RNA polymerase II acts as an RNA-dependent RNA polymerase to extend and destabilize a non-coding RNA

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Editor: Anne Nielsen

1st Editorial Decision 29 August 2012

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all three referees express great interest in your findings; however referees #1 and #2 both emphasize that a revised manuscript will have to demonstrate a functional role for the RdRP-mediated B2 elongation. We agree that additional functional data is required, but given the limited number of experiments outlined by the referees, it may not be directly clear how much additional data would be required to address the request for functional insight. I would therefore appreciate it if you could provide me with a short outline of the experiments you would add in a revision, before you start extensive additional experimentation beyond the specific suggestions in the referee reports.

Given the referees' positive recommendations, 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 or rejection 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:

In addition to its well-known role as a DNA-dependent RNA polymerase, RNA polymerase II has also been shown to have an RNA-dependent RNA polymerase activity. In the present paper, the authors characterize this activity further, focusing on the ability of mouse B2 RNA to serve as both the template and the substrate to which NTPs are added. This reaction itself has interesting properties, in particular the rather sharp limit of 18 nt as the maximum extension possible of the B2 RNA. There is a deeper biological significance to this system: this laboratory has previously shown that B2 RNA can inhibit transcription of DNA templates by pol II, presumably by blocking the ability of the polymerase to properly interact with the template DNA. They have also shown that upon heat shock, B2 RNA plays a role in downregulating selected genes by associating with the pol II at the promoters of those genes. The present study was initiated by the observation that B2-pol II complexes can be dissociated by an activity in nuclear extracts in a reaction that requires all four NTPs and is amanitin-sensitive. The results of inhibition of B2 synthesis by RNA polymerase III in cells indicate that B2*, the extended form of B2, is less stable than B2. The combination of this result with the strong increase in B2 levels upon treatment of cells with amanitin (which blocks extension of B2 to B2*) leads the authors to suggest that B2 levels are controlled through the pol II-mediated conversion of B2 to B2*.

I think the biochemical studies are very well done. However, as the authors have carefully noted, the basic observation of an RNA-dependent RNA polymerase activity for pol II, using native RNAs as template and substrate, was reported earlier. At least to me, the most interesting part of this section of the paper is the discovery of a strong barrier to extension of B2 by more than 18 nt. The location of B2 RNA within pol II and the mechanistic details of the extension reaction have not been explored extensively, but I suspect these will ultimately provide a useful tool to probe polymerase structure-function relationships. The particular length at which extension stops is just beyond the length of the RNA-DNA hybrid which results in destabilization of pol II complexes when polymerase transcribes a single-stranded template in the absence of a nontemplate strand (see Kireeva et al., (2000) JMB 299, p. 325). It may be inappropriate to make too much of the comparison of the two transcription systems but I do suggest the authors comment on this.

My main concern with this paper centers on the in vivo experiments and in particular the conclusions that the authors wish to make from them. It is clear that B2* exists in cells, but I was surprised that the authors did not determine the actual amounts of total B2 and B2*- isn't this necessary to interpret their results? Put another way: if the conversion to B2* affects only a very small fraction of B2, is it likely that blocking that conversion is important in controlling B2 levels? My understanding is that most pol II is not free in solution (see Kimura et al., (1999) MCB 19, p. 5383). The large majority of pol II that is "chippable" onto DNA (outside of heat shock) is already transcriptionally engaged and thus not available to B2. Is there a significant pool of pol II that can function in the B2 to B2* conversion in normally growing cells? It is certainly true that B2 RNA is remarkably induced by amanitin treatment. The authors assert that this results from a major stabilization of B2, since it can no longer be converted to the unstable B2* form. The critical control
for this assertion is the lack of effect of amanitin on 7SK RNA levels. This did not strike me as a conclusive control, since B2 uses an internal A/B box promoter while 7SK uses an upstream promoter. Particularly in light of this difference in promoter architecture, it seems reasonable to suggest as an alternative that B2 levels go up because the B2 promoter is induced by the stress imposed on the cells through poisoning with amanitin—after all, B2 is also induced upon heat shock. I would suggest that a direct measure of the rate of B2 transcription as a function of amanitin treatment (perhaps by a gro-seq sort of approach) would considerably strengthen the argument that the authors wish to make here.

A final, minor point: in the Methods, it was noted that TFIIIF was included in some of the B2 release assays when partially purified release factor was employed. Was IIF necessary to see release? If so, this might clarify (one of) the binding surface(s) on pol II that B2 uses. It would also be interesting in light of recent results suggesting that Gdown1 displaces TFIIIF at or shortly after preinitiation complex formation.

Referee #2:

The mechanism by which non-coding RNAs can influence gene expression is currently of great interest. Previous work from the Goodrich and Kugel labs has demonstrated that the Alu and B2 non-coding RNAs emanating from SINE elements can bind to RNA Pol II and inhibit transcription upon heat shock. This repression occurs at level of PIC assembly with the non-coding RNAs changing the contacts with the promoter DNA, importantly these effects can be reversed by removal of the RNA.

In the current study the authors address the mechanism by which the activity of the non-coding RNAs could be modulated. The authors describe that partially purified cell extracts in the presence of all four NTPs are able to dissociate the B2-RNA-Pol II complex in vitro in a manner dependent on RNA polymerase catalytic activity. The RNA Pol II extends the B2 RNA by ~20nts, however, titration experiments show that this does not result in the release of the RNA from the polymerase. The extension is dependent on the RNA template sequence, since omission of specific NTPs gives rise to defined products that correlate with the sequence of a template region towards the 3’ end of the B2 RNA. This is further supported by nucleotide substitution experiments. In vitro transcription assays demonstrate that under conditions where B2 can be extended it is still able to repress transcription, but ~3 fold less than the non-extended form. RT-PCR experiments identify the presence of the extended B2 RNA in mouse cells and inhibition of RNA Pol II activity increases the levels of total B2 RNA without affecting the levels of extended RNA. Inhibition of Pol II transcription demonstrates that the extended B2 RNA is less stable than the total B2 RNA.

This has the potential to be an important study showing that RNA Pol II has RdRP activity that can modify mammalian ncRNAs and potentially modulate their function. The authors should experimentally clarify the following points relating to the role of RdRP activity:

1. The study is currently preliminary since the role, and therefore the biological relevance, of the 3’end extension in modulating the activity of B2 is unclear. The modification has a relatively modest effect on the repression of transcription likely because the extended B2 RNA remains associated with the RNA polymerase.

2. It is also not clear how the RNA decay profile in Figure 7D corresponds to the repression mechanism, since it suggests that the total B2 RNA contains two pools one that corresponds to a similar rate of decay as the extended B2 and a more stable state, which of these pools relate to the population involved in repression is not clear.

3. Since the authors have previously nicely shown that the B2 RNA inhibits transcription by disrupting the interactions between the PIC and the promoter, the authors should check if the modification reaction may restore native PIC-DNA contacts to enable a transcription competent RNA Pol II complex.
Referee #3:

The authors analyze the metabolism of the Pol III-transcribed B2 regulatory RNA that regulates Pol II transcription initiation. They begin by finding an activity in cell extracts that, in combination with all 4 NTPs, causes Pol II-B2 dissociation. At least part of this NTP-dependent dissociation is due to Pol II extending B2 RNA by 18 nucleotides. This appears to be a single-round reaction. The authors determine the region of B2 that acts as a template for Pol II. Experiments with actinomycin D and alpha amanitin suggest that the extended form of B2 has a much lower half-life than the short form. This suggests that Pol II participates in destruction of this inhibitory RNA.

B2 regulatory RNA and its metabolism and cellular interactions are interesting and important. These authors have previously shown that it regulates expression of many genes in response to heat shock. The experiments are all high quality and, while some of the experiments are indirect, they are convincing in making the authors points. I recommend publication in EMBOJ.

Additional Author Correspondence 13 September 2012

Thank you for giving us the chance to outline the experiments we propose to perform to address the reviewer's points and to provide additional insight into the function of Pol II RdRP extension of B2 RNA. We believe that the studies presented in the initial manuscript provide evidence for several functions for the extension of B2 RNA. Most importantly, extension causes a dramatic increase in the rate at which B2 RNA decays in cells. Second, extension decreases the potency of B2 RNA as a transcriptional repressor. Third, extension causes the Pol II/B2 RNA complex to be less stable in the presence of a cellular factor.

The experiments we propose to do are:

1. Measure amounts of total B2 and extended B2 in cells. This addresses a point made by Reviewer 1. I will, however, point out that extension enhances the rate of decay of B2 RNA and about half of the total B2 RNA decays with the enhanced rate. Therefore, given the difference in apparent decay rates, at any given time the ratio of extended B2 RNA to total B2 RNA is likely to be quite low. This does not mean that the extension reaction is not important in cells, but rather is a reflection of the observation that extended B2 RNA is not kinetically stable and could therefore be present at low levels.

2. Determine the effect of treating cells with alpha-amanitin on transcription of other Pol III genes with A/B box promoters, such as tRNA genes. This will address the first Reviewer's concern that amanitin caused increased transcription of B2 RNA from SINEs in the genome due to a stress response. We agree this is possible and we will test the effect of amanitin on other Pol III transcripts, as well as clarify this point in the manuscript. Although it is worth pointing out that even if amanitin does increase transcription from other A/B box Pol III promoters, this does not change the main conclusion of Figure 7; that extension of B2 RNA increases the rate of decay in cells. With respect to using GRO-seq to investigate SINE transcription as suggested by Reviewer 1, this would be very challenging, due to the fact that SINEs are repeat elements. There are over three hundred thousand B2 SINEs in the genome and the redundancy in sequence will make it impossible to ascribe the short GRO-seq reads to specific SINEs without data from other orthogonal genome-wide studies. To our knowledge no lab has used GRO-seq to analyze SINE element transcription. Finally, we have not established the GRO-seq technique in our lab, do not currently have funds available to perform these experiments, and it would likely take us much longer than three months to obtain reproducible results.

3. Determine the ability of ectopically-expressed B2 RNA and extended B2 RNA to repress luciferase expression from a transfected reporter plasmid. This experiment addresses points 1 and 2 of Reviewer 2, who questioned the function of extension with respect to the transcriptional repression activity of B2 RNA, but did not propose specific experiments. We have used transient transfection assays in the past (Mariner, et al. Molecular Cell, 2008, 29:499) to measure the repression of Pol II transcription by ectopically-expressed B2 RNA in Cos cells (which themselves
do not have B2 RNA). If expressed extended B2 RNA (e.g. 1-196) does not repress the reporter to the same extent, this will provide cell-based evidence that part of the function of extension is to decrease the potency of B2 RNA as a repressor. This would complement the in vitro studies showing that extended B2 RNA decreases the potency of transcriptional repression (Figure 6).

4. Determine the effect of extension of B2 RNA on its ability to prevent Pol II from engaging promoter DNA, as assessed by crosslinking. This addresses the second Reviewer's point 3. We have previously shown that B2 RNA keeps Pol II from crosslinking to promoter DNA containing photoreactivatable crosslinkers (Yakovchuck, et al. PNAS, 2009, 106:5569). We will perform similar assays before and after Pol II is allowed to extend B2 RNA.

I look forward to hearing your thoughts on the proposed experiments and the potential for our submission of a revised manuscript.

Additional Editorial Correspondence 21 September 2012

Thank you very much for your email outlining the planned experiments for a revised version of your manuscript and my apologies for the delay in getting back to you.

I have looked at your outline and consulted with one of the referees, and we both find that your suggested experiments would provide the requested functional insight on B2 elongation. You are therefore invited to submit a revised manuscript containing these outlined experiments. With regard to the difficulties associated with measuring B2 transcription rates through GRO-seq analysis, we agree that this would be outside the scope of the manuscript. Should you be able to address the B2 transcription rate through a different approach this would in our view strengthen the manuscript, but it is not an absolute requirement from our side.

Thank you for giving us the opportunity to consider your manuscript. I look forward to your revision.

1st Revision - authors' response 17 December 2012

We thank the reviewers for their thoughtful and helpful comments that improved the quality of the manuscript. Our point-by-point responses to address the reviewers' concerns are in bold text below.

Referee #1:
In addition to its well-known role as a DNA-dependent RNA polymerase, RNA polymerase II has also been shown to have an RNA-dependent RNA polymerase activity. In the present paper, the authors characterize this activity further, focusing on the ability of mouse B2 RNA to serve as both the template and the substrate to which NTPs are added. This reaction itself has interesting properties, in particular the rather sharp limit of 18 nt as the maximum extension possible of the B2 RNA. There is a deeper biological significance to this system: this laboratory has previously shown that B2 RNA can inhibit transcription of DNA templates by pol II, presumably by blocking the ability of the polymerase to properly interact with the template DNA. They have also shown that upon heat shock, B2 RNA plays a role in down regulating selected genes by associating with the pol II at the promoters of those genes. The present study was initiated by the observation that B2-pol II complexes can be dissociated by an activity in nuclear extracts in a reaction that requires all four NTPs and is amanitin-sensitive. The results of inhibition of B2 synthesis by RNA polymerase III in cells indicate that B2*, the extended form of B2, is less stable than B2. The combination of this result with the strong increase in B2 levels upon treatment of cells with amanitin (which blocks extension of B2 to B2*) leads the authors to suggest that B2 levels are controlled through the pol II-mediated conversion of B2 to B2*.

I think the biochemical studies are very well done. However, as the authors have carefully noted, the basic observation of an RNA-dependent RNA polymerase activity for pol II, using native RNAs as
template and substrate, was reported earlier. At least to me, the most interesting part of this section of the paper is the discovery of a strong barrier to extension of B2 by more than 18 nt. The location of B2 RNA within pol II and the mechanistic details of the extension reaction have not been explored extensively, but I suspect these will ultimately provide a useful tool to probe polymerase structure-function relationships. The particular length at which extension stops is just beyond the length of the RNA-DNA hybrid which results in destabilization of pol II complexes when polymerase transcribes a single-stranded template in the absence of a non-template strand (see Kireeva et al., (2000) JMB 299, p. 325). It may be inappropriate to make too much of the comparison of the two transcription systems but I do suggest the authors comment on this.

We thank the reviewer for pointing out the Kireeva paper. We now comment on mechanistic biochemical studies of Pol II DNA-dependent transcription that provide insight into why RdRP extension of B2 RNA stops after 18 nt, including the Kireeva (2000) JMB paper. We also comment on the potential utility of RdRP extension of B2 RNA as a tool to investigate Pol II structure/function relationships.

My main concern with this paper centers on the in vivo experiments and in particular the conclusions that the authors wish to make from them. It is clear that B2* exists in cells, but I was surprised that the authors did not determine the actual amounts of total B2 and B2* - isn't this necessary to interpret their results? Put another way: if the conversion to B2* affects only a very small fraction of B2, is it likely that blocking that conversion is important in controlling B2 levels?

We have now used primer extension and RT-PCR to measure the amounts of B2 RNA and B2 RNA* in cells compared to standard curves obtained using purified in vitro transcribed B2 RNA and B2 RNA*. We found that 66 +/- 19% of the cellular B2 RNA exists in the extended form. This value is higher than we anticipated, despite it being within error of the estimate that 50% of the cellular B2 RNA is a form that is either extended or can be extended, based on the kinetic experiment in Figure 7D. We are concerned that the quantitation might be misleading due to complications with accurately measuring cellular B2 RNA using primer-based approaches since the population of B2 RNA in cells is likely heterogeneous, whereas the primers used are of a specific sequence. We are currently working on generating RNA-seq libraries to assess the relative level of 3' end extension of B2 RNA in cells. This will provide an orthogonal measurement of the fraction of the total B2 RNA that is extended, which does not require the use of specific primers against B2 RNA. We prefer not to publish the primer extension and RT-PCR quantitation of the fraction of B2 RNA that is extended at this time, and would rather wait to publish it later along with the results from the RNA-seq data we will obtain in the future. We do not want to risk publishing the 66% value at this point in case it does not correlate well with the RNA-seq data.

My understanding is that most pol II is not free in solution (see Kimura et al., (1999) MCB 19, p. 5385). The large majority of pol II that is "chippable" onto DNA (outside of heat shock) is already transcriptionally engaged and thus not available to B2. Is there a significant pool of pol II that can function in the B2 to B2* conversion in normally growing cells?

The Kimura et al. (1999) paper showed that ~40% of the Pol II (140,000 molecules) was soluble in HeLa cells (i.e. not engaged in transcription on chromatin). We imagine the situation would be similar in 3T3 cells, and that this amount of Pol II would be sufficient to extend the B2 RNA present in the non-stressed cells used in the experiments shown in the manuscript. We now comment on this in the Discussion and reference the Kimura paper.

It is certainly true that B2 RNA is remarkably induced by amanitin treatment. The authors assert that this results from a major stabilization of B2, since it can no longer be converted to the unstable B2* form. The critical control for this assertion is the lack of effect of amanitin on 7SK RNA levels. This did not strike me as a conclusive control, since B2 uses an internal A/B box promoter while 7SK uses an upstream promoter. Particularly in light of this difference in promoter architecture, it seems reasonable to suggest as an alternative that B2 levels go up because the B2 promoter is induced by the stress imposed on the cells through poisoning with amanitin - after all, B2 is also induced upon heat shock. I would suggest that a direct measure of the rate of B2 transcription as a function of amanitin treatment (perhaps by a gro-seq sort of approach) would considerably strengthen the argument that the authors wish to make here.

We agree with the reviewer that it was possible amanitin caused increased transcription of B2 RNA from SINEs in the genome due to a stress response. Therefore, we have now tested the effect of amanitin on other Pol III transcripts that, like B2 SINEs, contain A/B box Pol III
promoters. These new data are included in Figure 7B. We found that amanitin did not significantly affect levels of 7SL RNA and tRNA<sub>Leu</sub>. We also monitored the effect of amanitin on B1 RNA levels, which is a stress-induced SINE transcript with an A/B box promoter; however, B1 RNA cannot serve as a substrate for extension by the Pol II RdRP activity (Figure 2). Hence, B1 RNA levels are a good control for amanitin causing a transcriptional stress response. As shown in Figure 7B, levels of B1 RNA slightly decreased upon amanitin treatment.

Together the new data further support the model that amanitin inhibition of Pol II RdRP extension of B2 RNA leads to an overall stabilization of B2 RNA and higher steady-state levels. That said, it remains possible that amanitin does trigger an increase in Pol III transcription of select B2 SINEs. This, however, would not change the main conclusion of Figure 7 – that extension of B2 RNA increases the rate of decay of a large population of the B2 RNA in cells under non-stressed conditions. These points are now clarified in the manuscript.

Lastly, using GRO-seq to investigate SINE transcription is a great idea, however, this would be very challenging because SINEs are repeat elements. There are over 350,000 B2 SINEs in the genome and the redundancy in sequence would make it difficult and perhaps impossible given current technology to ascribe the short GRO-seq reads to specific SINEs. Any attempt to do this will require data from other orthogonal genome-wide studies. To our knowledge no lab has used GRO-seq to analyze SINE transcription. We have not yet established the GRO-seq technique in our lab, and believe doing so with repeat elements will require a long term commitment and likely a collaboration with a lab that has experience with the technology. As such, we were not able to perform GRO-seq during the 3 months allotted for revision of our manuscript.

A final, minor point: in the Methods, it was noted that TFIIF was included in some of the B2 release assays when partially purified release factor was employed. Was IIF necessary to see release? If so, this might clarify (one of) the binding surface(s) on pol II that B2 uses. It would also be interesting in light of recent results suggesting that Gdown1 displaces TFIIF at or shortly after preinitiation complex formation.

TFIIF was not necessary to observe release, however, did enhance the efficiency of release by the factor during purification. A previous publication from our lab found that TFIIF could destabilize the interaction between Pol II and B1 RNA, however, did not affect the kinetic stability of the Pol II/B2 RNA complex (Wagner, et al. 2000. MCB). The role of TFIIF in the facilitated dissociation of B2 RNA from Pol II by the unidentified factor, and whether Gdown1 plays a role in the process, will be topics of future research.

Referee #2:
The mechanism by which non-coding RNAs can influence gene expression is currently of great interest. Previous work from the Goodrich and Kugel labs has demonstrated that the Alu and B2 non-coding RNAs emanating from SINE elements can bind to RNA Pol II and inhibit transcription upon heat shock. This repression occurs at level of PIC assembly with the non-coding RNAs changing the contacts with the promoter DNA, importantly these effects can be reversed by removal of the RNA.

In the current study the authors address the mechanism by which the activity of the non-coding RNAs could be modulated. The authors describe that partially purified cell extracts in the presence of all four NTPs are able to dissociate the B2-RNA-Pol II complex in vitro in a manner dependent on RNA polymerase catalytic activity. The RNA Pol II extends the B2 RNA by ~20nts, however, titration experiments show that this does not result in the release of the RNA from the polymerase. The extension is dependent on the RNA template sequence, since omission of specific NTPs gives rise to defined products that correlate with the sequence of a template region towards the 3' end of the B2 RNA. This is further supported by nucleotide substitution experiments. In vitro transcription assays demonstrate that under conditions where B2 can be extended it is still able to repress transcription, but ~3 fold less than the non-extended form. RT-PCR experiments identify the presence of the extended B2 RNA in mouse cells and inhibition of RNA Pol II activity increases the levels of total B2 RNA without affecting the levels of extended RNA. Inhibition of Pol II transcription demonstrates that the extended B2 RNA is less stable than the total B2 RNA.
This has the potential to be an important study showing that RNA Pol II has RdRP activity that can modify mammalian ncRNAs and potentially modulate their function. The authors should experimentally clarify the following points relating to the role of RdRP activity:

1. The study is currently preliminary since the role, and therefore the biological relevance, of the 3’ end extension in modulating the activity of B2 is unclear. The modification has a relatively modest effect on the repression of transcription likely because the extended B2 RNA remains associated with the RNA polymerase.

   We believe that the manuscript addresses the biological relevance of the 3’ end extension: it functions to destabilize B2 RNA in cells prior to heat shock. To further address the effect of 3’ end extension on transcriptional repression by B2 RNA, we performed the new experiment in Figure 6E. In vitro, extended B2 RNA (B2 RNA*) is a less potent repressor compared to B2 RNA (Figure 6C). Moreover, the extension reaction makes B2 RNA a better substrate for facilitated dissociation from Pol II (Figure 1). These data suggest that in cells B2 RNA* would inhibit transcription to a lesser extent compared to B2 RNA. To test this we performed transient transfection assays in which we ectopically expressed B2 RNA or B2 RNA* in Cos cells (which themselves do not have B2 RNA) and measured the effect on expression from a luciferase reporter. B2 RNA* did not repress the reporter to the same extent as B2 RNA, which is now shown in Figure 6E. These data provide cell-based evidence that, in addition to its role in destabilizing B2 RNA, 3’ end extension also decreases the potency of B2 RNA as a transcriptional repressor.

2. It is also not clear how the RNA decay profile in Figure 7D corresponds to the repression mechanism, since it suggests that the total B2 RNA contains two pools that correspond to a similar rate of decay as the extended B2 and a more stable state, which of these pools relate to the population involved in repression is not clear.

   Figure 7D shows that cellular B2 RNA exists in two pools, one that decays rapidly and corresponds to extended B2 RNA, and one that decays more slowly under non-stressed conditions. This manuscript does not investigate the coupling between RdRP extension of B2 RNA and transcriptional repression during cell stress. We have previously shown that after heat shock, B2 RNA acts as a transcriptional repressor; we do not yet know if B2 RNA functions as a repressor prior to heat shock. Hence, we have no reason to believe that either of these pools of B2 RNA functions in transcriptional repression. B2 RNA levels increase significantly upon cellular stress in part due to increased transcription of B2 SINEs. We have not investigated the stability of total B2 RNA or extended B2 RNA after heat shock. Moreover, the heat shock induced B2 RNA that functions in repression may or may not be efficiently extended. It is also possible that the increase in B2 RNA levels during heat shock could result in part from decreased extension of B2 RNA. Future studies will be aimed at understanding the role of RdRP extension in controlling the levels of B2 RNA during heat shock and the transcriptional repression properties of this ncRNA during cellular stress. We now clarify these points in the text.

   That said, we speculate that the population of B2 RNA involved in transcriptional repression during heat shock will be more stable. This is based on the following observations: extended B2 RNA can be removed from Pol II by a cellular factor (Figure 1); B2 RNA* is a less potent transcriptional repressor than B2 RNA in vitro (Figure 6C); and the new data showing that ectopically expressed B2 RNA* represses a reporter in cells less effectively than B2 RNA (Figure 6E).

3. Since the authors have previously nicely shown that the B2 RNA inhibits transcription by disrupting the interactions between the PIC and the promoter, the authors should check if the modification reaction may restore native PIC-DNA contacts to enable a transcription competent RNA Pol II complex.

   We have now performed the crosslinking experiment shown in Figure 6D. We incorporated B2 RNA* (the extended transcript) into PICs and monitored Pol II-DNA crosslinking in these complexes compared to complexes containing B2 RNA or no RNA. We found that B2 RNA* substantially inhibited a Pol II-promoter crosslink, although not to the same extent as B2 RNA. These results correlate with the transcriptional inhibition data in Figure 6C, which show that transcriptional inhibition by B2 RNA* is less potent compared to B2 RNA.
Referee #3:
The authors analyze the metabolism of the Pol III-transcribed B2 regulatory RNA that regulates Pol II transcription initiation. They begin by finding an activity in cell extracts that, in combination with all 4 NTPs, causes Pol II-B2 dissociation. At least part of this NTP-dependent dissociation is due to Pol II extending B2 RNA by 18 nucleotides. This appears to be a single-round reaction. The authors determine the region of B2 that acts as a template for Pol II. Experiments with actinomycin D and alpha amanitin suggest that the extended form of B2 has a much lower half life than the short form. This suggests that Pol II participates in destruction of this inhibitory RNA.

B2 regulatory RNA and its metabolism and cellular interactions are interesting and important. These authors have previously shown that it regulates expression of many genes in response to heat shock. The experiments are all high quality and, while some of the experiments are indirect, they are convincing in making the authors points. I recommend publication in EMBOJ.

We thank the reviewer for the positive comments and recommendation.

2nd Editorial Decision 03 January 2013

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below. As you will see they both find that all criticisms have been addressed in an adequate manner and I am therefore pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

REFEREE REPORTS:

Referee #1

In my review of the original version of this manuscript, I expressed particular concern about the conclusion that the very strong induction of B2 RNA by amanitin was based on the inability of pol II to extend B2 to the much less stable B2* form. Since amanitin treatment stresses cells, I offered an alternative explanation: since B2 is known to be responsive to other stresses, perhaps that is the primary basis for the amanitin effect. The authors have now provided a strong response: B1 RNA, which is also stress responsive (and which also has the same promoter architecture as B2) does not increase with amanitin treatment. Crucially, B1 is not a substrate for the RNA-dependent RNA polymerase activity of pol II. While a direct measure of B2 transcription such as gro-seq would be conclusive, I agree with the authors that it is unreasonable to ask them to overcome the considerable technical difficulties for such an experiment as a condition for publishing the current manuscript.

The authors have responded appropriately to my other concerns as well. The combination of mechanistic biochemistry with a regulatory role for the RNA-dependent RNA polymerase activity makes a compelling case for publication.

Referee #2

We have read the revised manuscript and the authors responses and we think it is fine.