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Mg^{2+} facilitates leader peptide translation to induce riboswitch-mediated transcription termination

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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 15 October 2010

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

Should you be able to address these criticisms in full, we could consider a revised 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. This is particularly pertinent since the highly related Cell paper was published before submission of this manuscript, and the Cell paper would certainly have to be cited and discussed, in particular in light of the apparent discrepancies regarding the Proline data, as raised by all the referees.

In this case all referee comments will have to be addressed, by experimentation where appropriate. The only possible exception is referee 2's request for a full in vitro & in vivo analysis ('a comprehensive evaluation of both models in parallel to settle the above issues in a quantitative manner in vivo and in vitro'). I summarize some of the important referee issues raised:

ref 1 notes that your study presents an advance over the Cell paper, which should be cited, in showing vitro RNA structure probing data. The referee requests a broader discussion on this. The abstract in particular needs to be more clear.

ref 2 states the Cromie etal. paper predicted some of these findings. On the other hand, in the referee's opinion, Cromie was controversial as it was based on weak data and the current paper could

actually invalidate it if translational control was definitively shown to be the key.
The referee states 'Even though the manuscript identifies a new component of Mg2+ dependent control of mgtA at the post-transcriptional level, it does not give insight into whether translational control is the major mechanism of the proposed RNA sensing of Mg2+ concentrations. '
Notably, 'controls are often missing'. Again, you will need to refer to Cell paper and discuss discrepancies. The referee wants the stem loop opening mechanism better supported experimentally.

ref 3 again highlights differences to Cell paper, while this referee is not as perturbed as the other reviewers by this (s/he argues that the experiments were done very differently), s/he requires discussion. A number of detailed textural points must be addressed.

Ref 4 finds evidence for the key structure probing experiments weak, noting that this would be a key advance over the Cell paper. Controls need to be added. A key figure is wrongly numbered, as we already discussed earlier this week.

Please note that given the related publication, we cannot allow more than 6 weeks for a full revision (preferably less). Please contact us after 4 weeks to discuss progress. The revised data would be seen by two of our referee panel. In the hypothetical case of acceptance, we would certainly aim to pursue fast track publication.

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 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 COMMENTS

Referee #1 (Remarks to the Author):

The manuscript by Zhao et al. identifies translation of a 17 amino acid upstream ORF (uORF) in cis (and not the peptide itself) as necessary for control of gene expression by the magnesium ion responsive 5'-UTR of the Salmonella mgtA gene. Although a similar result was recently described in a paper published in Cell, these authors have discovered the phenomenon independently, and extend the work beyond that of the competitors with compelling in vitro RNA structure probing data that demonstrate SD-sequence occlusion induced by low magnesium ion concentrations, probably a key aspect of the function of this genetic switch. Furthermore, the results presented here allow the authors to resolve several puzzles scattered through the previous literature. Altogether, the results suggest the existence of a new type of gene-regulatory mechanism that should be of interest to a broad readership. We thus feel that this manuscript is suitable, in principle, for publication in the EMBO Journal.

Several issues that merit attention are the following.

1. The abstract should state explicitly the central finding that in-cis translation of the embedded ORF is required for attenuating downstream gene expression. The last sentence of the abstract is vague and confusing.

2. The full mechanism controlling the expression of the downstream mgtA ORF is not yet established to be premature termination of transcription, as opposed to other forms of elongation

interruptions including RNAP inactivation, extended pauses, or even mRNA processing or stability. This needs to be made clear in the abstract and throughout the text.

3. The result showing that expressing MgtF peptide in trans did not rescue the start codon substitution, and therefore indicating that it is the act of MgtF translation, as opposed to the peptide that elicits the transcription attenuation in high Mg2+ is important, and should be more prominently described (also see point 1).

4. The authors should make a connection in discussion, and possibly in the abstract, with uORFs from eukaryotic and viral gene regulatory systems.

5. The data presented here are largely in line with those of the recent Cell paper, except for the discrepancy on the effect of proline limitation on the regulatory outcome. Why were some of the in vivo experiments in this MS carried out in E.coli rather than Salmonella? What is the evidence that results obtained in E.coli apply to the Salmonella riboswitch? Is there a way to reconcile these findings with those described in Cell paper? We suggest the authors explicitly note this caveat in the text, or provide additional data addressing this discrepancy.

6. The "switch 1" and "switch 2" nomenclature is confusing, since what is happening is apparently the inter-conversion of two mutually exclusive secondary structures (comprising stems A/B or C/D). It may be more informative to describe stems or helices or paired regions, and where needed, "the 5' strand of helix A" etc.

7. Although semantic, it would be more in line with the rest of the riboswitch literature to refer to the regulatory RNA element as the 5'-untranslated region (5'-UTR) rather than the leader.

Referee #2 (Remarks to the Author):

The manuscript by Zhao et al. reports the discovery of a conserved proline-rich, short reading frame-referred to as mgtF-in the 5' leader sequence of the Salmonella mgtA gene, the latter of which was known to be regulated in response to intracellular Mg2+ concentrations. Specifically, it had been postulated that the 5' leader of mgtA directly senses Mg2+ at the RNA level. If Mg2+ is high, transcription will prematurely terminate in the leader sequence, whereas low Mg2+ promotes the formation of an alternative structure which allows RNA polymerase to read through the leader and transcribe the mgtA coding region (Cromie et al. 2006 Cell, co-authored by the corresponding author of the present manuscript).

The authors demonstrate, using mgtF overexpression from a constitutive promoter In E. coli, that the peptide is produced in a Mg2+ dependent manner; it can be detected as a fusion protein under growth in media with millimolar but not micromolar Mg2+ concentration. Using a lacZ reporter specific for 5' leader control of mgtA expression and suitable mutants thereof, the also show that translation of the MgtF peptide is required that achieve Mg2+ dependent control MgtA expression. If the mgtF start codon is inactivated, or a premature stop codon inserted in the reading frame, mgtA is no longer repressed under high Mg2+ conditions. RNA structure probing in vitro is then used to show that the translation initiation region of mgtF is more accessible under the high Mg2+ condition, suggesting that ribosome binding is determined directly by Mg2+ induced structure changes. Several mutations are used to infer that occupancy of this region by ribosomes promotes a structural switch in the 5' leader that will eventually lead to transcriptional termination. Lastly, the authors address whether the unusually high number of proline or arganine codons in mgtF has any biological meaning such that availability of these amino acids would regulate the translational efficiency of mgtF and thereby mgtA transcription. This is done by codon substitutions in the 5' leader and growth in different strains defective of amino acid import. The results suggest that changes in intracellular proline or arganine concentrations are unlikely to impact the control of mgtA transcription, both generally and with respect to the proposed Mg2+ sensing activity of this 5' leader sequence.

Even though the manuscript identifies a new component of Mg2+ dependent control of mgtA at the post-transcriptional level, it does not give insight into whether translational control is the major

mechanism of the proposed RNA sensing of Mg2+ concentrations. Specifically, the previous Cell paper by Cromie et al. 2006 made a very strong point that Mg2+ is sensed co-transcriptionally in a process that only requires RNA polymerase and established a framework of RNA structure changes that cause transcriptional termination or anti-termination. Note that this model has met with some skepticism, especially since the co-transcriptional sensing was based on results of in vitro transcription experiments that were not too compelling. It now appears that Mg2+ sensing occurs via translational control, though the authors dodge a clear statement as to whether the translational control is the key event (which would invalidate the former model) or a mere prerequisite for Mg2+ sensing by transcription. It is possible that both mechanisms operate in parallel; either way, for a paper in EMBO J one would expect to see a comprehensive evaluation of both models in parallel to settle the above issues in a quantitative manner in vivo and in vitro. Given the previous publication, it will be important to arrive at a clear picture of which of the two mechanisms controls allows Mg2+ control of the mgtA leader at the RNA level. For example, the authors should repeat the previous in vitro transcription experiments in the presence of ribosomes, and include mutant RNAs that favor one or the other mechanisms. Other than that, even though the quality of the presented data is generally good, essential controls are often lacking.

In summary, the manuscript as it stands would be better suited for a journal below the level of EMBO J, even though it would still require a major revision and additional work. However, if the above points were sufficiently addressed and additional experiments performed to hammer home the mechanism, it should be of interest to the readership of EMBO J as well. I could imagine that the manuscript would then be sufficiently different from and more informative than the related Cell papers from the Groisman lab on the mgtA 5' leader and models of Mg2+ sensing (Cromie et al. 2006; Park et al. 2010), even if contradictions remain and models need to be corrected.

More specific comments:

1. This manuscript directly competes with a recent paper from the Groisman lab (2010 Cell 142:737-748) which the authors have chosen to ignore although it has been out in print for a month. There is considerable overlap in results, which agree on the mgtF reading frame being important for the control of mgtA. However, the present manuscript contradicts the major finding of the Groisman lab who suggest that the proline codons serve an important function, integrating a sensing of proline levels and hyperosmoctic stress into mgtA control, independently of Mg2+. The authors need to clearly discuss the similarities and contradictions of the two studies. In addition, the Groisman lab already named the very same reading frame mgtL; I see little point in the authors' referring to it as mgtF as this will be confusing for future readers.

2. Is the mgtA leader really a riboswitch? As it stands now, it's a transcription attenuator whose activity if determined by Mg2+ dependent binding of ribosomes to an upstream ORF. By contrast, riboswitches are controlled by structural rearrangement of RNA that is solely determined by binding of the ligand.

3. Identification of the MgtF peptide (page 8 and Figure 2C): The experiment shown lacks the control by MALDI-TOF spectrum determined on a strain that does not express the peptide, otherwise it is hard to argue that the labeled peak is MgtF.

4. Lower paragraph on page 8 through first para on page 10: The text is very hard to comprehend, mainly because of the way the mutations are referred to. This will benefit from clearer writing and organizing reporter fusion data as tables or integrating them in Figure 3. In addition, Figure 3B will benefit from inclusion of more information regarding the consequences of the introduced mutations (for example, A71C is start codon inactivated, A71G is GUG start codon, ...).

5. Structure probing in Figure 4: The data is indicative of Mg2+ being able to open stem-loop structure D, but the evidence is insufficient. This experiment should include RNA mutants expected to perturb the Mg2+ response, as well as control RNAs to show that Mg2+ does not change the structure of just any RNA leader. Most importantly, the authors need to show that the Mg2+ dependent structure change in the mgtF RNA permits productive ribosome binding (for example, by 30S toeprinting). Again, appropriate mgtF and unrelated control RNAs will be needed for this experiment, including mgtF RNAs with mutations at the Shine-Dalgarno sequence or start codon, and in other regions of the leader that talk to stem-loop D.

Referee #3 (Remarks to the Author):

This manuscript demonstrates the existence of a short ORF in the 5' leader of the mgtA gene, previously described as containing a Mg-sensing riboswitch. In this work, the authors demonstrate that this conserved ORF has a profound effect on expression from the downstream gene. They find that expression of the ORF is itself regulated by Mg levels (possibly by the changes in RNA folding previously found for this leader) and that the down-regulation of mgtA/reporter expression at high Mg requires that the ORF be translated.
The data here differs from that recently published by Park et al in that they see a role for proline/hyperosmolarity sensing that is not seen in this study. However, these experiments were done in very different ways, so there is no real conflict between the findings. The major new point here is that Mg sensing needs ORF translation. In general, the results are clear, although the writing could be more precise and better integrated with previous work on this system.

Specific comments:

1. p. 4: sentence is unclear: mgtA was transcribed more in a high Mg condition (compared to what).

2. Fig. 2C: A control is really needed for this, of either an uninduced, or vector induced to confirm that the peak is in fact MgtF. If a FLAG tag is added to this construct, is the protein more abundant? This might help to confirm that the wild-type peptide is in fact unstable.

3. P.9, end of top paragraph: this sentence is unclear. Are you saying that when the ORF is not translated, stem-loop B is formed, or that it is usually formed in high Mg (dependent on translation)? Is formation of stem-loop B required for the usual down-regulation at high Mg.

4. Fig. 4: The data in this is central to the model in Fig. 4C. A bit more explanation/experimentation would help. Given that all but one of the DMS modification ratios in Fig. 4A are increased with high Mg, it is difficult to be certain that this isn't just an effect of Mg on DMS modification. For instance, nt 87-89 are apparently not paired in either SL D or A, according to Fig. 4, but do show an increase in DMS with Mg. Should the results all be normalized to this, and only larger effects considered? Extending the region to look more at the positions near 105 would help as well (does the DMS result agree with the RNase T1 cleavage result?). If one did normalize some of these, the opposite pattern for C56 and G105 would be even more striking.

5. Fig. 4: Switch 2 is not discussed at all in the text on p. 10-11, or in the figure legend. As noted below, if Fig. 4C is moved later in the paper, this could be addressed there. I assume the authors want to say that translation affects these conformations, keeping stem-loop C from forming, but this is not discussed at all. Are there earlier mutants that would speak to the role of the "switch 2" region forming that are worth mentioning?

6. P. 11, p. 16: The authors cite the fact that in Cromie et al, a UCUCC mutant (changed to AGAGG) disabled the response to Mg, and they say that is consistent with their model. However, I differ with their interpretation of both what would be expected and what the results actually were. While there is no Mg response in those cells, the level of expression is very low (Table 2, Cromie et al; the level at low Mg is equivalent to the WT at high Mg). Thus, regardless of what that paper said, I would say this not longer responds to Mg and is basically off. Looking at Fig. 4C, the mutation would have two effects: while it would disrupt A1, it would also disrupt the switch 1 sequence that binds to the SD in structure D. I would think this would lead to constitutive MgtF expression, (SD is not supposed to be sequestered in thir high Mg condition), and therefore should be off for mgtA under all conditions (what was seen).

7. The redundancy between p.11 and p. 16 might be helped by saving Fig. 4C for the discussion and only discussing it there).

8. Given the difference in findings here and in the publication by Park et al on the role of the proline codons, this section might profit from a bit more discussion, or at least pointing out the major

differences in how these experiments were carried out (long term, 10 mM proline, measuring fusion expression here, short term (15'), 1 M proline, measuring mRNA levels in Park et al).

9. P.17, Fig. 4: Since one might conclude from the work presented here that sensing of Mg for termination or readthrough depends in vivo on the ribosome and MgtF, a very clear discussion of how this is integrated with the previous model of in vitro Mg-dependent termination of transcription would be useful. I can imagine a number of models, and it wasn't clear to be which the authors favor and what was the data for each. A couple of the points needing clarification:

a. One model (the preferred one?) is that there is a Mg-dependent variation in mRNA folding; this allows the ORF to be read or not read (dependent on SL A folding), leading in turn to SL C forming or not forming. In this model, the 'riboswitch" is necessary only to regulate ribosome entry. However, as far as I can tell, this is not fully consistent with Cromie et al, finding termination in vitro, although one could argue that the in vitro situation is different enough (and regulation was much less dramatic in vitro) so that there is a bit of SL C in that condition.

b. As the authors state (p. 8), MgtF synthesis is a prerequisite for termination, based on the data showing that, in the absence of ORF translation, mgtA expression is on constitutively. If SL B is necessary for termination, the mutants suggest that translation to the termination codon allows B to form (by blocking C? no data really provided here). In this model, the ribosome could possibly be the Mg sensor, and something around the SD/initiation codon determines its dependence on Mg.

10. Minor comments: P.6, middle of page: amino acid residues are not underlined (highlighted instead).

Referee #4 (Remarks to the Author):

The present manuscript provided an interesting model demonstrating the influence of the translation of a small open reading frame on the expression of the downstream gene encoding a Mg transporter MgtA. The authors have shown that the sORF undergoes a conformational change induced by Mg2+ that facilitates the translation of the sORF and in turn induces transcription termination of the downstream gene MgtA.

However, the probing data are overinterpretated. I do not see how the reactivity pattern support so well the two secondary structure models and especially the effect of magneisum ions. The main effect of Mg is to enhance slightly the reactivity of bases in the region 73 to 86. The only significant changes in reactivity are located around position 30 (see the top of the gel) where several nucleotides appear protected when Mg concentration increased. It seems that this region become base paired. To further improve the structure model, additional experiment would be required such as CMCT modification to get information on U at N3 and G at N1. Why the authors did not perform the classical DNA sequencing ladders using reverse transcriptase on the four nucleotides? Some of the labels on the autoradiographiy are not correctly assigned: A55 instead of C56, A64 and G63 instead of A63 and G64, etc... This should be also corrected in the quantification of the results. In Figure 1A, incubaton controls have also to be performed in the presence and in the absence of Mg. The RNase T1 cleavage at position 105 at low Mg concentration is really weak. I am not sure that this is really relevant. In the latter experiment, there is no incubation control.

The reactivity of the nucleotide have to be reported on the secondary structure as shown in Figure 4C.

The authors have shown that the transcription of MgtA is unlikely dependent of the proline or arginine concentration in contrast to ther ecent results of Park et al. As such, this is potentially interesting. However, they have mutated single proline codon at positions 3, 5 or 7. Perhaps the effect of proline would be seen if the codons at position 3, 5, 7 and 9 would have been mutated all together, taking care that the structure of the hairpin D would not be altered by the modifications.

Referee #1

1. The abstract should state explicitly the central finding that in-cis translation of the embedded ORF is required for attenuating downstream gene expression. The last sentence of the abstract is vague and confusing.

We have rewritten the abstract to clarify vague themes. The last sentence of the abstract was modified in the revised manuscript.

2. The full mechanism controlling the expression of the downstream mgtA ORF is not yet established to be premature termination of transcription, as opposed to other forms of elongation interruptions including RNAP inactivation, extended pauses, or even mRNA processing or stability. This needs to be made clear in the abstract and throughout the text.

Although the full mechanism requires further development, we have now discussed possible new mechanisms regarding MgtF translation-coupled premature termination of *mgtA* transcription in the abstract and throughout the text.

3. The result showing that expressing MgtF peptide in trans did not rescue the start codon substitution, and therefore indicating that it is the act of MgtF translation, as opposed to the peptide that elicits the transcription attenuation in high Mg2+ is important, and should be more prominently described (also see point 1).

We have proposed a possible role for MgtF translation, which is comparable to a mechanism used by some uORFs in eukaryotes, in which the post-translation release of the ribosome subunit(s) influences the downstream coding region (p19). In addition, we have discussed the possibility of RNase E interacting with the *mgtA* 5'LR to promote degradation, possibly facilitated by MgtF translation (p20, the first paragraph).

4. The authors should make a connection in discussion, and possibly in the abstract, with uORFs from eukaryotic and viral gene regulatory systems.

We have discussed the uORF comparable mechanisms (see this Referee, Comment #3).

5. The data presented here are largely in line with those of the recent Cell paper, except for the discrepancy on the effect of proline limitation on the regulatory outcome. Why were some of the in vivo experiments in this MS carried out in E.coli rather than Salmonella? What is the evidence that results obtained in E. coli apply to the Salmonella riboswitch? Is there a way to reconcile these findings with those described in Cell paper? We suggest the authors explicitly note this caveat in the text, or provide additional data addressing this discrepancy.

We carried out quantitative determination of cytoplasmic proline concentrations, under the conditions used in the Cell 2010 paper, which they concluded to be important for the MgtF translation-dependent regulation of premature termination of *mgtA* transcription. We also constructed a *Salmonella* proline auxotroph to determine the *mgtA* expression in the prolinelimiting conditions. However, we did not find that proline concentration had any effect on the *mgtA* regulation suggested. In addition, we discuss the reason as to why they found that the MgtF translation is independent of Mg2+.

6. The "switch 1" and "switch 2" nomenclature is confusing, since what is happening is apparently the inter-conversion of two mutually exclusive secondary structures (comprising stems A/B or C/D). It may be more informative to describe stems or helices or paired regions, and where needed, "the 5' strand of helix A" etc.

We do not use "switch 1" and "switch 2" in the revised manuscript, but, instead, use the sequences to describe the stem-loop switching.

7. Although semantic, it would be more in line with the rest of the riboswitch literature to refer to the regulatory RNA element as the 5'-untranslated region (5'-UTR) rather than the leader.

In fact, the 5'UTR now contains the *mgtF* ORF that can be translated. Thus, we continue to use the 5' leader region (i.e., 5'LR).

Referee #2

... it does not give insight into whether translational control is the major mechanism of the proposed RNA sensing of Mg2+ concentrations. Specifically, the previous Cell paper by Cromie et al. 2006 made a very strong point that Mg2+ is sensed co-transcriptionally in a process that only requires RNA polymerase and established a framework of RNA structure changes that cause transcriptional termination or anti-termination...

We have discussed that the translation of MgtF is not responsible for the Mg2+ sensing of the riboswitch: "Based on this model, it is the Mg2+ signal that determines the translation of MgtF, not the translation of MgtF that leads to the Mg2+ sensing" (p18). In addition, we concur with the findings of the Cell 2006 paper in which Mg2+ is sensed by RNA itself. However, the truncated transcripts detected from the *in vitro* transcription assay could be derived from actual premature termination or strong pausing, which prevents the RNA polymerase from proceeding.

It now appears that Mg2+ sensing occurs via translational control, though the authors dodge a clear statement as to whether the translational control is the key event (which would invalidate the former model) or a mere prerequisite for Mg2+ sensing by transcription.

Again, we have discussed that Mg2+ sensing does not occur via translation of MgtF. However, translation of MgtF is required for the signal transduction from the actual Mg2+ sensor to its downstream effector domain(s).

...it will be important to arrive at a clear picture of which of the two mechanisms controls allows Mg2+ control of the mgtA leader at the RNA level. For example, the authors should repeat the previous in vitro transcription experiments in the presence of ribosomes, and include mutant RNAs that favor one or the other mechanisms...

In vitro transcription assays were repeated by supplementing ribosomes and using mutant RNAs which turned off translation of MgtF *in vivo* (Supplemental Figure 1 and p12 & 19).

Other than that, even though the quality of the presented data is generally good, essential controls are often lacking.

Several assays were repeated using sufficient controls, e.g., a vector control (pUHE) was added to the MS analysis along with the *mgtF* plasmid (pUHE-*mgtF*) (Figure 2C).

More specific comments:

1. This manuscript directly competes with a recent paper from the Groisman lab (2010 Cell 142:737-748) which the authors have chosen to ignore although it has been out in print for a month. There is considerable overlap in results, which agree on the mgtF reading frame being important for the control of mgtA. However, the present manuscript contradicts the major finding of the Groisman lab who suggest that the proline codons serve an important function, integrating a sensing of proline levels and hyperosmoctic stress into mgtA control, independently of Mg2+. The authors need to clearly discuss the similarities and contradictions of the two studies. In addition, the Groisman lab already named the very same reading frame mgtL; I see little point in the authors' referring to it as mgtF as this will be confusing for future readers.

These comments have been discussed above (see Referee #1, response #5).

2. Is the mgtA leader really a riboswitch? As it stands now, it's a transcription attenuator whose activity if determined by Mg2+ dependent binding of ribosomes to an upstream ORF. By contrast, riboswitches are controlled by structural rearrangement of RNA that is solely

determined by binding of the ligand.

The function of the *mgtA* 5'LR was established as a riboswitch in the Cell 2006 paper. Our results concur with that finding. Although different from known mechanisms of transcription attenuation, there is no Rho-independent terminator identified in this Mg2+ riboswitch.

3. Identification of the MgtF peptide (page 8 and Figure 2C): The experiment shown lacks the control by MALDI-TOF spectrum determined on a strain that does not express the peptide, otherwise it is hard to argue that the labeled peak is MgtF.

We have added a vector control (pUHE) in which the signal (*m/z*: 2171.38, Figure 2C) representing MgtF was not detected.

4. Lower paragraph on page 8 through first para on page 10: The text is very hard to comprehend, mainly because of the way the mutations are referred to. This will benefit from clearer writing and organizing reporter fusion data as tables or integrating them in Figure 3. In addition, Figure 3B will benefit from inclusion of more information regarding the consequences of the introduced mutations (for example, A71C is start codon inactivated, A71G is GUG start codon, ...).

We have revised these two sections and included a table (Figure 3C) to characterize the 5'LR substitutions.

5. Structure probing in Figure 4: The data is indicative of Mg2+ being able to open stem-loop structure D, but the evidence is insufficient. This experiment should include RNA mutants expected to perturb the Mg2+ response, as well as control RNAs to show that Mg2+ does not change the structure of just any RNA leader. Most importantly, the authors need to show that the Mg2+ dependent structure change in the mgtF RNA permits productive ribosome binding (for example, by 30S toeprinting). Again, appropriate mgtF and unrelated control RNAs will be needed for this experiment, including mgtF RNAs with mutations at the Shine-Dalgarno sequence or start codon, and in other regions of the leader that talk to stem-loop D.

We synthesized an RNA with substitutions at 91-95, which should lose its ability to respond to Mg2+ and thus promote the continuous translation of MgtF, to further demonstrate our model by carrying out additional experiments for RNA probing (p12). We also synthesized another RNA which carries a double substitution at 91-95 and 102-106 which could restore stem-loop A1 and restore the premature termination of the *mgtA* transcription in high Mg2+ (p12-13). Our results provide additional data to support our model.

Referee #3

1. p. 4: sentence is unclear: mgtA was transcribed more in a high Mg condition (compared to what).

We are unsure of the location of the comment, however, we have found "that substituted nucleotide 98 from C to U, resulted in expression of *Salmonella mgtA* in high Mg2+ concentrations (O'Connor et al, 2009)" suggesting that, compared to the wild-type, substitution of 98, which changed the Arg-10 codon to a stop codon, eliminated premature termination of *mgtA* transcription in high Mg2+ due to the early stop of MgtF translation.

2. Fig. 2C: A control is really needed for this, of either an uninduced, or vector induced to confirm that the peak is in fact MgtF. If a FLAG tag is added to this construct, is the protein more abundant? This might help to confirm that the wild-type peptide is in fact unstable.

We have added a vector control (pUHE) in which the signal (*m/z*:2171.38, Figure 2C) representing MgtF was not detected.

3. P.9, end of top paragraph: this sentence is unclear. Are you saying that when the ORF is not translated, stem-loop B is formed, or that it is usually formed in high Mg (dependent on translation)? Is formation of stem-loop B required for the usual down-regulation at high Mg.

We have changed “Thus, when the MgtF start codon is disrupted, *mgtA* transcription remains activated in high Mg2+, in which stem-loop B is formed.” to “Thus, if MgtF fails to be translated, high Mg2+ is not sufficient to induce premature termination of *mgtA* transcription.” (p10, first paragraph).

4. Fig. 4: The data in this is central to the model in Fig. 4C. A bit more explanation/experimentation would help. Given that all but one of the DMS modification ratios in Fig. 4A are increased with high Mg, it is difficult to be certain that this isn't just an effect of Mg on DMS modification. For instance, nt 87-89 are apparently not paired in either SL D or A, according to Fig. 4, but do show an increase in DMS with Mg. Should the results all be normalized to this, and only larger effects considered? Extending the region to look more at the positions near 105 would help as well (does the DMS result agree with the RNase T1 cleavage result?). If one did normalize some of these, the opposite pattern for C56 and G105 would be even more striking.

Mg2+ does have an effect on the DMS modification, however small. The average ratio of each modified nucleotide is 1.3 - 1.4, however all of the nucleotides shown in Figure 4 are at least 2.0, therefore, we can conclude that these increased ratios are, in fact, due to the conformation change. In addition, our results show that nucleotide 89 is base-paired in low Mg2+, consistent with our model. The results are normalized against nucleotide 70, which is not base-paired in low or high Mg2+. We previously found that the DMS modification in G105 had a slight decrease and therefore, was not previously included in the manuscript, however, is now included in the revised manuscript. In general, there is an agreement between the DMS and RNase T1 cleavage results.

5. Fig. 4: Switch 2 is not discussed at all in the text on p. 10-11, or in the figure legend. As noted below, if Fig. 4C is moved later in the paper, this could be addressed there. I assume the authors want to say that translation affects these conformations, keeping stem-loop C from forming, but this is not discussed at all. Are there earlier mutants that would speak to the role of the "switch 2" region forming that are worth mentioning?

We do not use the terms “switch 1” or “switch 2” in the revised manuscript. Instead, we have discussed the stem-switching (or base-pairing) by defining the conformational changes. Our conclusion suggests that Mg2+ concentration determines MgtF translation. We discuss that translation of MgtF unlikely facilitates the formation of stem-loop B (p19).

6. P. 11, p. 16: The authors cite the fact that in Cromie et al, a UCUCC mutant (changed to AGAGG) disabled the response to Mg, and they say that is consistent with their model. However, I differ with their interpretation of both what would be expected and what the results actually were. While there is no Mg response in those cells, the level of expression is very low (Table 2, Cromie et al; the level at low Mg is equivalent to the WT at high Mg). Thus, regardless of what that paper said, I would say this not longer responds to Mg and is basically off. Looking at Fig. 4C, the mutation would have two effects: while it would disrupt A1, it would also disrupt the switch 1 sequence that binds to the SD in structure D. I would think this would lead to constitutive MgtF expression, (SD is not supposed to be sequestered in thir high Mg condition), and therefore should be off for mgtA under all conditions (what was seen).

We want to thank this reviewer for the comment in which we are in agreement with. We determined expression using a reconstructed plasmid harboring the UCUCC to AGAGG (91-95) substitution. Our results repeated the data presented in the Cell 2006 paper in which the “switch 1” was disrupted (we eliminated the use of “switch 1” and “switch 2” from the revised manuscript). As the reviewer suggested, we believe that “while the substitution (91-95) would disrupt A1, it would also disrupt the switch 1 sequence that binds to the SD in structure D… this would lead to constitutive MgtF expression”. This would be why the transcription of *mgtA* is mostly turned off in low Mg2+ (Figure 4B and Cell 2006 paper). An additional substitution (at position 102-106) restores the response to Mg2+ (Figure 4B). As described in Referee #2, Comment #5, we mapped the RNAs with substitutions at these nucleotides, as well as RNA with a double substitution at 91-95 and 102-106 which could restore stem-loop A1 and restore the premature termination of the *mgtA* transcription (Figure 4C).

7. The redundancy between p.11 and p. 16 might be helped by saving Fig. 4C for the discussion and only discussing it there).

We discussed the original Figure 4C (now, Figure 6) in the discussion.

8. Given the difference in findings here and in the publication by Park et al on the role of the proline codons, this section might profit from a bit more discussion, or at least pointing out the major differences in how these experiments were carried out (long term, 10 mM proline, measuring fusion expression here, short term (15'), 1 M proline, measuring mRNA levels in Park et al).

We have expanded our discussion to report differences in our conclusions. We carried out quantitative determination of cytoplasmic proline concentrations and β-galactosidase assays, under the conditions used in the Cell 2010 paper, which they concluded to be important for the MgtF translation-dependent premature termination of *mgtA* transcription. However, we did not find that proline concentration had any effect on the *mgtA* regulation suggested. In addition, we discuss the reason as to why they found that the MgtF translation is independent of Mg2+ (p18-19).

9. P.17, Fig. 4: Since one might conclude from the work presented here that sensing of Mg for termination or readthrough depends in vivo on the ribosome and MgtF, a very clear discussion of how this is integrated with the previous model of in vitro Mg-dependent termination of transcription would be useful. I can imagine a number of models, and it wasn't clear to be which the authors favor and what was the data for each. A couple of the points needing clarification: a. One model (the preferred one?) is that there is a Mg-dependent variation in mRNA folding; this allows the ORF to be read or not read (dependent on SL A folding), leading in turn to SL C forming or not forming. In this model, the 'riboswitch" is necessary only to regulate ribosome entry. However, as far as I can tell, this is not fully consistent with Cromie et al, finding termination in vitro, although one could argue that the in vitro situation is different enough (and regulation was much less dramatic in vitro) so that there is a bit of SL C in that condition.

We believe that the truncated transcript (220-nt long) *in vitro* is derived from a product in which RNA polymerase (RNAP) is paused in high Mg2+, and not actual termination, because Rhoindependent terminator was not found from upstream of the nucleotide C220. Therefore, we believe that in high Mg2+, the RNA conformation favors the pausing of the RNAP at this nucleotide site. After the sample is processed, the paused RNA intermediate is visualized as a 220 nt band. MgtF translation and the ribosome, *in vivo*, may allow the premature termination to take place near this strong pausing site with the assistance of other cellular factors (e.g., RNase E). Consistent with this hypothesis, a previous study identified a truncated *mgtA* 5'LR with an apparent length ~ 240 nt (see Kawano, etc., 2005). We also discussed this possibility in the revised manuscript (p4, first paragraph).

b. As the authors state (p. 8), MgtF synthesis is a prerequisite for termination, based on the data showing that, in the absence of ORF translation, mgtA expression is on constitutively. If SL B is necessary for termination, the mutants suggest that translation to the termination codon allows B to form (by blocking C? no data really provided here). In this model, the ribosome could possibly be the Mg sensor, and something around the SD/initiation codon determines its dependence on Mg.

There is no evidence suggesting that translation of MgtF to the stop codon facilitates the formation of stem-loop B. In fact, the stem-loop B is induced by Mg2+ in an *in vitro* system without supplementing protein factors (Cell 2006 paper). We will develop a translation-coupled *in vitro* transcription system to address this question in the future.

10. Minor comments: P.6, middle of page: amino acid residues are not underlined (highlighted instead).

We corrected it in the revised manuscript.

Referee #4

…. However, the probing data are overinterpretated. I do not see how the reactivity pattern support so well the two secondary structure models and especially the effect of magneisum ions. The main effect of Mg is to enhance slightly the reactivity of bases in the region 73 to 86. The only significant changes in reactivity are located around position 30 (see the top of the gel) where several nucleotides appear protected when Mg concentration increased. It seems that this region become base paired.

Since the first submission, we have carried out additional probing assays using wild-type and RNA mutants involved in the stem-switching that determine the accessibility of the SD site. Our results further support our model (see Referee #2, Comment #5). Indeed the sequence around nucleotide 30 appears to be protected, however, the additional assays did not support this result (Figure 4C).

To further improve the structure model, additional experiment would be required such as CMCT modification to get information on U at N3 and G at N1. Why the authors did not perform the classical DNA sequencing ladders using reverse transcriptase on the four nucleotides?

We carried additional CMCT modification. In general, although U nucleotides can be modified well, G nucleotides are poorly modified. Unfortunately, there are very few U nucleotides throughout the studied sequence for analysis. In addition, many papers use the Maxam and Gilbert reaction ladder, including the Cell 2006 paper.

Some of the labels on the autoradiographiy are not correctly assigned: A55 instead of C56, A64 and G63 instead of A63 and G64, etc... This should be also corrected in the quantification of the results.

The A64/G63 incorrectly assigned label was corrected in the revised manuscript. The C56 label was correct in the original manuscript.

In Figure 1A, incubaton controls have also to be performed in the presence and in the absence of Mg. The RNase T1 cleavage at position 105 at low Mg concentration is really weak. I am not sure that this is really relevant. In the latter experiment, there is no incubation control.

I assume the reviewer means Figure 4A, although it was printed as Figure 1A. We have carried out an additional DMS modification assay to include the control incubated at each Mg2+ condition. For some unknown reason, RNase T1 cleavage of the G105 nucleotide is less efficient than others, not only in our manuscript, but in the Cell 2006 paper, as well.

The reactivity of the nucleotide have to be reproted on the secondary structure as shown in Figure 4C.

We have labeled the reactivity of the nucleotide on the secondary structure as shown in Figure 6 (original Figure 4C).

The authors have shown that the transcription of MgtA is unlikely dependent of the proline or arginine concentration in contrast to ther ecent results of Park et al. As such, this is potentially interesting. However, they have mutated single proline codon at positions 3, 5 or 7.
Perhaps the effect of proline would be seen if the codons at position 3, 5, 7 and 9 would have been mutated all together, taking care that the structure of the hairpin D would not be altered by the modifications.

We have determined attenuation using a double mutant with substitutions at positions 3 and 5, which encode two prolines in wild-type MgtF peptide, and are conserved in various bacteria species (Figure 1C). We did not find the proline effect on the MgtF translation-coupled transcription. More analyses on transcription of *mgtA* regarding the proline and arginine effects have been discussed (p13-16).

2nd Editorial Decision 28 December 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by our four referees whose comments are shown below. I am sorry for the delay in re-reviewing this time-sensitive manuscript

We were hoping to make a clear cut decision at this stage, given our policy to only undertake one significant round of revision and the fact that the related paper by Park et al. was published back in September.

Unfortunately, your revision appears to have been premature: three of the four referees continue to show interest in the dataset (the fourth referee is negatively disposed at this stage). However, referees 2, 3 and 4 all raise substantial experimental deficiencies, which in our view preclude publication at this point.

The key points that will have to be addressed experimentally are:

1) as requested by referees 3 and 4, perform the triple mutant at positions 5, 7 and 9, in order to address the discrepancies with the previous literature.
2) as requested by referees 2 and 3, repeat the in vitro transcription experiments in the presence of ribosomes, and include mutant RNAs that favor one or the other mechanisms
3) show native gels (ref 4).

Furthermore, referees 2 and 3 request that the nomenclaure be changed to mgtL, to reflect the previously published nomenclature. Since this is important to avoid uneccessarily confusing the community, we will have to insist on this point.

Also, please note that referees 1, 2 and 3 make a number of excellent suggestions for improving the text.

Should you be able to address these criticisms in full, we could consider a revised 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.

The revisions required are significant and would have to be completed very rapidly (within 5 weeks), given the September publication in Cell. They would have to convince the referees, in particular referees 2 and 3.

I would therefore understand it if you were to rather decide to publish the manuscript rapidly and elsewhere. If you decide to re-submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. When preparing your letter of response to the referees' comments, please bear in mind that this might 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

Yours sincerely and with the best wishes for the New Year,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have addressed my concerns. My only suggestion, for indexing purposes, is to edit the last sentence of the abstract so it says "... in a manner similar to upstream ORFs (uORFs) in eukaryotes."

Referee #2 (Remarks to the Author):

The authors have considerably improved their manuscript but a few crucial issues remain and should be addressed prior to publication in EMBO J.

Major issues:

1. For no apparent reason, the authors keep referring to the upstream ORF as mgtF, even though this ORF has already been published as mgtL. This will be very confusing for any reader, and I insist the name be changed to mgtL. Moreover, mgtL ("leader") is more in line with established nomenclature of attenuation systems of which the authors are describing one.

2. In my previous review, I argued that "it will be important to arrive at a clear picture of which of the two mechanisms controls allows Mg2+ control of the mgtA leader at the RNA level. For example, the authors should repeat the previous in vitro transcription experiments in the presence of ribosomes, and include mutant RNAs that favor one or the other mechanisms...". The authors state in their rebuttal that "In vitro transcription assays were repeated by supplementing ribosomes and using mutant RNAs which turned off translation of MgtF in vivo (Supplemental Figure 1 and p12 & 19)". They then decide not to interpret the result, which is that they fail to recapitulate in vitro the postulated translation-dependence of Mg2+ mediated termination. However, the experiment as it seems to have been done is ill-designed; as far as I understand, the authors just added some ribosomes from a commercial 70S translation system to their in vitro transcription assay. Of course, they must also add the initiator and other tRNAs, as well as translation factors (all available in this commercial PURESYSTEM as well), to achieve TRANSLATION. In other words, one cannot get anything meaningful out of this experiment if the ribosomes cannot translate mgtL. Thus, this crucial experiment to show that mgtL is translated in a Mg2+ dependent manner thereby impacts transcription termination is yet to be done properly. It should also include a means to calculate how much mgtL is translated in the mixture (could be easily done using one or more radio-labeled amino acid as published by others before).

Minor issues:

3. The revised abstract finishes with a strong statement as to a potential mechanistic similarity in the regulatory function of upstream ORFs in pro- and eukaryotes. I do not think that such strong statement is fully justified by the present results, and it should be softened accordingly. In addition, now that the authors have settled on this model, they should at least mention the intriguing work by the Bläsi lab who showed that a trans-encoded small RNA targets an upstream ORF to regulate the downstream RNA (Vecerek et al. EMBO J 2007, 26(4):965-75).

4. I suggest that authors drop the 5'LR, and just spell out 5' leader region. The manuscript is rich in alphabetic soup already. I bet it will not matter much for the character count.

5. Page 6, first para: The last sentence of the introduction is cryptic. Just state specifically where the two papers in question agree and disagree.

Referee #3 (Remarks to the Author):

This revised manuscript on the role of a short ORF in promoting premature termination within the mgtA leader is significantly improved and clarified, and many of the previous issues are addressed. The major finding - that this ORF is required for the proper in vivo regulation of mgtA - is clear, and in the revised paper the issue of whether or not there is an effect of proline and the proline codons within the ORF on regulation is much more clearly addressed. Also more clearly addressed is the

issue of Mg effects on the folding of the RNA itself, although whether the in vitro termination seen in a purified system is directly relevant to the in vivo regulatory events remains to be determined. This is of general interest in presenting a novel combination of a riboswitch (apparently affecting ribosome access) regulating gene expression by regulating synthesis of an upstream ORF.

1. Is there a clear explanation for why two different groups find different effects of proline? Because the experiments were done somewhat differently, and use somewhat different mutants (a direct test of fusions in this paper, RNA levels in the Park et al paper), it would be good to have included the mutation tested by Park et al (Pro5, 7, 9 mutated) to make sure the differences are not in the choice of mutants, or to carry out a parallel experiment measuring the fusion and the RNA levels either in the pro 3, 5 mutant or in the proline auxotroph to see if it is the readout that gives different results (as done by Park et al). I found the figure legend for Fig. 5 a bit difficult to follow - were cells pre-grown in high Proline (as for Park et al), before splitting to cultures with and without proline?

2. Minor suggestions for improving the presentation a bit further:

a. P. 4: Premature termination does not necessarily always require a rho-independent terminator. The phrasing here might be modified to say there are not sequences consistent with a rho-independent terminator, and that the mechanism of termination or pausing is not known.

b. P.6; When you name MgtL, it really is critical to say at every point that this is the same as MgtF. While I understand the reluctance to change your name to theirs, it really would simplify the literature and make it easier for readers.

c. The use of "Meanwhile" is not quite right (p. 4, p. 7). For instance, p. 7, it would be better to say" It is this right arm that is the switching sequence..."

d. P. 8: This details of the affinity chromatography would be better in Materials and Methods.

Referee #4 (Remarks to the Author):

The authors did correct some mistakes and produced some additional data. Despite these changes and although some of the chemical probing data are better, the effects are really weak and not particularly convincing (the single strong effect is at residue C56 that is paired in both structures shown). So one wonders whether there is a reality in the proposed two structures. The 2006 paper is not solid on this issue either.

The authors do not show native gels that would prove the presence of two states. Are the two states shown reversible in vitro? Why are the authors only looking at the short transcripts between 56 and 159? What are the states of the residues around 220 and 240 where termination occurs? Where are precisely the pause sites?

Most of the observations could be explained by a direct effect of the concentration of magnesium on the speed of the polymerase leading to alternative structures of the RNA with differential access for the ribosomes.

Further, Cromie et al have shown that mutation of codon at posiiton 3 has no effect. Here the authors make the double mutant at positions 3 and 5, which is equivalent to a single mutant. The authors have to perform the triple mutant at positions 5, 7 and 9 since those codons were shown to be important by Cromie et al.

2nd Revision - authors' response 28 January 2011

Referee #1:

The authors have addressed my concerns. My only suggestion, for indexing purposes, is to edit the last sentence of the abstract so it says "... in a manner similar to upstream ORFs (uORFs) in eukaryotes."

We have changed this sentence to “Presumably, *mgtL* ORF directs translation to localize a ribosome *in cis* to act on downstream RNA in a manner similar to some upstream ORFs in prokaryotes and eukaryotes”.

Referee #2:

The authors have considerably improved their manuscript but a few crucial issues remain and should be addressed prior to publication in EMBO J.

Major issues:

1. For no apparent reason, the authors keep referring to the upstream ORF as mgtF, even though this ORF has already been published as mgtL. This will be very confusing for any reader, and I insist the name be changed to mgtL. Moreover, mgtL ("leader") is more in line with established nomenclature of attenuation systems of which the authors are describing one.

We have changed the term “MgtF” to “MgtL” in this manuscript.

2. In my previous review, I argued that "it will be important to arrive at a clear picture of which of the two mechanisms controls allows Mg2+ control of the mgtA leader at the RNA level. For example, the authors should repeat the previous in vitro transcription experiments in the presence of ribosomes, and include mutant RNAs that favor one or the other mechanisms...". The authors state in their rebuttal that "In vitro transcription assays were repeated by supplementing ribosomes and using mutant RNAs which turned off translation of MgtF in vivo (Supplemental Figure 1 and p12 & 19)". They then decide not to interpret the result, which is that they fail to recapitulate in vitro the postulated translation-dependence of Mg2+ mediated termination. However, the experiment as it seems to have been done is ill-designed; as far as I understand, the authors just added some ribosomes from a commercial 70S translation system to their in vitro transcription assay. Of course, they must also add the initiator and other tRNAs, as well as translation factors (all available in this commercial PURESYSTEM as well), to achieve TRANSLATION. In other words, one cannot get anything meaningful out of this experiment if the ribosomes cannot translate mgtL. Thus, this crucial experiment to show that mgtL is translated in a Mg2+ dependent manner thereby impacts transcription termination is yet to be done properly. It should also include a means to calculate how much mgtL is translated in the mixture (could be easily done using one or more radio-labeled amino acid as published by others before).

The original thought regarding this issue was because we had results showing that *mgtL* could not be translated *in vitro* using full-length 5'LR RNA (as described in page 7 in the original and 1st revised versions, we were unable to detect MgtL peptide from an *in vitro* translation reaction using full-length *mgtA* 5'LR RNA, suggesting that a premade RNA template was unable to direct MgtL translation). One possibility could be that a cellular factor(s) is required for this process and was absent in the *in vitro* reaction. To test whether the ribosome could exert an *in trans* effect, we carried out an *in vitro* transcription of the *mgtA* 5' leader region by supplementing 70S ribosome (Fig. S1, the 1st revised manuscript). To determine if *mgtL* translation takes place during transcription elongation of the *mgtA* 5'LR, we carried out an *in vitro* transcription-translation coupled reaction, per the reviewer's request, that contained RNA polymerase, initiator and other tRNAs, as well as translation factors supplemented with NTPs, amino acids and the ^{35}S-labeled methionine and ^{32}P-UTP, respectively. There was still no detectable MgtL peptide in ^{35}S-labeled peptide collection although a truncated LacZ protein, as a positive control, could be detected in this reaction, further suggesting that *mgtL* translation should require additional cellular factor(s). We show some of our preliminary results that provide evidence to support our hypothesis (data not shown).

Minor issues:

3. The revised abstract finishes with a strong statement as to a potential mechanistic similarity in the regulatory function of upstream ORFs in pro and eukaryotes. I do not think that such strong statement is fully justified by the present results, and it should be softened accordingly.
In addition, now that the authors have settled on this model, they should at least mention the intriguing work by the Bläsi lab who showed that a trans-encoded small RNA targets an upstream ORF to regulate the downstream RNA (Vecerek et al. EMBO J 2007, 26(4):965-75).

We have changed the last sentence in the revised abstract to "Presumably, *mgtL* ORF directs translation to localize a ribosome *in cis* to act on downstream RNA in a manner similar to some upstream ORFs in prokaryotes and eukaryotes". In addition, we have added the following on p 20, "It has been suggested that high Mg^{2+} reduced the 5'LR *mgtA* transcript stability in a RNase E-dependent manner (Spinelli *et al*, 2008). Furthermore, RNase E cleaves the ferric uptake regulator *fur* mRNA when the ribosome cannot initiate translation of the upstream ORF due to its SD site base-paired with a *trans*-acting regulatory RNA RyhB (Većerek *et al*, 2007). We propose that, in high Mg^{2+} when the *cis*-acting anti-SD sequence is unpaired to its target, the SD site of *mgtL*, translation confers a novel function to bring a ribosome on site to form a complex with stem-loop B, subsequently facilitating RNase E to bind and degrade *mgtA* 5'LR".

4. I suggest that authors drop the 5'LR, and just spell out 5' leader region. The manuscript is rich in alphabetic soup already. I bet it will not matter much for the character count.

We have decided to continue the use of 5'LR as it has been used in current literatures and is replacing the commonly used 5'UTR.

5. Page 6, first para: The last sentence of the introduction is cryptic.
Just state specifically where the two papers in question agree and disagree.

We have changed this sentence to: "While the presence of the *mgtL* ORF is undisputed, our model of the Mg^{2+}-dependent/proline-independent *mgtL* translation via a novel stem-loop switch does not support their conclusions."

Referee #3:

This revised manuscript on the role of a short ORF in promoting premature termination within the mgtA leader is significantly improved and clarified, and many of the previous issues are addressed. The major finding - that this ORF is required for the proper in vivo regulation of mgtA - is clear, and in the revised paper the issue of whether or not there is an effect of proline and the proline codons within the ORF on regulation is much more clearly addressed. Also more clearly addressed is the issue of Mg effects on the folding of the RNA itself, although whether the in vitro termination seen in a purified system is directly relevant to the in vivo regulatory events remains to be determined. This is of general interest in presenting a novel combination of a riboswitch (apparently affecting ribosome access) regulating gene expression by regulating synthesis of an upstream ORF.

1. Is there a clear explanation for why two different groups find different effects of proline? Because the experiments were done somewhat differently, and use somewhat different mutants (a direct test of fusions in this paper, RNA levels in the Park et al paper)

As described in p21 "Our conclusions that the Mg^{2+}-dependent synthesis of MgtL is required for premature termination of *mgtA* transcription contradicts recent reports in which they found that *mgtL* transcription was Mg^{2+}-independent (Park *et al*, 2010). Their conclusions, however, are misleading because (i) their engineered *mgtL-lacZ* fusion contained a deleted *mgtL* stop codon which disrupted the stem A required for formation of the Mg^{2+} sensing domain of the 5'LR. (ii) Their "low Mg^{2+}" condition to test the *mgtL-lacZ* fusion was indeed a high Mg^{2+} condition (Cromie *et al*, 2006, Cromie & Groisman, 2010) and therefore could not be distinguished by the 5'LR whose Mg^{2+} sensing had been disrupted anyway. (iii) Different from the low-copy number plasmid (pYS1010) that we used to study regulatory function of the *mgtA* 5'LR in which transcription is only regulated by the 5'LR, they determined *mgtA* transcription, particularly its response to proline, from its chromosomal locus, which, in addition to the 5'LR (Cromie *et al*, 2006), is regulated by at least two independent promoters controlled by PhoP, in response to the extracytoplasmic Mg^{2+} (Garcia Vescovi *et al*, 1996), and Rob (Barchiesi *et al*, 2008). It is shown that, when the 5'LR is located in

its native chromosomal location, it appears to have an additional regulatory function because the C98T substitution in the 5'LR unexpectedly led to constitutive *mgtA* transcription even in high Mg^{2+} (O'Connor *et al*, 2009) in which transcription initiation is supposed to be repressed by the PhoP/PhoQ system. This result cannot be explained by the premature termination of *mgtL* translation which takes place after transcription is initiated, simply because transcription initiation does not occur. (iv) The regulatory activity of Rob might be changed in altered nutrient conditions they used, such as proline, which should mediate transcription initiated from 44 nt of the 5'LR (Barchiesi *et al*, 2008). Apparently, their real-time PCR assay could measure the transcripts of the *mgtA* coding region, but not the 5'LR due to a primer (Park *et al*, 2010) which corresponds to 7-31 nt of the 5'LR absent in Rob-stimulated transcripts, resulting in changed ratios of the 5'LR RNA to any other RNAs as shown in the results of Pro3-substituted 5'LR (Park *et al*, 2010)".

it would be good to have included the mutation tested by Park et al (Pro5, 7, 9 mutated) to make sure the differences are not in the choice of mutants, or to carry out a parallel experiment measuring the fusion and the RNA levels either in the pro 3, 5 mutant or in the proline auxotroph to see if it is the readout that gives different results (as done by Park et al).

In p15, we discussed our data using the Pro-5,7,9 triple-substitution tested by Park *et al* as well as a Pro-3,5,7,9 tetra-substitution mutation and found that neither influenced the regulatory activity of the 5'LR (Figure 5A and S3A).

I found the figure legend for Fig. 5 a bit difficult to follow - were cells pre-grown in high Proline (as for Park et al), before splitting to cultures with and without proline?

We have changed this legend (now is in Figure S3) "As described in Materials and Methods in a recent study (Park *et al*, 2010), *Salmonella* cells in **A**, **B** and **C** were grown in modified N-minimal medium with 0.005 mM (low) or 0.5 mM (high) Mg^{2+} in the presence of 1 mM proline for 1 hr, washed and then grown for 15 min and 2 h in medium containing or lacking proline".

2. Minor suggestions for improving the presentation a bit further:
a. P. 4: Premature termination does not necessarily always require a rhoindependent terminator. The phrasing here might be modified to say there are not sequences consistent with a rho-independent terminator, and that the mechanism of termination or pausing is not known.

The sentence in the first paragraph of p4 now reads "Since the *mgtA* 5'LR does not have sequences consistent with a Rho-independent terminator, the 220-nt transcript is unlikely a product generated *in vitro* through transcription termination, but a product from the strong pausing of the RNA polymerase in high Mg^{2+}. The mechanism of termination or pausing, however, is not known. It is possible that *mgtA* transcription is paused at nucleotide 220, probably by a RNA conformation induced in high Mg^{2+}, and subsequently terminated near nucleotide 240 *in vivo* by additional cellular

components".

b. P.6; When you name MgtL, it really is critical to say at every point that this is the same as MgtF. While I understand the reluctance to change your name to theirs, it really would simplify the literature and make it easier for readers.

We have changed the MgtF peptide name to MgtL in the revised manuscript.

c. The use of "Meanwhile" is not quite right (p. 4, p. 7). For instance, p. 7, it would be better to say" It is this right arm that is the switching sequence..."

We have removed "meanwhile" from both pages.

d. P. 8: This details of the affinity chromatography would be better in Materials and Methods.

We removed the specific details of the affinity chromatography on p. 8 so that it now reads "Affinity chromatography was carried out to isolate MgtL-FLAG (MW 3,164 daltons) from bacterial cultures grown in low and high Mg^{2+}. The peptide sample was separated and a band was detected from the bacterial cells grown in high Mg^{2+} (Figure 2B), which migrated to a position slightly slower than a control peptide, magainin 2 (MW 2,465 daltons)".

Referee #4:

The authors did correct some mistakes and produced some additional data.
Despite these changes and although some of the chemical probing data are better, the effects are really weak and not particularly convincing (the single strong effect is at residue C56 that is paired in both structures shown). So one wonders whether there is a reality in the proposed two structures. The 2006 paper is not solid on this issue either.
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Most of the observations could be explained by a direct effect of the concentration of magnesium on the speed of the polymerase leading to alternative structures of the RNA with differential access for the ribosomes.
Further, Cromie et al have shown that mutation of codon at position 3 has no effect. Here the authors make the double mutant at positions 3 and 5, which is equivalent to a single mutant. The authors have to perform the triple mutant at positions 5, 7 and 9 since those codons were shown to be important by Cromie et al.

Fig. 4A shows that, in wild-type 5'LR, nucleotides G63 and A64 belonging to the SD site is modified more in high Mg^{2+} than low Mg^{2+}, also the G65 and G66 nucleotides appear to be cleaved more by RNase T1 in high Mg^{2+} than in low Mg^{2+} (Figure S2). We have changed the description on p11, "A primer extension assay… ^{62}GGAGG^{66}, proposed to be the SD sequence here (Figure 1B), was located in a double-stranded region in low Mg^{2+}, however in a single-stranded region in high Mg^{2+} (Figure 4A). The nucleotides G63 and A64 in the SD sequence were modified 2.7 and 2.4-fold more in high Mg^{2+} (3 mM) than in low Mg^{2+} (0.1 mM), respectively (Figure 4A), indicating their locations in a single-stranded region in high Mg^{2+} regardless of simulated base-pairs (Cromie *et al*, 2006)"; and on p12, "Additional mapping of the full-length wild-type RNA with RNase T1…revealed that high Mg^{2+} facilitates the accessibility of this nuclease to G65 and G66 located in the SD sequence because they were cleaved 3.4-fold more in high Mg^{2+} than in low Mg^{2+} (Figure S2), suggesting that the SD site was localized in a single-stranded region in high Mg^{2+} making it more accessible. In contrast, G105 in the anti-anti-SD sequence was cleaved 3.7-fold more in low Mg^{2+} than in high Mg^{2+}, implying that it should be located in double-stranded region by base-paring with the anti-SD sequence in high Mg^{2+}, however, located in a single stranded region when the anti-SD sequence is switched to form stem-loop D in low Mg^{2+} (Figure 6)". These results support our current model in which the SD site is located in single stranded regions in high Mg^{2+}. Indeed the RNA structure model in Figures 1A, 2A, and 3A previously showed that a part of the SD site (nucleotides G63 and A64) were base-paired in the previous submission. Here, we have changed the solid line, representing the base-pairing, to a dotted line as our results do not support the base-pairing of these nucleotides in high Mg^{2+}. In regard to nucleotide C56, the reviewer may have misinterpreted our data in Figure 4A, in which the C56 nucleotide is modified more in low Mg^{2+} than in high Mg^{2+} which is consistent with our stem-loop switching model shown in Figure 1A in which C56 is base-paired in high Mg^{2+} and thus modified less by DMS, but not base-paired in low Mg^{2+}.

We are not sure what the reviewer is referring to when mentioning the "native gel" nor do we understand the "reversible *in vitro* states". We have presented the chemical probing data in its entirety (Figure 4A, C). As illustrated in the attached figure 2 (data not shown), in order to dissect the RNA structure around the *mgtL* open reading frame region between nucleotides 56 and 159, it was necessary to clearly present this region and observe a clear change by quantifying the bands. Therefore, it was necessary to run the gel for an extended period of time to clearly separate the bands in this region (Figure 4A, C), otherwise the area of interest would be too compact and thus poorly distinguishable for quantification, as was the case in the 2006 Cell paper (Cromie *et al*, 2006) and the attached Figure 2. In regards to the nucleotides around 220, our results implicate that the conformation is unchanged, regardless of Mg^{2+} concentration, according to the results in the attached Figure 2 and those in the Cell 2006 paper. In an *in vitro* transcription result, the transcription pausing site is at nucleotide C220 (Cell 2006 paper). However, it is unclear if premature transcription termination of *mgtA* is exactly at nucleotide 240 due to the low resolution of the RNA ladder (Kawano *et al*, 2005).

According to the discussion by the reviewer, the Mg^{2+} effect on the RNA polymerase, which allows the polymerase to run faster, should be relatively non-specific. As a possible result, other transcriptions, such as *lacZ* in pYS1000 (Cell 2006 paper) should also respond to Mg^{2+}, which is not the case.

At the top of page 16, we discussed our data using the Pro-5,7,9 triple-substitution tested by Park *et al* as well as a Pro-3,5,7,9 tetra-substitution mutation and found that neither influenced the regulatory activity of the 5'LR (Figure 5A and S3A).

In addition, we answer the highlighted questions from editor as follows:

Referees 2, 3 and 4 all raise substantial experimental deficiencies, which in our view preclude publication at this point.

The key points that will have to be addressed experimentally are:
1) as requested by referees 3 and 4, perform the triple mutant at positions 5, 7 and 9, in order to address the discrepancies with the previous literature.

At the top of page 16, we discuss our data using the Pro-5,7,9 triple-substitutiontested by Park *et al* as well as a Pro-3,5,7,9 tetra-substitution mutation and found that neither influenced the regulatory activity of the 5'LR (Figure 5A and S3A).

2) as requested by referees 2 and 3, repeat the in vitro transcription experiments in the presence of ribosomes, and include mutant RNAs that favor one or the other mechanisms

As described in page 7 in the original and 1st revised versions, we were unable to detect a MgtL peptide from an *in vitro* translation reaction using full-length *mgtA* 5'LR RNA, suggesting that a premade RNA template was unable to direct MgtL translation. One possibility could be that a cellular factor(s) is required for this process and was absent in the *in vitro* reaction. To test whether the ribosome could exert an *in trans* effect, we carried out an *in vitro* transcription of *mgtA* 5' leader region by supplementing 70S ribosome (Figure S1C, and Figure S1 in the 1st revised manuscript). Here, to determine if *mgtL* translation takes place during transcription elongation of the *mgtA* 5'LR, we carried out an *in vitro* transcription-translation coupled reaction, per the reviewer's request, that contained RNA polymerase, initiator and other tRNAs, as well as translation factors supplemented with NTPs, amino acids and the ^{35}S-labeled methionine and ^{32}P-UTP, respectively. There was still no detectable MgtL peptide in ^{35}S-labeled peptide collection although a truncated LacZ protein, as a positive control, could be detected in this reaction, further suggesting that *mgtL* translation should require additional cellular factor(s).

3) show native gels (ref 4). Furthermore, referees 2 and 3 request that the nomenclaure be changed to mgtL, to reflect the previously published nomenclature. Since this is important to avoid uneccessarily confusing the community, we will have to insist on this point.

We are unsure as to what referee 4 is referring to when suggesting "native gels". We have shown our gels in their entirety (the attached Figure 2).

We have changed the MgtF peptide name to MgtL to avoid confusion.

3rd Editorial Decision 20 February 2011

I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

I append the comments of referees 2 and 3 below. Please note ref 2's recommendation, which could be mentioned in the proof.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #2 (Remarks to the Author):

The authors have made a commendable effort to revise their manuscript, and I recommend it now be published.

For future in vitro work on the system, my advice is that labeled amino acids other than 35S-methionine are used to detect the MgtL peptide. This short reading frame does not contain internal methionine residues, and a methionine that comes in through initiator tRNA is not very stable, so it does not surprise me that the authors keep failing to detect the peptide in their in vitro translation (Figure S1). Better use 14C-labelled amino acids of internal positions.

Referee #3 (Remarks to the Author):

The final revision of this manuscript is improved, primarily by addressing more fully the differences in results between a previous publication on the regulation by this short ORF and results presented here. The use of common nomenclature is also a welcome improvement.

