THE EMBO JOURNAL

Manuscript EMBO-2009-70861

Conformational Changes in Switch I of EF-G Drive Its Directional Cycling On and Off the Ribosome

Cristina Ticu, Roxana Nechifor, Boray Nguyen, Melanie Desrosiers

Corresponding author: Kevin Wilson, University of Alberta

Review timeline:

Submission date:	16 March 2009
Editorial Decision:	15 April 2009
Revision received:	18 May 2009
Accepted:	26 May 2009

Transaction Report:

(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 15 April 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees, whose comments are enclosed. As you will see, all three referees recognise the value of your results in terms of providing insight into the EF-G cycle. While referee 1 is less enthusiastic, both reviewers 2 and 3 support publication after substantial revision to the text. Given these positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers.

In particular, referees 2 and 3 highlight the need to significantly extend the figure legends and the results section to adequately explain the data presented. This is critical - at the moment, the legends do not provide sufficient explanation of the figures. In addition, referees 1 and 2 both find that further discussion of the literature would be essential in order to put your study in better context. I would also remind you of the broad readership of the EMBO Journal, and encourage you to make clear the potential significance of your results in the wider context of GTPase cycles. Given that your manuscript is currently well under our character limit, I hope you should be able to incorporate these changes without too much difficulty. Also, in his/her confidential comments to the editor, referee 1 recommends a change to the title: given that your study focuses exclusively on the switch1 region, this should be referred to in the title. One final point: I notice that Figure 2A seems to consist of two separate gels spliced together. I would like to remind you that this should be clearly indicated with a black line.

I should add that it is EMBO Journal policy to allow only a single round of revision. Therefore, acceptance of your paper will depend on your ability to fully answer the points raised by the referees.

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

Yours sincerely,
Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Recent studies of cryo-EM studies of EF-Tu and EF-G bound ribosomes have examined the mechanism of GTP hydrolysis, and the role of switch 1 in tRNA selection and translocation, respectively. The current study focuses on positions of switch 1 of EF-G in various states of EF-G on and off the ribosome. The method used is cleavage by trypsin. Just as cryo-EM, this method cannot provide actual dynamic information or order events in time. Still, the study provides information that enriches the context in which existing structural information can be interpreted.

Detailed comments:

p. 4 first paragraph: Taylor et a., EMBO J. 2007 is not referenced, where EF2 movements linked to GTP hydrolysis and translocation are in fact addressed. This is relevant here and on p. 11 (middle of page; role of GTP hydrolysis on EF-G in translocation via rotation of superdomains).

p.6 last paragr.: Zaviolov and Ehrenberg's observations relate to GTP hydrolysis activity, and are not direct observations of rotation. More appropriate quote would be Valle et al. (2003) who look at rotation by cryo-EM and state that rotation is not observed unless the P-site tRNA is deacylated; i.e., the peptide bond has been transferred.

p.7 first paragr.: "allowing the ribosome subunits to undergo rotation" -- it will be helpful here to cite Valle et al., 2003 where the rotation has been observed under these conditions.

P.10 second paragr. in discussion: is the comparison meant to be analogous, or is it the comparison that establishes the analogy?

last paragr.: semicolon needed after "ribosome cavity"

p. 12: last sentence is difficult to understand because of the sequence of many clauses/qualifiers in the end with unspecific targets. As a consequence, the differing view is not clearly enunciated.

Referee #2 (Remarks to the Author):

The manuscript of Ticu et al., "Conformational Changes in EF-G Drive Its Directional Cycling On and Off the Ribosome," describes the use of multiple biochemical and biophysical methods to probe the position of EF-G and its sub-domains, in particular the switch-1 loop, in solution and during the process of translocation on the ribosome. Although the methods employed are limited to equilibrium studies, the authors utilize nucleotide analogues and antibiotics with known functions to stall each system examined in relatively homogeneous states. Thus, the work likely proves informative on conformation events within EF-G that are important to the process of uninhibited translocation. Specifically, the authors find that the switch-1 domain of EF-G likely opens after GTP hydrolysis, contributing to the GDP (as presumably Pi) release pathway as well as EF-G release from the post-translocation complex. The work is generally well written and adequately explained. Given that the findings are discussed in the context of other GTPases, it will likely be easily consumed and appreciated by even the general reader. The extensive nature of the methods employed adequately substantiates the new perspectives obtained for those in the ribosome and translation fields.

Upon minor revisions, I would recommend acceptance to EMBO. During revision, focus should be

given to making the figure legends of the manuscript significantly more detailed in nature. It seems that the authors were too brief in many instances and it is very difficult to follow the figures with the limited information presented. Thus, the reader is forced to go back and forth between the text and the figures when trying to obtain the necessary information to understand the reagents used in each figure. That being said, the information content is there, it is just a bit too difficult to obtain. Also, during revision efforts should be made by the authors to incorporate knowledge of the dynamic nature of the ribosome in discussion of previous research and in their interpretation of their findings.

Specific comments:

On page 3, paragraph 2, the authors state that the first step of translocation -wherein the acceptor stems of A- and P-site tRNA move with respect to the ribosome -is induced by EF-G. The authors reference Agirrezabala et al. to substantiate this claim. However, the findings of Agirrezabala et al. demonstrate that this step occurs in the ABSENCE of EF-G. Thus, either the authors to revise their use of the word "induce" and perhaps speak to the dynamic nature of the pre-translocation complex when discussing the mechanism of translocation.

On page 5, paragraph 1, the authors discuss the effects of GDPNP on the mechanism of translocation where it is stated that, "...GDPNP partially inhibited the extent of translocation, while having a modest effect on the reaction kinetics." The heterogeneity observed in this experiment is curious. Is this perhaps due to the purity of the GDPNP used? Some mention of this finding and its relevance to the later findings seems important? If I understand the results correctly, the trypsin probing experiments only really report on the sub-set of ribosomes that translocate in the presence of GDPNP (b/c experiments were performed on purified ribosome complexes where EF-G(GDPNP) would only be loosely associated with the pre-translocation complex.

On page 5, paragraph 1, the authors state that the GDP/vio complex is trapped in an intermediate state after 50S but before 30S translocation, referencing Ermolenko et al. To me, this seems like an unnecessary oversimplification of the literature. Cooperman et al. showed that viomycin stabilizes peptidyl-tRNA in an A/A position. Ermolenko et al. showed that viomycin stabilized deacylated P-site tRNA in a P/E position.

On page 5, the authors state that, "...free-EF-G is removed by ultrafiltration." What exactly is ultrafiltration?

On page 6, paragraph 2, the sentence, "We could plausibly dock....onto the entrance to cavity between..." I believe this should say, ...the entrance to THE cavity...

On page 6, final paragraph, the authors state that, "This movement requires prior peptide bond formation, transferring the peptidyl moiety from the P- to A-site tRNA." However, Cornish et al Mol. Cell 2008 showed that subunit ratcheting can occur even when P-site tRNA is acylated. Given recent evidence of the highly dynamic nature of the pre-translocation complex, caution should be used when making such statements as it seem more likely to be a shift in equilibrium or the rates of dynamics that are affected.

On page 7, paragraph 1, during the discussion of Figure 3, the authors concluded that the switch-1 conformational change does not depend on ribosome ratcheting. However, the experiments performed are inconclusive about subunit ratcheting. First, the implicit assumption is made that tRNA positions and hybrid state tRNA configurations are strictly coupled. To my knowledge, this conclusion cannot be made based on the current literature. Second, the position of tRNA is not probed in the EF-G bound ribosome complex. Are the authors implicitly using the results of Ermolenko et al. to substantiate this claim?

On page 7, final paragraph, the authors state that, "All three proteins..." when I believe only two BABE-coupled EF-G proteins were examined. In general, this section could use clarification.

On page 9, 2nd and 3rd paragraphs, the authors state that, "From the GDPNP complex EF-G(OG) is released very slowly, but 6-fold faster than mant-GDPNP. This statement seems very confusing. EF-G releases from the ribosome before nucleotide release? How does this affect the mant-GDPNP dissociation data? It would seem this finding is important. Yet, two sentences later, the authors state

that, ... "EF-G and GDP are released rapidly (nearly simultaneously) from the ribosome." This statement seems unjustified given the previous finding and should be clarified.

The final two sentences of the discussion seem relatively weak and inadequately address how their findings can fit with the work of Zavialov et al. I do not know how the authors conclude that GTP reloads onto EF-G after GDP release. This seems especially difficult to understand in the context of the sentence on page 9, where the authors state that, "Altogether, these results indicate that EF-G and GDP are release rapidly (nearly simultaneously) from the ribosome."

Referee #3 (Remarks to the Author):

This mms reports the results of some interesting experiments that bear on the mechanism of EF-G action during protein synthesis. The model they present for the mechanism of EF-G action is plausible, emminently testable. The authors might wish to revise their mms to clean up some lose ends.
1. On the bottom of page 3 the text suggests that formation of the hybrid state of the ribosome following peptide bond formation is dependent on EF-G. I am under the impression that it happens spontaneously.
2. Possibly because of length restraints (?) the text does not adequately describe the experiments that produced the data displayed in the figures. For example, I was unable to understand what Figure 1E is all about either from the text or from the legends. It is not reasonable to ask readers to make the effort to decipher the meaning of sketchily explained experiments. More care needs to be taken with this aspect of the text, and an order of magnitude more care taken with the figure legends, which are close to useless as they now stand.

1st Revision - authors' response 18 May 2009

Point-by point response:

Dear Dr. Wilson,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees, whose comments are enclosed. As you will see, all three referees recognise the value of your results in terms of providing insight into the EF-G cycle. While referee 1 is less enthusiastic, both reviewers 2 and 3 support publication after substantial revision to the text. Given these positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers.

In particular, referees 2 and 3 highlight the need to significantly extend the figure legends and the results section to adequately explain the data presented. This is critical - at the moment, the legends do not provide sufficient explanation of the figures.

Response: The figure legends are now substantially extended. The Results are explained in greater detail and clarified where needed, as pointed out by the referees (see below). Portions of the Methods, dealing with specific experiments in the Figures, have been moved to the Figure Legends. The Materials have been moved to the Supplementary Information, since the text is now close to the maximum limit of 55,000 characters (including spaces). Cross-references have been added between the various sections, so that readers can more easily find the necessary information.

In addition, referees 1 and 2 both find that further discussion of the literature would be essential in order to put your study in better context. I would also remind you of the broad readership of the EMBO Journal, and encourage you to make clear the potential significance of your results in the wider context of GTPase cycles. Given that your manuscript is currently well under our character limit, I hope you should be able to incorporate these changes without too much difficulty.

Response: The Discussion is extensively revised, again in response to the refereesí comments. It is

now divided into four sections. Each starts with a general short paragraph that relates EF-G to general features of GTPases. Section 1 relates our work to the relevant crystallographic and cryo-EM literature (as requested by referee #1). Section 2 relates our new model to older ideas on the role of GTP hydrolysis as well as switches in GTPases in general. Section 3 addresses the relationship between ribosomal subunit rotation and sw1 accessibility (as requested by referee #2). Section 4 summarizes our findings and again sets them into the broader context of previous studies and possible future directions.

Also, in his/her confidential comments to the editor, referee 1 recommends a change to the title: given that your study focuses exclusively on the switch1 region, this should be referred to in the title.

Response: "Switch 1" is added to the title and to the running title.

One final point: I notice that Figure 2A seems to consist of two separate gels spliced together. I would like to remind you that this should be clearly indicated with a black line.

Response: Black line is added in this figure.

Referee #1 (Remarks to the Author):

Recent studies of cryo-EM studies of EF-Tu and EF-G bound ribosomes have examined the mechanism of GTP hydrolysis, and the role of switch 1 in tRNA selection and translocation, respectively. The current study focuses on positions of switch 1 of EF-G in various states of EF-G on and off the ribosome. The method used is cleavage by trypsin. Just as cryo-EM, this method cannot provide actual dynamic information or order events in time. Still, the study provides information that enriches the context in which existing structural information can be interpreted.

Response: We generally agree with the first sentence by referee #1. Throughout our manuscript, we have acknowledged the substantial prior structural insights from cryo-EM, and in fact make extensive use of the work by Connell et al for interpreting our data. At the same time, the complexity and multi-step nature of the EF-G catalytic cycle cannot be fully characterized by any single method. Dynamic elements of translation complexes are particularly challenging to track, yet they are arguably among the most critical for driving protein synthesis. The prior structural studies (both by cryo-EM and crystallography) are the starting point from which our study evolved.
Nevertheless, in response to the detailed comments by this referee (below), our revised manuscript now includes a longer summary of the previous structural contributions. In the Introduction (p.4), two paragraphs summarize more comprehensively the prior structural studies on both EF-G and its eukaryotic homolog eEF2, on and off their respective ribosomes. The final paragraph in the Introduction links our new results to the prior structural structures (p.4): "Öwhich we could interpret in the context of previous structural studies." In the Discussion, two paragraphs are devoted to how our results relate to prior cryo-EM studies (p.12-13). The final section of the Discussion makes a final acknowledgment (p.16): "The approaches we have used for tracking sw1 movements complement prior structural investigations of free and ribosome-bound EF-G."
We donít generally agree with the remarks: "The method used is cleavage by trypsin. Just as cryo-EM, this method cannot provide actual dynamic information or order events in time." First, while our methods of necessity probe trapped complexes, using well-characterized antibiotics or nonhydrolyzable GTP analog, we started by carefully assessing the functional state of all complexes (Figure 1), and we continued to check the functional states of complexes we probed throughout our study (Supplementary Figures S1, S4, and S5). Second, while we admit that such experiments do not address actual dynamics, they do indicate the equilibrium conformational state of sw1 in relation to the functional state of the complexes being probed. We hope that these points are made convincingly and more clearly in the revised manuscript. Finally, we should point out that our study uses a combination of biochemical, chemical, and physical methods. It is not just limited to EF-G cleavage by trypsin. Together, we believe that they provide persuasive evidence for a substantial conformational movement in switch 1, in the functional context of the EF-G cycle.

Detailed comments:

p. 4 first paragraph: Taylor et a., EMBO J. 2007 is not referenced, where EF2 movements linked to GTP hydrolysis and translocation are in fact addressed. This is relevant here and on p. 11 (middle of page; role of GTP hydrolysis on EF-G in translocation via rotation of superdomains).

Response: The paragraphs (p.4) in the Introduction, which summarizes the prior structural investigations of EF-G, has been revised. A new paragraph, which summarizes the structural investigations in eEF2, has been added. The paper by Taylor et al is cited here. Also, in a new paragraph in the first section of the Discussion (p.12-13), this paper has been cited again.

p.6 last paragr.: Zaviolov and Ehrenberg's observations relate to GTP hydrolysis activity, and are not direct observations of rotation. More appropriate quote would be Valle et al. (2003) who look at rotation by cryo-EM and state that rotation is not observed unless the P-site tRNA is deacylated; i.e., the peptide bond has been transferred.

Response: The introductory paragraph to this section (p.7-8) is revised. In its original version, we had cited a review article by Frank et al (2007), with reference to ribosome subunit rotation. In the revised version, we have replaced this citation with Frank & Agrawal (2000), the original paper reporting subunit rotation. The following sentence cites Valle et al (2003), the primary reference that correlates the acylation state of the P-site tRNA and ribosomal rotation. Zavialov et al is cited in the following paragraph with respect to EF-Gís ability to bind to the ribosome in both the rotated and unrotated conformations (as assessed by its GTPase).

p.7 first paragr.: "allowing the ribosome subunits to undergo rotation" -- it will be helpful here to cite Valle et al., 2003 where the rotation has been observed under these conditions.

Response: Valle et al (2003) is cited again, with reference to EF-G binding (p.8).

P.10 second paragr. in discussion: is the comparison meant to be analogous, or is it the comparison that establishes the analogy?

Response: The comparison is meant to establish (or more precisely, extend) the analogy between Ras and the elongation factors. This sentence has been clarified (p.12): "This principle can be illustrated by comparing crystal structures of EF-Tu and EF-G, off the ribosome (Supplementary Figure S6)." A new sentence has been added at the end of the paragraph: "Thus, these comparisons suggest that, like Ras, sw1 adopts dissimilar conformations in EF-GïGDP and EF-TuïGDP."

last paragr.: semicolon needed after "ribosome cavity"

Response: The paragraph that formerly included this run-on sentence is revised.

p. 12: last sentence is difficult to understand because of the sequence of many clauses/qualifiers in the end with unspecific targets. As a consequence, the differing view is not clearly enunciated.

Response: This confusing passage in the original manuscript made reference to a paper by Zavialov et al (2005). Because this paper has been controversial, convincingly discounted by subsequent work by Wilden et al (2006), and not essential for our model, we have removed its reference from the revised manuscript. Also, we should note again that the end of the Discussion is completely revised (p.16). Two paragraphs are added, which summarize our main conclusions, and place them in the context of previous studies and possible future directions.

Referee #2 (Remarks to the Author):

The manuscript of Ticu et al., "Conformational Changes in EF-G Drive Its Directional Cycling On and Off the Ribosome," describes the use of multiple biochemical and biophysical methods to probe the position of EF-G and its sub-domains, in particular the switch-1 loop, in solution and during the process of translocation on the ribosome. Although the methods employed are limited to equilibrium studies, the authors utilize nucleotide analogues and antibiotics with known functions to stall each

system examined in relatively homogeneous states. Thus, the work likely proves informative on conformation events within EF-G that are important to the process of uninhibited translocation. Specifically, the authors find that the switch-1 domain of EF-G likely opens after GTP hydrolysis, contributing to the GDP (as presumably Pi) release pathway as well as EF-G release from the post-translocation complex. The work is generally well written and adequately explained. Given that the findings are discussed in the context of other GTPases, it will likely be easily consumed and appreciated by even the general reader. The extensive nature of the methods employed adequately substantiates the new perspectives obtained for those in the ribosome and translation fields.

Upon minor revisions, I would recommend acceptance to EMBO. During revision, focus should be given to making the figure legends of the manuscript significantly more detailed in nature. It seems that the authors were too brief in many instances and it is very difficult to follow the figures with the limited information presented. Thus, the reader is forced to go back and forth between the text and the figures when trying to obtain the necessary information to understand the reagents used in each figure. That being said, the information content is there, it is just a bit too difficult to obtain.

Response: The figure legends are now substantially extended. They now describe in detail the specific conditions and experimental procedures leading to each figure panel. In some cases, information that was previously found in the Materials and methods is now found in the legend of the figure to which to it specifically refers. In other cases, where the procedures apply to multiple figures, cross-references have been added between the legends and the Materials and methods.

Also, during revision efforts should be made by the authors to incorporate knowledge of the dynamic nature of the ribosome in discussion of previous research and in their interpretation of their findings.

Response: The dynamic nature of the ribosome is described further throughout the revised manuscript. In the Results (p.12-13), the section dealing with how sw1 cleavage by trypsin relates to the 50S translocation step, and the accompanying rotational movement of the ribosomal subunits, has been revised. In the first section of the Discussion (p.12-13), we describe how our results relate to disordered sw1 in recent cryo-EM studies of EF-G, eEF2, and EF-Tu. We have added a section later in the Discussion ("Coupling of sw1 to ribosomal translocation"), which is devoted specifically to how ribosome conformational changes may regulate EF-G conformational changes (in particular, the flipped-out sw1).

Specific comments:

On page 3, paragraph 2, the authors state that the first step of translocation -wherein the acceptor stems of A- and P-site tRNA move with respect to the ribosome -is induced by EF-G. The authors reference Agirrezabala et al. to substantiate this claim. However, the findings of Agirrezabala et al. demonstrate that this step occurs in the ABSENCE of EF-G. Thus, either the authors to revise their use of the word "induce" and perhaps speak to the dynamic nature of the pre-translocation complex when discussing the mechanism of translocation.

Response: The word "induce" is revised to "stabilize" (p.3). The citation of Agirrezabala et al (2008) has been replaced with Speigel et al (2007), where it was shown that EF-G-GDPNP binding to the ribosome thermodynamically stabilizes the first (50S) step of translocation.
While 50S translocation can occur without EF-G (and indeed, even 30S translocation, very slowly under certain conditions), our paper focuses on the physiological mechanism in which EF-G is essential. Thus, our emphasis in this paragraph is on the role of EF-G binding and GTP hydrolysis on the two-step mechanism of translocation.

On page 5, paragraph 1, the authors discuss the effects of GDPNP on the mechanism of translocation where it is stated that, "...GDPNP partially inhibited the extent of translocation, while having a modest effect on the reaction kinetics." The heterogeneity observed in this experiment is curious. Is this perhaps due to the purity of the GDPNP used? Some mention of this finding and its relevance to the later findings seems important? If I understand the results correctly, the trypsin probing experiments only really report on the sub-set of ribosomes that translocate in the presence of GDPNP (b/c experiments were performed on purified ribosome complexes where EF-G(GDPNP) would only be loosely associated with the pre-translocation complex.

Response: The role of GTP hydrolysis in the EF-G cycle has been investigated for many years, and remains a controversial issue. Originally, Kaziro and coworkers in the 1970s first showed that EF-G can catalyze translocation with nonhydrolyzable GTP analogs. More recently, a widely cited paper by Rodnina et al (1997) showed that GTP hydrolysis precedes the 30S translocation step, and that GTP hydrolysis accelerates this translocation by 50-fold. But here, it should be mentioned that they also found that EF-G binding to the ribosome alone accounted for 1000-fold acceleration in translocation. Two subsequent studies, by Pan et al (2007) and Zavialov & Ehrenberg (2003), have challenged the work of Rodnina et al (1997). They observed that GTP hydrolysis stimulated the rate of 30S and 50S translocation by only 3- to 5-fold acceleration. The source of the controversy seems to lie with the complexity of the translocation mechanism, coupled to GTP hydrolysis. Currently, there is unfortunately no consensus on which of the many assays for monitoring various aspects of tRNA/mRNA translocation are most reliable.
Our present study is based on two independent assays for translocation, which we developed in previous studies (Wilson & Nechifor, 2004; Nechifor et al, 2007). We have examined the purity of GDPNP (and other nucleotides) by thin-layer chromatography (Nechifor et al 2007) as well as anion-exchange FPLC (Resourse Q; unpublished data), and cannot detect other contaminating nucleotides in GDPNP, and small amounts (<5%) of GDP in our GTP stocks. In addition, we cannot detect contaminating nucleotides in our EF-G and ribosome preparations. Thus, it seems unlikely that our observed heterogeneity in translocation with GDPNP arises from these impurities.
In response: First, we have expanded the first section of the Results, to describe our translocation assays in greater detail and to mention the above discrepancy (p.5): "Second, we monitored 30S translocation by two complementary assays. The extent of 30S translocation was determined by toeprinting, which tracks ribosome movement along a defined mRNA (Figure 1D). The kinetics of 30S translocation was followed by using mRNA 3í-labeled with pyrene, whose fluorescence becomes quenched upon its entry into the ribosome (Figure 1E). EF-GïGTP catalyzed rapid and efficient translocation, whereas EF-GïGDPNP catalyzed partial translocation, as we reported previously (Wilson & Nechifor, 2004). However, GDPNP slowed the kinetics by only three-fold, a much smaller effect than previously reported (Rodnina et al, 1997; Katunin et al, 2002) but in agreement with another study (Pan et al, 2007)." [Note: Pan et al. provide the more precise quantitative kinetics, over Zavialov & Ehrenberg.] Second, Figure 1E itself has been re-scaled in terms of time (x-axis), to more clearly show the kinetics of translocation. Third, the commercial sources of our nucleotides are listed in the Supplementary Information (p.1). Finally, the potential implications of our results are further discussed in the section on coupling between GTP hydrolysis, sw1 movement, and translocation (p.15-16).

On page 5, paragraph 1, the authors state that the GDP/vio complex is trapped in an intermediate state after 50S but before 30S translocation, referencing Ermolenko et al. To me, this seems like an unnecessary oversimplification of the literature. Cooperman et al. showed that viomycin stabilizes peptidyl-tRNA in an A/A position. Ermolenko et al. showed that viomycin stabilized deacylated P-site tRNA in a P/E position.

Response: Ermolenko et al (2007) show tRNA movement by chemically probing the ribosomal 50S P and E sites. Their results were interpreted in the simple 50S translocation (hybrid state) model (Moazed & Noller, 1989). This model is now well established, with many subsequent papers from many labs in support of it (see Introduction, p.3).
Cooperman et al presumably refers to the paper by Pan et al (2007), from Coopermanís lab. This paper provided evidence that 50S translocation may be subdivided into two steps, in which the acceptor ends of the two tRNA move independently on the 50S subunit. Their conclusions were based on time-dependent changes in fluorescently labeled tRNAs, and effects of viomycin on these changes, which were interpreted as independent tRNA movements in the 50S subunit. While the Cooperman model has received independent support (Munro Blanchard 2007, Mol Cell 25, 505), other studies (Cornish et al and Agirrezabala et al, cited in our manuscript) have questioned it. In any case, we do not feel that the precise mechanism of 50S translocation is critical to our results, since sw1 movement appear to be independent of 50S translocation and instead depend on GTP hydrolysis and 30S translocation.

On page 5, the authors state that, "...free-EF-G is removed by ultrafiltration." What exactly is ultrafiltration?

Response: "ultrafiltration" is replaced with "filtration" (p.6). This procedure is described more specifically in the Materials and methods (p.17): "Free EF-G was removed from ribosome-bound EF-G by centrifugal filtration (Millipore Microcon YM-100): reactions were diluted in 500 µl buffer A (supplemented, as appropriate, with 20 µM fus or 100 µM vio) at 0oC, and re-concentrated to ~10 l."

On page 6, paragraph 2, the sentence, "We could plausibly dock....onto the entrance to cavity between..." I believe this should say, ...the entrance to THE cavity...

Response: "the" is added (p.7).

On page 6, final paragraph, the authors state that, "This movement requires prior peptide bond formation, transferring the peptidyl moiety from the P- to A-site tRNA." However, Cornish et al Mol. Cell 2008 showed that subunit ratcheting can occur even when P-site tRNA is acylated. Given recent evidence of the highly dynamic nature of the pre-translocation complex, caution should be used when making such statements as it seem more likely to be a shift in equilibrium or the rates of dynamics that are affected.

Response: According to Valle et al (2003), this movement requires prior peptide bond formation. The recent study of Cornish et al (2008) confirmed the qualitative correlation between subunit ratcheting and the acylation state of the P-site tRNA. However, referee #2 raises a good point: Cornish et al were surprised to find that subunit ratcheting can occur with N-Ac-Phe-tRNAPhe in the P site, although it was inefficient (25%), compared to the ratcheting of ribosomes containing deacylated tRNAPhe (85%). [From Table 2 of their paper.]
In response, we have revised this sentence, now stated more cautiously (p.8): "Previous studies have correlated subunit rotation with peptide bond formation (Valle et al, 2003) and 50S translocation (Ermolenko et al, 2007; Cornish et al, 2008)." The results from this section are further discussed (p.16): "...recent studies suggest that 50S translocation and subunit rotation can be decoupled (Cornish et al, 2008; Marshall et al, 2008)."

On page 7, paragraph 1, during the discussion of Figure 3, the authors concluded that the switch-1 conformational change does not depend on ribosome ratcheting. However, the experiments performed are inconclusive about subunit ratcheting. First, the implicit assumption is made that tRNA positions and hybrid state tRNA configurations are strictly coupled. To my knowledge, this conclusion cannot be made based on the current literature. Second, the position of tRNA is not probed in the EF-G bound ribosome complex. Are the authors implicitly using the results of Ermolenko et al. to substantiate this claim?

Response: We monitored 50S translocation by the movement of tRNA from the P/P state to P/E state (p.8): "By chemically probing tRNA interactions in the 50S P site, we confirmed that the former ribosome contained tRNA in the P/P state, while the latter ribosome underwent 50S translocation, corresponding to tRNA movement to the P/E state (Figure 3B; Moazed & Noller, 1989)." According to the correlation stated above, we assume that subunit rotation follows 50S translocation. We find that sw1 movement out of the ribosome cavity occurs, irrespective of 50S translocation. However, referee #2 raises another good point.
In response, we have revised this section to be more cautious (p.8): "Taken together, these results favor the interpretation (ii, above) that GTP hydrolysis induces a conformational change in sw1, making it more accessible to cleavage by trypsin in the GDP/fus state, rather than an indirect effect of ribosomal subunit rotation."

On page 7, final paragraph, the authors state that, "All three proteins..." when I believe only two BABE-coupled EF-G proteins were examined. In general, this section could use clarification.

Response: This has been revised to state (p.9): "... EF-G proteins 58C-FeBABE and 196C-FeBABE. These proteins ..." The third protein was Cys-free EF-G, which we used as a negative control for the hydroxyl radical-induced cleavages in rRNA (Figure 4A). The Cys-free protein was functionally characterized in the paper by Wilson & Noller (1998).

On page 9, 2nd and 3rd paragraphs, the authors state that, "From the GDPNP complex EF-G(OG) is released very slowly, but 6-fold faster than mant-GDPNP. This statement seems very confusing. EF-

G releases from the ribosome before nucleotide release? How does this affect the mant-GDPNP dissociation data? It would seem this finding is important. Yet, two sentences later, the authors state that, ... "EF-G and GDP are released rapidly (nearly simultaneously) from the ribosome." This statement seems unjustified given the previous finding and should be clarified.

Response: The point we are trying to make here is that there is a very large acceleration in the rate of GDP release (7500-fold) and EF-G release (860-fold) after sw1 flips out from the ribosome cavity. The comparatively small 6-fold discrepancy in their accelerated rates can be attributed to stabilizing effect of the mant probe attached to GDP (or possibly a destabilizing effect of the OG probe attached to EF-G). Ideally, we wanted to monitored release of EF-G and GDP from the same complex (i.e., with both OG and mant probes attached), but technical problems (interference of the fluorescent signals of the two probes) prevented us from obtaining useful results.
In response, we have added a new paragraph (p.11): "Thus, both nucleotide and EF-G release from the ribosome strongly correlate to the predicted effects of sw1 conformational changes. Release of mant-GDP and EF-G(OG), with GDP in the complex, occurred at nearly the same rapid rate. The 5.8-fold difference between kd values for mant-GDPNP and EF-G(OG), with GDPNP in the complex, may be attributed to the stabilizing effect of the mant group (~3-fold; Wilden et al, 2006). We should note, however, that we could not monitor release of both nucleotide and EF-G in the same experiment, due to technical issues of fluorescence interference between the mant and OG probes.."

The final two sentences of the discussion seem relatively weak and inadequately address how their findings can fit with the work of Zavialov et al. I do not know how the authors conclude that GTP reloads onto EF-G after GDP release. This seems especially difficult to understand in the context of the sentence on page 9, where the authors state that, "Altogether, these results indicate that EF-G and GDP are release rapidly (nearly simultaneously) from the ribosome."

Response: The last section of the Discussion has been revised (p.16). Because the Zavialov et al paper remains controversial, and not essentially important for the model we present, it has be omitted.

Referee #3 (Remarks to the Author):

This ms reports the results of some interesting experiments that bear on the mechanism of EF-G action during protein synthesis. The model they present for the mechanism of EF-G action is plausible, emminently testable. The authors might wish to revise their mms to clean up some lose ends.

1. On the bottom of page 3 the text suggests that formation of the hybrid state of the ribosome following peptide bond formation is dependent on EF-G. I am under the impression that it happens spontaneously.

Response: Indeed, hybrid state formation (50S translocation) can happen spontaneously (Moazed & Noller, 1989; Agirrezabala et al, 2008). However, EF-G has been shown to drive this step forward (Speigel et al, 2007). See our further comments in response to Referee #2 on the same issue.

2. Possibly because of length restraints (?) the text does not adequately describe the experiments that produced the data displayed in the figures. For example, I was unable to understand what Figure 1E is all about either from the text or from the legends. It is not reasonable to ask readers to make the effort to decipher the meaning of sketchily explained experiments. More care needs to be taken with this aspect of the text, and an order of magnitude more care taken with the figure legends, which are close to useless as they now stand.

Response: The figure legends are now substantially extended and describe the specific conditions and procedures of each experiment. The main text of the Results have been expanded in many places, to describe more fully both the experimental approaches and the important results. In a few cases, links have been made to the Materials and methods, where more appropriate. Sections of the Materials and methods have been clarified.
In response: With respect to Figure 1E in particular, the experiment is described better in the Results (p.5): "The kinetics of 30S translocation was followed by using mRNA 3í-labeled with

pyrene, whose fluorescence becomes quenched upon its entry into the ribosome (Figure 1E)." It is also described more fully in the legend to Figure 1 (p.23): "Kinetics of ribosome translocation, measured by using 3í-pyrene-mRNA (Studer et al, 2003; Nechifor et al, 2007). Fluorescence traces (offset for clarity) monitor translocation kinetics. In Panels D and E, reactions contained: pretranslocational ribosome (0.5 M; see Materials and methods), EF-G (1.0 M), and GTP (1 mM). Inhibitors were: GDPNP (1 mM), vio (0.5 mM), or fus (1 mM)." Finally, Figure 1E itself has been re-scaled in terms of time (x-axis), to more clearly show the kinetics of translocation. (See also our response to referee #2 on a related point.)

