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Active site electrostatics protect genome integrity by blocking abortive hydrolysis during DNA recombination

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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 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three reviewers, whose comments are attached below. As you will see, all of these referees find your results interesting and the study generally well executed. Nevertheless, they also raise some specific points that would need to be addressed before the publication in The EMBO Journal may be warranted. I would thus like to invite you to prepare a revised manuscript, taking into account the various comments and suggestions of the reviewers. Pending adequate revision, we should be happy to consider a revised manuscript for publication. Please be reminded, however, that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you answer all the points raised at this stage. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Dr. Jayaram presents a technically and conceptually elegant story of how Flp recombinase guards against hydrolysis of its DNA target site - and how there are two flavors of hydrolysis reactions. The results reveal, by comparison to previous studies of vaccinia TopIB, that different transesterifying enzymes can exploit different strategies to achieve the goal of protecting against frivolous breakage. The findings are of general interest and wide significance, given the prevalence of such transesterification steps in many aspects of DNA and RNA metabolism. The manuscript is very well written also.

Comments and requested revisions:

1) The authors should specify the solution conditions for the cleavage-hydrolysis reactions in Methods on p. 24, rather than referring to a previous publication. Chemically alert readers will want to know the pH and other parameters. Also, please state how the reactions were quenched.

2) p. 11, line 11: typo "methylphosphate"

3) The starting labeled substrates for Sp and Rp MeP experiments (Fig. S4A) and mixed MeP experiments (Fig. 5B) are somewhat "dirty" and contain a shorter contaminant. This is quite pronounced in Fig. S4, where the authors acknowledge that it is likely to be shorter by a nucleotide. The argument that "given its low amount, its contribution to the reaction would not be significant" depends on how low the amount really is. It looks to be maybe 10% of the input to me. I'd like to see it quantified. The important point is that one might expect to see a correspondingly shorter hydrolysis product. It looks to me that such product might be cut off the bottom in Fig, S4A and 5B. Is the product there? If so, please show it and comment, or else state/show that it is not there.

4) p. 14, last line: typo "topoisomerase"

Referee #2 (Remarks to the Author):

The manuscript presents a study of aberrant hydrolysis reactions promoted by Flp recombinase, and discusses how the Flp active site excludes water molecules which could act as inappropriate nucleophiles.

The results and conclusions presented here are novel and will be of significant interest to others in the field. The manuscript should encourage researchers working on the mechanisms of related enzymes to think of their own systems in a new light, and the clever use of methylphosphonate substitutions coupled with active site mutants (pioneered by the Shuman group's work on topo I, and developed here) is proving to be a very useful approach. The challenging experimental work has clearly been competently carried out, and, apart from a fair number of trivial typos and the occasional over-flowery use of language (which can get addressed in editing), the manuscript is a model of clarity; I wish they were all as easy to read as this! My comments below are mostly of a minor nature.

Comments:

1. Page 6, line 13. I don't think 'ostracize' is a verb applicable to water.

2. Page 9, last sentence of first paragraph ("These findings..."). The fact that hydrolysis is much more apparent in half-site than in full-site reactions is interesting, and it might be useful to have a little more discussion of this. For example, could it be due to persistent lack of the tyrosine residue that should be donated by a partner subunit, leaving a 'gap' in the active site constellation? Is the crystallographic evidence informative; i.e. could the structural consequences of lack of a partner subunit or nucleophile be predicted?

3. Page 11, line 12; sentence beginning "The transfer of the scissile phosphate...is being tested." This is quite cryptic; maybe should be more explicit about how this will be done.

4. Somewhere in the Discussion (e.g. 3rd paragraph on page 14), it might be helpful to explicitly compare the rates of the hydrolysis reaction (on the phosphotyrosyl intermediate) to that of the 'normal' reaction, i.e. attack of 5'-hydroxyl.

5. Page 16, line 10. Is the mutant evidence enough to say that the effect of Arg-308 is not simply steric? Arginine is the biggest residue.

6. Figure 2D. It would be useful to label the figure to show what is meant by 'Type I' and 'Type II' hydrolysis.

7. Figure 4B and C. I think the figure would benefit from more explanation in the legend; it wasn't clear to me what was in some of the lanes. In Lane 1 of Figure 4B (labelled 'MG'), are there cleavage products? All I can see clearly is the band labelled S(26-mer). Also, will Maxam-Gilbert cleavage at C not remove the C residue, making the product a 3'-phosphorylated 22-mer? I'm confused by this (also Figure S4); the predicted product should be stated.

8. It should be made clear at the beginning of the Results section that Flpe, not wild-type Flp, is being used in the experiments.

Referee #3 (Remarks to the Author):

This manuscript from the Jayaram laboratory presents new insight into how the tyrosine recombinase-topoisomerase family of proteins regulates catalysis. Specifically, processes that normally limit hydrolysis of the phospho-tyrosyl intermediate are uncovered, as is a process by which the OH of water is used to cleave the scissile DNA phosphoester rather than the normal recombinase tyrosine. This work follows on from, and contrasts with, comparable experiments from the Shuman lab on Vaccinia topoisomerase IB [Tian et al, 2003, 2005]. The conception and execution of the work is impeccable, as is the presentation of the manuscript [with a few small exceptions; below]. The work will be of particular interest to those that work on site-specific recombination and topisomerases.

Specific comments
1. page 11 para 2 line 5, phosphate not phopshate

2. page 11 last paragraph- argue that leaving 3'OH amenable to ligase re-sealing nick but 1) there is a 5'OH (OK they mention this) and 2 the FLP is now permanently innactive as tyrosine-phosphate. Could this be examined by using a radioactive phosphate at the scissile position?

3. page12 5 lines from bottom FLP R308A 3 is missing

4. R308A allows type II nuclease activity, but is it electrostatics or steric hinderance that excludes water--what if a large uncharged residue is including at position 308, rather than an alanine substitution?

5. When an intact 5'OH is used on the half-site, hyrolysis on the normal phosphate late on becomes significant. But is this hydrolysis of the covalent intermediate or of the hairpin--if you use a hairpin substrate and the Y343F mutant what happens? Supplementary Figure 2B suggests it is the hairpin that is being hydrolysed--i.e. similar to the topoisomerase reaction and it may be dimerisation that protects from hydrolysis. The level of unreacted substrate is very small already by 2 hours and most is in the rejoined 54mer form, yet the hydrolysis product increases without much obvious change in the very low level of unreacted substrate. If this is the case it allows dissection of the relative roles of exclusion of water within one active site and the contribution of dimerisation to the exclusion of water. Perhaps dimerisation hides the direct nuclease activity?

6. R308 repulsion of water??? exclusion would be a better word--surely water isn't repulsed by R308?

1st Revision - point-by-point response 17 April 2009

We are submitting the revised version of the above manuscript, taking into account the general and specific comments of the three referees. We explain below how each point raised by an individual referee has been addressed during revision.

Referee 1

1. The reaction conditions are now explicitly described under methods (P. 24; line 9-11). The pH of the reaction mixtures, which is the most critical parameter in regards to hydrolysis of the phosphodiester bonds, has been stated in the first section under 'Resultsí: Experimental layout for assaying Flp activity on MeP substrates (P. 7; line 15 and 16). This was the reason why other details were not spelled out in the original manuscript but abridged to a reference. The reactions were quenched by adding SDS to a final concentration of 0.1% before DNA extraction. The quenching procedure is also described under methods (P. 24; line 13).

2. P.11; line 11. 'Methylphosphate'; the typo has been corrected (now P. 12; line 23). 'Phosphate' spelling has now been corrected in other places as well.

3. Figure S4A, Figure 5. The SP substrate has been purified to get rid of the contaminant, and the data from the clean substrate are included as the new Figure S4A, replacing the old one. The strong preference for the SP form over the RP form was not obfuscated by the small amount of the shorter oligonucleotide present in the unpurified substrate.
The mixed MeP substrate in Figure 5B was also purified to remove the one nucleotide shorter chain. The new Figure 5B displays the results obtained using the contaminant-free substrate.
[In the old Figure 5B (now replaced) the level of the contaminant substrate (one nucleotide shorter at the 3'end) was roughly 7.3%. The faint hydrolysis product band from the shorter substrate was visible only upon longer phosphorimaging, and was approximately 4.1% of the normal product.]

4. P 14; last line. The typo in 'topoisomerase'here (now P. 17; line 9) and elsewhere has been corrected.

Referee 2

1. Page 6; line 13. The word 'ostracize'has been replaced (with some regret) by 'expel'(P. 6; line 13).

2. Page 9, last sentence of first paragraph. We have added the following sentence to suggest that the weaker Flp dimer interface in half-site complexes may cause higher susceptibility to hydrolysis: 'The dimer interface formed by adjacent Flp subunits in half-site complexes may be more pliant than in full-site complexes, and thus more yielding to water entry (see also Discussion) (P. 10; lines 15-17). The role of the interface in keeping water out is considered in more depth under 'Discussion'(P. 17; lines 13-22 and P. 18; lines 1-3).
Since the target of the Type I Flp endonuclease is the 3í-phosphotyrosyl bond and the covalently bonded tyrosine is donated by the Flp neighbor in trans, it is not quite true that there 'is a gap in the active site constellationí. The notion of the gap is more appropriate for the Type II Flp endonuclease activity that targets the DNA phosphodiester bond, and is independent of the tyrosine nucleophile. The structure of a Flp monomer bound by a half-site or even a Flp dimer complexed with two associated half-sites would be very useful. Their comparison to the tetramerñDNA structures might reveal the 'Achilles heel'in the half-site reactions. Perhaps it is the stability of the inter-subunit interactions in the tetramer that promotes crystallization.

3. Page 11, line 12. The sentence has been modified as follows, to be more explicit about the methods being followed: The transfer of the scissile phosphate from DNA to Tyr-343 of Flp, predicted by the latter reaction, is being probed by labeling the DNA substrate internally with 32P at this position as well as scouting for the signal for the phosphotyrosine containing tryptic peptide from Flp in mass spectrometry (P. 13; lines 2-5). The success of identifying the phosphotyrosine would depend on its being stable, and not being susceptible to further hydrolysis by Flp (as indicated in the following sentence; P. 13; lines 5-6).

4. Discussion (3rd paragraph; page 14). We have now given the relative rates for the joining reaction versus hydrolysis when water has to compete with the 5í-hydroxyl for the phosphotyrosyl target (P. 17; lines 2 and 3).

5. Page 16; line 10. Reactions with a spectrum of mutants at the 308 position support the electrostatic explanation. The example of Arg-308 to glutamine substitution mentioned in the paper (as unpublished data) is only one of several such substitutions tested. For example, lysine at 308 is protective, but is less effective than arginine, and others are not. These results, which will form part of a separate study, suggest that the choice of Arg-308 was an evolutionary optimization between its catalytic power and its anti-hydrolytic efficacy. We have now indicated that other substitutions, in addition to R308A and R308Q, also fail to block hydrolysis, but without going into details (P. 19; lines 2-4).

6. The type I and type II hydrolysis reactions are so indicated in Figure 2, and described in the legend (P. 27; lines 16-18).

7. Figure 4B and C (also Figure S4). Figure 4 legend has been expanded, and panels B and C are now explained separately for clarity (P. 28; lines 12-22 and P. 29; lines 1-3). Higher intensity lanes of the Maxam-Gilbert sequence ladders are now added to panels B and C of Figure 4, and individual bands are labeled. The chemical modification and cleavages were C + T reactions, and the relevant band was a 23-mer with a C at the 3'end. For the standard phosphate oligo, this product contained a 3'-phosphate. For the MeP containing oligo, the chemical reactions produced enhanced strand scission at the substituted position to yield a 23-mer ending in C with a 3'-Me phopshate or a 3'-hydroxyl in roughly equal amounts. A higher intensity lane for the sequence ladder is also included in Figure S4.

8. Flpe: The last paragraph in the first section of 'Results'makes it clear that Flpe and its mutant derivatives were used in this study. Flp and Flpe behaved nearly identically under the reaction conditions employed (P. 8; lines 12-15).

Referee 3

1. Page 11, para 2, line 5. 'phosphate'spelling has been corrected here and elsewhere.

2. Page 11, last paragraph. The significance of the 3í-OH end in repair has been explained (P. 13; lines 10-19). Yeast apparently lacks an obvious 5í-kinase, and a 5í-phosphate, competent for ligation, is thought to be generated by repair synthesis.
Yes, the suggestion that the transfer of the scissile phosphate, radioactively labeled, to Tyr-343 of Flp may be assayed is a valid one. We are attempting to follow this method as well as seek the predicted phosphotyrosine containing peptide by mass spectrometry. The caveat, though, is that this phosphate could be potentially removed by a Flp assisted hydrolytic reaction. These points are now stated in the revised manuscript (P. 13; lines 2-5).

3. Page 12, 5 lines from bottom. The missing 'A'in R308A has been added.

4. The effect of arginine is not simply steric from the mutational evidence that we have. As noted in the manuscript, glutamine at this position does not protect against hydrolysis. From unpublished data, we know that lysine is protective, but less effective than arginine, while others tested so far are not. We have now indicated that substitutions in addition to R308A and R308Q also fail to block hydrolysis, but without going into details (P. 19; lines 2-4).
An exhaustive mutational analysis of the charged residues of the catalytic pentad in MeP reaction is being carried out as a separate study.

5. The suggested hairpin experiments are interesting. From early work, we know that the hairpin is not cleaved (or cleaved quite inefficiently) by Tyr-343. The primary block to the reaction appears to be the loop formed by the spacer strand, which will impede dimerization of [Flp-half-site] (and the trans-donation of the Tyr-343 nucleophile). Even if the scissile phosphate in the hairpin is activated by bound Flp, direct hydrolysis is not expected with Flp or Flp(Y343F) because of the presence of Arg-308 as the protector. Removal of Arg-308 will not help, because phosphate activation will be blocked. A feasible experiment would be to use MeP containing hairpin, perfectly matched or with mismatches in the loop, and react it with Flp, Flp(R308A) and Flp(R308A, Y343F or G). These will not be terribly different from the half-site assays shown in the manuscript. The prediction would be that in the hairpin substrate, we will see the type II endonuclease activity with Flp(R308A) and Flp(R308A, Y343For G) but not with Flp. A valid question is whether the type II endonuclease is occurring from a monomeric Flp-half-site complex or a dimeric Flp-half-site complex. From previous work, it is known that the Flp(Y343F) mutation affects dimerization of the half-site-monomer complex, and Flp(Y343F) assisted hydrogen peroxide cleavage or type II RNase cleavage occurs from the monomeric complex. One would guess that this is also true for the type II endonuclease activity of Flp(R308A) or Flp(R308A, Y343F or G). We will think more carefully about how the MeP hairpin might be exploited to glean new mechanistic information.
Supplementary Figure, S2B. The joining reaction is more rapid relative to hydrolysis, their first order rate constants being 3.1 x 10-3 s-1 and 9.7 x 10-5 s-1, respectively. These values are now mentioned under Discussion (P. 17; lines 2 and 3). The main point of this figure is that an active 5'hydroxyl strongly competes out water.
The 'very low level of unreacted substrate'does decrease during the 2hr to 8hr period, and is almost completely gone by 8 hr. Yes, the joined product also appears to contribute to hydrolysis. As noted above, the hairpin is not a substrate (or at least a very poor one) for cleavage by Tyr-343. The joined product contains (in addition to the majority hairpin) a finite fraction of intermolecular joined products, which would be equivalent to pseudo-full-sites. The sequence of the hairpin and that of the pseudo-full-site strands would be identical, therefore indistinguishable by mobility in a denaturing gel. The contribution to hydrolysis from the joined product is almost all due to cleavage of the pseudo-full-sites and subsequent breakdown of the tyrosyl intermediate. These points are explained under Figure S2 in 'Supplemental data'(P.3; lines 11-17).

6. 'Repulsion'has been replaced by 'exclusion'. 'Repulsion' appears to be a standard expression in physics and electrochemistry to describe the force that tends to keep bodies of like charges apart. The title of the Shuman paper in Mol Cell (Tian et al.) is: 'Guarding the genome: electrostatic repulsion of water by DNA----'.

