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Cellular pathways controlling integron cassette site folding

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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 07 January 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. First of all, please let me apologise for the delay in getting back to you with a decision on your manuscript. This was due firstly to some difficulty in finding appropriate referees, and then to the Christmas/New Year period. However, your study has now been seen by three referees whose comments are enclosed. As you will see, all three referees are broadly supportive of publication, contingent upon a number of issues being resolved in a revised version of the manuscript. In particular, I would like to draw your attention to the comments of reviewer 1 (and also alluded to by reviewer 2) regarding the evidence for cruciform extrusion. In points 1 and 2, referee 1 outlines his/her concerns with these experiments, and suggests additional experiments to more rigorously demonstrate that this occurs and is important. In the assessment of this referee, such data would be critical for the eventual acceptance of your manuscript in the EMBO Journal. In addition, referees 2 and 3 both highlight various aspects of the text that would benefit significantly from additional discussion in order to better place your results in the broader context, and to make them more easily understood by the non-specialist reader. From an editorial point of view, this is also an issue that we picked up on, and I would therefore strongly encourage you to revise the text accordingly.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Loot et al have investigated cellular pathways influencing the site-specific recombination of integron encoded attI and attC sites. It was previously shown that the recombination reaction is unconventional in that the reaction requires a folded single stranded attC site and that only the bottom strand of attC binds integrase and serves as a recombination substrate. This sets up the question as to what cellular processes regulate/promote this recombination reaction. The authors provide convincing evidence that conjugation and DNA replication promote attI x attC recombination and are able to strongly correlate this with the bottom strand of attC being present (at least transiently) as ssDNA. In addition, the authors argue that extrusion of the attC site into a cruciform provides an alternative mechanism for forming ss DNA. This would then point to DNA supercoiling as playing an important role in integron cassette capture. Relationships between recombination frequencies and attC site length are also considered. Overall, the paper is well written and easy to follow. Specific concerns are listed below.

Major concerns

1. I have some concern with the conclusion that cruciform extrusion is a major factor in recombination. First, the authors don't rigorously prove cruciform formation in vitro with their attC containing plasmids. This requires high-resolution structure mapping and/or two dimensional gel electrophoresis. The latter would provide an estimate of the free energy of supercoiling required to drive the cruciform transition for a given attC plasmid substrate - this is useful to know with regards to how likely cruciform formation is in vivo. With this information one would be in a stronger position to interpret the results in Figure 6B. That is, would the decrease in superhelix density in the top(-) gyr(-) strain be sufficient to negatively impact on the structural transition and therefore be held accountable for the difference in recombination frequencies observed?

2. Second, does the experiment in Figure 6B take into account attC plasmid transformation efficiencies. My experience is that upon relaxing a plasmid DNA with topo I a significant amount of nicked plasmid is generated. Might the decrease in recombination frequency observed for supercoiled versus topo I treated (relaxed) plasmid be due to a reduced input of transformable DNA?

3. One general concern is the lack of correlation between recombination frequencies and the 'opportunity' of attC DNA to achieve 'single strandedness'. For example, a much higher recombination frequency (almost 2-orders of magnitude) is observed for the attCaadA7 site in theta-replication assay versus the conjugation assay. Yet, the opportunity for the attC site to adopt the single strand state should be much higher in the conjugation assay. Is this a real difference or an artifact of the recombination assay set-up in the two experiments? This type of issue should be dealt with in the Discussion. Similarly, one might expect that the recombination under non-replicating conditions (and inferred to be driven by cruciform extrusion) would occur at a lower frequency than under conjugation conditions, yet the frequencies are very similar. Given these apparent inconsistencies, I am left wondering if in fact there may be an important host factor requirement (i.e. other than replication, conjugation or supercoiling) for generating ss attC sequences.

4. In Figure 5, the authors show a correlation between the potential of an attC plasmid to form an S1 sensitive structure and recombination frequency. That is, the attCaadA7 plasmid shows S1 sensitivity whereas the VCR2/1 plasmid does not and the former exhibits a higher recombination

frequency compared to the latter. I was left wondering how tight this correlation is? The authors have many other attC site plasmids and it would be useful to attempt to extend this correlation. For instance, VCR180, which has a recombination frequency intermediate to that of aadA7 and VCR2/1 might show an intermediate level of S1 sensitivity.

Minor Comments

1. Figures 1 and 2 could easily be combined.

2. Pg 5, line 7: I'm not sure what is meant by 'ss DNA constraints'.

3. Pg 9, line 12: What is meant by 'active site'.

4. Pg 12, line 5: It is not topo I that prevents cruciform extrusion rather it is relaxation of the plasmid DNA that prevents cruciform extrusion. Accordingly, in Figure 6 it would be more relevant to indicate that the input attC plasmid DNA was either supercoiled or relaxed.

5. Discussion of the possible mechanism of cruciform extrusion (C- or S-type pathways) (Figure 7) is premature as in my view a stronger case for this type of structural transition needs to be made.

6. Pg 20, line 15: 'efficiency' of what?

7. There is an elegant way to test for structural transitions in supercoiled plasmids in vivo, although it requires uncoupling DNA and protein synthesis. If a plasmid undergoes a structural transition in vivo that involves DNA unwinding (such as cruciform formation) then topoisomerases inside the cell will restore the steady-state level of supercoiling by further reduces the linking number. Upon purifying the DNA two distributions of topoisomers will be produced; one with an average linking number indicative of molecules that haven't undergone the transition and one with a lower average linking number from molecules that have undergone the transition. The difference in the distributions provides information regarding how many turns of DNA are unwound by the structural transition [see Haniford, D.B. & Pulleyblank, D.E. J. Biomol. Str. Dyn. 1, 593 (1983)]. The authors may wish to perform this analysis on attC plasmids to see if they can provide additional support for their contention that attC sites undergo a cruciform transition in vivo.

Referee #2 (Remarks to the Author):

The manuscript describes experiments showing how different processes that generate single-stranded (ss) DNA in the cell can promote attC folding and recombination in integron cassette recombination. The conclusions of the work are somewhat diffuse, as they do not provide a single large 'quantum leap' in the science of integrons, but collectively I do think that they make a significant and valuable contribution to the field. In particular, the demonstrations that attC folded structures can be formed in cells containing normal plasmids, i.e. without conjugation, are important for those wishing to understand the biology of these systems.

I have a number of comments on issues that I think require attention:

1. The claim in the abstract that cellular processes making ssDNA such as conjugation and replication favour proper attC folding is (I think) justified by the results, but is not explained and defended very clearly in the Discussion etc. The evidence supporting this claim should be summarized.

2. Throughout, there are some minor grammatical problems, which should get fixed.

3. Page 5, line 7. I don't know what "(21)" means.
This paragraph is quite hard for the reader. It might be better to include a well-constructed figure in the main part of the manuscript, showing how the attC derivatives were constructed from existing sites. From Figure S1 I can't work this out. The parts that are "natural" and the parts that have been altered should be indicated somehow.

4. Page 8, line 5-6. A reference should be given for the evidence that very little ssDNA is produced on the leading strand.

5. Page 9, top of page (etc.). To call the extruded attC sites cruciforms might possibly be confusing to some, as the strands are annealed in a complex way unlike standard cruciform structures. Maybe the use of the terminology should be explained.

6. Page 11, centre. I agree that the results suggest 'cruciform' formation from dsDNA, but another explanation might be that the non-replicating plasmids eventually get degraded by nucleases, transiently creating ssDNA. This possibility should be discussed.

7. Page 12, line 8. Figure 6A shows an S1 nuclease assay, not recombination as the text suggests. The apparent error should be fixed. Line 16; the meaning of "...blunt the effect of supercoiling." is unclear.

8. Page 21. A reference for the UNAFold software should be given. I could not understand from this paragraph how the folding probability was being calculated; in particular the statement "it is sufficient to constrain the pairing of A17 for the proper fold to be energetically the most favorable." What does this mean?

9. In Figures 3A and 4A, it is not possible to work out the relationship between the points on the graphs and the sites that provided the data. I realise that this could be too complex to include in the figure itself or the legend, but the information could at least be provided in Supplementary Table 3; that is, the calculated folding probability for each attC site, and the VTS size for each site.

10. Legend to Figure 4. Please confirm that the G values quoted are for cruciform formation in dsDNA as seems to be implied here, and not for folding of a ssDNA.

11. Figure 5C. The legend should state more clearly what this experiment is for, i.e. what these results show.

12. Figure 1B. It would be helpful to label the attC parts referred to in the text, including the VTS and UCS; also to indicate where the cut site is in the folded attC.

13. Supplementary figure 1. The diagrams here are quite basic. As noted above, it would be helpful to show which nucleotides are "natural" and which have been added or altered by mutation; also where the L and R motifs are in each folded attC.

Referee #3 (Remarks to the Author):

Loot, Bikard, Rachlin and Mazel
Cellular pathways controlling integrons cassette site folding

When I began reviewing this manuscript I felt sure I would find a major caveat to the claim in the abstract that supercoiling causes large interrupted palindromic sequences to extrude as cruciform structures from dsDNA, and that these are substrates for the integron recombinase. Despite this skepticism I find that the data is internally consistent, and the author's interpretation entirely reasonable. I am therefore happy to give this paper my full support if the authors would discuss their work in a wider biological context, as outlined below, and thereby allay my misgivings.

The reason for my initial skepticism is that it has been widely accepted that interrupted palindromes (ie. those with substantial spacer sequences in the middle) do not extrude from the E. coli chromosome in vivo to a significant extent in the absence of replication. The authors interpret their results in terms of integron recombination. But this seems to miss the bigger picture in which their results appear to show that interrupted palindromes exist in vivo as extruded cruciforms, contrary to a long-held belief. This would be an important contribution and this aspect of the work needs to be dealt with in the introduction and the discussion.

I am not very familiar with the palindrome literature and will stand corrected if my understanding

has been superseded. However, if this is the case, there will be others who hold this believe and the authors should probably make some explicit statements on the subject. Neither can I offer a detailed discussion of the evidence against extrusion from dsDNA in vivo. Perhaps the authors could consult reviews by David Leach or the following paper by Sue Lovett, which is an excellent starting point and source of references:
Dutra, B.E. and Lovett, S.T. (2006) Cis and trans-acting effects on a mutational hotspot involving a replication template switch. J Mol Biol, 356, 300-311.

As I understand the arguments: Uninterrupted palindromes can not be maintained in E. coli because the extruded structure is cleaved by an enzymatic activity. The fact that a 3-6 bp spacer sequence in the middle is sufficient to stabilize a palindrome suggests that this prevents extrusion. The authors do discuss the energetics of extrusion but it is not placed in a wider context.

Palindromes are thought to be maintained by the co-evolution of the two half-sequences owing to a template switching mechanism during replication. Thus, an imperfect palindrome is converted to a perfect palindrome. This process is easy to measure in a genetic assay and has been studied extensively (eg. Lovett paper above). As far as I can see there is little evidence in either prokaryotes or eukaryotes for the existence of interrupted cruciform/hairpins in non replicating DNA. Evidence against the extrusion of cruciform/hairpins is that mismatch repair mutations do not influence the frequency at which imperfect inverted repeats are 'corrected' to perfect repeats. Quoting from the Lovett paper: "The effects of mismatch repair were inconsistent with a proposal that mismatch correction within a hairpin formed by the inverted repeats gives rise to the mutation. Rather, our results suggested that the mutation arises by mispairing during chromosomal replication, with the mutation susceptible to removal by subsequent methyl-directed mismatch repair." Granted, this paper is dealing with a special situation not exactly analogous to the present argument. However, I think it would certainly make for a more rounded manuscript if the authors were to discuss more fully the views in the field concerning the extrusion of palindromes in vivo.

SPECIFIC POINTS

Throughout the results and figures frequencies are quoted without any units. A frequency is always a something per something. For example, oscillations per second or events per cell per generation.

In Fig 3A I need my glasses and a magnifying glass to read the frequencies. The font size should be increased in all of the figures as they cause considerable aggravation to the reader.

The term cointegrate is used several times. Do the authors really mean "cointegrate" in the sense of a fusion of the donor and target replicon with a copy of the mobile element at each of the TWO junction points? Or do they mean a simple replicon fusion.

In many places the authors quote the density of negative supercoiling in vivo as close to -0.06. In fact, this is the density in a purified plasmid. It has been established that proteins constrain a superhelical density of about 0.03. The respective densities in vivo and vitro are therefore 0.03 and 0.06. The authors should state this and cite the reference where this was first shown. These inaccuracies of terminology introduce a potential confusion for the non-aficionados.

Is pTSC replication completely unidirectional?

The paper is generally very well written, but there are small sections where it seems to lack polish, for example, page 5 was particularly bad.

Table S3 is mentioned before TS1 and TS2. Also it was not clear to me if TS3 contained the raw data for Fig. 3A?

1st Revision - authors' response 07 May 2010

Referee #1 (Remarks to the Author):

Loot et al have investigated cellular pathways influencing the site-specific recombination of integron encoded attI and attC sites. It was previously shown that the recombination reaction is unconventional in that the reaction requires a folded single stranded attC site and that only the bottom strand of attC binds integrase and serves as a recombination substrate. This sets up the question as to what cellular processes regulate/promote this recombination reaction. The authors provide convincing evidence that conjugation and DNA replication promote attI x attC recombination and are able to strongly correlate this with the bottom strand of attC being present (at least transiently) as ssDNA. In addition, the authors argue that extrusion of the attC site into a cruciform provides an alternative mechanism for forming ss DNA. This would then point to DNA supercoiling as playing an important role in integron cassette capture. Relationships between recombination frequencies and attC site length are also considered. Overall, the paper is well written and easy to follow. Specific concerns are listed below.

Major concerns

1. I have some concern with the conclusion that cruciform extrusion is a major factor in recombination. First, the authors don't rigorously prove cruciform formation in vitro with their attC containing plasmids. This requires high-resolution structure mapping and/or two dimensional gel electrophoresis. The latter would provide an estimate of the free energy of supercoiling required to drive the cruciform transition for a given attC plasmid substrate - this is useful to know with regards to how likely cruciform formation is in vivo. With this information one would be in a stronger position to interpret the results in Figure 6B. That is, would the decrease in superhelix density in the top(-) gyr(-) strain be sufficient to negatively impact on the structural transition and therefore be held accountable for the difference in recombination frequencies observed?

As requested we performed an additional experiment in order to obtain a high-resolution structure map. In this experiment, we precisely determined the S1 nuclease cleavage sites by sequencing a representative sample of cleaved plasmids. This shows that most cleavages target the foreseen stem-loop structure and that predicted structural elements are preferably cut by the nuclease. The precise experimental description has been added to the material and methods section. The results are shown in figure 5D, and the following sentences were added in the results (p11, line 260 to 268):

We obtained a high-resolution structure map of S1 cleavage positions in the attCaadA7 site to strengthen our hypothesis of cruciform extrusion. We determined the precise boundaries of the S1 nuclease generated fragments from a representative sample by sequencing. Figure 5D shows the distribution of the cleavage sites within the attCaadA7 site and its neighboring sequences. Among 91 sequenced clones, 73% (66/91) of the S1-cleavage sites clearly map to the attCaadA7 cruciform extrusion and its structural features. The 25 remaining clones revealed S1-cleavage sites on both sides of the attCaadA7 site up to a distance of 40 bases and could be explained by non attC-specific extrusions of nearby inverted repeats (Figure S4).

Concerning the 2D gels, we do not think that this technique is appropriate for the detection of such cruciforms. Indeed, we believe that spontaneous attC site extrusion occurs with a very low probability as these imperfect cruciforms are in most cases the least stable form, even in highly supercoiled DNA. In addition, it is very likely that the integrase stabilizes cruciform extrusion. The following paragraphs have been added in the discussion (p19, line 459 to 484), to clarify these points:

Until now, extrusions from only perfect palindromes have been observed in vivo (Pearson et al, 1996). Extrusion from an imperfect palindrome has only been observed in AT-rich sequences in vitro using two-dimensional gel electrophoresis (Benham et al, 2002). It has been shown that imperfections have major effects on the overall energetics of cruciform extrusion and on the course of this transition.
It had been previously shown that intrastrand base-pairing aptitude (ie the palindromic structure), not primary sequence, conditions the attC site recombination (Bouvier et al, 2005 and 2009). Since modification of the structural properties of the attC site directly affects the recombination efficiency, attC site folding is likely the limiting step in the recombination process. Consequently, the frequencies obtained in the transformation assay (recombination from ds plasmid) suggest a low probability of attC cruciform formation. This corroborates the fact that cruciform structures were

never observed for imperfect palindromes that are not particularly AT-rich.
We propose a stabilizing effect of the integrase, which could capture attC sites upon their extrusion and recombine them efficiently. This type of stabilizing effect on cruciform formation has been previously observed. For instance, in a study of the effect of S1 nuclease on cruciform extrusion, Singleton and Wells (Singleton and Wells, 1982) obtained data supporting the fact that the nuclease may exert a transient stabilizing effect upon cruciform formation. Similarly, Noirot et al, showed the ability of the initiator RepC protein to enhance cruciform extrusion from the pT181 origin of replication (Noirot et al, 1990).
In this study, we chose to develop an in vivo assay (recombination from ds plasmid) which, contrary to the other classical assays previously used, allowed us to observe low probability attC site cruciform extrusion (from 10-3 down to 10-5), and directly demonstrated the implication of cruciform structures in integron recombination. Moreover the fact that only the attC bottom strand can recombine allowed us to separate hairpin formation occurring on the lagging strand from hairpin formation in cruciform structures (see figure 3C).

2. Second, does the experiment in Figure 6B take into account attC plasmid transformation efficiency? My experience is that upon relaxing a plasmid DNA with topo I a significant amount of nicked plasmid is generated. Might the decrease in recombination frequency observed for supercoiled versus topo I treated (relaxed) plasmid be due to a reduced input of transformable DNA?

The experiment takes both elements into account: the efficiency of transformation of each recipient strain, as well as the transformation efficiency of topo I treated and non-treated attC plasmids. We added the following sentences in the results to clarify the experiment (p14, line 333 to 337), and gave detailed explanations in the material and methods section.

To establish a recombination frequency, we transform in parallel a pir+-permissive strain (UB5201-Pi) with the same negatively supercoiled and relaxed plasmids samples. Here again, the transformation efficiency of the both JTT1 and UB5201-Pi strains are determined beforehand and used to adjust the final ratio and normalize the results (see Material and methods).

3. One general concern is the lack of correlation between recombination frequencies and the 'opportunity' of attC DNA to achieve 'single strandedness'. For example, a much higher recombination frequency (almost 2-orders of magnitude) is observed for the attCaadA7 site in theta-replication assay versus the conjugation assay. Yet, the opportunity for the attC site to adopt the single strand state should be much higher in the conjugation assay. Is this a real difference or an artifact of the recombination assay set-up in the two experiments? This type of issue should be dealt with in the Discussion. Similarly, one might expect that the recombination under non-replicating conditions (and inferred to be driven by cruciform extrusion) would occur at a lower frequency than under conjugation conditions, yet the frequencies are very similar. Given these apparent inconsistencies, I am left wondering if in fact there may be an important host factor requirement (i.e. other than replication, conjugation or supercoiling) for generating ss attC sequences.

We understand the referee's concern. It was unclear in the text that one cannot rigorously compare the three different in vivo approaches. To clarify this we added the following sentences in the Discussion (p16, line 379 to 386):

For these, we used three different in vivo approaches, which differ significantly in terms of attC copy numbers and replicative status of the DNA. The "suicide conjugation assay" delivers by conjugation one copy of single-stranded attC-containing plasmid (non replicative) in the recipient cell. The "non-replicative assay" delivers by transformation, one or more copies of the attC-containing non replicative plasmid in the recipient cell. In the "theta-replicative assay", a large number of replicative copies of the attC-carrying plasmid is contained in the recipient cell. Note, that the experimental procedures of these approaches are different and that it would be unwise to compare their respective recombination efficiencies.
See also our answer to the "SPECIFIC POINTS" raised by Referee 3.

4. In Figure 5, the authors show a correlation between the potential of an attC plasmid to form an S1 sensitive structure and recombination frequency. That is, the attCaadA7 plasmid shows S1 sensitivity whereas the VCR2/1 plasmid does not and the former exhibits a higher recombination

frequency compared to the latter. I was left wondering how tight this correlation is? The authors have many other attC site plasmids and it would be useful to attempt to extend this correlation. For instance, VCR180, which has a recombination frequency intermediate to that of aadA7 and VCR2/1 might show an intermediate level of S1 sensitivity.

The point we want to make with this experiment is that attC sites with a small VTS can extrude cruciforms with a probability high enough to be detected with the S1 nuclease assay whereas sites with a large VTS, as expected, cannot. As a matter of fact, we do not expect to see, as the referee suggests, a correlation between S1 sensitivity and the recombination frequency. For instance VCR180 has an intermediate recombination frequency to that of attCaadA7 and VCR2/1 but has the same VTS as VCR2/1 and should therefore not be sensitive to the S1 nuclease. Its higher recombination frequency compared to VCR2/1 is explained by the absence of strong parasite structures. We nevertheless performed two supplementary controls. The following sentences have been added in the "results" (p11, line 251 to 257). We also added new panels (B and C) in Figure 5.

To confirm this observation, we performed the same experiment but using a mutant derivative of attCaadA7, the attCaadA7Mut3 site and a mutant derivative of VCR, the VCRGAA site (Figure 5A and C). The mutations of the attCaadA7Mut3 disrupt the base pairing of the upper part of the stem and have been shown to decrease the recombination by more than 100 fold (Bouvier et al, 2005). As expected, we failed to detect bands indicating cruciform extrusion of this attCaadA7 site derivative. On the contrary, VCRGAA which contains a small VTS, ensures cruciform extrusion at a higher level than the natural VCR site.

Minor Comments

1. Figures 1 and 2 could easily be combined.
Done

2. Pg 5, line 7: I'm not sure what is meant by 'ss DNA constraints'.

To clarify our point, we modified the sentence as follows (page 5, line 106 to 108):

In order to determine if these size limits result from the constraints linked to the folding of ssDNA, we made a series of 21 attC site derivatives (Figure 2 and S1, Table S1 and S2).

3. Pg 9, line 12: What is meant by 'active site'.

The active attC site corresponds to the recombinogenic site (which contains the R and L boxes able to be bound by the integrase). To avoid confusion we substituted "active site" by "recombinogenic site" in all instances in the ms.

4. Pg 12, line 5: It is not topo I that prevents cruciform extrusion rather it is relaxation of the plasmid DNA that prevents cruciform extrusion.

We agreed and modified the sentence as follows (page 14, line 323 to 327):

For these, we used topoisomerase I which catalyzes the relaxation of negatively supercoiled DNA by introducing single strand breaks that are subsequently religated. Relaxed DNA cannot stabilize cruciform structures.

Accordingly, in Figure 6 it would be more relevant to indicate that the input attC plasmid DNA was either supercoiled or relaxed.

Done

5. Discussion of the possible mechanism of cruciform extrusion (C- or S-type pathways) (Figure 7) is premature as in my view a stronger case for this type of structural transition needs to be made.

We deleted these speculations in the discussion.

6. Pg 20, line 15: 'efficiency' of what?

Sorry, it is efficiency of transformation. We modified the text.

7. There is an elegant way to test for structural transitions in supercoiled plasmids in vivo, although it requires uncoupling DNA and protein synthesis. If a plasmid undergoes a structural transition in vivo that involves DNA unwinding (such as cruciform formation) then topoisomerases inside the cell will restore the steady-state level of supercoiling by further reduces the linking number. Upon purifying the DNA two distributions of topoisomers will be produced; one with an average linking number indicative of molecules that haven't undergone the transition and one with a lower average linking number from molecules that have undergone the transition. The difference in the distributions provides information regarding how many turns of DNA are unwound by the structural transition [see Haniford, D.B. & Pulleyblank, D.E. J. Biomol. Str. Dyn. 1, 593 (1983)]. The authors may wish to perform this analysis on attC plasmids to see if they can provide additional support for their contention that attC sites undergo a cruciform transition in vivo.

We thank the Referee for this suggestion. However, this needs a lot of additional work and we prefer keep it for our future studies.

Referee #2 (Remarks to the Author):

The manuscript describes experiments showing how different processes that generate single-stranded (ss) DNA in the cell can promote attC folding and recombination in integron cassette recombination. The conclusions of the work are somewhat diffuse, as they do not provide a single large 'quantum leap' in the science of integrons, but collectively I do think that they make a significant and valuable contribution to the field. In particular, the demonstrations that attC folded structures can be formed in cells containing normal plasmids, i.e. without conjugation, are important for those wishing to understand the biology of these systems.

I have a number of comments on issues that I think require attention:

1. The claim in the abstract that cellular processes making ssDNA such as conjugation and replication favour proper attC folding is (I think) justified by the results, but is not explained and defended very clearly in the Discussion etc. The evidence supporting this claim should be summarized.

We modified the discussion in its first part to include the following paragraph, which summarize our points (p17, line 405 to 411).

We carefully analyzed the impact of conjugation and theta replication processes on the folding of the bs of the attC site and on the integron recombination. We showed that conjugation ensures the folding of attC sites containing a larger VTS (e.g. VCR) or a shorter VTS (e.g. attCaadA7) with even efficiency. We also demonstrate that when carried on the lagging strand of the replicated DNA, the attC bs is recombined at a higher rate, showing that the availability of ssDNA impacts the recombination frequency of attC sites. These results demonstrate that replication can regulate the efficiency of integron recombination.

2. Throughout, there are some minor grammatical problems, which should get fixed.
The ms has been corrected by a native English speaker.

3. Page 5, line 7. I don't know what "(21)" means.

We defined this in the text (p5, lines 107 and 108): we made a series of 21 attC site derivatives

This paragraph is quite hard for the reader. It might be better to include a well-constructed figure in the main part of the manuscript, showing how the attC derivatives were constructed from existing sites.

Done, we added a new figure (Figure 2).

From Figure S1 I can't work this out. The parts that are "natural" and the parts that have been altered should be indicated somehow.

Done. We added additional informations in this figure. Specifically, we annotated the base additions, deletions or substitutions in the different VCR derivatives.

4. Page 8, line 5-6. A reference should be given for the evidence that very little ssDNA is produced on the leading strand.

Done, (Wolfson and Dressler, 1972)

5. Page 9, top of page (etc.). To call the extruded attC sites cruciforms might possibly be confusing to some, as the strands are annealed in a complex way unlike standard cruciform structures. Maybe the use of the terminology should be explained.

We added the following sentence in the "Introduction" (p4, line 82 to 84) to explain the use of the cruciform terminology which seems very appropriate for the attC sites.

Indeed, inverted repeats (perfect or imperfect) have the potential to form branched structures called cruciforms, in which interstrand baseñpairing within the symmetric region is replaced by intrastrand base-pairing (Courey, 2008).

6. Page 11, centre. I agree that the results suggest 'cruciform' formation from dsDNA, but another explanation might be that the non-replicating plasmids eventually get degraded by nucleases, transiently creating ssDNA. This possibility should be discussed.

This is an interesting suggestion. We performed a control assay (see supplementary materials and methods) that ruled out this possibility and added the following sentence in the results (p12, line 282 to 302):

Nevertheless, we cannot exclude the presence of nicked/damaged molecules in our plasmid preparation. Those molecules could allow attC site folding from the single-stranded DNA generated during their repair and explain the obtained recombination events. However, as we failed to detect these nicked molecules by electrophoretic gel analysis, we concluded that, if they exist, these molecules represent a very minor part of the supercoiled plasmid preparation. If this minor part accounts for the majority of the recombination events, we should observe a much higher recombination frequency when all molecules are damaged. To test this hypothesis, we transformed the same cellular setup with identical quantities of either nicked or supercoiled molecules containing the attCaadA7 site (see supplementary information, Table S1 and S2). We modified the plasmids to introduce a single Nb-Bts1 endonuclease site either at 33nt or 296 nt away from the attC site, and in the two orientations at each locations. This endonuclease only cuts one strand, producing plasmids carrying a nick on either the bottom or top strand, depending on the orientation of the endonuclease site. The nicked molecules showed recombination frequencies similar to the supercoiled plasmid (Figure S3). As none of these nicked molecules presented a higher frequency of recombination than the supercoiled ones, these results confirmed that the presence of a minor part of damaged/nicked molecules couldn’t account for the frequency of recombination obtained in this assay. It is yet to be determined how these nicked molecules recombine. The most likely explanation is that nicked plasmids are rapidly ligated, allowing the introduction of supercoils and recombination through attC site cruciform extrusion.

7. Page 12, line 8. Figure 6A shows an S1 nuclease assay, not recombination as the text suggests. The apparent error should be fixed.

Sorry for this mistake, we corrected it as follows (p14, lines 326 and 327):

As expected, we observed an inhibitory effect of topoisomerase I treatment on attCaadA7 cruciform extrusion in vitro (Figure 6A).

Line 16; the meaning of "...blunt the effect of supercoiling." is unclear.

We replaced this sentence by the following (p14, line 339 to 341):

However in this experiment, the topoisomerases and gyrases of the WT strain could act on the transformed plasmids by changing their supercoiling state before they might be recombined.

8. Page 21. A reference for the UNAFold software should be given.

Done, (Zuker, 2003)

I could not understand from this paragraph how the folding probability was being calculated; in particular the statement "it is sufficient to constrain the pairing of A17 for the proper fold to be energetically the most favorable." What does this mean?

We expanded the calculation description to make it clearer as follows (see supplementary information):

If we constrain the proper pairing of A17, we can observe that the most energetically favorable fold is by far the proper fold (data not shown). Based on the assumption that we are in an equilibrium state, this means that the majority of the molecules that pair the A17 properly should have this proper fold. We computed the probability to pair the A17 properly, using the hybrid-ss function of UNAFold.

9. In Figures 3A and 4A, it is not possible to work out the relationship between the points on the graphs and the sites that provided the data. I realise that this could be too complex to include in the figure itself or the legend, but the information could at least be provided in Supplementary Table 3; that is, the calculated folding probability for each attC site, and the VTS size for each site.

Done

10. Legend to Figure 4. Please confirm that the dG values quoted are for cruciform formation in dsDNA as seems to be implied here, and not for folding of a ssDNA.

We added the calculation of the dGc (for cruciform formation) in figure 4 and in material and methods.

11. Figure 5C. The legend should state more clearly what this experiment is for, i.e. what these results show.

Done, see figure 6A (numbering of figures has been changed).
Briefly, we specified the type of experiment in the legend; as follows:

Recombination frequencies of different attC sites after transformation of attC-containing plasmids in non-permissive permissive recipient cells (see Material and methods: recombination assay with a non-replicative substrate).

12. Figure 1B. It would be helpful to label the attC parts referred to in the text, including the VTS and UCS; also to indicate where the cut site is in the folded attC.

Done, see new figure 1B

13. Supplementary figure 1. The diagrams here are quite basic. As noted above, it would be helpful to show which nucleotides are "natural" and which have been added or altered by mutation; also where the L and R motifs are in each folded attC.

Done, see figure S1

Referee #3 (Remarks to the Author):

When I began reviewing this manuscript I felt sure I would find a major caveat to the claim in the abstract that supercoiling causes large interrupted palindromic sequences to extrude as cruciform structures from dsDNA, and that these are substrates for the integron recombinase. Despite this skepticism I find that the data is internally consistent, and the author's interpretation entirely reasonable. I am therefore happy to give this paper my full support if the authors would discuss their work in a wider biological context, as outlined below, and thereby allay my misgivings.

The reason for my initial skepticism is that it has been widely accepted that interrupted palindromes (ie. those with substantial spacer sequences in the middle) do not extrude from the E. coli chromosome in vivo to a significant extent in the absence of replication. The authors interpret their results in terms of integron recombination. But this seems to miss the bigger picture in which their results appear to show that interrupted palindromes exist in vivo as extruded cruciforms, contrary to a long-held belief. This would be an important contribution and this aspect of the work needs to be dealt with in the introduction and the discussion.

We modified the text, mostly in the abstract and discussion, to clarify our observations. We also tried to emphasize the relevance of our observations in the broader context of interrupted palindromes and their possible extruded cruciforms. Here are the main modifications we made

ABSTRACT (p1, line 20 to 23)
By developing a very sensitive in vivo assay, we also provide evidence that attC sites can recombine as cruciform structures by extrusion from double-stranded DNA. Moreover, we show an influence of DNA superhelicity on attC site extrusion in vitro and in vivo.

DISCUSSION (p19, line 459 to 484)

Until now, extrusions from only perfect palindromes have been observed in vivo (Pearson et al, 1996). Extrusion from an imperfect palindrome has only been observed in AT-rich sequences in vitro using two-dimensional gel electrophoresis (Benham et al, 2002). It has been shown that imperfections have major effects on the overall energetics of cruciform extrusion and on the course of this transition.
It had been previously shown that intrastrand base-pairing aptitude (ie the palindromic structure), not primary sequence, conditions the attC site recombination (Bouvier et al, 2005 and 2009). Since modification of the structural properties of the attC site directly affects the recombination efficiency, attC site folding is likely the limiting step in the recombination process. Consequently, the frequencies obtained in the transformation assay (recombination from ds plasmid) suggest a low probability of attC cruciform formation. This corroborates the fact that cruciform structures were never observed for imperfect palindromes that are not particularly AT-rich.
We propose a stabilizing effect of the integrase, which could capture attC sites upon their extrusion and recombine them efficiently. This type of stabilizing effect on cruciform formation has been previously observed. For instance, in a study of the effect of S1 nuclease on cruciform extrusion, Singleton and Wells (Singleton and Wells, 1982) obtained data supporting the fact that the nuclease may exert a transient stabilizing effect upon cruciform formation. Similarly, Noirot et al, showed the ability of the initiator RepC protein to enhance cruciform extrusion from the pT181 origin of replication (Noirot et al, 1990).
In this study, we chose to develop an in vivo assay (recombination from ds plasmid) which, contrary to the other classical assays previously used, allowed us to observe low probability attC site cruciform extrusion (from 10-3 down to 10-5), and directly demonstrated the implication of cruciform structures in integron recombination. Moreover the fact that only the attC bottom strand can recombine allowed us to separate hairpin formation occurring on the lagging strand from hairpin formation in cruciform structures (see figure 3C).

Note that we interpret our results in terms of integron recombination, because in the case of integrons only the bottom folded attC site is involved in recombination. This means that if the attC site bottom strand is not carried by the lagging strand, the attC site has to use another pathway to recombine (such as cruciform extrusion). For all other imperfect palindrome sites, there is no strand selectivity involved and therefore the major pathway of folding most probably involves replication.

I am not very familiar with the palindrome literature and will stand corrected if my understanding has been superseded. However, if this is the case, there will be others who hold this believe and the

authors should probably make some explicit statements on the subject. Neither can I offer a detailed discussion of the evidence against extrusion from dsDNA in vivo. Perhaps the authors could consult reviews by David Leach or the following paper by Sue Lovett, which is an excellent starting point and source of references:
Dutra, B.E. and Lovett, S.T. (2006) Cis and trans-acting effects on a mutational hotspot involving a replication template switch. J Mol Biol, 356, 300-311.

As I understand the arguments: Uninterrupted palindromes can not be maintained in E. coli because the extruded structure is cleaved by an enzymatic activity. The fact that a 3-6 bp spacer sequence in the middle is sufficient to stabilize a palindrome suggests that this prevents extrusion.

It has indeed been observed that perfect palindromes are genetically unstable, presumably because they disturb replication and are cleaved by SbcCD (Leach, 1994). We observed this phenomenon when attempting to construct the VCR180 (see Discussion p20 and 21). But what we observe is that natural attC sites are genetically stable suggesting that they fold into cruciform at low frequencies. In addition, once structured, they are most probably stabilized by the integrase and SbcCD might not be able to reach them. As such, SbcCD would not represent a threat to their genetic stability.

Palindromes are thought to be maintained by the co-evolution of the two half-sequences owing to a template switching mechanism during replication. Thus, an imperfect palindrome is converted to a perfect palindrome. This process is easy to measure in a genetic assay and has been studied extensively (eg. Lovett paper above).

We wish to thank the referee for pointing out this very interesting paper. This paper shows the ability of a quasi-palindrome to fold during replication (independent of the lagging and leading strand position). By a replication template switch, this imperfect palindrome can become perfect.

We do not know if this template-switch mechanism exists for the attC sites. Nevertheless, mutations that would remove imperfections in the attC palindrome would render the site non-functional. It is indeed known that the extrahelical bases and the unpaired central spacer in the hairpin are crucial for the integrase recognition (Bouvier et al, 2009). Thus, the selective pressure to maintain imperfect palindromes probably counteracts the drift mediated by the template switch mechanism. It is also worth noting that template switching cannot have an impact on recombination frequency since the rate of mutations (10-7) is largely lower than the frequency of attC recombination (10-1 to 10-4 depending of the attC site).

As far as I can see there is little evidence in either prokaryotes or eukaryotes for the existence of interrupted cruciform/hairpins in non replicating DNA.

Yes, indeed, the only evidence for extrusion of an imperfect palindrome is an in vitro analysis (visualization by 2D gel electrophoresis) performed by Benham and coll (Benham et al, 2002). Moreover, in this case, the imperfect palindrome is very AT rich probably ensuring the success of the in vitro cruciform detection. In our study, we succeed in showing the ability of imperfect attC palindromes to extrude from dsDNA using an in vivo assay based on integron recombination. Our assay is much more sensitive than all the classical cruciform detection assays previously used. Indeed, we can detect cruciform extrusion down to a frequency of 10-5 (such as for VCR sites).

See also our answer to point 1.

Evidence against the extrusion of cruciform/hairpins is that mismatch repair mutations do not influence the frequency at which imperfect inverted repeats are 'corrected' to perfect repeats. Quoting from the Lovett paper: "The effects of mismatch repair were inconsistent with a proposal that mismatch correction within a hairpin formed by the inverted repeats gives rise to the mutation. Rather, our results suggested that the mutation arises by mispairing during chromosomal replication, with the mutation susceptible to removal by subsequent methyl-directed mismatch repair." Granted, this paper is dealing with a special situation not exactly analogous to the present argument. However, I think it would certainly make for a more rounded manuscript if the authors were to discuss more fully the views in the field concerning the extrusion of palindromes in vivo.

For us, the fact that the attC sites are not corrected to perfect repeats is not evidence against the

extrusion of cruciforms in vivo. Indeed, the Lovett paper shows that the MMR system preferentially targets the replicating (hemi-methylated) dsDNA after template switching over the ss folded DNA. Consequently, we do not expect mismatch repair to render imperfect cruciforms perfect. Moreover, it has been shown that palindrome sites could be stabilized by host proteins (Singleton and Wells, 1982, Noirot et al, 1990, see also discussion). These proteins could prevent the mismatch repair proteins from binding the cruciform structures.
In the revised manuscript we discuss more extensively the different views about the ability of cruciforms to extrude from dsDNA in vivo. Please, see the modifications presented at the beginning of our answer to referee 3 (see above) and also the two following paragraph that we added in the discussion.

1) p18, line 427 to 433
In particular, numerous in vivo methods have indicated that the superhelical density must be between -0.025 and -0.05 (Zheng et al, 1991). These values may be too low for cruciform formation. However, many factors (transcription, growth conditions, stress, topoisomerase IÖ) may transiently increase the local superhelical density to a critical level sufficient for cruciform extrusion (see review (Pearson et al., 1996)). Indeed, evidence exists for the formation of cruciforms in vivo and implicates these non B-DNA structures in diverse cell functions (see review (Pearson et al., 1996)).
2) p18, line 437 to 453
This assay consists of the transformation of supercoiled pSW::attCs plasmids into a recipient strain where they cannot replicate. The attC sites are carried by ds DNA and would mostly recombine after cruciform extrusion. In these conditions, we obtained significant recombination frequencies for all the tested attC sites. As expected, we observed a correlation between recombination frequency of the attC site as a cruciform structure and the length of the VTS. The propensity to form non-recombinogenic structures also seems to influence the formation of the proper cruciform. Nevertheless it is quite surprising that sites with large VTS recombine at all. Those sites have a very unfavorable energy of cruciform formation even in highly supercoiled DNA, and spontaneous transitions to a cruciform state would be expected to occur with a much smaller probability than the observed recombination frequencies. This suggests that host proteins can favour cruciform formation in a process that is yet to be identified. It is important to note that in the conjugation assay, these two parameters (VTS size and the presence of non-recombinogenic structures) do not seem to influence integron recombination. This is probably due to the fact that the attC sites are delivered as ss DNA and that in these conditions they have ample time to fold and be captured by the integrase even though they might have large VTS and non-recombinogenic structures.

SPECIFIC POINTS

Throughout the results and figures frequencies are quoted without any units. A frequency is always a something per something. For example, oscillations per second or events per cell per generation.

This is an interesting remark. The calculation of the frequency of recombination events is a little bit different for each assay, and this is why units are not given in figures and tables. They are however precisely given for each assay in the material and methods section. Indeed, in the suicide conjugation assay, the recombination frequency represents the number of cells containing one recombined plasmid per total number of recipient cells. In the recombination assay with a replicative double-stranded substrate, the products of the recombination reaction are extracted and used to transform competent cells. Therefore, the absolute recombination frequency is influenced by the copy number and transformation efficiency of both the recombined and receptor plasmids. The frequency of recombination represents the number of transformed recombined plasmids per number of transformed receptor plasmids. In such conditions, recombination frequencies can only be taken as relative measures within a specific assay and cannot be used to compare the different assays.

For the two following assays, one can admit that the frequency of recombination could be underestimated since each cell probably contains more than one copy of donor and receptor plasmids.
For the recombination assay using unidirectional replicative substrate, the recombination frequency represents the number of cells containing at least one event of recombination per total cell number.
For the recombination assay using a non-replicative substrate, the recombination frequency corresponds to the number of cells containing at least one event of recombination per total number of transformed cells, estimated independently.

For more details on the calculation of recombination frequency for each assay, see material and methods and supplemental material and methods.

In Fig 3A I need my glasses and a magnifying glass to read the frequencies. The font size should be increased in all of the figures as they cause considerable aggravation to the reader.

Done

The term cointegrate is used several times. Do the authors really mean "cointegrate" in the sense of a fusion of the donor and target replicon with a copy of the mobile element at each of the TWO junction points?

No

Or do they mean a simple replicon fusion:

Yes, a fusion of the plasmid containing the attC site with the plasmid containing the attI site.

In many places the authors quote the density of negative supercoiling in vivo as close to -0.06. In fact, this is the density in a purified plasmid. It has been established that proteins constrain a superhelical density of about 0.03. The respective densities in vivo and vitro are therefore 0.03 and 0.06. The authors should state this and cite the reference where this was first shown. These inaccuracies of terminology introduce a potential confusion for the non-aficionados.

We apologize for this mistake. We corrected the sentence in the results ("The influence of superhelicity on integron recombination") p14, line 341 to 345:

Therefore, we repeated the experiment in the SD7 strain, a topA10 gyrB266 derivative of JTT1, which due to its mutations in topoisomerase I and DNA gyrase, has a lower intracellular level of negative supercoil density (Napierala et al, 2005). Calculation of the average superhelical density (-av) of the pUC19 reference plasmid isolated from JTT1 and SD7 strains were 0.057 and 0.049, respectively (Napierala et al., 2005).

And also the sentence in the discussion ("Folded attC sites as sensors of environmental stress") p21, line 508 to 510:

Indeed, the average supercoil density of a pBR322 reporter plasmid extracted from mid-log cultures of WT Salmonella is 13% lower (=-0.060) than that from E. coli (=-0.069) (Champion and Higgins, 2007).

We also added the following sentence in the discussion ("Double strand extrusion") p18, lines 427 and 428:

In particular, in vivo, numerous methods have indicated that the superhelical density must be between -0.025 and -0.05 (Zheng et al, 1991). Is pTSC replication completely unidirectional?

Yes, since it's a derivative of the pSC101 plasmid. We added a reference: Philips, 1999 (p7, line 162)

The paper is generally very well written, but there are small sections where it seems to lack polish, for example, page 5 was particularly bad.

We tried to improve the text of page 5. Moreover, the entire manuscript has been read and corrected by a native English speaker.

Table S3 is mentioned before TS1 and TS2.

Table S1 and S2 are mentioned page 5, line 108 and table S3, page 5, line 120.

Also it was not clear to me if TS3 contained the raw data for Fig. 3A?

Yes, TS3 contains the raw data of both fig 3A and fig 4A. We added these details in Table S3.

2nd Editorial Decision 07 June 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-73250R. Please let me first apologise for the slight delay in getting back to you with a decision; this was due to the need to seek an additional opinion on your manuscript before making a final decision. However, I am now in a position to make a decision on your study, and am pleased to be able to tell you that we will be able to accept it for publication in the EMBO Journal, pending minor revision.

I sent your study back to referee 1, whose comments are enclosed below. As you will see, he/she still questions the conclusiveness of the evidence for cruciform extrusion and is therefore not fully supportive of publication. Therefore, I wanted to get input from an editorial advisor as to the validity of these concerns. The assessment of this expert advisor was a positive one, and he/she finds that you do provide good evidence for cruciform extrusion. Given this, as well as the positive reports from referees 2 and 3 in the initial review, we are in a position to be able to offer to publish your manuscript. First, however, I would like to give you the opportunity to respond to the comments of referee 1, and it may be valuable to make some adjustments to the text to recognise his/her concerns. In addition, the referee recommends a final editing of the text: while I find his/her comments as to the quality of the writing to be an exaggeration, there are a number of places where this could perhaps be improved (e.g. p12 line 278-279 should read "determined beforehand" not "beforehand determined"; p14 second paragraph is written in present not past tense).

I would therefore like to invite you to send me a final version of the manuscript addressing these points; the easiest way to do this would be by e-mail, and we can then replace the previous version of the text in our system. Once we have this, we should then be able to accept the manuscript for publication without further delay.

Many thanks,
Editor

The EMBO Journal

Referee 1 comments:

My feeling remains that the authors do not provide convincing evidence for cruciform extrusion under conditions where attC substrate plasmids don't replicate but recombination still occurs. Their evidence is largely circumstantial, which is ok as long as they present it in this way. I agree that cruciform extrusion is the most logical explanation for seeing recombination but there is no strong experiment even in the revised version to prove this beyond a reasonable doubt. My biggest concerns are: (1) To get around the problem that many attC sequences do support recombination but look unlikely to form cruciforms the authors invoke the idea that Int may help promote cruciform extrusion. This is a reasonable idea but they don't do an experiment to test this. (2) The authors discuss the possibility that small changes in superhelix density (perhaps caused by stress conditions) could induce recombination but since they have not performed any experiment to establish a threshold level of supercoiling required to drive cruciform formation for any attC site it is impossible to know if their discussion is meaningful.

Finally, the writing is still not up to the quality expected for a journal like EMBO. This is particularly true for some of the new passages.

2nd Revision - authors' response 11 June 2010

Here is our modified ms and our answers to the ref.1 comments

First of all, we would like to thank you for your positive decision about our revised manuscript. We were very happy to see that your expert advisor found that we did provide good evidence for cruciform extrusion.

We corrected the two points, p12 line 278-279 and p14 second paragraph, according to your suggestions. We also asked an English native speaker to read it again and further improve the English. He made a few minor corrections.

Concerning the comments of referee 1, here are our answers to the 2 points raised:

(*1) Int may help promote cruciform extrusion. This is a reasonable idea but they don't do an experiment to test this.*
We effectively propose in the ms that Int may help to stabilize cruciform extrusion. In fact, we already performed experiments in collaboration with V. Parissi (CNRS, UMR 5234 Bordeaux, France) showing an in vitro stabilization effect of the attC site folding by the integrase. The attC folding is followed through absorbance difference between ss and ds DNA and we observe a clear Int stabilization effect on all tested attC site derivatives.

These results will be described in a second ms, that we are currently writing, but we added a sentence about these unpublished observations in the discussion (p20).

"...We propose a stabilizing effect of the integrase, which could capture attC sites upon their extrusion and recombine them efficiently. We obtained preliminary results that support this hypothesis (Loot, Parissi, Bikard and Mazel, in preparation) and we are currently exploring the parameters of this stabilization process. This type of stabilizing effect on cruciform formation has been previously observed. For instance, in a study of the effect of S1 nuclease on cruciform extrusion,...

(2) The authors have not performed any experiment to establish a threshold level of supercoiling required to drive cruciform formation for any attC site.

We first attempted to perform 2D gel electrophoresis, but we failed to detect any cruciform formation. We then realized that the very low frequency of recombination obtained for the integron recombination in non replicative conditilons (~10-3) suggests that the cruciform is not the predominant form in vivo, and probably in vitro too. This is also in agreement with the scientific literature. Indeed cruciform extrusion from imperfect palindromes have never been observed at significant rate in vitro, unless they are very AT rich (Benham et al, 2002).

Even if we failed to establish a threshold level of supercoiling required to drive cruciform formation, we clearly showed an influence of supercoiling on the extrusion of attC sites in vitro and on their recombination frequency in vivo. For instance, we observed a significant decrease of the attC recombination aptitude in the top gyr deficient strains. Furthermore, we do not expect the cruciform topology to be the most stable form even at very low superhelix densities apart maybe for those sites that have no VTS (attCaadA7, VCR-GAA). Thus, there is no threshold of supercoiling to measure, we only expect the transition toward cruciform to be more frequent at lower superhelix densities, even if it is never the most stable form. The S1 nuclease assay shows that even for attCaadA7 the cruciform is not predominant. Furthermore, this S1 assay probably exaggerates cruciform extrusion, since the S1 cleavage is irreversible and could thus displace the equilibrium between the fully paired and the cruciform DNA.

