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Conformational flexibility of RNA polymerase III during transcriptional elongation

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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 18 August 2010

Thank you for submitting your manuscript for consideration at The EMBO Journal. Two referees have evaluated the study and I enclose their reports below. Both referees find it interesting and important and recommend publication in The EMBO Journal once the requested validation and clarification on the interpretation of the data is incorporated. Should be able to address these issues we would be happy to consider a revised version of the manuscript.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The manuscript presented by Fernandez-Tornero et al. described a cryo electron microscopy study of RNA polymerase III in the apo form and in the DNA-bound elongation state. The authors describe the enzyme at an outstanding resolution and identify unambiguously the RNA pol III specific subunit, their contacts with the core subunits and propose the position of conserved domains based on homology modeling. The analysis of the elongation complex identified the position of the DNA duplex and of the RNA exit path at unprecedented precision. The work meets very high standards but the following questions should be carefully answered before publication.
I am surprised by the "spiky" appearance of the map at 9.9 A resolution as compared to the map used later at 11.5A This appearance reflects obviously the higher resolution but the difference between 11.5 and 9.9 A is not expected to be that prominent. In addition, these spikes show some directionality which makes me think of a reconstruction artifact. I suggest that the authors try different back projection algorithms to validate their result.
The interpretation of the extra density present in the DNA binding cleft need to be clarified. The density difference map (supplemental figure S6), shows a number of additional densities in the cleft most of which are explained by the DNA duplex and the nascent RNA. However several other densities are present and in particular, the authors should notably comment on the density present at the end of the upstream DNA where an almost circular thread of density is detected. Is there any explanation for this additional density? The authors should also discuss why the single coding DNA strand and the RNA are visible whereas the non coding stand is not although according to the authors it should be hold in place by subunit C37 (page12). In any case it is unexpected that a single DNA strand can be detected at a resolution of 16.5 A. The authors should justify that they are not over interpreting the difference maps.
Figure4B is hard to digest; the authors could add it to the supplemental material and summarize the moving parts of the enzyme where positive and negative densities are associated, the additional densities and the missing densities in a more synthetic figure.
Finally I do not understand the authors concluding remarks on the importance to strengthen the interaction around the downstream DNA through the Pol III -specific subcomplexes for facilitated reinitiation. Reinitiation requires that the enzyme is able to jump back to the transcription start site and transcription factor TFIIIC bound to the internal promoter element plays certainly a major role in this event. The authors should elaborate on their ideas.

Minor points:
Legend to Figure S6. The modeling of the RNA according to its sequence is supposed to be described in Methods and I could not find any such description. If the authors want to refer to Kettenberger et al they can do so directly in the legend to figure S6.
The word "Sub-classification" on page 5 does not describe accurately the sorting process. The maximun likelihood methods should be mentioned.
Authors use either cryoEM or electron cryo microscopy, should be homogenized
It is not clear in supplemental figure 1 that the third goup (noted class 4) is lacking the specific subunits as stated in the text on page 5
On page 12 the sentence containing "the two densities barely contact each other" is not clear enough, the authors should recall that these are the two Pol III specific densities.

P12 of supplemental methods, what is a biotwin ice microscope? Is this meant to be a cryo electron microscope equipped with a biotwin lens?

Referee #2 (Remarks to the Author):

The manuscript by Fernandez-Tornero and colleagues report the structure of yeast RNA polymerase (Pol) III at around 10 angstroms resolution and that of elongating Pol III at 16.5 angstroms. The new structure of the apo Pol III is a very significant improvement over their previous structure allowing to distinguish many new features on the structure. Moreover, using homology modeling for the heterotrimer C31/C34/C82 and the heterodimer C37/C53, they can position the subunits quite precisely. These subunit are equivalent to some of the Pol II general transcription factors but are permanently associated with the Pol III. The structure of the elongating complex indicates the position of the DNA and possibly the exiting RNA (see Minor remarks below).

Altogether, this manuscript, which is clearly written and correctly referenced, adds much structural information to the biology of Pol III transcription and should of interest to the wide audience of EMBO Journal.

Minor remarks:
-Since the heterotrimer subunits have 6 winged helices, I was wondering if the cryo-EM structure could allow their unambiguous placement. A sense of this should be given in the paragraph on the second half of page 10.
-In the description of the Pol III elongation complex on page 11, I would like to have a description of how the elongation were prepared. I know it is in the Materials and Methods but nevertheless one or two lines are warranted.
-The structure of the elongation complex is compared with that proposed by Gnatt in 2001. It used a staggered template. Comparison with more recent Pol II structures from the Kornberg or Cramer laboratories were a transcription bubble with an RNA is present would be better.
-In the elongating structure, it is not clear in the text how the authors identify what additional density comes from the exiting RNA. I looked at figure S6 were an exiting RNA is indicated in some density. However, in the Gnatt paper only the heteroduplex is present. The paper thus does not help in that respect. How is the exiting RNA positioned in Pol III structure and what are the arguments for it should be described in more details in the text.
-The authors hypothesizes that the closing of Pol III upper opening cleft by the dimer might facilitate transcription reinitiation. I am not convinced. I would rather suggest that it stabilizes the ternary complexe and, hence, processivity, in which the dimer is also known to be involved. I guess that facilitated reinitiation would require an undetermined interaction between Pol III and its general transcription factors, possibly via the dimer, but this view is more speculative than a role in transcription elongation.

1st Revision - authors' response 21 September 2010

Referee #1 (Remarks to the Author):

"The authors describe the enzyme at an outstanding resolution and identify unambiguously the RNA pol III specific subunit, their contacts with the core subunits and propose the position of conserved domains based on homology modeling. The analysis of the elongation complex identified the position of the DNA duplex and of the RNA exit path at unprecedented precision. The work meets very high standards but the following questions should be carefully answered before publication."

Response: We are very pleased that the referee appreciates the high quality of the work. We have addressed the questions of the referee as outlined below.

Referee 1: "I am surprised by the "spiky" appearance of the map at 9.9 A resolution as compared to the map used later at 11.5 A. This appearance reflects obviously the higher resolution but the difference between 11.5 and 9.9 A is not expected to be that prominent. In addition, these spikes show some directionality which makes me think of a reconstruction artifact. I suggest that the authors try different back projection algorithms to validate their result."

Response: Following the referee's suggestion we have tried different back projections (SIRT with constrains, conjugate gradients, weighted Fourier), all giving similar results as it is now also stated at page 9 in the Supplementary Methods. We therefore conclude that the "spiky" appearance of exclusively the peripheral regions of the map (which accommodate the Pol III-specific subunits) is not a problem of the reconstruction algorithm, but rather reflects noise correlation. The common core, which seems to be more rigid and less variable, gets better during the last cycle of refinement, allowing the identification of some helices, as expected at 9.9 ≈ resolution, at the expense of a worse representation of the peripheral parts (no helices here and probably worse resolution). This is why we decided to use the 9.9 ≈ map to describe the Pol III core and the 11.5 ≈ map for the more peripheral regions, as stated on page 7, second paragraph, last sentence.

Referee 1: "The interpretation of the extra density present in the DNA binding cleft needs to be clarified. The density difference map (supplementary figure S6) shows a number of additional densities in the cleft most of which are explained by the DNA duplex and the nascent RNA. However several other densities are present and in particular, the authors should notably comment on the density present at the end of the downstream DNA where an almost circular thread of density is detected. Is there any explanation for this additional density?"

Response: Additional densities in the reconstruction of the Pol III elongation complex different from the DNA duplex and the nascent RNA are in most cases concomitant with negative densities presumably reflecting conformational changes as described on page 12 of the original manuscript. We think that the quasi-circular thread of positive density observed in the front view of Figure 4A (inset) results from the downstream DNA itself and movements of several subunits (or parts of them) towards the DNA. Therefore, the "circular" thread can be divided into several regions: the first (top right) corresponds to the downstream DNA as can be seen from the fitting (Figure 4A, front, inset), the second (left) corresponds to a conformational change of the Pol III C37/C53 heterodimer (presumably mainly subunit C53 as explained in the text), whereas the remaining additional density (bottom right) corresponds to the movement of subunit Rpb5 maybe together with subunit C82. The revised text now includes a more thorough description of the additional density features on page 12 together with a revised Figure 4. The revised manuscript now states:

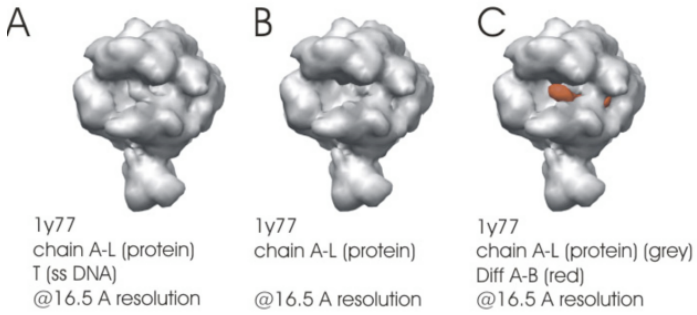
Page 12: "Apart from the major piece of positive density corresponding to the transcription bubble, the structure of Pol III elongation complex presents several differences with respect to the apo form of the enzyme, principally in the surroundings of the most downstream DNA duplex. Because they appear as pairs of negative and positive densities in the difference map (Figure 4B), they are likely to reflect conformational changes occurring in the transition from transcriptional initiation to elongation. One important change occurs for the C31/C82/C34 heterotrimer, which essentially moves away from the stalk towards the DNA binding site. Concomitant with this movement, the stalk rotates in the opposite direction leading to a weaker connection with the heterotrimer, whereas subunit Rpb5 approaches C31/C82/C34 (Figure 4B)."

Referee 1: "The authors should also discuss why the single coding DNA strand and the RNA are visible whereas the non coding stand is not although according to the authors it should be hold in place by subunit C37 (page12)."

Response: We think that we are able to observe the single coding DNA strand and the RNA because their binding modes to the Pol III enzyme are very stable and homogeneous, i.e. their positions are identical in the entire dataset. In contrast, the single, non-coding DNA strand presumably interacts less tightly with Pol III, although it is contacted by subunit C37. Because of fewer interactions most of the non-coding DNA strand presumably is mobile and cannot be observed as continuous density. Our results are similar to the results observed by Kettenberger et al. (Mol Cell, 2004) and Westover et al. (Cell, 2004) for the Pol II enzyme, who also observe continuous density for the coding, but not the non-coding DNA strand.

Referee 1: "In any case it is unexpected that a single DNA strand can be detected at a resolution of 16.5 A. The authors should justify that they are not over interpreting the difference maps."

Response: To prove that the density of a single nucleotide strand can be detected at 16.5 ≈ resolution, we have calculated maps of RNA polymerase II at 16.5 ≈ resolution with (A) and without (B) a single strand of bound RNA using the crystal structure of Pol II in complex with a transcription bubble (Kettenberger et al., 2004; PDB-code: 1Y77). As seen on the figure enclosed for the referee, the difference map clearly identifies the corresponding density (C, red). We therefore do not think that we over-interpret our data.



Referee 1: "Figure 4B is hard to digest; the authors could add it to the supplemental material and summarize the moving parts of the enzyme where positive and negative densities are associated, the additional densities and the missing densities in a more synthetic figure."

Response: The new manuscript now includes a modified, more synthetic version of Figure 4B, where we have only represented the most solid pieces of difference density and added arrows to indicate coordinated movements of the different regions.

Referee 1: "Finally, I do not understand the authors concluding remarks on the importance to strengthen the interaction around the downstream DNA through the Pol III -specific subcomplexes for facilitated reinitiation. Reinitiation requires that the enzyme is able to jump back to the transcription start site and transcription factor TFIIIC bound to the internal promoter element plays certainly a major role in this event. The authors should elaborate on their ideas."

Response: Both referees (see also referee 2) question the relationship between strong binding to DNA and facilitated reinitiation. We agree with both referees that a better grip on DNA is only indirectly linked to facilitated reinitiation. We speculate that the closure of the upper tip of the DNA-binding cleft and the full enclosure of downstream DNA likely prevents the enzyme from easily falling off the DNA at the transcription terminator. Slower detachment from the DNA template may allow the enzyme to more easily re-initiate a new round of transcription (certainly involving TFIIIC as mentioned by the referee). However, we admit that this hypothesis has little experimental support. To address the concerns of both referees, we have removed our statement concerning facilitated re-initiation in the abstract and introduction. Also, as pointed out by referee 2, the impact on the enzyme's processivity is more straightforward, and we have modified the text accordingly. Pages 2, 4 & 13 now read:

Page 2: "…, which likely increases processivity and provides structural insights into the conformational switch between Pol III-mediated initiation and elongation."

Page 4: "…thereby providing insight into Pol III transcriptional initiation and processivity."

Page 14: "This is likely to hinder the enzyme from falling off the DNA and, therefore, enhances its processivity. Moreover, Pol III directs several rounds of transcription on the same gene once attached to DNA, an event known as facilitated reinitiation (Dieci and Sentenac, 1996) in which subunits C37 and C53, together with C11, are involved (Landrieux et al., 2006). The enclosure of downstream DNA likely decreases the speed at which the enzyme detaches from the template once the transcription terminator is reached thereby providing time that may allow facilitated re-initiation to occur more easily."

Minor points:

Referee 1: "Legend to Figure S6. The modeling of the RNA according to its sequence is supposed to be described in Methods and I could not find any such description. If the authors want to refer to Kettenberger et al. they can do so directly in the legend to figure S6."

Response: Our previous statement ("..modeling according to its real sequence…") has been misleading. Due to the 16.5 ≈ resolution of the elongation complex structure, we have only taken

into account the length of the single-stranded RNA by following the path of the positive difference density in order to show that the length of this density perfectly fits the length of the RNA strand present in our synthetic transcription bubble. As suggested by the referee, we have now included a more detailed explanation in the legend to figure S6.

Referee 1: "The word "Sub-classification" on page 5 does not describe accurately the sorting process. The maximum likelihood methods should be mentioned."

Response: Following the request of this referee we now mention the use of the maximum-likelihood sorting method on page 5 (see below). The detailed description of the methods is found in the Supplementary Data.

Page 5: "Two-dimensional sub-classification of particles with certain orientations followed by three-dimensional maximum likelihood sorting (Scheres et al. 2008) of the two-dimensional class-averages were employed to sort out the different populations in the dataset (Supplementary Methods). Initial sorting by sub-classification and maximum-likelihood methods was followed by further refinement by projection matching and supervised classification."

Referee 1: "Authors use either cryoEM or electron cryo microscopy, should be homogenized."

Response: We now employ the term "electron cryomicroscopy" throughout the revised version of the manuscript.

Referee 1: "It is not clear in supplemental figure 1 that the third group (noted class 4) is lacking the specific subunits as stated in the text on page 5"

Response: In the revised version we modified our statement on page 5 as follows:

Page 5: "The three resulting volumes share the same core but two of them, representing one fourth of the particles each, show either no density for both Pol III-specific subcomplexes (Supplementary Figure S1, volume 1) or only a fraction of them (volume 4)."

Referee 1: "On page 12 the sentence containing "the two densities barely contact each other" is not clear enough, the authors should recall that these are the two Pol III specific densities."

Response: We have modified this sentence on page 13, which now reads:

Page 13: "Whereas in the apo form of Pol III the two densities, each corresponding to one of the Pol III-specific subcomplexes, barely contact each other (arrowed star in Figure 3A)Ö"

Referee 1: "P12 of supplemental methods, what is a biotwin ice microscope? Is this meant to be a cryo electron microscope equipped with a biotwin lens?"

Response: Yes, the referee is correct. The microscope is a CM 120 microscope equipped with a Biotwin lens with an accelerating voltage of 120 kV. We have modified the text in the Supplementary Methods, page 12 to clarify this point.

Referee #2 (Remarks to the Author):

"The new structure of the apo Pol III is a very significant improvement over their previous structure allowing to distinguish many new features on the structure. Moreover, using homology modeling for the heterotrimer C31/C34/C82 and the heterodimer C37/C53, they can position the subunits quite precisely. These subunits are equivalent to some of the Pol II general transcription factors but are permanently associated with the Pol III. The structure of the elongating complex indicates the position of the DNA and possibly the exiting RNA (see Minor remarks below). Altogether, this manuscript, which is clearly written and correctly referenced, adds much structural information to the biology of Pol III transcription and should of interest to the wide audience of EMBO Journal."

Response: We appreciate that the referee is so positive about the manuscript.

Minor remarks:

Referee 2: "Since the heterotrimer subunits have 6 winged helices, I was wondering if the cryo-EM structure could allow their unambiguous placement. A sense of this should be given in the paragraph on the second half of page 10."

Response: The small size of the individual winged helix (WH) domains together with the large size of the cryo-EM density corresponding to the C31/C34/C82 heterotrimer makes unambiguous fitting of individual WH domains difficult. In the revised version we explain this in more detail.

Page 10: "Based on these results we have fitted individual WH domains in our major additional EM density. Although the large size of this density makes the unambiguous assignment to specific domains difficult, several rounded features of appropriate size are apparent next to the DNA-binding cleft that could harbour globular WH domains."

Referee 2: "In the description of the Pol III elongation complex on page 11, I would like to have a description of how the elongation complex was prepared. I know it is in the Materials and Methods but nevertheless one or two lines are warranted."

Response: In the Results & Discussion section of the revised manuscript, we now included a brief explanation on how the elongation complex was prepared. The manuscript now states:

Page 11: "Purified Pol III was saturated with a 5-fold molar excess of the transcription bubble that after incubation was removed by size exclusion chromatography yielding a homogeneous elongation complex (see Material and methods)."

Referee 2: "The structure of the elongation complex is compared with that proposed by Gnatt in 2001. It used a staggered template. Comparison with more recent Pol II structures from the Kornberg or Cramer laboratories where a transcription bubble with an RNA is present would be better."

Response: The structure of a complete transcription bubble (Kettenberger et al., 2004) has now been used for comparison yielding the same conclusions. The manuscript has been modified to include the new comparison.

Referee 2: "In the elongating structure, it is not clear in the text how the authors identify what additional density comes from the exiting RNA. I looked at figure S6 were an exiting RNA is indicated in some density. However, in the Gnatt paper only the heteroduplex is present. The paper thus does not help in that respect. How is the exiting RNA positioned in Pol III structure and what are the arguments for it should be described in more details in the text?"

Response: Our assignment of the density to exiting RNA is based both on the difference density and known biochemical results. The additional density is continuous from the RNA/DNA heteroduplex and follows a path along the saddle that coincides with the RNA exit path in Pol II (Andrecka et al., 2007). Moreover, the additional density is continuous up to the stalk, which for Pol III was demonstrated to interact with RNA (Jasiak et al., 2006). In the new version of the manuscript we have described in detail the rational behind our hypothesis.

Page 11/12: "Moreover, the large positive density shows a continuous extension that runs from the DNA/RNA heteroduplex to the stalk (Figure 4A), just above the saddle element (formed by subunits C160 and C128), a region that has been identified as the RNA exit path in Pol II (Andrecka et al., 2007). Modelling an extended RNA comprising 10 additional nucleotides, present in our synthesized bubble, exactly fits the length of the continuous density (Supplementary Figure S6) and reaches the external part of the tip domain in the stalk (Figures 2E and 4A), consistent with the RNA-binding activity of the Pol III stalk observed in vitro (Jasiak et al., 2006)."

Referee 2: "The authors hypothesize that the closing of Pol III upper opening cleft by the dimer might facilitate transcription reinitiation. I am not convinced. I would rather suggest that it

stabilizes the ternary complex and, hence, processivity, in which the dimer is also known to be involved. I guess that facilitated reinitiation would require an undetermined interaction between Pol III and its general transcription factors, possibly via the dimer, but this view is more speculative than a role in transcription elongation."

Response: Following both referee's recommendation we have modified the text referring to facilitated re-initiation (see above for the response to referee 1).

Editorial decision 24 September 2010

Your revised manuscript has been evaluated once more by referee #1, who finds that you have satisfactorily addressed all the concerns raised. I am therefore, happy to accept your study for publication in the The EMBO Journal. You will receive the official acceptance letter early next week.