THE EMBO JOURNAL

Manuscript EMBO-2011-77297

The Structure of a Transcription Activation Sub-Complex Reveals How σ^{70} is Recruited to PhoB Promoters

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Review timeline:

Submission date:	14 February 2011
Editorial Decision:	22 March 2011
Revision received:	20 June 2011
Editorial Decision:	15 July 2011
Accepted:	15 June 2011

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 22 March 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see the referees find the sigma4-PhoBE-DNA structure to be interesting but they require some further analysis to support the model and to make the study suitable for The EMBO Journal.

While the authors are in general positive about the study they raise a number of issues that need to be addressed prior to publication in The EMBO Journal. Referee #1 finds that since the structure is of modest resolution, complementary experimental data demonstrating the integration on of Sigma-4 into the PhoB-DNA complex should be provided. I have further discussed this issue with this referee who has stated:

"I suggest that the DNA binding results on the binary complex are substituted for the same (or similar) experiments on the ternary complex. More specifically, I recommend that the authors test the binding (ideally showing the Kd values) of sigma-4 to (i) PhoB(E)/DNA; (ii) pho box DNA (that contains the nearly consensus -35 element); and (iii) DNA containing consensus -35 promoter box."

This referee also agrees with the issue raised by referee #2 concerning the removal of the data describing PhoBE binding to DNA, otherwise the remaining concerns of referee #2 are with the presentation of the data. Referee #3, who does not require additional experiments, suggests that the impact of the paper would be enhanced by in vitro transcription assays to support the conclusions from crystal structure, I would like to encourage you to add these data. Given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this manuscript, Blanco et al. report the original crystal structure of the transcription activator PhoB(E) bound to its cognate DNA binding site (pho box) and to the region 4 of the RNAP sigma subunit (sigma-4) known to recognize the -35 promoter element in the transcription initiation complexes. In the complex, the PhoB(E) dimer binds the DNA pho-box in the head-to-tail fashion, while the RNAP sigma-4 domain interacts with both the pho-box DNA and PhoB(E) activator. In particular, sigma-4 binds DNA in a distinct manner as compared to that in the binary sigma-4/DNA complex and the authors suggest that the contacts with the activator account for this difference. Based on the structure and modeling, Blanco et al. provide several important implications/predictions concerning the mechanism and role of PhoB(E) during transcription initiation. Thus, the subject and major conclusions of the work are of high general interest.

However, the structure is determined at quite a modest resolution (4.3A) that did not allow the authors to reveal the major binding determinants between sigma-4 and PhoB(E)/DNA in the complex while the overall interface looks quite local. In addition, the available biochemical data seem to be not entirely supportive to the structural results (page 10). This raises a major concern on the specificity of the observed interactions and subsequently on the validity of the mechanistic implications. The fact that the ternary complex sample in many cases provided the crystals of only the binary, PhoB(E)/DNA complex (page 8) additionally augments this concern. There are two questions in this regard. First, is the sigma-4 domain involved in the crystal contacts? Second, the downstream region of the pho box contains nearly consensus -35 sequence (TTGTCA) that seems to be well accessible from the major grove in the structure. How do authors explain that sigma-4 does not bind to this site in the complex?

Altogether, in my opinion, the work would greatly benefit from the complementary experimental data (for example, gel shift or some other convincing binding assay) showing that sigma-4 is indeed specifically integrated in the PhoB/DNA complex with reasonable (physiological) affinity.

Referee #2

This manuscript describes extremely interesting structural results from crystals of a transcription initiation sub-complex that includes the σ4 domain of E. coli σ70 fused to the RNA polymerase (RNAP) β subunit flap tip-helix, the PhoB effector domain (PhoE), and pho box DNA. The X-ray data extends to only 4.3 Å-resolution, but this is sufficient to position all of the protein components (all with previously determined high-resolution structures). The results reveal that the σ4 domain likely makes some contacts with the DNA (where the -35 element would be, but pho box DNA lacks

a -35 element), but is reoriented to also make contacts with PhoBE (compensating for the lost -35 element contacts). These results are of great interest, but unfortunately the manuscript has many serious presentation problems that need to be addressed before publication:

1) In addition to the transcription initiation sub-complex described above (and described in the abstract of the manuscript), the manuscript also presents structural results and some DNA binding studies on PhoBE alone (summarized in Figs. 1 and 2A). These results are completely superfluous to the transcription sub-complex structure. I believe the authors realize this since this aspect of the study is not mentioned at all in the abstract. The new PhoBE/26-mer DNA structure provides no new information that is relevant to the main story (the initiation sub-complex) since a high-resolution crystal structure of PhoBE/DNA (on a different DNA) has already been determined and published by this group. In addition, the binding studies (presented in Fig. 1) do not lead to any conclusions or insights that have any bearing on understanding the initiation sub-complex. Therefore, these data (the data presented in Figs. 1, 2A, and 2B) should be removed from this manuscript and presented elsewhere.

2) Fig. 3A - Because of the nature of these structural results (only 4.3 Å-resolution, little refinement), there need to be more views of the electron density presented. These extra images can be put in the supplementary information if necessary. Furthermore, the orientation shown in Fig. 3A (with the end-on view of the DNA) is not particularly informative.

3) Fig. 3B - I don't really understand the purpose of Fig. 3B, it can probably be removed.

4) Figs. 4A, 4B, 4C:
- there is very poor color discrimination between the upstream PhoBE (purple) and σ4 (dark red) - a different color for one of these should be chosen.
- please label some positions on the DNA so the viewer can understand the relative position of the proteins with respect to the DNA sequence (as shown in Fig. 4D).
- it would also be helpful to label the N- and C-termini (when visible) of the various protein domains.

5) Again, it is difficult to tell PhoBE (purple) from σ4 (red). The amino acid side chain labels in Figs. 5A and 5B are very difficult to understand and relate to the structure. It is difficult to tell where the various labeled side chains are supposed to be on the protein backbones, and it is even difficult to tell which protein the side chain labels are referring to since the labels are all just black text and jumbled together in the interface of the two domains. I would suggest:

- mark the α-carbons of the relevant side chains with a colored sphere

- the text for an amino acid label should be colored the same as the protein to which it's referring

- put the label text in an ordered fashion to the side and use lines or arrows to point to the relevant α-carbon sphere. If this is done carefully it will be much easier to understand.

Fig. 5C is very informative.

6) In general, Fig. 6 is overly complex and is therefore unecessarily difficult to understand what the authors are trying to illustrate. I would suggest:

- eliminate the shadows in the rendering - this makes complex images even more complex and difficult to digest.

- better color discrimination between σ in the holoenzyme (pinkish purple) and σ in the PhoBE-complex (reddish purple).

- it would be much easier, I think, to see the change in orientation if thin, α-carbon backbone worms for σ4 were used instead of the helix cylinders.

- the backbone and secondary structure of the entire RNAP is shown, making the image very dramatic, but most of this is completely irrelevant to the region around σ4 where the authors whish

to focus. I would suggest showing most of the RNAP as a molecular surface (except for probably the flap domain).

- In the RNAP holoenzyme, where is σ3? It seems to be missing.

7) On pg. 9, in the only full para., it says that Figure 4D illustrates how the σ4 domains in the RNAP holoenzyme (on the -10/-35 promoter) and on the PhoBE complex reported here are located exactly at the same position from the transcription origin - but Fig. 4D doesn't show any information about where σ4 is located (this information would be useful, however).

8) Some of the text is very concise, clear, and well written (for instance, the Introduction does an excellent job of setting up the study), but other parts of the manuscript need to be edited more carefully for proper English usage. Just a few examples:

- pg. 7, last sentence - 'Mutations and C-terminal deletions of σ70 showed the implication of the σ4 subdomain...'

- pg. 8, sentence beginning on line 3 - 'However, protein production of the σ4 domain resulted very difficult because of lack of expression...'. In fact the rest of this para. that follows has multiple problems.

Referee #3

Bacterial transcription factor directory contacts with RNA polymerase (RNAP) and activates transcription. PhoB is a transcription activator of Pi-uptake genes and interacts with sigma domain 4 (sigma4) of RNAP holoenzyme. PhoB dependent promoters lack canonical sigma factor recognizing -35 promoter DNA element, therefore, an interesting question is how does holoenzyme contact with the DNA bound PhoB protein as an alternative to the -35 DNA recognition. This study provides a structural framework to answer this question.

Authors solved the sigma4, PhoB and DNA tertiary complex crystal structure and revealed a first molecular description of the sigma4 and activator interaction supplements to the lack of canonical sigma4-DNA interaction.

In addition, authors found that the PhoB-sigma interaction moves a position of sigma4 relative to the DNA compared with ordinary sigma-DNA complex, therefore, authors proposed a model that the PhoB remodels the sigma4 position within the holoenzyme and it enhances the transcription by making polymerase easy to start the transcription with less amount of abortive transcripts. This is an interesting hypothesis and it should be addressed by relatively easy in vitro transcription experiment (this reviewer does not request doing this experiment by authors, but if they will carry out the experiment and report a result in this paper, it will be a high impact paper in transcription field).

This paper also provides a technical advance for crystallization. For making a stable tertiary complex for crystallization, authors used a cleaver approach; making a chimera protein containing sigma4 and a flap tip alpha-helix of beta subunit. The flap tip helix fits a hydrophobic cleft of sigma4 and stabilizes the sigma4 folding in solution. This approach can be used for other crystallization projects of sigma4 and transcription factor complexes.

This paper reports interesting structures for understanding a mechanism of factor-dependent bacterial transcription, provides an interesting hypothesis and also gives an important technical advance. This reviewer recommends this paper is suitable for publication in EMBO J.

Minor comments:

Authors may try to change the title. In stead of telling what structure authors solved, telling what authors found from the structures.

p.3, l.12; add "domain" after "sigma4".

p.3, l.18; 2.3->2,4 and 2.4->2.3
p.3, l.20; delete "channel"
p.4, l.20; "RNAP subunit" -> factor
p.4, l.21; "sigma" -> "sigma4 domain"
p.4, l.22-23; "a reoriented structure of the sigma4 domain." explain relative to what??
p.5, first paragraph; show a gel-filtration column chromatography elution profile in the supplemental material
p.8, l.8; "transcription initiation" -> holoenzyme formation
p.8, l.10-12; don't say data not shown. Show a gel-filtration column chromatography elution profile and SDS-PAGE in the supplemental material
p.8, l.14-15; delete "after many attempts where the crystals systematically turned out to contain only PhoB-DNA binary complexes (like the 26 bp shown in Figure 2A)"
p.8, l.21; "Figure 2C" -> "Figures 2C and 3B"
p.9, l.21; "Figure 5A and B" -> "Figure 5A"
p.9, l.22; "Figures 4, 5A and B" -> "Figures 4 and 5B"
p.14, l.18-21; Figure 6C and D are not sufficient for explaining authors prediction. Make a better figure for this.

Figure 1B and C do not match their legends. Legends say concentration but I believe numbers above gels are ratio between labeled and non-labeled DNA.
Figures 3A and 3B do not have to be stereo views.
Figure 3 legend; swap 3B and 3C
Figure 3C; explain relative orientations of these three views
Figure 4D; explain in the figure legend what are magenta colored A bases in the figure
Figure 5A; not clear where are these amino acids on sigma4 and where are T-34 and A-35 bases in DNA.
Figure 5B; not clear where are these amino acids on sigma and PhoB.

Table 1, PhoB/26-mer DNA structure; authors reported this structure is 2.8A resolution, however, a completeness of the highest resolution shell is only 53%, which is very low. The 2.8A resolution is overestimated number. This reviewer suggests authors determine the highest resolution based on its completeness higher than 80%.

Table 1, sigma4-bflap/PhoB/28-mer DNA structure; refinement statistic for this structure is missing

1st Revision - authors' response 20 June 2011

All comments of the referees have been addressed as follows:

Referee 1

1) *Is the sigma 4 domain envolved in crystal contacts?*

The position and orientation of s_4 is unlikely to be driven by crystal contacts. They are very scarce for this molecule and identical structure is observed for the different s_4 molecules of the asymmetric unit having diverse environments in the crystal. The crystal lattice is basically built by PhoB-PhoB, PhoB-DNA and DNA-DNA contacs.

2) *Second, the downstream region of the pho box contains nearly consensus -35 sequence (TTGTCA) that seems to be well accessible from the major grove in the structure. How do authors explain that sigma-4 does not bind to this site in the complex?*

s_4 cannot bind to the -29 to -24 TTGTCA in the downstream pho box because it would not be correctly placed for the interaction with the downstream PhoB protomer, which is necessary for the s_4 to bind to the DNA (The new EMSA experiments (Fig. 1) confirm that s_4 cannot bind to the pho box in the absence of PhoB). In order to get a similar interaction between $PhoB^E$ and s_4 in a downstream complex, as the observed upstream complex, s_4 would cover the region -25 to -20

(please check Figure 4A and B) which has the sequence CACGGC (Fig. 4D), far from the -35 consensus. Note the the position of PhoBE dimer is fixed, and in tandem, as observed in several crystal structures solved so far. This is explained now in page 8, 1rst paragraph.

3) *Altogether, in my opinion, the work would greatly benefit from the complementary experimental data (for example, gel shift or some other convincing binding assay) showing that sigma-4 is indeed specifically integrated in the PhoB/DNA complex with reasonable (physiological) affinity.*

"*I suggest that the DNA binding results on the binary complex are substituted for the same (or similar) experiments on the ternary complex. More specifically, I recommend that the authors test the binding (ideally showing the Kd values) of sigma-4 to (i) PhoB(E)/DNA; (ii) pho box DNA (that contains the nearly consensus -35 element); and (iii) DNA containing consensus -35 promoter box*."

New band shift experiments with the ternary complex have been performed as suggested. They clearly show that s_4 binds to the Pho box once the PhoB/DNA complex is formed. The experiments are described in page 5, last paragraph, in page 6, 1st paragraph and the new Fig. 1.

Referee 2

1) *In addition to the transcription initiation sub-complex described above (and described in the abstract of the manuscript), the manuscript also presents structural results and some DNA binding studies on PhoBE alone .... The new PhoBE/26-mer DNA structure provides no new information that is relevant to the main story .... these data (the data presented in Figs. 1, 2A, and 2B) should be removed from this manuscript and presented elsewhere.*

We have removed this part from the manuscript.

2) *... there need to be more views of the electron density presented. These extra images can be put in the supplementary information if necessary. Furthermore, the orientation shown in Fig. 3A (with the end-on view of the DNA) is not particularly informative*

Supplementary figure 1A, B and C has been included showing different views of the experimental and after rigid body refinement maps, with the DNA clearly visible in lateral views.

3) *Fig. 3B - I don't really understand the purpose of Fig. 3B, it can probably be removed.*

The purpose of the figure is to illustrate the fitting of the b-flap tip helix in the hydrophobic crevice of s_4 supporting what is stated in the text in page 7, 1rst paragraph. We have now labelled the different elements to make the figure clearer. It could be moved to supplemental material, but we believe that it is useful for the reader as it is now.

4) *Figs. 4A, 4B, 4C:*
- there is very poor color discrimination between the upstream PhoBE (purple) and σ4 (dark red) - a different color for one of these should be chosen.
- please label some positions on the DNA so the viewer can understand the relative position of the proteins with respect to the DNA sequence (as shown in Fig. 4D).
- it would also be helpful to label the N- and C-termini (when visible) of the various protein domains.

- The color of the $PhoB^E$ protomer interacting with s_4 has been changed to blue.
- Some position of DNA has been labeled.
- N- and C-termini have been labeled.

5) *Again, it is difficult to tell PhoBE (purple) from σ4 (red). The amino acid side chain labels in Figs. 5A and 5B are very difficult to understand and relate to the structure. It is difficult to tell where the various labeled side chains are supposed to be on the protein backbones, and it is even difficult to tell which protein the side chain labels are referring to since the labels are all just black text and jumbled together in the interface of the two domain.*

The color of one $PhoB^E$ protomer has been changed from purple to blue to distinguish it from the red s_4. All labels have been changed and are now depicted inside rectangles with different colours according to what molecule they belong to. Only few have been labeled in the $PhoB^E/s_4$ interface for clarity. The protein domains and some DNA nucleotides have been labeled.

6) *In general, Fig. 6 is overly complex and is therefore unecessarily difficult to understand what the authors are trying to illustrate. I would suggest:*

- eliminate the shadows in the rendering - this makes complex images even more complex and difficult to digest.

- better color discrimination between σ in the holoenzyme (pinkish purple) and σ in the PhoBE-complex (reddish purple).

- it would be much easier, I think, to see the change in orientation if thin, α-carbon backbone worms for σ4 were used instead of the helix cylinders.

- the backbone and secondary structure of the entire RNAP is shown, making the image very dramatic, but most of this is completely irrelevant to the region around σ4 where the authors whish to focus. I would suggest showing most of the RNAP as a molecular surface (except for probably the flap domain).

- In the RNAP holoenzyme, where is σ3? It seems to be missing.

All panels of Fig. 6 have been changed following the referee's suggestions:

- The shadows have been eliminated in panel A

- Colors have been changed to enhance discrimination.

- The view of the entire RNAP has been substituted by a zoom on the region of interest, with the different subunits shown as molecular surfaces with different colors and worm-like representations. An additional zoomed stereo panel (C) with worm-like representation showing the movement of s_4 and the b-flap tip helix has been included

- σ_3 is not visible in this focused view.

7) *On pg. 9, in the only full para., it says that Figure 4D illustrates how the σ4 domains in the RNAP holoenzyme (on the -10/-35 promoter) and on the PhoBE complex reported here are located exactly at the same position from the transcription origin - but Fig. 4D doesn't show any information about where σ4 is located (this information would be useful, however).*

Fig. 4D has been changed indicated the areas covered $PhoB^E$ dimer and s_4 on the *pho box* sequence, coloring the -35 to -30 sequence of covered by s_4 in both the -35 and the pho box sequences (pink rectangle). The Fig legend has been changed accordingly.

8) *Some of the text is very concise, clear, and well written (for instance, the Introduction does an excellent job of setting up the study), but other parts of the manuscript need to be edited more carefully for proper English usage. Just a few examples:*

- pg. 7, last sentence - 'Mutations and C-terminal deletions of σ70 showed the implication of the σ4 subdomain...'

- pg. 8, sentence beginning on line 3 - 'However, protein production of the σ4 domain resulted very difficult because of lack of expression...'. In fact the rest of this para. that follows has multiple problems.

Both sentences have been corrected and the English has been checked throughout by a professional English corrector.

Referee 3

In addition, authors found that the PhoB-sigma interaction moves a position of sigma4 relative to the DNA compared with ordinary sigma-DNA complex, therefore, authors proposed a model that the PhoB remodels the sigma4 position within the holoenzyme and it enhances the transcription by making polymerase easy to start the transcription with less amount of abortive transcripts. This is an interesting hypothesis and it should be addressed by relatively easy in vitro transcription experiment (this reviewer does not request doing this experiment by authors, but if they will carry out the experiment and report a result in this paper, it will be a high impact paper in transcription field).

We agree with the referee's comment. In vitro transcription experiment would certainly help to increase the impact of the results. The referee also points out that that he is not requesting the authors to do this experiment. Indeed this is an experiment a bit away from our expertise and the scope of this paper, which would include assays with the whole RNAP. Furthermore, the statement that it may enhance transcription by remodeling is just a hypothesis at the end of the discussion. Further work by us or others may confirm or not the hypothesis but we believe it is worth putting it forward.

Minor comments:
Authors may try to change the title. In stead of telling what structure authors solved, telling what authors found from the structures.

Title changed, including what the structure reveals.

p.3, l.12; add "domain" after "sigma4".

Corrected

p.3, l.18; 2.3->2,4 and 2.4->2.3

Corrected

p.3, l.20; delete "channel"

Corrected

p.4, l.20; "RNAP subunit" -> factor

Corrected

p.4, l.21; "sigma" -> "sigma4 domain"

Corrected

p.4, l.22-23; "a reoriented structure of the sigma4 domain." explain relative to what??

With respect to the binding of s_4 to canonical -35 sequence. Explained in now in the text (page 4, last paragraph)

p.5, first paragraph; show a gel-filtration column chromatography elution profile in the supplemental material

Gel filtration profile included as suppl. Fig. 1

p.8, l.8; "transcription initiation" -> holoenzyme formation

Corrected

p.8, l.10-12; don't say data not shown. Show a gel-filtration column chromatography elution profile and SDS-PAGE in the supplemental material

Gel filtration profile and SDS-PAGE included as supl. Fig. 1

p.8, l.14-15; delete "after many attempts where the crystals systematically turned out to contain only PhoB-DNA binary complexes (like the 26 bp shown in Figure 2A)"

Sentence eliminated.

p.8, l.21; "Figure 2C" -> "Figures 2C and 3B"

Corrected

p.9, l.21; "Figure 5A and B" -> "Figure 5A"

Corrected

p.9, l.22; "Figures 4, 5A and B" -> "Figures 4 and 5B"

Corrected

p.14, l.18-21; Figure 6C and D are not sufficient for explaining authors prediction. Make a better figure for this.

Figure 6 has been completely re-done as explained in the answer to ref 2.

Figure 1B and C do not match their legends. Legends say concentration but I believe numbers above gels are ratio between labeled and non-labeled DNA.

These figures have been eliminated as suggested by ref 1 and 2.

Figures 3A and 3B do not have to be stereo views.

Stereo view eliminated

Figure 3 legend; swap 3B and 3C

Corrected

Figure 3C; explain relative orientations of these three views

The relative orientation is now shown in the figure and explained in the fig. legend.

Figure 4D; explain in the figure legend what are magenta colored A bases in the figure

Explained now in the Fig. legend.

Figure 5A; not clear where are these amino acids on sigma4 and where are T-34 and A-35 bases in DNA.

Bases and amino acids have been labeled in a better and clearer way.

Figure 5B; not clear where are these amino acids on sigma and PhoB.

Acids have labeled in a better and clearer way, with different colors according to what protein they belong to.

Table 1, PhoB/26-mer DNA structure; authors reported this structure is 2.8A resolution, however, a

completeness of the highest resolution shell is only 53%, which is very low. The 2.8A resolution is overestimated number. This reviewer suggests authors determine the highest resolution based on its completeness higher than 80%.

This structure (and data in Table 1) has been eliminated from the paper as suggested by ref. 1 and 2.

Table 1, sigma4-bflap/PhoB/28-mer DNA structure; refinement statistic for this structure is missing

Usual crystallographic refinement statistics have no meaning in a low resolution structure as the one reported here were only a couple of rigid body refinement cycles have been performed to fit the structures of known proteins and DNA in the experimental maps. This is the case of other similar resolution structures reported in the literature.

2nd Editorial Decision 15 July 2011

Thank you for submitting your revised manuscript to The EMBO Journal, it has now been re-evaluated by two of the original referees who find that you have satisfactorily addressed the previous concerns raised. I am happy to accept the manuscript for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1
The authors properly addressed the major concerns of this reviewer and I believe that the manuscript is now suitable for publication.

Referee #2
In my previous review, I stated that this manuscript presented very interesting results but suffered from many serious presentation problems. This revision has addressed most if not all (at least partially) of my concerns and is dramatically improved, and in my opinion is suitable for publication.

