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**Super DksAs: Substitutions In DksA Enhancing Its Effects On Transcription Initiation**


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**Review timeline:**

- Submission date: 10 February 2009
- Editorial Decision: 09 March 2009
- Revision received: 06 April 2009
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**Transaction Report:**

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

**1st Editorial Decision** 09 March 2009

Thank you for submitting your research manuscript for consideration to The EMBO Journal editorial office. As rather obvious from the enclosed reports, the referees indicate potential interest in the identified DksA mutants that seem to bypass ppGpp requirement under certain growth conditions. Nevertheless, the reports also reveal current shortcomings that will have to be resolved during a single round of major amendments. Specifically, ref#1 requests clarifications on figure 3 (ppGpp-dependency of DksA variants in vitro). This should include a DksA concentration curve to solidify the current conclusions, providing the necessary biochemical evidence for the observed mutant phenotypes. Rather more importantly, ref#2 requests a more sensitive binding assay to confirm the current conclusion. This appears to be a very significant concern, as alternative interpretations (change of location rather than affinity due to the mutations) cannot be excluded from the indirect cleavage assay employed. Finally, I am certainly also aware of the only minor points raised by ref#3.

Although these assessments leave hesitations about the conclusiveness of the current paper at least at this stage, we still would like to offer you the chance for one round of major revision. I do have to remind you that the decision on acceptance or rejection still depends on the content of the final version of your manuscript.

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

Yours sincerely,

Editor
The EMBO Journal
REFEREE REPORTS:

Referee #1 (Remarks to the Author):

These experiments establish very clear further evidence that DksA can alone mediate the reactions of the ppGpp pathway, as was also implied by previous DksA overproduction experiments; furthermore, the inhibitory effect of the overactive mutants strengthens evidence for the role of DksA in regulation of ribosomal RNA synthesis. The results further provide a hint of mechanism, in that the mutants have independent and additive effects on function but not on binding of DksA to core RNA polymerase, suggesting a functional change beyond simple binding.

It's an inherently good idea to look for these mutants, but it's not quite clear how the activity of TraR in the absence of ppGpp provides a rationale. It was already known that overproduction of DksA circumvents the ppGpp requirement, from which the existence of super DksA that act by binding better might have been expected.

Most of the data is quite convincing, although one central experiment (Figure 3) leaves some unease, namely the effect of the mutationally altered DksA versus wild type DksA on transcription from the amino acid promoters in vitro in the absence of ppGpp - it's not completely clear that the effect without ppGpp is as negative as the authors conclude. Partly this may be due to the very low signal and apparently high background, leaving uncertainty about the margin of error in each determination. For livJ in particular, there is a three fold effect in the absence of ppGpp. These differences are just as great as the mutants provide in the presence of ppGpp with his and thr, values with which the authors feel comfortable in concluding a positive effect. For there may be a 20-40% effect of the mutations in the absence of ppGpp. How reproducible are these numbers? Would a 1-3 to 3-fold effect be sufficient to explain the in vivo effects in Figure 2? Was it not worth doing a DksA concentration curve with this experiment, even though the chosen 2 micromol concentration is saturating by other criteria? In a way this is a critical biochemical test of the mutant phenotypes, and one wonders if the authors are completely happy with quality of this experiment.

The lines in Figure 6 are not correctly drawn. In particular, the -ppGpp curves level off, whereas the data shows them still decreasing between 100 and 500 nM DksA, for all three mutants. This gives the impression that there is some component of the inhibition that requires ppGpp, whereas neither the data nor the text says this.

Referee #2 (Remarks to the Author):

This manuscript describes the isolation of DksA mutants that bypass the ppGpp requirement for growth in minimal medium lacking amino acids. Extensive and elegant biochemical analyses of the effect of ppGpp and DksA on transcription have, unfortunately, not been bolstered by structural analysis. Therefore, the approach undertaken in this manuscript is welcome. I find one major problem in the manuscript: the use of Fe-induced DksA cleavage as a measure of RNAP affinity to DksA. Cleavage is induced due to the proximity of the DksA tip to the active site of RNAP. The authors do not consider the possibility that the amino acid substitutions might not change the affinity of DksA to RNAP but the location of the DksA tip with respect to the RNAP active site. The authors mention that "a more sensitive binding assay will be required to confirm their conclusions" Page 21, end of 2nd para). This assay should be performed before this manuscript is published.

Minor changes

1- Page 3, 1st para, line 13: Change tRNA to tRNAs
2- Page 7, line 6, delete "the pDksA plasmid"
3- Page 8, 2nd para, line 4. The promoter that not code for a transporter. Change to: ...a promoter that directs the transcription of a transporter...
4- Page 10, 1st para, line 3. Change to: N881- and L15F-DksAs
The authors describe the genetic isolation and properties of novel DksA mutants that suppress aminoacid auxotrophy of E. coli lacking ppGpp. Given the extensive involvement of these two non-classical transcription modulators in bacterial transcriptional control, the findings are likely to be of broad interest. I have only a few minor comments for the author's consideration.

1. P 4 line 2. I am not sure "shifting the occupancy" is the best phrase to use here; perhaps "lowering the transition state energy between" might be better....

2. P9 line 8: correct to: Fig. 2C, lanes 2-3)

3. P12 line 2 from bottom: pDks needs correcting to pDksA (two places)

4. P17, last two lines from bottom: need some minor rephrasing; as it stands it implies transcription from rRNA operons is down regulated by reduced translational capacity (rather than being down regulated as part of the mechanism that down regulates translational capacity).

5. Fig. 1: text at the bottom of panel B appears out-sized as compared to the remainder of the figure.

6. This is a figure rich manuscript; I would recommend moving panels A to D of Fig. 8 (to supplementary materials, since it is the final panel (E) that allows the reader to follow the argumentation/conclusion.

We would like to thank you and the referees for the thorough reviews of our ms and for the opportunity to submit a revised version to the EMBO Journal. We hope the changes below will be sufficient to address the referees' concerns, but feel free to contact us again should questions remain.

Referee #1.

We would like to thank this reviewer for his comments on our experiments and conclusion.

1. It's an inherently good idea to look for these mutants, but it's not quite clear how the activity of TraR in the absence of ppGpp provides a rationale. It was already known that overproduction of DksA circumvents the ppGpp requirement, from which the existence of super DksA that act by binding better might have been expected.

We agree. This paragraph in the Introduction has been removed, and we have included a short revised paragraph about TraR in the Discussion (2nd paragraph).
2. Most of the data is quite convincing, although one central experiment (Figure 3) leaves some unease, namely the effect of the mutationally altered DksA versus wild type DksA on transcription from the amino acid promoters in vitro in the absence of ppGpp - it's not completely clear that the effect without ppGpp is as negative as the authors conclude. Partly this may be due to the very low signal and apparently high background, leaving uncertainty about the margin of error in each determination. For livJ in particular, there is a three fold effect in the absence of ppGpp. These differences are just as great as the mutants provide in the presence of ppGpp with his and thr, values with which the authors feel comfortable in concluding a positive effect. For there may be a 20°30% effect of the mutations in the absence of ppGpp. How reproducible are these numbers? Would a 1-3 to 3-fold effect be sufficient to explain the in vivo effects in Figure 2? Was it not worth doing a DksA concentration curve with this experiment, even though the chosen 2 micromolar concentration is saturating by other criteria? In a way this is a critical biochemical test of the mutant phenotypes, and one wonders if the authors are completely happy with quality of this experiment.

Part of the confusion here may have resulted from comparison of the wrong lanes. The referee should compare lane 1 with lanes 3, 5, 7, and 9 in each panel of Fig. 3, rather than lanes 3 with lanes 5, 7, and 9. We have now directed the reader in line 7 of the section "DksA Variants Require ppGpp for Transcription Activation In Vitro" to compare the appropriate lanes. We also modified Fig. 3 by inserting "1.0" under lane 1 to emphasize this point.

The referee also wants to see concentration-dependence curves. We showed the data at the saturating DksA concentration, because this is where the effects of DksA alone should have been the greatest. Nevertheless, we now provide concentration curves as requested for livJ and thrABC in Figures S2A and S2B. As requested, we also show the quantitation of Fig. 3 and additional repeats of Fig. 3 as Supplemental Table 1. Hopefully, it should now be more obvious that effects of the DksA proteins by themselves are very small on positive control of the amino acid promoters, compared to when ppGpp is also present. The contrast should also be more obvious for effects of DksA alone on negative control of rRNA promoters.

3. The lines in Figure 6 are not correctly drawn. In particular, the -ppGpp curves level off, whereas the data shows them still decreasing between 100 and 500 nM DksA, for all three mutants. This gives the impression that there is some component of the inhibition that requires ppGpp, whereas neither the data nor the text says this.

The referee wants us to redraw the curves shown in Fig. 6B-6E. The curve-fitting was done without weighting different points in the curve more than others. Forcing the curves to go through the points corresponding to the highest DksA concentrations would unduly weight the least reliable points in the curve (i.e. the points where transcription is closest to background). The point made by the text and figures is simply that complexes containing the DksA mutants still respond to ppGpp, not that inhibition "requires" ppGpp. The validity of this conclusion is apparent no matter how one draws the curves. Furthermore, these results imply that changes in ppGpp concentration could affect transcription in vivo even when the mutant DksA proteins are present, a point now stated explicitly in the next to the last paragraph of the section entitled "The DksA Variants Do Not Require ppGpp to Inhibit rrnB P1 In Vitro".

Referee #2.

This manuscript describes the isolation of DksA mutants that bypass the ppGpp requirement for growth in minimal medium lacking amino acids. Extensive and elegant biochemical analyses of the effect of ppGpp and DksA on transcription have, unfortunately, not been bolstered by structural analysis. Therefore, the approach undertaken in this manuscript is welcome. I find one major problem in the manuscript: the use of Fe-induced DksA cleavage as a measure of RNAP affinity to DksA. Cleavage is induced due to the proximity of the DksA tip to the active site of RNAP. The authors do not consider the possibility that the amino acid substitutions might not change the affinity of DksA to RNAP but the location of the DksA tip with respect to the RNAP active site. The authors mention that "a more sensitive binding assay will be required to confirm their conclusions" Page 21, end of 2nd para). This assay should be performed before this manuscript is published.
We thank the referee for the compliments about our past work. However, the comment that "The authors do not consider the possibility that the amino acid substitutions might not change the affinity of DksA to RNAP but the location of the DksA tip with respect to the RNAP active site" indicates that we did not explain our assay well enough leading to some confusion from this referee about the binding assay. Therefore, we have added some text, primarily to the binding assay section in the Methods, to explain the assay better. Previously, localized cleavage of DksA (or Gre factors) by Fe++ substituted for Mg++ in the RNAP active site has been used only as a measure of proximity of the coiled-coil tip to the RNAP active site. Here we use the cleavage reaction for measuring binding affinity by keeping the DksA concentration constant and varying the RNAP concentration. The concentration of RNAP where half-maximal cleavage is obtained is a measure of the apparent KD of RNAP for DksA. The DksA concentration is kept well below that of RNAP and the experimentally-derived KDs are independent of the DksA concentration.

In contrast to what the referee states, the apparent binding constants would be exactly the same whatever the cleavage efficiencies of the mutant and whether the positions of the cleavages were the same or not. The point is that the binding constants are determined from the fraction of DksA cleaved, normalized to the fraction cleaved at saturating RNAP concentration. Thus, even if the position of the cleavage were different from that in the wild-type protein, or even if the fraction cleaved were less for the mutants than for the wild-type protein, this would not affect the calculation of the apparent KD. (Actually, these mutants get cleaved to the same extent as the wild-type protein and they get cleaved at the same position as the wild-type, but this is irrelevant for the KD calculation).

We have also removed the sentence near the end of the Discussion (end of second paragraph on original page 20) about how a more sensitive binding assay might be required to answer a subtle question we are making concerning whether or not the substitutions might also alter a later step in the mechanism in addition to binding of DksA to RNAP. The present assay is quite sufficient to justify the major conclusion made here, so we have deleted this sentence to eliminate confusion.

Minor changes:

- We made all the requested changes except the first one: "tRNA" should not be plural in this case ("tRNA promoters").

Referee #3.

We would like to thank the reviewer for the kind comments.

Minor changes.

1. P 4 line 2. I am not sure "shifting the occupancy" is the best phrase to use here; perhaps "lowering the transition state energy between" might be better.....

Actually, we think that "shifting the occupancy" is the more appropriate term. As indicated in Rutherford et al. 2009, DksA shifts RPI to RPC, although it may do so by lowering the transition state energy, as implied by the referee's comment. We clarified the sentence by changing it to "by shifting the occupancy of certain intermediates on the pathway to open complex formation, perhaps by lowering the energy of the transition state between them."

2. P9 line 8: correct to: Fig. 2C, lanes 2-3).

Changed as requested

3. P12 line 2 from bottom: pDks needs correcting to pDksA (two places)

Changed as requested.
4. P17, last two lines from bottom: need some minor rephrasing; as it stands it implies transcription from rRNA operons is down regulated by reduced translational capacity (rather than being down regulated as part of the mechanism that down regulates translational capacity).

*Changed to "adjusting rRNA promoter activity to changes in the nutritional status of the cell."*

5. Fig. 1: text at the bottom of panel B appears out-sized as compared to the remainder of the figure.

*Changed as requested.*

6. This is a figure rich manuscript; I would recommend moving panels A to D of Fig. 8 (to supplementary materials, since it is the final panel (E) that allows the reader to follow the argumentation/conclusion.

*Although we agree with the referee that the ms is quite "figure rich" and that the summary curves (panel E) are sufficient to illustrate the conclusion, we feel that simplifying Fig 8 to include only panel E would exacerbate the potential for misinterpretation illustrated by the comments of referee #2. Therefore, we would prefer to leave the figure intact to illustrate how the KDs were determined and to emphasize the quality of the data.*