Functional characterization of an active Rag-like transposase

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The formation of diverse immunoglobulin genes results in part from Rag protein–mediated DNA double-strand breaks at the edges of immunoglobulin gene segments, followed by combinatorial reassembly of these segments. We report that a Transib transposase from the insect Helicoverpa zea is active in vitro and that its breakage and joining activities mimic those of Rag, providing strong evidence that Rag and Transib transposases were derived from a common progenitor.

The assembly of an immunoglobulin gene mimics transposon excision, followed by joining of the flanking donor sites to form the coding gene segments1,2 (Supplementary Fig. 1). The excised intervening segment from an immunoglobulin gene can also mimic transposon insertion in vitro by inserting into a target DNA3,4. Sequence similarities between the Rag1 subunit of the Rag protein, which mediates immunoglobulin breakage and joining, and the transposases of the fossil Transib transposon superfamily suggested that the core region of Rag1 was derived from a Transib transposon5. We have characterized an active Transib transposase and have found that the transposase from the Transib transposon called Hztransib, of Helicoverpa zea6, is active in vitro. Notably, the mechanism of its breakage and joining activities are like those of Rag, providing strong evidence that Rag1 and Transib transposases were derived from a common progenitor.

Hztransib was found in the insect pest Helicoverpa zea6 (cotton bollworm or corn earworm) by genome sequencing of the region around a cytochrome P450 gene of interest. The fact that there is variation in its genomic position and copy number in different populations suggested that it might be a still-active element7. To analyze its activity directly, we isolated the Hztransib transposase by adding a His6 tag sequence to the open reading frame, expressing it in Escherichia coli and affinity purifying the protein.

To evaluate Hztransib’s ability to excise the transposon end from flanking DNA and then join it to a target, we used a DNA fragment containing a 51-base-pair (bp) Hztransib end sequence and 37 bp of flanking DNA, in which the interior 5′ end of the transposon was labeled, as a substrate in reaction mixtures using a plasmid as target DNA, and then we displayed the products on a native agarose gel (Fig. 1). Two products are visible: one generated when a single transposon-end oligonucleotide joins to the plasmid target DNA, giving a nicked-circle product (single-end join, SEJ), and the other created when two end oligonucleotides join at the same target position, resulting in linearization of the plasmid (double-end join, DEJ) (Fig. 1b, lane 2).

Mutational analysis of many transposases, integrases and the Rag1 subunit of Rag has identified three conserved acidic amino acids, essential for catalytic activity, that lie on an RNase H–like fold that closely juxtaposes them to bind essential divalent metal, usually Mg2+, ions at the catalytic center8–11. These positions are conserved in Transib transposases5. Hztransib residues Asp126, Asp225 and Glu436 align with the cognate amino acids in the Rag1 subunit (Supplementary Fig. 2), and mutation of these conserved amino acids in Hztransib significantly decreased the excision and target-joining reaction (Fig. 1b, lanes 3–5). Such active site mutations also block Rag-mediated DNA end cleavage and target joining8–11. We suspect that the lower transposase-dependent band results from end binding.

The double-strand breaks at the recombination signal sequences (RSSs) promoted by Rag involve both a hydrolysis reaction and a direct transesterification that occur through mechanisms used by

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Received 20 February; accepted 31 May; published online 8 July 2012; doi:10.1038/nsmb.2338
other transposases and integrases. Rag DNA breakage initiates with a nick that frees the 3' end of the RSS from the flanking coding DNA segment. The newly exposed coding segment 3'-OH then attacks its complementary strand, generating a hairpin on the coding DNA and introducing the double-strand break that frees the RSS-bounded segment.

To analyze the mechanism by which Hetransib makes the double-strand break at the transposon end to separate it from the flanking DNA, we used a DNA-substrate fragment containing a 51-bp Hetransib end sequence and 37 bp of flanking DNA in which the interior 5' end of the transposon and the flanking DNA were labeled, and we displayed the reaction products on a denaturing acrylamide gel (Fig. 2a). The 37-nucleotide (nt) species is the donor top-strand fragment resulting from nicking at the junction of the flanking DNA with the top transposon strand (Fig. 2b, lane 2). This nicking position is identical to that of Rag and distinct from that of hAT transposases that are also excised via hairpin formation, which is initiated by a nick one nucleotide into the flanking donor DNA. Present in a lower amount is the 51-nt, 3'-OH transposon bottom-strand fragment that results from the double-strand break that separates the transposon end from the flanking donor DNA. We also assayed the Hetransib transposases with active siteD126A, D225A and E436A mutations and found that nicking was not detectable (Fig. 2b, lanes 3, 4) or was reduced (lane 5). We suspect that the residual nicking activity with the Hetransib mutant E436A results from the use of Mn$^{2+}$ rather than Mg$^{2+}$ as a cofactor (see below). No recombination was observed when Mg$^{2+}$ was used as a cofactor (data not shown).

With Rag, cleavage of the bottom strand occurs when the 3'-OH on the newly released donor top strand attacks its complementary strand, forming a hairpin on the flanking DNA. Such a hairpin is faintly visible in an incubation carried out using wild-type Hetransib transposase and an intact oligonucleotide as a substrate (Fig. 2b, lane 2). The hairpin was much more prominent, however, when a substrate ‘prenicked’ at the 5’ end of the transposon was used as a substrate (Fig. 2b, lane 7). A series of species is evident, the most slowly migrating of which comigrates with an authentic 74-nt hairpin, indicating that the Hetransib transposase generates a double-strand break via a hairpin. The ladder of species below the authentic hairpin may reflect degradation at the 3’ end of the hairpin or imprecise hairpin formation; the use of the recessed 3'-OH in the 32-nt product may contribute to such imprecise hairpin formation. Such species are not evident with a substrate that is prenicked to expose the 3'-OH end of the transposon (data not shown).

As is also true with Rag containing mutations in Rag1 (refs. 8–11), reduced hairpin formation was observed using the Hetransib transposase active site mutants D126A, D225A and E436A (Fig. 2b, lanes 8–10). We suggest that the residual nicking activity with the Hetransib mutant E436A results from the use of Mn$^{2+}$ as a cofactor. We also found that Hetransib can promote target joining in vitro to form SEJs and DEJs using a ‘precleaved’ transposon-end substrate in which the terminal 3'-OH is already exposed (Fig. 3a). We observed the same amounts of SEJ and DEJ products when using a 50-bp Hetransib end as with a 294-bp Hetransib end (data not shown). As occurs with Rag1 mutants, less target joining was observed using the active site mutant D126A, D225A and E436A Hetransib transposases (Fig. 3b). We suggest that the residual target-joining activity with the Hetransib mutants results from the use of high amounts of Mn$^{2+}$ as a cofactor (Supplementary Fig. 3). When a reaction using a transposon fragment labeled at its interior 5’ end was displayed on a denaturing gel (Fig. 3c), a product
the length of the plasmid plus the transposon end was observed, consistent with joining of the 3′-OH end to the target DNA.

Finally, to further analyze the structure of the products formed by the joining of Hztransib ends to a target DNA, we constructed a mini-Hztransib transposon in which 50-bp Hztransib ends flanked a kanamycin-resistance (kan8) gene fragment, incubated it with a target plasmid and transposase, and recovered insertion products by selection for kan8 following transformation in E. coli. DNA sequence analysis of 44 insertions at different sites revealed that all had perfect 5-bp target-site duplications that were enriched with 5′-CGnCG, as occurs with Rag1,2,4 (Supplementary Fig. 4). Transposition was reduced by a factor of more than 20 when the Hztransib transposases mutated at the conserved RNase H residues D126A, D225A and E436A were used in the transformation transposition assay (Supplementary Fig. 4).

These experiments have revealed that a Transib transposase uses the same mechanism for the same breakage and joining reactions as does the immunoglobulin gene recombinase Rag. The nick that initiates the double-strand break occurs exactly at the junction of the transposon end and the flanking donor DNA, the subsequent double-strand break occurs by hairpin formation between the exposed 3′-OH donor end and its complementary strand, and the released 3′-OH end of the transposon directly attacks the target DNA. Moreover, mutation of a conserved catalytic triad of amino acids in both Rag1 and Hztransib transposase block breakage and joining. This functional analysis provides strong evidence that Rag and Transib transposases are descended from a common progenitor.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We thank the other members of the Craig lab for fruitful discussions, H. McComas for her assistance with the figures and text, and S. Desiderio for his comments on the manuscript. N.L.C. is supported as a Howard Hughes Medical Institute Investigator.

AUTHOR CONTRIBUTIONS

N.L.C. conceived the project; C.G.H., X.L. and N.L.C. designed the experiments; C.G.H. and X.L. carried out the experiments; C.G.H., X.L. and N.L.C. analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nsmb.2338. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Matthews, A.G. & Gettinger, M.A. Nat. Immunol. 10, 817–821 (2009).
2. Schatz, D.G. & Swanson, P.C. Annu. Rev. Genet. 45, 167–202 (2011).
3. Hiom, K., Melek, M. & Gellert, M. Cell 94, 463–470 (1998).
4. Agrawal, A., Eastman, Q.M. & Schatz, D.G. Nature 394, 744–751 (1998).
5. Kapitonov, V.V. & Jurka, J. PLoS Biol. 3, e181 (2005).
6. Chen, S. & Li, X. Gene 408, 51–63 (2008).
7. Du, E., Ni, X., Zhao, H. & Li, X. Insect Mol. Biol. 20, 291–301 (2011).
8. Hickman, A.B., Chandler, M. & Dyda, F. Crit. Rev. Biochem. Mol. Biol. 45, 50–69 (2010).
9. Kim, D.R. et al. Genes Dev. 13, 3070–3080 (1999).
10. Landree, M.A., Wibbenmeyer, J.A. & Roth, D.B. Genes Dev. 13, 3059–3069 (1999).
11. Fugmann, S.D., Villey, I.J., Ptaszek, L.M. & Schatz, D.G. Mol. Cell 5, 97–107 (2000).
12. Zhou, L. et al. Nature 432, 995–1001 (2004).
13. Yang, W., Lee, J.Y. & Nowotny, M. Mol. Cell 22, 5–13 (2006).
ONLINE METHODS

Cell growth and transposase purification. An E. coli codon-optimized version of the *Htxtransib* transposase gene was synthesized by DNA2.0, cloned into the Ncol–EcoRI sites of the pBAD Myc/His (Invitrogen) vector and expressed in Top10 cells. One liter of cells was grown by [10] in LB at 100 µg/mL carbencillin until reaching an OD₆₀₀ of about 0.7. Arabinose was added to 0.01%, and cell growth continued overnight at 16 °C. The cells were chilled, harvested by centrifugation and then resuspended in 10 ml column buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 7.8, 10% glycerol). The cells were lysed in a French press, the lysate was spun at 36,000 r.p.m. for 70 min, and the resulting supernatant was filtered through a 0.45-µm syringe filter and then diluted 1:1 with column buffer. The lysate was then loaded onto a 1 ml nickel Sepharose column, washed with wash buffer (40 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 7.8, 10% glycerol), and eluted in storage buffer (500 mM NaCl, 1 mM EDTA, 2 mM DTT, 20 mM Tris, pH 7.8, 25% glycerol). The final protein concentration was 1 mg/ml, and *Htxtransib* transposase was about 5–10% of the total protein.

*Htxtransib* transposase mutants D126A, D225A and E436A were generated using oligonucleotides CGH10, 5'-GGTACAGATCTTACCGGCGATCATGGTCATC; Hz Emut-For, 5'-GATGACCATGATCGCCGGTAAGATCTGTACC and Hz D2mut-Rev, 5'-32P[ATP] and T4 polynucleotide kinase. A 294-bp fragment used in transposition reactions. The reaction mixtures were phenol extracted, ethanol precipitated and resuspended in 10 µL H₂O. Five microliters were added to 10 µL of the addition of stop buffer (10 mM EDTA, 10% SDS, 0.15% bromophenol blue, 0.25% xylene cyanol). Denatured samples were displayed on denaturing acrylamide gels containing 8% ExplorER acrylamide and 7 M urea in 1× TBE. The 294-bp *Htxtransib* left-end fragment used in Figure 3b was generated by PCR from the *Htxtransib* left end in pTJ201 by PCR amplification with the 5′-end-labeled primers (*Htxtransib*, bold) 5′-CACGGTGAGATGCTCATGCTGCTC; and 5′-CCCAAGATGATGCTGCTC; and 5′-CGCGCGCTCGCCTCTTC. The precleaved 50-bp *Htxtransib* end fragment used in Figure 3c was assembled from oligonucleotides 5′-CACGG TGAGATGCTCATGCTGCTC; and 5′-CGCGCGCTCGCCTCTTC.

Native samples were displayed on 1% agarose gels in 1× TAE. For alkaline agarose gels, in vitro transposition reactions were ethanol precipitated and resuspended in 50 ml NaOH and 1 ml EDTA following the addition of 6× loading dye (300 mM NaOH, 6 ml EDTA, 18% Ficoll, 0.15% bromophenol blue, 0.25% xylene cyanol). Denatured samples were displayed on a 1% agarose gel in 50 ml NaOH and 1 ml EDTA. For denaturing acrylamide gels, samples were phenol extracted, ethanol precipitated and resuspended in 12 µl 95% formamide, 20 ml EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol, boiled and then placed on ice. Samples were displayed on denaturing acrylamide gels containing 8% ExplorER acrylamide and 7 M urea in 1× TBE.

Transformation assay for *Htxtransib* integration. Three hundred nanograms of *Htxtransib* L50-kan⁵-R50 fragment and 500 ng pUC19 were used as substrates in in vitro transposition reactions. The reaction mixtures were phenol extracted, ethanol precipitated and resuspended in 10 µl H₂O. Five microliters were transformed into 25 µl One Shot Mach I chemically competent cells (Invitrogen), LB was added to 500 µl and transposition products were recovered by selection for kanamycin resistance.