

Manuscript EMBO-2011-79085

A pRNA-induced structural rearrangement triggers 6S-1 RNA release from RNA polymerase in *Bacillus subtilis*

Benedikt M. Beckmann, Philipp G. Hoch, Manja Marz, Dagmar K. Willkomm, Margarita Salas and Roland K. Hartmann

Corresponding author: Roland K. Hartmann, Philipps-Universität Marburg

Review timeline:

Submission date:	08 August 2011
Editorial Decision:	09 September 2011
Revision received:	23 December 2011
Accepted:	18 January 2012

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

09 September 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. I have now received the final report from the two referees who evaluated your study and I enclose their comments below. As you will see they are very positive regarding the study but both request mutational analysis to confirm the change in conformation and formation of the hairpin within the 6S-1 RNA. Given the support from the referees I would like to invite you to submit a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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 initiative, please visit our website: <http://www.nature.com/emboj/about/process.html>

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

This very exciting manuscript describes a key advance in understanding the function of a universally conserved noncoding RNA, 6S RNA. In bacteria, 6S RNA binds the housekeeping RNAP tightly and specifically, preventing transcription during stationary phase. Exit from stationary and into exponential growth requires release of 6SRNA. How is RNAP released to transcribe? Release occurs when [NTP] concentrations increase, allowing 6S RNA to template transcription of small, product RNAs (pRNA). However, to date, it is been completely unclear how transcription leads to observed dissociation of the 6S RNA:pRNA hybrid from RNAP.

Here Beckmann et al. present a series of experiments that suggest that the key event is the formation of a hairpin in the noncoding RNA strand, and that this hairpin can only form once a certain length of pRNA is made. Interestingly, there is an interplay between the off rates of various pRNA lengths; as they increase from 8 to 14, the off rate of the pRNA greatly decreases, leading to stable hairpin formation and thus release from RNAP. Sequence analysis suggests that this mechanism may be universal for all bacterial 6S RNAs.

Overall, the experiments, and their logic are well-described and explained. My suggestions (below) are that the MS needs to broaden the discussion to make connections to the structure of RNAP, and other regulatory RNA processes, to clarify the cartoons/models shown in some of the figures, and to do one additional experiment. These changes will strengthen/clarify the importance of the authors' findings, and will help the manuscript appeal to a broader audience.

1. While I understand that this may be semantics, Fig. 5 top panel presents how many in the field typically view transcription - that RNA synthesis proceeds from upstream to downstream. But all the 6SRNA figures are shown in the opposite orientation, with transcription proceeding from downstream to upstream. For clarity and recognition by a broader audience - it would be best to reorient the 6S figures (e.g Figs. 3 and 5, SFig1B and Sfig. 4) so that the coding strand is on the bottom. In Fig. 5, I would recommend adding a red ball in the RNAP ovals to highlight the active site. Top panel, I would replace the closed complex as shown with a cartoon of an open DNA complex. This will reinforce the comparison with 6S RNA (mimic of DNA open complex). In the bottom panel, it would be better if the pRNA hybrid was shown inside the RNAP oval (see comment 2), and the released product outside. It would be very helpful in illustrating their model if the authors added an additional step in the mechanistic cartoon. This model would precede kconf and show what they think the 6SRNA:p14:RNAP complex looks like with the hairpin formed pre-release.

2. The authors are encouraged to expand their discussion to put their hairpin results in the context of the high resolution structures of RNAP and the results from the Hsu and Ebright labs regarding how DNA "scrunches" during initiation from DNA open complexes, leading to promoter escape. Also, their kinetic competition model between different RNA structures (e.g. duplex RNA vs hairpin) is reminiscent of how some riboswitches work, and possibly deserves some comparison, discussion as a theme of regulatory RNAs.

3. Ideally the authors would mutate the sequence of 6S-1 to test their hairpin hypothesis. While the paper is significant as it is, the evidence for the hairpin formation is limited to interpretations of differential cleavage and mobility shifts, and computer predictions. By mutating the sequence to prevent hairpin formation, the authors would greatly solidify their hypothesis and the overall impact of this paper.

Referee #2

This manuscript by Hartmann and co-workers reports a new finding on the life cycle of 6S RNA, a ubiquitous regulator of RNA polymerase (RNAP) in eubacteria that has served as a paradigm of a conserved noncoding RNA that targets the transcription machinery by mimicking the nucleic acid (DNA) template of the latter. Using a variety of biochemical techniques, the authors address the key question of how RNAP can disentangle itself from the stable 6S RNA::RNAP complex. Previous work by others (Wassarman and Wagner labs) had implicated short RNA species, the so-called

pRNAs which RNAP synthesizes using 6S RNA as the template, as important players in the release phase.

The present paper identifies the molecular basis for the RNAP release, showing that pRNAs of a certain length (14mers) induce a structural change in the 6S RNA upon which it can no longer bind and sequester RNAP. The switch is proposed to involve a collapse of the central bulge of 6S RNA, such that the lower (non-template) strand can form a hairpin structure, diminishing the ability of 6S RNA to mimic an open promoter structure of RNA. By contrast, shorter pRNAs of 8-9 nucleotides length which are observed *in vivo* too, cannot trigger this switch.

This is an excellent manuscript that addresses an important molecular question in RNA biology; it will surely be of general interest and should be published in the EMBO Journal. The work resulted from attention to details in the interpretation of high-throughput sequencing data generated in a previous study, in which the authors had observed several different sizes of pRNA from 6S-1 RNA. As such, it is one of the first studies in bacteria in which a stringent analysis of RNA deep sequencing results has been carried on to the level of a new molecular mechanism of a regulatory RNA.

The presented experimental data is of high-quality and involves some excellent ideas such as the 8mer LNA probe to block 6S RNA, and the manuscript generally is well-written. I have one point (analysis of hairpin mutants) that should be addressed experimentally prior to publication; the other criticism can be addressed by amendments to the text.

Major criticism:

1. A central element of the authors' model how pRNAs induce a structural arrangement of 6S RNA to incapacitate it as an inhibitor of RNAP is the formation of a hairpin in the lower part of the central bulge of 6S RNA (130-150 nt region in Figure 3E, lower panel). This model is supported by structure probing data for *Bacillus* 6S-1 RNA (Fig. 3) and an experiment with the 8mer LNA (Fig. S2). Additional support may come from observations that a mutant in *E. coli* 6S RNA carrying a nucleotide exchange in the corresponding bulge region shows a release defect as well as that many 6S RNA species can be predicted *in silico* to have the propensity to form that short hairpin. However, to firmly validate their model, the authors should introduce point mutations in the 138-148 region of the *Bacillus* 6S-1 RNA in order to suppress hairpin formation, and determine how these changes affect inhibition and release. This could be done with the established *in vitro* assays though *in vivo* data with mutated endogenous 6S RNA genes would be stronger (albeit less straightforward in terms of experimentation).
2. Some 6S RNA species, e.g. of *Helicobacter pylori*, have been reported to produce pRNAs from both strands of the central bulge, implications of which for the generality of the model presented here should be discussed.

Minor criticism:

3. Page 3, line 9 from bottom: suggest to change to "... initiated at nucleotide C40 ...".
4. Page 4, line: replace "in a mirror-like setup" with "reciprocally" or "conversely".
5. Page 4, line 8 from bottom: "opened the perspective to transcribe" sounds a little awkward; better write "enable/permitted to".
6. Page 8, first line of second para: add original reference to dRNA-seq.
7. Page 10: The last sentence of Discussion is not clear and might as well be omitted.

Reviewer 1

We broadened the discussion to make connections to what is known about RNAP in mechanistic terms (abortive transcription), we compared the role of 6S RNA with other regulatory RNA processes (e.g. riboswitches), we clarified and further specified the cartoons/models shown in some of the figures, and we conducted the requested additional experiment(s).

Comment 1: We have reoriented the 6S RNA structure in all figures as requested. We have redrawn the previous Fig. 5 (now Fig. 6), incorporating the suggestions of this reviewer, and further refined the model based on our findings with the mutant 6S-1 RNA having a disrupted hairpin in the 3'-part of the central bulge. We also added a kinetic scheme to this figure, which has been adapted from a corresponding scheme developed for abortive transcription at DNA promoters.

Comment 2: We have now discussed our data in the context of the RNAP "scrunching" mechanism operational during abortive transcription initiation, and cited some of the work by the Hsu and Elbricht labs. Likewise, we included a discussion part that compares the mechanistic parallels and differences between 6S RNA and riboswitches. We have also discussed the suggestion "*The authors are encouraged to expand their discussion to put their hairpin results in the context of the high resolution structures of RNAP.*" After intense discussions, we have refrained from doing so because we feel that too little is known on 6S RNA tertiary structure and its spatial organization when bound to RNAP, which would make such a discussion rather speculative.

Comment 3: Thanks a lot to both reviewers for suggesting this experiment, which indeed revealed that things are less simple than initially anticipated. Our results indicate that the hairpin structure in the 3'-part of the central bulge is neither absolutely essential for pRNA synthesis, nor for the conformational rearrangement or 6S-1 RNA release from RNAP, but nonetheless contributes to the overall efficiency of the process (see new Fig. 4). These additional experiments made clear that the central bulge collapse of 6S-1 RNA is the key event in the structural rearrangement of this type of 6S RNA.

Reviewer 2

Comment 1: This comment is congruous with comment 3 of reviewer 1 (see above).

Comment 2: *H. pylori* 6S RNA is indeed an interesting case, thanks to the reviewer for stimulating us to take a deeper look at this 6S RNA. Intriguingly, folding analyses predict that both, pRNA or pRNA* duplex formation with 6S RNA, stabilize the extended hairpin in the 3'-bulge. This could be a common key mechanistic component for the structural rearrangement and release from RNAP. This interesting case is now discussed in the revised version and documented in the new Fig. S5.

Minor comments 3-7 have been addressed as requested.

Thank you for submitting your revised manuscript to the EMBO journal. Your revision has now been seen by referee #2. As you can see below, the referee appreciates the introduced changes and support publication in the EMBO Journal. I am therefore very pleased to proceed with the acceptance of the paper for publication here. Referee #2 suggests improving the abstract in order to highlight the novel aspects of the paper. I think that the referee has a point and this can be easily fixed. You can send me a modified abstract by email and we will change it in the file.

Thank you for submitting your interesting manuscript to the EMBO Journal.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Referee #2:

The authors have satisfactorily addressed all my previous criticism, and I recommend the paper now be accepted for publication. However, the authors should try to improve the final version of their abstract; the current version makes it difficult for the reader to grasp what was known before, and what are the new findings of the present study. A few words along the lines of "We show here that ...", added to the beginning of the second sentence, might remedy the problem.