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The mechanism of translation initiation on Type 1 picornavirus IRESs

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 09 August 2013

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the unusually long duration of the review period in this case. Your study has now been seen by three referees whose comments are shown below.

As you will see, all three refs highlight the quality and impact of your findings, although they also all raise a number of minor points that you would need to address before they can support the publication of a revised manuscript.

At the more conceptual level, ref #3 does express concerns about the general relevance of the reported findings as well as the accessibility for the non-specialist reader, but in light of the positive endorsement from the other two referees, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may

be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

The manuscript by Sweeney et al describes a biochemical study on a reconstituted translation initiation system with type I picornaviral IRESs.The manuscript describes a substantial body of work that greatly advances our knowledge on the mechanism of initiation on these viral sequences, by characterising factor requirements and factor interaction sites for IRESs from several different virus species in great detail. I found the manuscript generally very interesting and well written.

I have two points where I think clarification would strengthen the conclusions drawn and would also help readers understand the implications of the manuscript better.

1) I am surprised by the complete lack of reference to eIF5 in the manuscript. In yeast, eIF5 is clearly a core component of 43S complexes and although the case seems less clear in mammalian cells, there is at least no evidence against a similar role in such cells. Recent reviews (eg Lorsch and Hinnebusch 2013, Pubmed ID 22815232) clearly discuss eIF5 as a universal core translation factor in all eukaryotes, which is involved in correct start codon localisation in an interplay with eIF1 and eIF1A. Thus, according to my best knowledge, eIF5 should be mentioned as a 43S component in the first sentence of the introduction. On the other hand, if there is evidence that eIF5 is not a canonical factor in mammalian cells, it would help readers like me if this could be specifically stated with a relevant citation.
Unless the authors can justify why eIF5 does not need to be mentioned, the omission of eIF5 in the reconstitution assays also needs to be explained and the implications of this need to be covered in the discussion. This is particularly the case where start codon selection is discussed, where eIF5 would be likely to make a difference (ideally, the relevant gels eg Fig 2F should include a sample with eIF5 added).

2) The authors discuss differences in signals with reference to the qualitative figures shown, which is fine for most signals where there is clear absence or presence of bands. However, in some cases the authors discuss qualitative differences in band strengths where significance of the differences should be determined. In the most extreme case (the effect of PTB in figure 2D) the authors state that there is a difference in the AUG743 footprint with and without PTB which is not evident to me from looking at the figure. Other examples are clearer (eg the effect of the C299A mutation in figure 1D) but proper stats would still be useful. On page 12, last paragraph, the authors state that "no significant differences between the levels of translation of wt and the mutant PV and EV71 mRNAs were observed..." implying that they did statistical analyses, in which case p-values or other relevant parameters should be indicated together with the qualitative statements in the text.

Minor comment
on page 12, last paragraph, the authors state "data not shown" - I thought this was not permissible for the EMBO Journal?

Referee #2

This manuscript describes an in vitro reconstitution of translation initiation on Type I Picornavirus IRESs, which include all enterovirus, and human rhinovirus IRESs. All the IRESs in this group share a common structure, which has been divided into five domains named II - VI.

The authors use Poliovirus (type1/Mahoney), entorovirus 71 and bovine enterovirus IRESes as examples of Type 1 IRESs and using purified initiation factors and ITAFs they show a common set of canonical initiation factors needed for efficient 43S recruitment on these IRESs. They also show that PCBP2 protein is the ONLY crucial ITAF for these IRESs, which is somewhat surprising result,

however, the data are very clean.

The experimental design and execution is of a very high standard and the quality of the toeprint/HRC gels is immaculate. I find the results very informative and support publishing in EMBO Journal.

I have few points that I would like the authors to respond to:

1. Figure 1 shows that ribosomes can see the AUG586 in PV dVI, but all these experiments are done with a construct that has AUG586 in a good-context (rather than in the semi-poor context that is seen in the wt). I would like to the authors to show what effect the AUG586 in good context construct (shown in Figure1) has on the toeprint signal at the AUG743 (as shown for the EV71 in Fig 3E).

2. Figure 2G, the text describing panel G (page 9, 14 lines up) states "Irrespective of the presence of HeLa cell extract...", however, there is no mention of HeLa cell extract or how it was made in the figure legend or in the M & M section.

3. Figure 3E shows one EV71-mutant in which the AUG592 is in good-context with another mutant that only differs by one nucleotide (ACCAUGGUG → ACCAUGGAG). The latter mutant shows a huge increase in toeprint signal at the AUG592, while only a small decrease in the toeprint signal at the AUG744. As mentioned above, there is no similar experiment for PV dVI mutant, however, the text (page 11, 12 lines up) states "Thus, as for the PV IRES, efficient inspection of dVI..."

These results suggest to me that majority of the 43S ribosomes are 'landing' to the 5'-side of the dVI, after which there might be two alternative routes to the main ORF AUG codon. Please could the authors like to comment?

4. The dVI in the BEV IRES is more unstructured than the dVI in PV/EV71 IRESs and although initiation of BEV IRES is dependent on eIF1, there seems to be a many 48S complexes in near-cognate codons (Fig3). Do any of the other ITAFs clear these signals?

5. Figure 4 shows in-vitro translation assays both in HeLa cell extract and in RRL, but there is no information about in which K+/Mg ++ concentrations these translation assays were performed. As there is much less signal in the translation assays in HeLa than in RRL only, this would be important to know.

Referee #3

In this work Sweeney and colleagues elegantly and comprehensively define the initiation factor requirements and ITAFs needed for initiation on type I picornavirus IRESes using toeprinting. The roles of other ITAFs and initiation factors (eIF1/1a) are also assessed. They determine that recruitment of eIF3 by eIF4G is essential for translation initiation, in contrast to type II picornavirus IRESs. Furthermore, using hydroxyl radical cleavage assay in conjunction with toeprinting, they determine the positioning of PCBP2 and eIF3 on the IRES domains. They test the hypothesis that the domain VI structure functions as a ribosome 'landing pad' by introducing structural changes and find that the specific shape/structure is not required, and so a 'landing pad' function is unlikely. The region scanned by the 43S ribosome complex upstream of the natural AUG was also determined.

The manuscript is very well written, though assumes that a non-specialist is familiar with the fairly specialized techniques used (toe printing, HRC). All experiments are executed to the highest standard and all conclusions made completely supported by the presented data. However, while in vitro reconstitution of translation on this type of IRES has not been achieved previously, this work does not reveal a surprising and novel mechanism or have particular significance beyond identifying differential requirements for translation initiation directed by type 1 vs type 2 viral IRESs. As such, it could be seen as highly specialized and of limited interest to a broad audience.

Minor points:
P9, line 9 - explanation of cycloheximide function ("inhibits elongation after the first round") should

be clarified to make clear this is a round of peptidyl transfer during translation elongation, not a round of translation on the mRNA

P6, figure 1 - a single sentence explaining toeprinting would assist a less experienced reader.

P7- HRC technique is not explained. This is a highly specialized technique and prior knowledge by the reader should not be assumed.

If the manuscript requires shortening, the discussion on P12 (final paragraph) and in the discussion section of the differences observed between translation of the IRESs in RRL and hela-cell free extracts seems particularly niche.

1st Revision - authors' response 18 September 2013

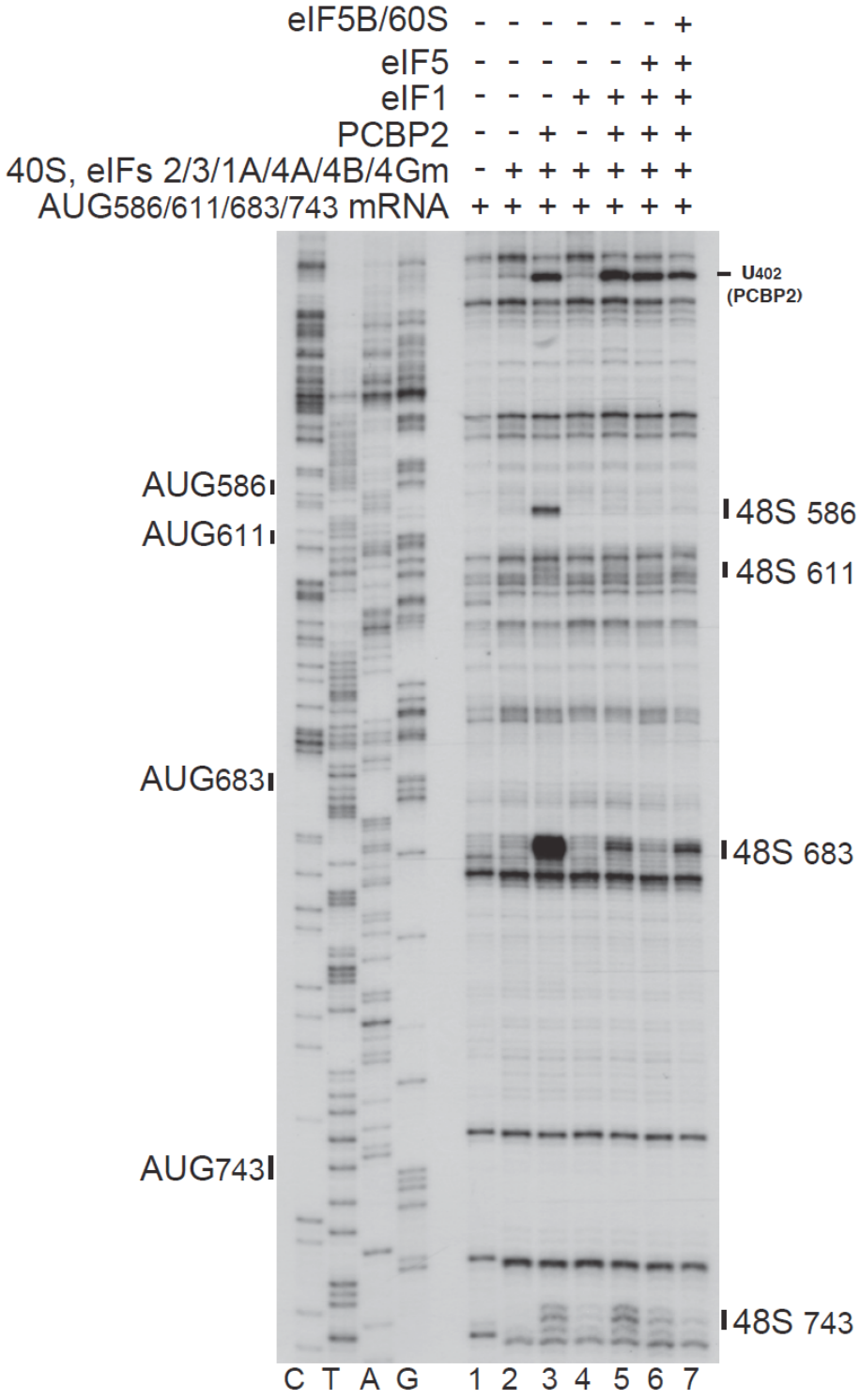
Reviewer 1

Specific comment #1:
I am surprised by the complete lack of reference to eIF5 in the manuscript. In yeast, eIF5 is clearly a core component of 43S complexes and although the case seems less clear in mammalian cells, there is at least no evidence against a similar role in such cells. Recent reviews (eg Lorsch and Hinnebusch 2013, Pubmed ID 22815232) clearly discuss eIF5 as a universal core translation factor in all eukaryotes, which is involved in correct start codon localisation in an interplay with eIF1 and eIF1A. Thus, according to my best knowledge, eIF5 should be mentioned as a 43S component in the first sentence of the introduction. On the other hand, if there is evidence that eIF5 is not a canonical factor in mammalian cells, it would help readers like me if this could be specifically stated with a relevant citation.
Unless the authors can justify why eIF5 does not need to be mentioned, the omission of eIF5 in the reconstitution assays also needs to be explained and the implications of this need to be covered in the discussion. This is particularly the case where start codon selection is discussed, where eIF5 would be likely to make a difference (ideally, the relevant gels eg Fig 2F should include a sample with eIF5 added).

Response: We have added a section to the Introduction to describe the role of eIF5 in initiation, as suggested (p3, lines 12-16). eIF5 is a canonical initiation factor in all eukaryotes, whose function is to activate GTP hydrolysis by eIF2 (and particularly subsequent Pi release) following establishment of codonanticodon base-pairing and dissociation of eIF1. GTP hydrolysis reduces eIF2's affinity for Met-tRNAi Met, leading to partial dissociation of eIF2-GDP from 40S subunits. Subsequent joining of 60S subunits and dissociation of eIF1, eIF1A, eIF3 and residual eIF2-GDP are mediated by eIF5B. Thus, the role of eIF5 in initiation codon selection is to commit an arrested 48S initiation complex to initiation at the site of arrest. The key role in selection of the codon on which 48S complex is arrested, on the other hand, belongs to eIF1. Whereas eIF1 associates with 43S complexes from the very beginning of the initiation process, the stage at which eIF5 joins mammalian 43S complexes (before or after establishment of codon-anticodon interaction) has not yet been conclusively established. The observation that over-expression of eIF5 in human cells causes initiation to occur prematurely at near-cognate or poor-context initiation codons upstream of the normal locus (Loughran et al. (2012) Nucl. Acids Res. 40, 2898-906) suggests that under normal conditions, eIF5 may not be a constitutive component of scanning 43S complexes *in vivo*. Importantly, in the presence of non-hydrolysable GTP analogue GMPPNP, eIF5 did not influence either the efficiency of 48S complex formation or the selection of the initiation codon (Abaeva et al., EMBO J, 2011), whereas its inclusion in 48S complex assembly reactions lacking eIF5B and 60S subunits in the presence of GTP resulted in overall reduction of 48S complex formation and leaky scanning because of destabilization of ribosomal association of Met-tRNAi
Met due to hydrolysis of eIF2-bound GTP when it is not immediately followed by subunit joining (Abaeva et al., EMBO J, 2011), which is consistent with the leaky scanning phenotype observed on deletion of eIF5B in yeast (Choi et al., Science, 1998). Thus, taking into account the role of eIF5 in the eukaryotic translation process, eIF5 was not included in reaction mixtures for 48S complex assembly.

Consistent with the above, addition of eIF5 alone to 48S complex assembly reactions shown in

Fig. 2F (as suggested) in the presence of GTP resulted in an overall reduction in the efficiency of 48S complex formation and leaky scanning past AUG683, resulting in the relative decrease in 48S complex formation at AUG683 and its concomitant increase at AUG743 (Figure below, compare lanes 5 and 6). As expected, additional inclusion into the reaction mixture of eIF5B and 60S subunits reduced leaky scanning, thereby increasing initiation at AUG683 (Figure below, lane 7), consistent with subunit joining resulting in ribosomal arrest, preventing further scanning downstream to AUG743. Neither of these experiments showed anything unexpected or functionally significant, particularly because AUG683 does not occur in native PV mRNA, and we therefore did not include them in our revised manuscript, which is already very dense.



Specific comment #2:

The authors discuss differences in signals with reference to the qualitative figures shown, which is fine for most signals where there is clear absence or presence of bands. However, in some cases the authors discuss qualitative differences in band strengths where significance of the differences should be determined. In the most extreme case (the effect of PTB in figure 2D) the authors state that there is a difference in the AUG743 footprint with and without PTB which is not evident to me from looking at the figure. Other examples are clearer (eg the effect of the C299A mutation in figure 1D) but proper stats would still be useful. On page 12, last paragraph, the authors state that "no significant differences between the levels of translation of wt and the mutant PV and EV71 mRNAs were observed..." implying that they did statistical analyses, in which case p-values or other relevant parameters should be indicated together with the qualitative statements in the text.

Response: We have quantified the intensity of toe-print signals corresponding to 48S complexes formed under different conditions relative to full-length cDNA, to provide a quantitative measure of the influence of PTB and the C299A mutation on 48S complex formation on the poliovirus IRES as requested. We did not perform statistical analysis of differences between the levels of translation of *wt* and the mutant PV, EV71 and BEV mRNAs (Figs. 4D-F), and the numbers underneath correspond to those particular gels that are shown. However, these experiments were done several times using different batches of lysates, and they all yielded qualitatively similar results. We have replaced the word “significant” by the word “major” on p12, line 20 to avoid any potential confusion.

Minor comment:
on page 12, last paragraph, the authors state "data not shown" - I thought this was not permissible for the EMBO Journal?
Response: The reference to ‘data not shown’ has been deleted.

Reviewer 2

Specific comment #1:
I would like to the authors to show what effect the AUG586 in good context construct (shown in Figure1) has on the toeprint signal at the AUG743 (as shown for the EV71 in Fig 3E).

Response:
We conducted the requested experiment, which is now included as Supplementary Figure S6 and is discussed on p11, line 21 – p12, line 3 of the revised text. As in the case of EV71 mRNA (Fig. 3E), strong enhancement of 48S complex formation on PV dVI AUG586 due to improvement of its nucleotide context had only a minor effect on initiation at authentic initiation codon AUG743. These data are consistent with earlier results obtained by in vitro translation in cell-free extracts, in which optimization of nucleotide context of poliovirus AUG586 had only a minor (two-fold reduction) effect on initiation at AUG743 (Pestova et al. (1994) Virology 204: 729-37).

Specific comment #2:
Figure 2G, the text describing panel G (page 9, 14 lines up) states "Irrespective of the presence of HeLa cell extract...", however, there is no mention of HeLa cell extract or how it was made in the figure legend or in the M & M section.

Response:
We thank the reviewer for noting this error, and have corrected the text appropriately. Since we are not showing this part of the figure and it is not essential for the manuscript, the statement concerning addition of HeLa cell extract was deleted.

Specific comment #3:
Figure 3E shows one EV71-mutant in which the AUG592 is in good-context with another mutant that only differs by one nucleotide (ACCAUGGUG → ACCAUGGAG). The latter mutant shows a huge increase in toeprint signal at the AUG592, while only a small decrease in the toeprint signal at the AUG744. As mentioned above, there is no similar experiment for PV dVI mutant, however, the

text (page 11, 12 lines up) states "Thus, as for the PV IRES, efficient inspection of dVI..." These results suggest to me that majority of the 43S ribosomes are 'landing' to the 5'-side of the dVI, after which there might be two alternative routes to the main ORF AUG codon. Please could the authors like to comment?

Response:
See response to the Specific comment #1.

Specific comment #4:
The dVI in the BEV IRES is more unstructured than the dVI in PV/EV71 IRESs and although initiation of BEV IRES is dependent on eIF1, there seems to be a many 48S complexes in near-cognate codons (Fig3). Do any of the other ITAFs clear these signals?

Response: We have addressed this question experimentally, and the results are now presented as Fig. S5 and mentioned on p11, lines 1-2 of the revised text. Other ITAFs did not influence initiation on near-cognate codons.

Specific comment #5:
Figure 4 shows in-vitro translation assays both in HeLa cell extract and in RRL, but there is no information about in which K+/Mg++ concentrations these translation assays were performed. As there is much less signal in the translation assays in HeLa than in RRL only, this would be important to know.

Response: The RRL and Hela cell-free translation extracts that we used are commercial products, and the manufacturers unfortunately did not make any information available to us concerning K+ and Mg++ concentrations in the HeLa extract and the K+ concentration in the RRL. All mRNAs were translated in conditions, which were experimentally optimized by inclusion of additional K+ and Mg++, but the final concentration of these ions cannot be calculated since the original concentrations in extracts are not known.
We disagree with the reviewer's conclusion that the translation signal in HeLa extract was substantially lower than in RRL, and think that the reviewer may have got this impression because of the slightly different lengths of exposure of different gels. However, when samples from HeLa and RRL translation were run on the same gel e.g. Fig. 4F, levels of translation are comparable, as stated on p13, lines 14-15. We think that this statement addresses the question clearly and directly.

Reviewer 3

Minor comment #1:
P9, line 9 - explanation of cycloheximide function ("inhibits elongation after the first round") should be clarified to make clear this is a round of peptidyl transfer during translation elongation, not a round of translation on the mRNA
Response: We have clarified this sentence as requested.

Minor comment #2:
P6, figure 1 - a single sentence explaining toeprinting would assist a less experienced reader.
Response: We have inserted an explanatory sentence as requested (p6, lines 14-16).

Minor comment #3:
P7- HRC technique is not explained. This is a highly specialized technique and prior knowledge by the reader should not be assumed.
Response: We have inserted explanatory text concerning the HRC technique as requested (p7, lines 9-11).

Minor comment #4:
If the manuscript requires shortening, the discussion on P12 (final paragraph) and in the discussion section of the differences observed between translation of the IRESs in RRL and hela-cell free extracts seems particularly niche.

Response: We thank the reviewer for these suggestions, and have accordingly truncated the

paragraph in question.

Accepted 02 October 2013

Thank you for submitting your revised manuscript. It has been seen by one of the original referees, whose comments are included below, and I am pleased to inform you that your study has now been accepted for publication in the EMBO Journal.

REFEREE REPORT

Referee #1:

Of the two major points I raised, the authors have addressed the first (on lack of mention of eIF5) well and I would thank them for the explanation of eIF5 requirements under various conditions, which I was not fully aware of.

Regarding the second point, on providing some statistical analysis of band strengths, the authors claim in their rebuttal letter that quantitative information was generated "as requested" but all I can find is that they now state % change in band strength for the two specific examples I highlighted. This is not exactly a concise statistical treatment... However, overall, I think the data support the conclusions drawn and so I think the authors changes are acceptable.

