot elements.

were unexpected because earlier studies with *OPRL1* RT context did not provide evidence for such effects on reporter levels which were found to be not substantially different in the presence or absence of *OPRL1* context (Loughran *et al.*, 2017). The main difference in the experimental approach in the previous study compared to our current analysis is the absence of the EMCV IRES in the constructs used previously.

Therefore, to clarify these contradicting observations, we next tested the sequences shown in Figure 1A in a monocistronic vector that encodes Rluc and has no EMCV IRES (Figure 3A). Cells were transfected with these constructs along with a separate Fluc expressing vector to control for transfection efficiency. Because Fluc is expressed from a separate vector in this setup we do not account for RNA stability levels.

In the absence of EMCV IRES, the *OPRL1* UAA context resulted in a similar drop in reporter levels with *AMD1*, *ACTB* and *ODC1* PEST (Figure 3B and 3C, *OPRL1* UAA). This is consistent with the *OPRL1* context destabilising effect as revealed in the EMCV IRES vector (Figure 1); however, the

similarities between constructs with and without EMCV IRES end there. With *OPRL1* constructs that support efficient readthrough, *ACTB* had reporter levels only slightly reduced consistent with it being a neutral sequence. The small but significant drop might be due to reduced Rluc activity in the fusion product. The more significant drop with *ODC1* PEST is consistent with its degon properties. *AMD1* exhibited the greatest reduction of more than 10-fold. Though slightly higher than those of *OPRL1*, the reporter levels generated with the truncated context in *trOPRL1* showed the same trend, i.e. *ACTB* had almost no reduction, small reduction with PEST and the largest reduction of about 5-fold with *AMD1*. The different effects that *AMD1*, *ACTB* and *ODC1* PEST had on reporter levels in the constructs with readthrough, *OPRL1* and *trOPRL1*, suggest that these effects are related to the nature of the translated sequence downstream of the stop codon in the RT context. Critically, for *AMD1* the reduction of Rluc activity in *OPRL1* RT in comparison with *OPRL1* UAA construct greatly exceed what would be expected if this was due to degradation of the readthrough product only, supporting our earlier claim (Yordanova *et al.*, 2018).

Figure 3. Effect of *OPRL1* readthrough context on Rluc levels in a vector without an IRES. (A) Schematic of the *AMD1*, *ACTB* and *ODC1* PEST constructs where Rluc stop codon context is varied. Fluc is expressed by a separate vector. (B) Normalized (Rluc/Fluc) luciferase activities. The leftmost blue box represents reporter levels with the empty Rluc expressing vector. (C) Normalized (Rluc/Fluc) activities were calculated for the constructs from (B) as percentages of the corresponding UGA construct. See Methods for box plot elements.

Reporter expression levels from the empty Rluc vector were in the range of those from *OPRL1* and *OPRL1* UAA constructs for *ACTB* and *ODC1* PEST. This is consistent with the previous study, which showed no change in reporter expression levels in the presence of *OPRL1* context (Loughran *et al.*, 2017) (Figure 3B).

Conclusions

In our investigation of ribosome stalling following stop codon readthrough in the human *AMD1* gene, we proposed a ribosome queuing model to explain downregulation of reporter genes fused with *AMD1* tail (Yordanova *et al.*, 2018). To test the model, we varied readthrough context at the *AMD1* stop codon and observed disproportionately high inhibition of upstream reporters in response to increased readthrough efficiency as predicted by the model. Here we report that the observed reduction of upstream reporter levels is due to the RT context rather than due to *AMD1* tail translation, contrary to our initial interpretations of the experiments presented in Figure 3C of the original publication. We also found that this inhibition is observed only in the reporter vector where the downstream reporter is under the control of an EMCV IRES.

This result helped us to uncouple the inhibitory effects of RT contexts and *AMD1* tail translation on reporter's expression. In a vector not using EMCV IRES initiation, reporter expression is reduced further when *AMD1* tail is translated (due to readthrough). This reduction is not observed when *AMD1* tail is replaced with unrelated sequences supporting our original claim that translation of *AMD1* tail has an inhibitory effect on expression of upstream ORFs.

While the nature of molecular mechanisms responsible for the reported effects remains to be elucidated, our work extends

the list of unexpected properties of IRES elements (Payne *et al.*, 2013; Shikama *et al.*, 2010) and thus reinforces the need for caution in interpretation of data obtained with IRES containing reporters.

Data availability

Underlying data

Figshare: Stop codon readthrough contexts influence reporter expression differentially depending on the presence of an IRES. <https://doi.org/10.6084/m9.figshare.12824483> (Yordanova *et al.*, 2020a).

This project contains the following underlying data:

- dataFigure_1.csc. (Raw data dual luciferase assay for Figure 1.)
- dataFigure_2. (Raw data dual luciferase assay for Figure 2.)
- dataFigure_3. (Raw data dual luciferase assay for Figure 3.)
- western_700. (Original unannotated western blot image.)

Extended data

Figshare: Stop codon readthrough contexts influence reporter expression differentially depending on the presence of an IRES. <https://doi.org/10.6084/m9.figshare.12833762> (Yordanova *et al.*, 2020b).

This project contains the following extended data:

- Extended_Data_File_1.csv. (List of test sequences and primers.)

Data are available under the terms of the Creative Commons Attribution 4.0 license (CC BY 4.0).

References

- Arribere JA, Cenik ES, Jain N, *et al.*: **Translation readthrough mitigation.** *Nature*. 2016; **534**(7609): 719–723.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Chamond N, Deforges J, Ulryck N, *et al.*: **40S recruitment in the absence of eIF4G/4A by EMCV IRES refines the model for translation initiation on the archetype of Type II IRESs.** *Nucleic Acids Res.* 2014; **42**(16): 10373–10384.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Dyer BW, Ferrer FA, Klinedinst DK, *et al.*: **A noncommercial dual luciferase enzyme assay system for reporter gene analysis.** *Anal Biochem.* 2000; **282**(1): 158–161.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Grentzmann G, Ingram JA, Kelly PJ, *et al.*: **A dual-luciferase reporter system for studying recoding signals.** *RNA.* 1998; **4**(4): 479–486.
[PubMed Abstract](#) | [Free Full Text](#)
- Ikeuchi K, Izawa T, Inada T: **Recent Progress on the Molecular Mechanism of Quality Controls Induced by Ribosome Stalling.** *Front Genet.* 2018; **9**: 743.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Joazeiro CAP: **Mechanisms and functions of ribosome-associated protein quality control.** *Nat Rev Mol Cell Biol.* 2019; **20**(6): 368–383.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lashkevich KA, Shlyk VI, Kushchenko AS, *et al.*: **CTELS: A Cell-Free System for the Analysis of Translation Termination Rate.** *Biomolecules.* 2020; **10**(6): 911.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Loetscher P, Pratt G, Rechsteiner M: **The C terminus of mouse ornithine decarboxylase confers rapid degradation on dihydrofolate reductase. Support for the pest hypothesis.** *J Biol Chem.* 1991; **266**(17): 11213–11220.
[PubMed Abstract](#)
- Loughran G, Chou MY, Ivanov IP, *et al.*: **Evidence of efficient stop codon readthrough in four mammalian genes.** *Nucleic Acids Res.* 2014; **42**(14): 8928–8938.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Loughran G, Howard MT, Firth AE, *et al.*: **Avoidance of reporter assay distortions from fused dual reporters.** *RNA.* 2017; **23**(8): 1285–1289.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Payne AJ, Gerdes BC, Kaja S, *et al.*: **Insert sequence length determines transfection efficiency and gene expression levels in bicistronic mammalian expression vectors.** *Int J Biochem Mol Biol.* 2013; **4**(4): 201–208.
[PubMed Abstract](#) | [Free Full Text](#)
- Shikama Y, Hu H, Ohno M, *et al.*: **Transcripts expressed using a bicistronic**

vector pIRESHyg2 are sensitized to nonsense-mediated mRNA decay. *BMC Mol Biol.* 2010; **11**: 42.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Shoemaker CJ, Green R: **Translation drives mRNA quality control.** *Nat Struct Mol Biol.* 2012; **19**(6): 594–601.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Wangen JR, Green R: **Stop codon context influences genome-wide stimulation of termination codon readthrough by aminoglycosides.** *eLife.* 2020; **9**: e52611.

[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

Yordanova MM, Loughran G, Zhdanov AV, *et al.*: **AMD1 mRNA employs**

ribosome stalling as a mechanism for molecular memory formation.

Nature. 2018; **553**(7688): 356–360.

[PubMed Abstract](#) | [Publisher Full Text](#)

Yordanova M, Loughran G, Atkins J, *et al.*: **Stop codon readthrough contexts influence reporter expression differentially depending on the presence of an IRES.** *figshare.* Dataset. 2020a.

<http://www.doi.org/10.6084/m9.figshare.12824483.v1>

Yordanova M, Loughran G, Atkins J, *et al.*: **Stop codon readthrough contexts influence reporter expression differentially depending on the presence of an IRES.** *figshare.* Dataset. 2020b.

<http://www.doi.org/10.6084/m9.figshare.12833762.v1>

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This study sought to test the hypothesis that the severe suppression of reporters fused to *AMD1 tail* was due to stop codon readthrough in *AMD1* mRNA followed by ribosome stalling in the *AMD1 tail*. To do this, the authors placed the reporter stop codon in the context of readthrough permissive sequences in a dual reporter vector with downstream reporter expression driven by the EMCV IRES. Consistent with their hypothesis, they observed an inverse correlation between upstream reporter activity in response to increased readthrough levels. They also found that this inhibition is observed only in the reporter vector where the downstream reporter is under the control of an EMCV IRES.

The authors concluded that the observed reduction of upstream reporter levels correlates with the RT context rather than to AMD1 tail translation, contrary to the hypothesis they derived from data they obtained in a prior publication. However, this study was not really satisfying at any level, and no strong evidence was presented to support most of the conclusions made. As a result, we are left with a correlative study that really doesn't add much to our understanding of how read through may inhibit translation of an ORF.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I have worked on translation termination and stop codon read through for 25 years. As such, I think I am qualified to review this study.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 04 Oct 2021

Pavel Baranov, University College Cork, Cork, Ireland

Comment:

This study sought to test the hypothesis that the severe suppression of reporters fused to AMD1 tail was due to stop codon readthrough in AMD1 mRNA followed by ribosome stalling in the AMD1 tail.

To do this, the authors placed the reporter stop codon in the context of readthrough permissive sequences in a dual reporter vector with downstream reporter expression driven by the EMCV IRES. Consistent with their hypothesis, they observed an inverse correlation between upstream reporter activity in response to increased readthrough levels.

They also found that this inhibition is observed only in the reporter vector where the downstream reporter is under the control of an EMCV IRES.

Response:

We apologise for insufficient clarity of our original manuscript, the Reviewer's summary does not reflect the content of the manuscript. We have already shown in our original publication on AMD1 that translation of AMD1 tail results in suppression of upstream reporters and that increasing readthrough efficiency at the reporters stop codon results in increased reporter suppression. We have also demonstrated that ribosomes stall at the end of AMD1 tail.

We have already used the readthrough permissive sequences in our AMD1 paper but as explained in the response to Reviewer 1 we aimed at using this approach for an expanded study of various stalling sequences. It was during the early stage of this work that we made the current observations.

The statement "They also found that this inhibition is observed only in the reporter vector where the downstream reporter is under the control of an EMCV IRES." is a key

misunderstanding. The inhibitory effect non-specific to AMD1 tail was observed with the IRES vector while the AMD1 specific inhibition was observed with the monocistronic vector.

Comment:

The authors concluded that the observed reduction of upstream reporter levels correlates with the RT context rather than to AMD1 tail translation, contrary to the hypothesis they derived from data they obtained in a prior publication.

Response:

In the IRES vector indeed reduction of upstream reporter levels correlates with the RT context, however in the monocistronic vector the reporter reduction is specific to AMD1 tail. Taken together these results are supportive of our conclusion published previously. However, our current observations also introduce new aspects specific to the IRES vector and unrelated to AMD1 tail.

Comment:

However, this study was not really satisfying at any level, and no strong evidence was presented to support most of the conclusions made. As a result, we are left with a correlative study that really doesn't add much to our understanding of how read through may inhibit translation of an ORF.

Response:

Indeed, we fail to provide a mechanistic explanation of our findings. Due to the unusual nature of the observations it might not be straightforward to uncover the mechanism behind the differential effects in the two vectors. However, we think it is very important to present these observations as they will no doubt be of use to researchers using similar reporter systems or for anyone who will study the mechanism of AMD1 translation control that we reported earlier.

Competing Interests: No competing interests were disclosed.

Reviewer Report 05 October 2020

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Adam Geballe

Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Yordanova and colleagues report follow-up studies of their Nature paper in which they found that translational readthrough at the AMD1 stop codon, leading to translation of a C-terminal extension (AMD1 tail), inhibited translation of the coding region. The authors had proposed that the inhibitory mechanism was due ribosome stalling at the downstream stop codon and queuing of ribosomes on the mRNA. A more recent paper using ribosome profiling from another group confirmed that ribosomes translate the AMD1 tail, but found no evidence to support ribosome queuing. To evaluate their model further, these authors now test the prediction that increasing readthrough past the stop codon should increase the queuing and potentiate the inhibitory effect of the AMD1 tail. Using dicistronic constructs, they found that increasing the readthrough at the end Rluc results in lower Rluc/Fluc expression, but the effect does not depend on the AMD1 tail nor does it even require that readthrough onto the tail. They then tested another set of stop codon elements that allow readthrough and found that a higher level of readthrough efficiency resulted in more inhibition, but again, the effect was not dependent on the AMD1 tail. However, when they tested these stop codon contexts and tail sequence in monocistronic constructs, they then found that the AMD1 tail may be important for inhibition.

The data are interesting but unconvincing in support of any clear conclusions. The results do suggest that the impact of the dicistronic constructs does not depend on the AMD1 tail, and there are modest differences in the impacts of the AMD1 tail vs the other tails in the monocistronic constructs, but the other tails have intermediate effects. There are multiple uncertain assumptions and other findings that are not well explained.

Specific comments:

- Abstract: “Monocistronic reporters with the same readthrough context sequence exhibit only a modest reduction” is confusing since there is a strong inhibitory effect with the AMD1 tail in the OPRL1 monocistronic construct and the other two do inhibit moderately.
- The authors report as “personal communication” that the truncated ORFL1 stop codon is sufficient for maximal readthrough, but this finding is quite important for interpreting many of the results and so the data should be presented.
- The authors state that the LDHB, AQP4, and OPRL1 RT sequences cause 2.5%, 6%, and 17% readthrough, but the paper cited (Loughran et al 2014) reports the latter OPRL1 cause 31% readthrough. A later paper revised that number to 17%. Might these numbers vary depending on the precise construct and context of the experiment? If so, it could impact the interpretation of some of the data (e.g. in Figure 2).
- Page 5. The argument “against a C-terminal degron activity” mediated by the ORFL1-derived peptide is not convincing. The fact that both the termination and RT products are lower in the OPRL1 and ORPL1UAA constructs does not rule out the possibility that the ORFL1 peptide (more so than the truncated version) is a degron that acts to degrade the short and longer proteins.
- In Fig. 1D, the variation in Fluc expression is hard to explain by any simple model. Among other confusing results, how do the authors explain the differing effects on Fluc expression of *trOPRL1* in the AMD1 construct vs. in the *ACTB* and *ODC1 PEST* constructs? It may be true that the EMCV IRES is unreliable as they propose, but it is not clear it is really the problem

nor how it might be misleading. There could be effects on IRES structure and function or on mRNA degradation of the dicistronic RNAs that results from upstream ORF, but they don't provide direct evidence for any model. The authors generally interpret changes in the Fluc levels as being measures of mRNA, but that may not be incorrect, even for the monocistronic constructs. Some additional data, such as measuring RNA levels might help evaluate these alternatives. Alternatively, other methods might be needed.

- Interpretation of the UAA constructs depends on the UAAUAA always being a strong terminator, which seems likely, but is unproven. The UAAUAA does reduce expression of the larger proteins in Fig. 1E but what are the other bands in the immunoblot that appear larger than the main protein? Are they background artifacts or other readthrough products?
- How many independent replicates of the transfections were done? Presumably at least several, but given the vagaries of transfection assays, coupled with the possibility that the Fluc may not be a good internal control, it is important to know that the data presented are highly reproducible.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I have been studying protein synthesis regulatory mechanism for >30 years. I discovered and characterized the mechanism of one of the early examples of ribosomal stalling in eukaryotes and have remain interested in this area. I think I am quite well qualified to evaluate this report.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 04 Oct 2021

Pavel Baranov, University College Cork, Cork, Ireland

Comment:

Yordanova and colleagues report follow-up studies of their Nature paper in which they found that translational readthrough at the AMD1 stop codon, leading to translation of a C-terminal extension (AMD1 tail), inhibited translation of the coding region. The authors had proposed that the inhibitory mechanism was due ribosome stalling at the downstream stop codon and queuing of ribosomes on the mRNA. A more recent paper using ribosome profiling from another group confirmed that ribosomes translate the AMD1 tail, but found no evidence to support ribosome queuing.

To evaluate their model further, these authors now test the prediction that increasing readthrough past the stop codon should increase the queuing and potentiate the inhibitory effect of the AMD1 tail. Using dicistronic constructs, they found that increasing the readthrough at the end Rluc results in lower Rluc/Fluc expression, but the effect does not depend on the AMD1 tail nor does it even require that readthrough onto the tail.

They then tested another set of stop codon elements that allow readthrough and found that a higher level of readthrough efficiency resulted in more inhibition, but again, the effect was not dependent on the AMD1 tail. However, when they tested these stop codon contexts and tail sequence in monocistronic constructs, they then found that the AMD1 tail may be important for inhibition.

The data are interesting but unconvincing in support of any clear conclusions. The results do suggest that the impact of the dicistronic constructs does not depend on the AMD1 tail, and there are modest differences in the impacts of the AMD1 tail vs the other tails in the monocistronic constructs, but the other tails have intermediate effects. There are multiple uncertain assumptions and other findings that are not well explained.

Response:

We thank the reviewer for their comments which together with those of reviewer 2 suggest that we have failed to clearly communicate the aims of our study.

This current study is not a further examination of AMD1 tail properties although the presented observations were made during such an investigation. The current study reveals the observation that some sequences fused 3' to that encoding Rluc, affect this reporter levels differentially in a monocistronic vector and in a bicistronic vector wherein the second reporter Fluc is expressed via the EMCV IRES.

The reviewer mentions that "To evaluate their model further, these authors now test the prediction that increasing readthrough past the stop codon should increase the queuing and potentiate the inhibitory effect of the AMD1 tail".

This is not so and we apologise for not expressing the aim of this report clearly. This experiment has been done already for our original publication on AMD1 using the three different readthrough (RT) promoting contexts featured in the current study too. There we

did not test control sequences alongside AMD1 tail and, in hindsight, erroneously interpreted the results of enhanced reporter inhibition with increasing RT efficiencies as evidence for ribosome queuing in AMD1 tail.

Based on these previously published experiments we thought that using readthrough enabled ribosome titration was a good approach to study the dynamics of ribosome stalling (and potential queuing) not only in AMD1 tail but in other verified or candidate stalling sequences. We therefore designed a comparative study where among other approaches we planned to use RT contexts to further examine AMD1 tail and other selected sequences. ACTB and ODC PEST were chosen to serve as control sequences where stalling was not reported. Early on within this work we made the unusual observation presented here.

In this manuscript we report our finding that when AMD1 tail, ACTB and ODC PEST test sequences were fused 3' to Rluc via an RT promoting context, they support similar inhibition of Rluc levels when tested in a dual luciferase vector where the second reporter Fluc is expressed via the EMCV IRES. This suggests that our interpretation of the experiments with three different stop codons contexts was incorrect in the original paper because the inhibition observed in figure 3d of our original study is not specific to AMD1 tail.

However, when these same Renilla-RT context-test sequence cassettes were tested in a monocistronic vector without the EMCV IRES, the test sequences caused the expected differential effects on Rluc levels. These effects are consistent with the AMD1 tail translation-mediated inhibitory effects already described. Therefore, with respect to AMD1 tail properties, the current study does not add new information, nor does it alter our conclusions from the earlier paper.

We are interested in further investigating the mechanism responsible for the link observed here but this is not straightforward, and it might take a while to elucidate. However, we consider it important to present these findings for other researchers who may be using similar experimental systems. Apart from being helpful for the avoidance of potential artefacts, the results could also be stimulatory for others to research the underlying mechanism.

Prompted by the reviewer's comments we amended the manuscript in order to describe our findings and the purpose of the manuscript more clearly. We have also performed more experiments including RT-qPCR to probe the accuracy of an IRES controlled expression of a reporter as a proxy for mRNA levels.

Comment:

Specific comments:

Abstract: "Monocistronic reporters with the same readthrough context sequence exhibit only a modest reduction" is confusing since there is a strong inhibitory effect with the AMD1 tail in the OPRL1 monocistronic construct and the other two do inhibit moderately.

Response:

Thank you for pointing this out, it was indeed not clearly described and we have now

changed the abstract to address this. The AMD1 tail has a strong inhibitory effect in the monocistronic vector unlike ACTB and ODC PEST that have only a modest effect. This is unlike in the bicistronic IRES vector where all three test sequences have the same inhibitory effect.

Comment:

The authors report as “personal communication” that the truncated ORFL1 stop codon is sufficient for maximal readthrough, but this finding is quite important for interpreting many of the results and so the data should be presented.

Response:

This finding is a part of another study that focuses on investigation of OPRL1 readthrough context. However it is currently under preparation. We would like to avoid duplicate publication of the same data and therefore made it available through figshare (doi:10.6084/m9.figshare.16661023).

Comment:

The authors state that the LDHB, AQP4, and OPRL1 RT sequences cause 2.5%, 6%, and 17% readthrough, but the paper cited (Loughran et al 2014) reports the latter OPRL1 cause 31% readthrough. A later paper revised that number to 17%. Might these numbers vary depending on the precise construct and context of the experiment? If so, it could impact the interpretation of some of the data (e.g. in Figure 2).

Response:

Initially the OPRL1 readthrough promoting context was tested in a dual luciferase vector pDluc (with no IRES) where the RT product is a fusion of Rluc and Fluc and with this method the RT efficiency was shown to be 31% (Loughran et al 2014 (PMID: 25013167)).

Later, we developed a new vector (pSGDLuc) that eliminates the reporter distortions caused by fused test sequences where the two luciferase reporters are flanked by two StopGo elements that prevent formation of an Rluc-Fluc fusion and the RT efficiency was calculated at 17% (Loughran et al 2017 (PMID: 28442579)). Western blotting and densitometry confirmed 17% readthrough for OPRL1 in both pDluc and pSGDLuc.

In both cases, RT efficiency is calculated from the Fluc/Rluc ratio of a readthrough construct normalised to the Fluc/Rluc ratio of an inframe control construct. The variation (17% and 31%) between the two vector systems was due to the fact that the inframe control from the earlier study had distorted Fluc/Rluc ratio likely due to the altered activity within the fusion product.

In addition, the OPRL1 UGA absolute Rluc values were not significantly different in pDluc, pSGDLuc and Rluc expressed from a monocistronic vector (figure 2A from Loughran et al 2017 paper (PMID: 28442579)). This together with the current results from the

monocistronic vector suggested that the differences we observed with the bicistronic vector might be due to the presence of the EMCV IRES – a feature absent in the other vector systems (pDluc, pSGDluc and monocistronic).

We first cited the Loughran et al 2014 paper (PMID: 25013167) because this is where the three RT candidates were first experimentally verified. We now cite the Loughran et al 2017 paper (PMID: 28442579) as well.

Comment:

Page 5. The argument “against a C-terminal degron activity” mediated by the ORFL1-derived peptide is not convincing. The fact that both the termination and RT products are lower in the OPRL1 and ORPL1UAA constructs does not rule out the possibility that the ORFL1 peptide (more so than the truncated version) is a degron that acts to degrade the short and longer proteins.

Response:

We agree, it is possible that OPRL1 derived peptide destabilizes the protein. However, we suspect that an Rluc fused degron would affect Rluc levels similarly in the IRES vector and the monocistronic vector used in this study as well as in the pDluc vector used in the previous Loughran et al. 2014 and 2017 studies. However, there is no indication that the OPRL1 signal acts as a degron in pDluc (figure 2A from Loughran et al 2017 (PMID: 28442579)).

Comment:

In Fig. 1D, the variation in Fluc expression is hard to explain by any simple model. Among other confusing results, how do the authors explain the differing effects on Fluc expression of trOPRL1 in the AMD1 construct vs. in the ACTB and ODC1 PEST constructs? It may be true that the EMCV IRES is unreliable as they propose, but it is not clear it is really the problem nor how it might be misleading.

Response:

We agree, unfortunately we have no simple explanatory model. The strikingly different Fluc behaviour in AMD1 construct is very surprising and unexpected. Therefore, we repeated the cloning of this construct independently to be sure that this is not an artifact of a mutation somewhere in the vector. We obtained the same result with an independently made construct. While we failed to explain this surprising observation, it shouldn't deflect us from the main message of this manuscript that the inhibition observed is not specific to AMD1 tail.

Comment:

There could be effects on IRES structure and function or on mRNA degradation of the dicistronic RNAs that results from upstream ORF, but they don't provide direct evidence for any model. The authors generally interpret changes in the Fluc levels as being measures of mRNA, but that may

not be incorrect, even for the monocistronic constructs. Some additional data, such as measuring RNA levels might help evaluate these alternatives. Alternatively, other methods might be needed.

Response:

We agree with the reviewer that it is important to verify if IRES driven Fluc expression reliably reflects mRNA levels. To address this, we performed RT-qPCR for all twelve test constructs from Figure 1 with two sets of primers targeted to both Rluc and Fluc coding sequences (new Figure 2).

For this experiment transfections were performed in 6 well plates with one well per construct. Part of the lysate was used for luciferase assay (Figure 2,B and C). Two independent experiments were performed on separate days. The results of one of these experiments are shown on Figure 2. The other experiment is available as Extended Data Figure 1.

The Fluc activities observed with AMD1 and ACTB remained as observed before. Moreover, these were confirmed by the RT-qPCR experiments that showed very good correlation between IRES driven Fluc expression levels and mRNA levels as measured with RT-qPCR with both primers for Rluc and Fluc (Figure 2,D and Extended Data Figure 1,D). Based on this we can conclude that the IRES-driven absolute values of Fluc expression reliably reflect mRNA levels.

With one of the replicate experiments, we did not observe the same Fluc activities as seen previously with ODC PEST, Extended Data Figure 1. It is important to emphasise that absolute luciferase values fluctuate due to variable transfection efficiencies which are very difficult to reliably control (only the ratio is reliable). However, within an individual experiment IRES-driven Fluc activities and RT-qPCR-determined FLuc levels are in a very good agreement.

Comment:

Interpretation of the UAA constructs depends on the UAAUAA always being a strong terminator, which seems likely, but is unproven. The UAAUAA does reduce expression of the larger proteins in Fig. 1E but what are the other bands in the immunoblot that appear larger than the main protein? Are they background artifacts or other readthrough products?

Response:

The heavier bands are unspecific with an exception of ACTB in OPRL1 contexts which are indeed likely readthrough products. They disappear in both UGA and UAAUAA constructs.

Comment:

How many independent replicates of the transfections were done? Presumably at least several, but given the vagaries of transfection assays, coupled with the possibility that the Fluc may not be a good internal control, it is important to know that the data presented are highly reproducible.

Response:

Indeed, this is an important point, see above regarding reproducibility of absolute values. To increase our confidence in these data we cloned again from scratch the AMD1 IRES constructs and transformed again these together with the ACTB and ODC PEST constructs. In addition, we sequenced long stretches containing the EMCV IRES and full length Fluc to exclude the possibility that a mutation has occurred in these areas that is affecting differentially Fluc expression of AMD1 OPRL1 UAAUAA and we saw no evidence for this.

The data for Figure 1 is from 12 independent transfections which were performed in the same day with cells that were split in three plates the day before. We have now repeated the transfections in 96 well plates several times and on different days and with cells at different number of passages (Extended Data Figure 2).

Competing Interests: No competing interests were disclosed.