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Linking the branchpoint helix to a newly found receptor allows lariat formation by a group II intron

Cheng-Fang Li, Maria Costa and François Michel

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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 23 March 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by two referees and I enclose their reports below. As you will see the referees find that defining a role for the iota-iota' interaction in the branching reaction is interesting and potentially important, however, they require further analysis to make it suitable for The EMBO Journal. Both referees raise a number of important issues regarding experiments to support the proposed model and request a number of clarifications. 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,

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Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

This is an important study because it raises questions about a generally accepted structural feature of group II introns, the coordination loop. The coordination loop was proposed by the Pyle lab to be the receptor for the branch point A in the first step of splicing. However, the coordination loop does not have a clear analog among different intron types, as one would expect for such a conserved element. The present manuscript proposes a different position for domain VI during the branching reaction that is incompatible with the purported role of the coordination loop. This study will probably not convince everyone immediately, but it sets forth a plausible alternative that is supported by at least as much data as the coordination loop.

The study begins with the examination of a subset of mitochondrial introns that have lost the branch site motif and an adjacent bulge motif. Conservation profiles suggested that when the branch site is lost, the residues in domain IC1 are free to mutate. This led to the hypothesis that the bulge motif in domain VI forms an interaction with two base pairs of domain IC1, which is named iota-iota'. Domain VI and IC1 were mutated to demonstrate that the predicted motifs have a role in the branching reaction. The interaction was modeled, which led to the successful testing of the juxtaposed arrangement of domains VI and IC1 using an oligonucleotide splint assay.

Francois Michel has an impressive record in identifying interactions using such methods, and this alone makes the study and conclusions compelling. On the other hand, the conclusions would be strengthened by additional experimental evidence, because there are weaknesses in some of the lines of evidence. For example, the strength of the statistical arguments is unclear because an alignment is not provided. Mutation of the iota-iota' motifs do not have the consequences one would expect: mutation of the loop motif of domain VI had no discernible effect, and progressive deletions of domain VI showed specific disruptions for branching only for the most extreme deletions where splicing is nearly abolished, raising the possibility that other factors may influence the ratios. The strongest evidence is definitely the oligonucleotide splint assay. It validates the logic that led to the hypothesis for the interaction. It is difficult to rationalize how the oligonucleotide data could be produced unless domain VI has the proposed position during the branching reaction.

Other:

For the domain IC1 UA:UA and IC1-2bp mutants, the branching rate decreases dramatically while hydrolysis does not, yet the ratio of lariat:linear products remains nearly the same as wild type. Why?

In the entropy analysis, why were the bulged A in domain V and A104 identified? Is it reasonable that they may contact the iota components in domain VI?

What exactly are the nucleotides involved in the iota-iota' interaction? In Figure 5, the interaction is shown as four base pairs in domain IC1 and four nucleotides in domain VI. This is based presumably on modeling rather than experimental evidence. The entropy analysis suggested that a larger portion of domain VI interacts with two base pairs of domain IC1. Please specify what the proposed interaction consists of, and the reasons.

In Figure 5, a 90 degree rotation of domain VI is depicted between the steps of splicing. In the model, however, the positions of the eta-eta' components in DII and DVI seem to be close enough that both eta-eta' and iota-iota' might form simultaneously even without DVI movement. Do the authors propose a twist of the DVI helix rather than a rotation? Please explain in more detail what conformational change is considered likely to occur for domain VI.

Minor suggestions:

p. 2 line 8 and throughout manuscript. Change "distal of" to "distal to"
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p. 32 Fig. 4 legend. Is the error measurement the standard deviation or standard error of the mean?
p. 33 Fig. 4 legend Change "full curve" to "solid curve"

Referee #2:

In this work Li et al., aimed at identifying a receptor site for the branch-point adenosine in group IIB1 introns. By combining phylogenetic and mutational analysis with a rational design of a "molecular tether", the authors were able to identify such a receptor site for D6. Strikingly, this site is located close to the ε′ and λ sites within stem c1 in domain 1. Importantly, this receptor site is specific for the first transesterification step of splicing. The proposed novel interaction was termed ι-ι′ and it has been suggested that this interaction is disrupted after branching and D6 has to undergo a significant rearrangement for the η-η′ contact to occur, which is known to be essential for performing the second step of splicing. Impressively, the authors went on and demonstrated that D6 can be modeled into the crystal structure the group IIC intron from Oceanobacillus iheyensis to allow the ι-ι′ contact. In light of previous findings, this manuscript presents a very interesting, elegant, but also in part controversial study on an aspect of group II intron splicing that finds its parallels in spliceosomal intron splicing.

Major comments

1. As mentioned by the authors, Pyle and coworkers demonstrated a few years ago that the branch-point adenosine is coordinated to the asymmetric internal loop composed of Jd″/d‴ and Jd‴/d″ in the ai5γ group IIB1 intron (Hamill, 2006). The Pyle lab had applied cross-linking to identify residues in spatial proximity to the branch-point. These residues included G1 and C-1 together with two nucleotides in J2/3 (G588 and U590; the counterpart of former and of A589 are part of the triple helix in the active site of the Oi. Intron (Toor et al., 2008).) All other cross-linked residues were located in the coordination loop, which harbors EBS 3 as well. Since Pyle and coworkers used a trans-branching system, the obtained data should have been specific for the branching pathway of splicing. While cross-links to G1, C-1, G588 and U590 can be readily explained by the fact that they are active-site constituents, the phylogenetic data in the current study do not seem to support the coordination loop as docking site for the branch-point. How can the cross-links from A880 (ai5γ branch-point) to the coordination loop be reconciled? Also, looking at Fig. 3A the branch-point A in D6 is not even remotely close to the coordination loop in the model. On the other hand, Hamill and Pyle did not observe any cross-links to stem c1 in D1. Is there any explanation for this apparent discrepancy between the two studies? Since the ai5γ intron has often been referred to as "weirdo" among group II introns, do the authors consider it a possibility that the coordination loop functions as receptor for the branch-point in the ai5γ intron only? Or, is more likely that cross-linking possibly produced in part erroneous data (as it had happened before).

Since there is this controversy, this reviewer is of the opinion that it would be an critical control experiment to mutate the corresponding tandem GU wobble pairs in stem c1 of the ai5γ intron and test for its ability to perform branching (despite data on the Bc. intron from Stabell et al., 2009).

2. Hydroxyl radical footprinting has been done on the Pylaiella intron (Costa et al., 2000). Have the authors also mapped D6 and stem c1? At least from the ai5γ footprinting data it appears that both GU wobble pair are internalized (except G87; Swisher et al., 2001). In other words, how does the model in Fig. 3A correlate with such footprinting data?

3. Assuming that ι-ι′ takes place in the ai5γ intron, the available NAIM data on this yeast mitochondrial intron support that the minor groove of stem c1 is involved in the ι-ι′ interaction: the exocyclic amine of G87 and the 2'hydroxyl groups of U86 and U110 were described to be important for branching (Boudvillain et al., 1998) - as stated by the authors. In the same paper a 2-aminopurine and 7-deaza effect were observed for A861 and A863, suggesting an involvement of the major groove (N6, N7). Can one infer any interaction from the available data and your model?
The presented data unambiguously demonstrate that D6 branch site and stem c1 are spatially very close, however, in order to definitively state that these structural elements are in physical contact (i.e. a novel interaction), it is preferable to have an idea about potential H-bonds in the ι-ι′ pairing (in addition to the phylogenetic evidence).
As stated by the authors, the D6 internal loop can be replaced with canonical base pairs without abolishing branching. What is the advantage of maintaining an internal loop throughout evolution?

4. The oligonucleotide tether is a very elegant way to further support the spatial proximity of D6 bulge and the tandem GU pairs in stem c1. The different variants were compared for their relative branching rates. However, it would be helpful to enlist the absolute kobs values together with the wt activity from Table I. Along the same line, I urge the authors to show a representative gel for splicing of the wt and at least the IC1 UA::UA mutant and the anchor 2 with 1 T only.

5. It is my understanding that the coordination loop is poorly conserved among group II introns (Michel et al., TIBS 2009), but what about the tandem GU pairs in stem c1? How well are these and in turn the ι-ι′ contact conserved among group II introns (possessing a branch-point) of different phylogenetic families.

6. Another tertiary contact has been proposed by Pyle and coworkers a few years ago (Fedorova et al., 2005): μ-μ′ in ai5γ. As this contact has not been included into the schematic drawing of Fig. 1A, I am wondering whether there is a specific reason for it. Is such a contact not supported by phylogeny in the Pylaiella intron?

Minor comments:

- I suggest highlighting Domain 2 in Fig. 3A to be able to imagine the conformational switch of D6 shown in Fig. 5.
- In the methods section, please name and cite the program used for the alignment.
- In Table II setup 2, please explain the 3-fold enhancement compared to setup 1.
- In the last section of the Discussion the authors mention "costly". I suggest removing or rephrasing this sentence. There is no need to explain why the authors have not performed NAIS (yet), as this would go beyond the scope of this manuscript.

1st Revision - authors' response 15 April 2011

Referee #1

This is an important study because it raises questions about a generally accepted structural feature of group II introns, the coordination loop. The coordination loop was proposed by the Pyle lab to be the receptor for the branch point A in the first step of splicing. However, the coordination loop does not have a clear analog among different intron types, as one would expect for such a conserved element. The present manuscript proposes a different position for domain VI during the branching

reaction that is incompatible with the purported role of the coordination loop. This study will probably not convince everyone immediately, but it sets forth a plausible alternative that is supported by at least as much data as the coordination loop.

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Comment: *Francois Michel has an impressive record in identifying interactions using such methods, and this alone makes the study and conclusions compelling. On the other hand, the conclusions would be strengthened by additional experimental evidence, because there are weaknesses in some of the lines of evidence. For example, the strength of the statistical arguments is unclear because an alignment is not provided.*

Answer: As stated in 'Materials in Methods', the alignment that we used for the statistical analyses in Figure 1 is available in Li et al. (RNA Journal, 2011, in press); the final version of that paper contains a 'Supplementary Dataset', which consists of our entire alignment of mitochondrial subgroup IIB1 ribozyme sequences in FASTA format. Please note that 577 sites were actually aligned and analyzed; the value wrongly quoted in the original version of this manuscript – 526 – was the number of sites used to build the phylogenetic tree in Fig.1C, after removal of the IBS and EBS nucleotides and a few highly variable segments (see Li et al., 2011).

Comment: *Mutation of the iota-iota' motifs do not have the consequences one would expect: mutation of the loop motif of domain VI had no discernible effect, and progressive deletions of domain VI showed specific disruptions for branching only for the most extreme deletions where splicing is nearly abolished, raising the possibility that other factors may influence the ratios.*

Answer: In our potassium-containing buffer, replacement of the internal loop motif by canonical base pairs or truncation of the distal section of domain VI down to four base pairs (which removes part – but only part – of what we propose to be the iota' motif) does have significant effects: the fraction of branched products is reduced from at least 70 percent in the wild-type down to 41 and 15 percent, respectively (bottom part of Table I). Moreover, the latter change affects the rate constant for branching both significantly and specifically (hydrolysis is not affected). In order to explain why relatively mild structural changes had no discernible effect in the ammonium-containing buffer, we proposed that in that buffer, docking of domain VI into its IC1 receptor may not be rate-limiting in wild-type precursor molecules.

Comment: *The strongest evidence is definitely the oligonucleotide splint assay. It validates the logic that led to the hypothesis for the interaction. It is difficult to rationalize how the oligonucleotide data could be produced unless domain VI has the proposed position during the branching reaction.*

Other:
For the domain IC1 UA:UA and IC1-2bp mutants, the branching rate decreases dramatically while hydrolysis does not, yet the ratio of lariat:linear products remains nearly the same as wild type. Why?

Answer: That is true only in ammonium buffer, in which about 90 per cent of molecules remain committed to forming lariats, and unable to hydrolyze the 5' splice site, even in mutants with a 10-fold reduced rate constant for branching (the only exception is the dVI-2bp mutant). In potassium buffer, however, changes in rate constants for branching and hydrolysis tend to be reflected in correspondingly altered proportions of branched and linear molecules among reaction products (see bottom part of Table I): what we believe to be the ability to dock domain VI into its receptor affects both observed rates of reaction and the partition of refolded precursor molecules into branching-compatible and hydrolysis-compatible conformations. As we pointed out in the 'Kinetic analyses' section of Materials and Methods, it already was noted by others that refolded precursor molecules

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do not form a single population with respect to the ability to initiate splicing by branching versus hydrolysis.

Comment: *In the entropy analysis, why were the bulged A in domain V and A104 identified? Is it reasonable that they may contact the iota components in domain VI?*

Answer: The ten intron sequences that lack a discernible branchpoint also have additional nucleotides at their 5' extremity; none of these sequences begins with G and in only five of them does the GUGCG group II conserved motif actually start with G – see Supplementary Dataset). The loss of both G1 and the ability to form lariats may have relieved in turn constraints on the base of A2389 (A376 in the Oceanobacillus ribozyme), whose location in Toor et al.'s crystal structure is compatible with a contact with G1 during the branching reaction. On the other hand, we have no plausible explanation for the statistical signal at A104, unless that nucleotide were to be oriented quite differently in the branching-ready configuration of the ribozyme.

Comment: *What exactly are the nucleotides involved in the iota-iota' interaction? In Figure 5, the interaction is shown as four base pairs in domain IC1 and four nucleotides in domain VI. This is based presumably on modeling rather than experimental evidence. The entropy analysis suggested that a larger portion of domain VI interacts with two base pairs of domain IC1. Please specify what the proposed interaction consists of, and the reasons.*

Answer: Yes, our tentative delimitation of the iota-iota' interaction in Figure 5 is based primarily on modeling, as we now make clear in the legend to that figure: the nucleotides we included are the ones that may directly contact one another according to our provisional model. In domain VI, sequence conservation is expected to extend beyond these nucleotides: most mutations within and immediately next to the internal loop are likely to have been counterselected because they would affect the conformation of that loop and, indirectly, the optimal positioning of nucleotides involved in the iota-iota' interaction (inversely, involvement of the IC1 helical backbone distal to the two G:U pairs is expected to remain undetected by our type of statistical analysis as long as only non-canonical base pairs need to be avoided).

Comment: *In Figure 5, a 90 degree rotation of domain VI is depicted between the steps of splicing.*

Answer: Figure 5 is intended as a mere sketch; as now explicitely stated, a 90 degree rotation was chosen for convenience of drawing and in order to convey the impression that this is a major translocation (although not quite as extensive as in J. Wang's model or A.M. Pyle's latest sketch, as indicated in our Discussion); see also below.

Comment: *In the model, however, the positions of the eta-eta' components in DII and DVI seem to be close enough that both eta-eta' and iota-iota' might form simultaneously even without DVI movement.*

Answer: Figure 3A is to be viewed in three dimensions and it should be noted that in the a5gamma and Pylaiella LSU2 ribozymes, the eta receptor is located far beyond the tip of what was left of domain II – hardly more than a stump – in the molecule that was cristallized by Toor et al. and whose atomic coordinates we used to build our model. We now point to the location of eta in Figure 3A and mention, in the legend to that Figure, the need to extend mentally the helix.

Comment: *Do the authors propose a twist of the DVI helix rather than a rotation? Please explain in more detail what conformational change is considered likely to occur for domain VI.*

Answer: Precise modeling of domain VI in its second-step conformation is beyond the scope of this work, if only since it involves some speculations. Nevertheless, let us assume that stems II and IIA in Figure 1A are coaxially stacked, domain VI (assuming it is rigid) would then need to rotate by about 50 degrees and undergo a 150-160 degree twist after the branching reaction in order to dock into its eta receptor; as a consequence, its tip should move by some 55 angstroms, which is a major translocation indeed (note that this value should not depend too much on the exact angle between helices II and IIA, since the latter is far shorter than the former).

Minor suggestions:

Comment: *p. 2 line 8 and throughout manuscript. Change "distal of" to "distal to"*

Answer: OK

Comment: *p. 2 line 13 Change "Noteworthingly" to "It is noteworthy that".*

Answer: We changed it to 'Interestingly' in order to remain at the 175 word limit

Comment: *p. 3 line 7 Change "ribozyme component" to "ribozyme components"*

Answer: OK

Comment: *p. 3 line 13 What is meant by "seemingly unique"? Unique among ribozyme active sites? A single active site within the group II ribozyme?*

Answer: We have replaced 'unique' by 'single'

Comment: *p. 4 line 14 Change "dubbed coordination loop" to "dubbed the coordination loop"*

Answer: OK

Comment: *p. 6 line 8 Change "sequence and structure" to "sequences and structures"*

Answer: OK

Comment: *p. 7 seven lines from bottom Change "with an altered" to "have an altered"*

Answer: OK

Comment: *p. 8 line 4 Change "Still, replacement" to "Unexpectedly" or "Contrary to this hypothesis"*

Answer: We replaced 'Still' by 'However'

Comment: *p. 10 line 3 Change "3' splice" to "3' splice site"*

Answer: Yes

Comment: *p. 10 line 4 Change "though" to "although"*

Answer: OK

Comment: *p. 11 line 11 and throughout manuscript. Change "shallow groove" to "minor groove" .*

Answer: In an A-type helix, the counterpart of the minor groove of the DNA B-type helix is actually somewhat wider than the so-called 'major' groove. We, and a number of our colleagues – just search for 'RNA helix shallow groove' on the Web – rather believe that the two grooves of an RNA double helix should be designated by the terms 'shallow' and 'deep'. We have added 'minor' between brackets after our first mention of 'shallow' [groove].

Comment: *p. 14 line 5 Change "must come truly" to "must truly come"*

Answer: OK

Comment: p. 14 *line 13 What does "prior modeling" refer to? If it is published it should be cited. If it is not published it should be "data not shown." If it is the modeling in this manuscript, then omit "prior."*

Answer: Yes, we were referring to the modeling in this manuscript, 'prior' was inappropriate and we removed it.

Comment: *p. 15 line 17 Change "supernumary" to something else, perhaps "supernumerary". "Appended" would be clearer.*

Answer: We changed this word to 'additional'

Comment: *p. 16 lines 17-20. The sentence is contradictory because it says there is both general agreement and debate.*

Answer: Yes, there is agreement on the fact that domain VI interacts at some stage with domain II, but debate on whether this interaction contributes to positioning the 3' splice site for exon ligation. We have rewritten that sentence accordingly.

Comment: *p. 18 line 5 What is meant by "costly"? Expensive? Experimentally difficult?*

Answer: We replaced 'costly' by 'difficult'

Comment: *p. 32 Fig. 4 legend. Is the error measurement the standard deviation or standard error of the mean?*

Answer: All errors are standard errors of parameters, which were estimated by fitting experimental data (see Figure S2) to equations, as explained in Materials and Methods (we have added 'and their standard errors' after 'initial rates at t=0').

Comment: *p. 33 Fig. 4 legend Change "full curve" to "solid curve"*

Answer: Yes

Referee #2

In this work Li et al., aimed at identifying a receptor site for the branch-point adenosine in group IIB1 introns. By combining phylogenetic and mutational analysis with a rational design of a "molecular tether", the authors were able to identify such a receptor site for D6. Strikingly, this site is located close to the ε' and λ sites within stem c1 in domain 1. Importantly, this receptor site is specific for the first transesterification step of splicing. The proposed novel interaction was termed ι-ι' and it has been suggested that this interaction is disrupted after branching and D6 has to undergo a significant rearrangement for the η-η' contact to occur, which is known to be essential for performing the second step of splicing. Impressively, the authors went on and demonstrated that D6 can be modeled into the crystal structure the group IIC intron from Oceanobacillus iheyensis to allow the ι-ι' contact. In light of previous findings, this manuscript presents a very interesting, elegant, but also in part controversial study on an aspect of group II intron splicing that finds its parallels in spliceosomal intron splicing.

Major comments

Comment: *1. As mentioned by the authors, Pyle and coworkers demonstrated a few years ago that the branch-point adenosine is coordinated to the asymmetric internal loop composed of Jd''/d''' and Jd'''/d'' in the ai5γ group IIB1 intron (Hamill, 2006). The Pyle lab had applied cross-linking to identify residues in spatial proximity to the branch-point. These residues included G1 and C-1 together with two nucleotides in J2/3 (G588 and U590; the counterpart of former and of A589 are part of the triple helix in the active site of the Oi. Intron (Toor et al., 2008).) All other cross-linked residues were located in the coordination loop, which harbors EBS 3 as well. Since Pyle and coworkers used a trans-branching system, the obtained data should have been specific for the branching pathway of splicing. While cross-links to G1, C-1, G588 and U590 can be readily explained by the fact that they are active-site constituents, the phylogenetic data in the current study do not seem to support the coordination loop as docking site for the branch-point. How can the cross-links from A880 (ai5γ branch-point) to the coordination loop be reconciled? Also, looking at Fig. 3A the branch-point A in D6 is not even remotely close to the coordination loop in the model.*

On the other hand, Hamill and Pyle did not observe any cross-links to stem c1 in D1. Is there any explanation for this apparent discrepancy between the two studies?

Answer: What Hamill and Pyle actually observed is (1) when D56 molecules with a photoactivable group at either the branch point or one of the two flanking nucleotides are incubated with the rest of the ribozyme (exD123) under conditions compatible with a folded structure, they crosslink to a diversity of sites, including the coordination loop; (2) when the unreacted, 'XL1' mixture of crosslinks is reincubated under conditions conducive to splicing, some of it reacts, and yields the free 5' exon, as well as another product, which might be a branched molecule, although that was not established. Importantly, crosslinks were mapped before, not after reincubation, so that it is not known which of the crosslinks in the mixture were compatible with activity (the same is true of the fraction of molecules that were able to carry out both steps of splicing – Fig. 5B).
When attempting to interpret these data, the authors implicitely assumed that domain VI should be stably docked into its first-step binding site in unreacted molecules. However, in Costa et al. (1997a) we had previously shown that yeast intron Sc.cox1/1 precursor molecules exist in (at least) two distinct, about equally abundant conformations, one which leads to branching and the other one to 5' splice site hydrolysis; these conformations must differ by the location of domain VI, since disrupting the eta-eta' interaction between domains II and VI suppressed hydrolysis, whereas molecules in which the same interaction was reinforced reacted exclusively by that mechanism. In Fig. 10 of that paper, domain VI of unreacted molecules was accordingly depicted as toggling between a DII-bound state and another one in which it was poised for the branching reaction (that is, bound to its then hypothetical, first-step receptor; obviously, domain VI should also remain unbound for some length of time in between two docking events). Our working model, which remains compatible with all data we know of (and in particular, the absence of domain VI in Toor et al.'s structure; see discussions in Pyle, 2010) was that as a general feature of group II self-splicing, domain VI always keeps toggling between its first- and second-step binding sites, even though the exact equilibrium between the two conformations must depend on each particular intron and intron form.
These ideas may now be put to test by mapping Hamill and Pyle's sites of crosslinking on Toor et al.'s atomic resolution model of ribozyme domains I to V. When that is done, it becomes apparent that essentially every nucleotide that would have been accessible to the branch site and its two flanking nucleotide in a dVI molecule that could freely rotate around the dV-dVI junction did give rise to a crosslink. Only residues in the proximal helix of domain V are missing in the list: since crosslinks at these sites would be internal to the D56 piece, they were not recovered in Hamill and Pyle's experiments.
It is also apparent from three-dimensional modeling that the IC1 helix is essentially out of reach of the branchpoint and its immediate neighbors (this can be checked with the help of the stereo drawings in Fig. 3A of this manuscript, even though the angle of view is not ideal). When domain VI is bound to its IC1 receptor, photoactivable bases at the branchpoint and its two neighbors are predicted to crosslink instead to the first two nucleotides of the intron and the last nucleotide of the 5' exon: these three positions were indeed among those recovered by Hamill and Pyle. Moreover, among the latter crosslinks, those to the G1 nucleotide (and perhaps also to the second residue of the intron) are liable to be compatible with splicing, which provides a ready explanation for the (limited) reactivity of the XL1 material.
To summarize, as long as they are not overinterpreted, Hamill and Pyle's data do not contradict in any way our own findings and conclusions.

Comment: *Since the ai5γ intron has often been referred to as "weirdo" among group II introns, do the authors consider it a possibility that the coordination loop functions as receptor for the branch-point in the ai5γ intron only? Or, is more likely that cross-linking possibly produced in part erroneous data (as it had happened before).*
Since there is this controversy, this reviewer is of the opinion that it would be an critical control experiment to mutate the corresponding tandem GU wobble pairs in stem c1 of the ai5γ intron and test for its ability to perform branching (despite data on the Bc. intron from Stabell et al., 2009).

Answer: As just stated, any possible controversy should not be about data, but their interpretation. In spite of its high A:U content and elevated magnesium requirements for in vitro activity, which do not make it such a good model system, the ai5γ intron looks fairly typical of organelle members of subgroup IIB1, and we see no reason why mutation of the tandem G:U pairs in the IC1 stem of that intron would not affect the ability of the ribozyme to carry out branching, as reported in this work for the Pl.LSU/2 ribozyme and also by Stabell et al., using a molecule from a different structural

subgroup (the experimental setup must of course ensure that branching is rate-limiting).

Comment: *2. Hydroxyl radical footprinting has been done on the Pylaiella intron (Costa et al., 2000). Have the authors also mapped D6 and stem c1? At least from the ai5γ footprinting data it appears that both GU wobble pair are internalized (except G87; Swisher et al., 2001). In other words, how does the model in Fig. 3A correlate with such footprinting data?*

Answer: Our data for the IC1 stem were published in Fig. 7 of that paper. In the lariat intron, there is a small zone of partial protection from hydroxyl radicals centered on G79, whereas nucleotides around position 100 are moderately accessible. However, these data should be regarded as irrelevant to our model as long as there is no evidence that domain VI is stably docked into its first-step receptor. As we explained on p.17 l.4-9, it is our hope that our system of anchoring nucleotides will make it possible to lock the ribozyme into its first-step conformation, which would in turn make 'footprinting' pertinent.

Comment: *3. Assuming that ι-ι' takes place in the ai5γ intron, the available NAIM data on this yeast mitochondrial intron support that the minor groove of stem c1 is involved in the ι-ι' interaction: the exocyclic amine of G87 and the 2'hydroxyl groups of U86 and U110 were described to be important for branching (Boudvillain et al., 1998) - as stated by the authors. In the same paper a 2-aminopurine and 7-deaza effect were observed for A861 and A863, suggesting an involvement of the major groove (N6, N7). Can one infer any interaction from the available data and your model?*

Answer: The information provided by NAIM experiments may reasonably be interpreted in terms of specific, direct atomic contacts only as long as one is dealing with components the structure of which is known (or believed to be so), as is the case for the IC1 distal helix. We believe that the structure, either in isolation or in interaction, of the AAA:CUA, DVI internal loop of mitochondrial subgroup IIB1 introns can not be predicted from currently available data and accordingly, we do not wish to take stands on what it might be. As already stated in our Text, we provisionally modeled the distal part of domain VI as a continuous helix because (i) that is by far the most commonly encountered situation in intron subgroups that have a conserved G:U pair in IC1 at positions equivalent to Pl.LSU/2 79 and 100; (ii) replacement of that loop by canonical base pairs is compatible with branching (even though it is not optimal; this work and Chu et al., 1998).

Comment: *The presented data unambiguously demonstrate that D6 branch site and stem c1 are spatially very close, however, in order to definitively state that these structural elements are in physical contact (i.e. a novel interaction), it is preferable to have an idea about potential H-bonds in the ι-ι' pairing (in addition to the phylogenetic evidence).*

Answer: As can be checked by modeling, the fact that a single-nucleotide tether is optimal (and the lack of it is tolerated) is hardly compatible with anything but a direct contact.

Comment: *As stated* (**Authors:** observed, in fact) *by the authors, the D6 internal loop can be replaced with canonical base pairs without abolishing branching. What is the advantage of maintaining an internal loop throughout evolution?*

Answer: Admittedly, the internal loop of mitochondrial members of subgroup IIB1 is replaced by a continous helix in the vast majority of bacterial members of this subgroup. However, close examination of secondary structure models reveals that the location of the predicted eta receptor may not be exactly the same in the two subsets and in fact, the total length of the distal DVI stem differs (by two base pairs): the need to simultaneously ensure efficient docking into the IC1 receptor and maintain the geometry between the base and tip of domain VI appropriate for the eta-eta' interaction may be the key to structural conservatism in the middle part of domain VI.

Comment: *4. The oligonucleotide tether is a very elegant way to further support the spatial proximity of D6 bulge and the tandem GU pairs in stem c1. The different variants were compared for their relative branching rates. However, it would be helpful to enlist the absolute kobs values together with the wt activity from Table I.*

Answer: At a 5 μM concentration of anchoring oligonucleotide, k_{obs} values (calculated from the fraction of unreacted precursor molecules) ranged from 0.0042 ± 0.0002 min^{-1} (for a 4-nucleotide

tether) to 0.0093 ± 0.0005 min^{-1} (for a 1–nucleotide tether).

Comment: *Along the same line, I urge the authors to show a representative gel for splicing of the wt and at least the IC1 UA::UA mutant and the anchor 2 with 1 T only.*

Answer: Yes, we now have three supplementary figures: Figure S1 shows a representative gel autoradiograph of wt and IC1 UA:UA splicing reactions in the presence of potassium; in Figure S2, reaction time courses in ammonium- and potassium-containing buffers are compared; and Figure S3 shows a gel with splicing reactions of the construct in Figure 3C in the presence of oligonucleotides with a 1T-tether and either a matched or mismatched handle for binding IC1; next to these lanes a wt splicing reaction was run on the same gel for comparison purposes.

Comment: *5. It is my understanding that the coordination loop is poorly conserved among group II introns (Michel et al., TIBS 2009), but what about the tandem GU pairs in stem c1? How well are these and in turn the ι-ι' contact conserved among group II introns (possessing a branch-point) of different phylogenetic families.*

Answer: The (counterpart of the) G79:U100 IC1 base pair is generally conserved in ribozyme structural subgroups IIB1, IIB3, IIB4 and IIC (see Toro, 2003, Environ. Microbiol. 5, 143-151, for nomenclature). Most importantly, in subgroup IIA, lengthening of the epsilon' loop from 4 to 11 nucleotides generates a ready candidate for a domain VI receptor, whereas the section of the ribozyme that would be expected to host a counterpart to the coordination loop (rather, to what would be left of that loop after migration of EBS3 to the δ position, next to EBS1) is not conserved, whether in terms of sequence or structure.

Comment: *6. Another tertiary contact has been proposed by Pyle and coworkers a few years ago (Fedorova et al., 2005): μ-μ' in ai5γ. As this contact has not been included into the schematic drawing of Fig. 1A, I am wondering whether there is a specific reason for it. Is such a contact not supported by phylogeny in the Pylaiella intron?*

Answer: Loop IIIA, with a GUAAU consensus sequence (the two adenines were proposed to constitute the μ site), has a scattered distribution in subgroups IIB and IIC – there is no evidence of its, or a counterpart of it, being present in the Oceanobacillus ribozyme, or in most of the many subgroup IIC members. In fact, μ-μ' is often omitted from secondary structure models (for instance from Fig. 3 of Hamill and Pyle, 2006). However, we agree that that is no good reason to overlook this proposed contact and since it is potentially present in the Pl.LSU/2 ribozyme, we have now included it in Figure 1A.

Minor comments:

Comment: *I suggest highlighting Domain 2 in Fig. 3A to be able to imagine the conformational switch of D6 shown in Fig. 5.*

Answer: Yes, we now point to the location of the eta receptor in Fig. 3A, and also stress in the legend to that figure that it is necessary to extend mentally the DII helix by about one helical turn when trying to imagine the position of the tip of domain VI when it is bound by the eta-eta' interaction (see also our answer to a similar comment by referee #1).

Comment: *In the methods section, please name and cite the program used for the alignment.*

Answer: The usefulness of alignments destined to be exploited in comparative sequence analyses depends for a large part on human expertise and accordingly, they should best be generated manually (see discussions in Michel and Costa, 'Inferring RNA structure by phylogenetic and genetic analyses', in 'RNA Structure and Function', Cold Spring Harbor Laboratory Press, R. Simons and M. Grunberg-Manago eds., pp. 175-202, 1998). We have made our alignment of mitochondrial subgroup IIB1 introns available as a Supplementary Dataset in Li et al. (RNA Journal, 2011).

Comment: *In Table II setup 2, please explain the 3-fold enhancement compared to setup 1.*

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Answer: Yes, the anti-DVI 7-mer included in setup 2 (and also in setups 4 and 6) provides limited compensation, and only so at very high oligonucleotide concentrations; in fact, the relative rate of branching remains too small to allow a Km to be estimated (see Figure 4A, lozenges and dotted curve). We have added a sentence to make it explicit that that is true not merely in a mutated IC1 context, but also in the presence of the wild-type IC1 sequence.

Comment: *In the last section of the Discussion the authors mention "costly". I suggest removing or rephrasing this sentence. There is no need to explain why the authors have not performed NAIS (yet), as this would go beyond the scope of this manuscript.*

Answer: We replaced 'costly' by 'difficult'.

2nd Editorial Decision 20 May 2011

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. It has been now been evaluated the two original referees and I enclose their reports below. As you will see from the comments both referees find that the revised manuscript has been significantly strengthened and both remain positive regarding publication. However, there are a couple issues that have not been fully addressed, one of these raised by referee #1. This referee would like to see additional evidence for the role of the iota-iota' interaction such as crosslinking or protection experiments. While this referee requested additional experimental evidence in the first round of review, I appreciate that they did not ask for these explicit experiments. It seems that these experiments would significantly strengthen the study and make it more convincing in light of the previous work from the Pyle lab. My opinion is that if these additional experiments would make the study overall more convincing and if they can be conducted in a reasonable timeframe it would be extremely beneficial for the paper and the field to include them. I am happy to discuss this matter further. I would also be grateful if you could include the additional changes suggested by the referees.

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 #1:

The manuscript is improved from the previous version, although some issues were not fully addressed.

-The alignment upon which the comparative data are based is stated to be available as supplementary data for a manuscript in press; however, the reviewers have not had access to it.

-I still think the domain VI mutagenesis experiments provide weak evidence. If the domain VI motif is so noticeably conserved over evolution, then one would expect a significant effect when it is mutated. Instead the significant effects seem to be for mutations in domain IC. It would be

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preferable to provide an additional type of experimental evidence for the contact between DVI and DIC. Cross-linking experiments would be an obvious choice, or protection experiments, which the Michel lab has used quite successfully in the past. The oligonucleotide experiment provides the only direct evidence for a contact. An additional source of data might cement the existence of the iota interaction, rather than making a strong case.

-p. 8 "Only by bringing the length of the helix distal to the branchpoint down to two base pairs do consequences suddenly become dramatic, with splicing proceeding almost exclusively by hydrolysis." A dramatic difference is not seen for the ammonium conditions, but for the potassium conditions, which have not yet been explained in the text.

-The explanation in Table 1 for the discrepancy between the fraction of product branched and the kbr/khy ratio is reasonable, but it would be helpful to include this explanation in the manuscript, perhaps as a footnote to the table.

-My point about the position of the eta interaction was not that it should be modeled, but that it is nearby. Given the uncertainties in modeling, it seems possible that one could model the eta and iota interactions simultaneously. This is unlikely to occur of course, but it suggests that the conformational change for domain VI may be subtle rather than dramatic.

-The detailed explanation accounting for data in the Hamill et al paper is informative, and in my opinion should be included in the Discussion. The present study directly contradicts the conclusions of the Hamill paper, and for the sake of clarity in the literature, it would be helpful for this to be acknowledged more directly with a rational explanation provided, since the Hamill data are not disbelieved.

-The added three supplementary figures are all improvements. I suggest including Supplementary Figure 1 in the main text.

-The phrase "fully consistent" is used in several places to describe how the experimental data relates to the comparative data. This seems an overstatement, because it implies that the theoretical inferences were entirely correct. There is noise in the comparative data though. "Consistent" would be better.

-p. 5 six lines from bottom "chloroplasts" not "chloroplats"

Referee #2:

As the authors have addressed all my previous comments in the revised version of their manuscript and accompanying letter, I do not have any further comments.

2nd Revision - authors' response 30 May 2011

Answers to the referee comments

Referee #1:

Comment: *The manuscript is improved from the previous version, although some issues were not fully addressed.*
-The alignment upon which the comparative data are based is stated to be available as supplementary data for a manuscript in press; however, the reviewers have not had access to it.

Answer: As now indicated in Materials and Methods, in the 'Sequence analyses' subsection, the alignment of intron sequences that we used for our comparative analyses can now be downloaded at http://rnajournal.cshlp.org/content/suppl/2011/05/05/rna.2655911.DC1.html

Comment: *I still think the domain VI mutagenesis experiments provide weak evidence. If the domain VI motif is so noticeably conserved over evolution, then one would expect a significant effect when it is mutated. Instead the significant effects seem to be for mutations in domain IC. It would be preferable to provide an additional type of experimental evidence for the contact between DVI and DIC. Cross-linking experiments would be an obvious choice, or protection experiments, which the Michel lab has used quite successfully in the past. The oligonucleotide experiment provides the only direct evidence for a contact. An additional source of data might cement the existence of the iota interaction, rather than making a strong case.*

Answer: With regard to mutational effects, it is essential to distinguish between ammonium and potassium ions, as was done in Table I. Even in subdomain IC1, point mutations have only limited effects as long as ammonium-containing solutions are used for self-splicing tests: substitution of the two consecutive G:U pairs by U:A pairs does not change detectably the fraction of molecules that will react by branching (Table 1) and our interpretation (p.8 l.12-14) was that docking of domain VI into its proposed IC1 receptor is not limiting for the ability to carry out branching under these conditions. In contrast, when branching is challenged by the presence of potassium ions, the fraction of molecules that react by branching becomes quite sensitive to structural alterations in both the iota and iota' motifs: precise removal of the DVI internal loop (dVI-4b) brings that fraction down to 0.15, a value that is close indeed to that observed (0.10) for the IC1 UA:UA mutant (Table I; the kbr/khy ratio is also the same for the two mutants).

Cross-linking or protection experiments on the wild-type intron are unlikely to detect the contact between DVI and IC1, since the molecule is predominantly in a second-step conformation, as revealed by the fact that disruption of eta-eta' greatly increases either the rate of branching (Chanfreau and Jacquier, 1996) or the fraction of molecules that react by branching (Costa et al., 1997). Performing similar experiments on the complex between one of our mutated ribozymes and an oligonucleotide that restores branching through complementarity to both DVI and IC1 will merely confirm the existence of this complex (provided its stability is sufficient to make it the new ground state of the system). For future experiments to be truly informative, they should be able to probe the active state of the branching complex, and as we already suggested (now on p.19 l.1-5), we believe that the best possible approach at present is NAIS (Strobel, 1999). However, as previously pointed out by referee #2, 'this would go beyond the scope of this manuscript'.

Comment: *p. 8 "Only by bringing the length of the helix distal to the branchpoint down to two base pairs do consequences suddenly become dramatic, with splicing proceeding almost exclusively by hydrolysis." A dramatic difference is not seen for the ammonium conditions, but for the potassium conditions, which have not yet been explained in the text.*

Answer: Our sentence referred to mutant dVI-2bp (Table I, line 4), which dramatically differs from the wild-type indeed, since splicing is seen to proceed almost exclusively by hydrolysis, even in ammonium conditions. In order to remove any possible source of confusion, we have now added an explicit reference to the mutant and corresponding line in Table I within the sentence in question.

Comment: *The explanation in Table 1 for the discrepancy between the fraction of product branched and the kbr/khy ratio is reasonable, but it would be helpful to include this explanation in the manuscript, perhaps as a footnote to the table.*

Answer: Yes, we have now inserted our explanation for this observed discrepancy at what we believe to be the most appropriate place – in the 'Kinetic analyses' subsection of Materials and Methods.

Comment: *My point about the position of the eta interaction was not that it should be modeled, but that it is nearby. Given the uncertainties in modeling, it seems possible that one could model the eta and iota interactions simultaneously. This is unlikely to occur of course, but it suggests that the conformational change for domain VI may be subtle rather than dramatic.*

Answer: As we previously explained, reasonable assumptions about the position of the eta receptor imply a large translocation. Nevertheless, we have now added bried notes of caution in the Discussion and legend to Fig. 3A so as to warn the reader that the exact location of eta is a bit uncertain.

Comment: *The detailed explanation accounting for data in the Hamill et al paper is informative, and in my opinion should be included in the Discussion. The present study directly contradicts the conclusions of the Hamill paper, and for the sake of clarity in the literature, it would be helpful for this to be acknowledged more directly with a rational explanation provided, since the Hamill data are not disbelieved.*

Answer: We have inserted in the Discussion an additional paragraph in which we explain that we believe Hamill and Pyle's data are compatible with our work indeed, as long as it is not assumed that domain VI is stably docked into its receptor site prior to branching.

Comment: *The added three supplementary figures are all improvements. I suggest including Supplementary Figure 1 in the main text.*

Answer: We are grateful to referee #2 for pointing out the necessity to give interested readers access to representative examples of our raw data and for helping us to select the gels to be provided as Supplementary Materials. On the other hand, we do not think it necessary to include alongside our main text gels and graphics that were not conceived for illustrative purposes, but for quantitation, and the main information content of which we believe to be appropriately summarized in the Tables and Figure 4.

Comment: *The phrase "fully consistent" is used in several places to describe how the experimental data relates to the comparative data. This seems an overstatement, because it implies that the theoretical inferences were entirely correct. There is noise in the comparative data though. "Consistent" would be better.*

Answer: Ok, we removed 'fully' every time it appeared in front of 'consistent'.

Comment: *p. 5 six lines from bottom "chloroplasts" not "chloroplats"*

Answer: Thank you.

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