Ty3 reverse transcriptase complexed with an RNA-DNA hybrid shows structural and functional asymmetry

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Retrotransposons are a class of mobile genetic elements that replicate by converting their single-stranded RNA intermediate to double-stranded DNA through the combined DNA polymerase and ribonuclease H (RNase H) activities of the element-encoded reverse transcriptase (RT). Although a wealth of structural information is available for lentiviral and gammaretroviral RTs, equivalent studies on counterpart enzymes of long terminal repeat (LTR)–containing retrotransposons, from which they are evolutionarily derived, is lacking. In this study, we report the first crystal structure of a complex of RT from the Saccharomyces cerevisiae LTR retrotransposon Ty3 in the presence of its polypurine tract–containing RNA-DNA hybrid. In contrast to its retroviral counterparts, Ty3 RT adopts an asymmetric homodimeric architecture whose assembly is substrate dependent. Moreover, our structure and biochemical data suggest that the RNase H and DNA polymerase activities are contributed by individual subunits of the homodimer.

Retrotransposons are mobile genetic elements that replicate through an RNA intermediate. They are divided into two groups, depending on whether flanking LTR sequences are present. Retrotransposons represent one of the main forces shaping the architecture of eukaryotic genomes1. For example, ~40% of the human genome is derived from retroelements, with 8% corresponding to the LTR class2, while in maize ~75% of the genome is derived from retroelements, mainly of the LTR class3. Retroviruses, such as human immunodeficiency virus (HIV), evolved from LTR elements through acquisition of an envelope gene, which allowed their egress from infected cells to initiate a subsequent round of infection4.

The Ty3 element of S. cerevisiae belongs to the Gypsy family5,6, and its RT is perhaps the most extensively characterized LTR retrotransposon enzyme with respect to its enzymatic activities7-9 and the architecture of nucleic acid duplexes with which it interacts10-15. Although the structural motifs mediating substrate recognition and catalysis generally resemble those of vertebrate retroviral RTs, a notable difference between the Ty3 and retroviral enzymes is the separation of Ty3’s DNA polymerase and RNase H active sites by ~13 bp (ref. 9), as opposed to 17 or 18 bp for lentiviral and gammaretroviral enzymes. Although the structural basis for such spatial separation is established for HIV-1 RT16,17, the origin of the shorter distance for Ty3 RT is difficult to explain from the retroviral structures. Ty3 RT lacks the connection, or tether, between its DNA polymerase and RNase H domains. Structural similarity between the RNase H subdomain of HIV-1 RT and its connection subdomain (which lacks the catalytic carboxylates found in the RNase H domain) originally suggested the former arose through domain duplication, while an alternative theory proposes that the functional RNase H domain was acquired from a source outside the LTR retrotransposons18.

Another well-characterized LTR element from S. cerevisiae is Ty1 of the Copia-like group, which is more distantly related to retroviruses than Ty3. The polypurine tract (PPT) primers for plus-strand synthesis for both the Copia and Gypsy families differ in length and composition from retroviral PPTs. LTR retroelement PPTs generally contain shorter, less homogeneous tracts of purines, implying differences in PPT recognition. LTR retrotransposon PPTs are accurately processed by their cognate RT in vivo19,20 and in vitro9,21, and it has also been proposed that a Ty3 RT–integrase fusion protein participates in reverse transcription in vivo19,22.

Despite extensive biochemical characterization of LTR retrotransposon RTs, detailed structural information is lacking. We therefore set out to structurally characterize Ty3 RT, and we report here the first structure of a retrotransposon RT in complex with its cognate PPT RNA-DNA hybrid at 3.1-Å resolution. The active enzyme is an asymmetric homodimer of 55-kDa subunits that associate in the presence of the nucleic acid substrate. Modeling of the spatial separation between the DNA polymerase and RNase H active sites, in addition to phenotypic mixing experiments, suggest that DNA polymerase and RNase H catalytic activities reside in separate subunits.

RESULTS

Overall structure

Details of RT purification, crystallization and structure solution can be found in the Online Methods. Selenomethionine-substituted protein was purified by immobilized metal-affinity, ion-exchange and

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Table 1 Data collection and refinement statistics for Ty3 RT–RNA-DNA complex crystals

|                         | Ty3 RT SeMet |
|-------------------------|-------------|
| **Data collection**     |             |
| Space group             | P2₁2₁2₂     |
| Cell dimensions         |             |
| a, b, c (Å)             | 320.7, 75.1, 108.3 |
| α, β, γ (°)             | 90, 90, 90 |
| Resolution (Å)          | 50.0–3.1 (3.29–3.1) |
| Rmerge                  | 0.10 (0.85) |
| I / σI                 | 11.9 (2.1)  |
| CC1/2                   | 99.8 (72.9) |
|Completeness (%)         | 99.6 (98.4) |
| Redundancy              | 5.1 (5.1)   |
| **Refinement**          |             |
| Resolution (Å)          | 3.1         |
| No. reflections         | 90,832      |
| Rmerge / Rfree          | 22.7 / 29.6 |
| No. atoms               | 14,105      |
| Protein                 | 12,734      |
| Ligand / ion            | 1360 / 10   |
| Water                   | 1           |
| B factors               | 131.7       |
| Protein                 | 129.3       |
| Ligand / ion            | 153.5 / 158.0 |
| Water                   | 79.7        |
| r.m.s. deviations       |             |
| Bond lengths (Å)        | 0.016       |
| Bond angles (°)         | 1.069       |

The data collection statistics are based on a single crystal.

*Values in parentheses are for highest-resolution shell. **CC1/2, correlation coefficient between the average intensities in two parts of the unmerged data, each with a random half of the measurements of each unique reflection21.

Resolution (Å) 50.0–3.1 (3.29–3.1)

Ty3 RT with the cognate RNase H cleavage site located 12 nt from the RNA strand, the sequence of which corresponded to the Ty3 logistically relevant PPT-U3 junction within the RNase H active site9.

The atomic model suggested that the biological unit in our crystals was a right-hand, with the ‘palm’ subdomain housing the active site, the ‘fingers’ stabilizing the RNA template strand and the ‘thumb’ interacting mainly with the DNA strand. In contrast, the position of the Ty3 RNase H domain corresponds with that of the retroviral connection subdomain. This supports the hypothesis that evolution of retroviral RTs from LTR retrotransposon enzymes involved conversion of their RNase H domain to a ‘connector’ with loss of catalytic function and recruitment of a new RNase H1 domain18.

Ty3 RT subunits A and B are identical in sequence, and the structures of individual subdomains are very similar. Their pairwise superpositions result in low r.m.s. deviations for the positions of pairs of Cα atoms: 0.5 Å for 95 Cα atom pairs in the fingers subdomains, 0.9 Å for 116 pairs in the palm subdomains, 1.3 Å for 54 pairs in the thumb subdomains and 1.0 Å for 80 pairs in the RNase H domains. Both of the fingers–palm fragments are also structurally similar (r.m.s. deviation of 2.6 Å over 227 pairs of Cα atoms). However, pronounced differences are apparent in the positioning of the RNase H and thumb subdomains (Fig. 1b). The difference in the position of the RNase H domain in the two subunits corresponds to a large, ~90° rotation around an axis running roughly through the contact point between the palm and thumb of subunit A. Consequently, the subunit B RNase H domain is positioned between its fingers and palm, blocking the DNA polymerase substrate binding cleft and causing the thumb subdomain to be displaced from the palm and rotated relative to the RNase H domain. Surprisingly, the conformation of subunit B resembles that of p51 HIV-1 RT, which lacks an RNase H domain23.

The subunit interface of the Ty3 RT dimer is highly polar, involving two main contact points. The first is formed by insertion of the subunit B fingers between the palm and RNase H domains of subunit A (Fig. 1a). Prominent interactions in this area involve (i) Arg203 (subunit A) and Ser175 (subunit B; hereafter, letters in parentheses represent dimer subunits), (ii) Asp127(A) and Lys177(B), and (iii) a salt bridge between Arg140(A) and Glu71(B) (Fig. 1c). The other contact region involves both RNase H domains (Fig. 1d): Arg413(A) interacts with the backbone of His68(B), Thr452(A) and His417(B), and Arg441(B), and Asp448(A) interacts with Ser429(B) and Arg442(B). Arg413 and Arg442 are conserved among other Gypsy retroelements (Supplementary Fig. 2), and Arg442 may be also essential for interaction with the DNA backbone.

RNase H domain

The RNase H domain and the retroviral connection subdomains adopt the RNase H fold, the most important element of which is the five-stranded, central β-sheet26. The first three strands are longer and run antiparallel to one another, whereas the last two are shorter and parallel to the first. The fold also contains two or three α-helices on one side of the central sheet and a single helix on the other. Cellular RNase H1 enzymes and closely related retroviral RNase H domains (collectively referred to as ‘cellular RNase H1 enzymes’) have two main differences from the Ty3 RNase H and retroviral connection subdomains (Fig. 2 and Supplementary Figs. 3 and 4). First, there is a deletion of about ten residues between the first two strands of the central β-sheet of the LTR retrotransposon enzyme, shortening the first strand in its C terminus (Fig. 2a,c). A second difference is the arrangement of α-helices between strands 4 and 5. The Supplementary Note provides a detailed comparison of substrate-binding residues between cellular and Ty3 RNase H enzymes. The Ty3 RNase H active site resembles cellular enzymes, likely functioning through the same mechanism (Supplementary Fig. 5a). However, equivalents of many residues mediating substrate binding in bacterial, human and HIV-1 RNase H1 could not be identified in Ty3 RNase H, especially those forming the phosphate-binding pocket27,28.
As neither of the dimer’s RNase H active sites interacts with the RNA, an important mechanistic question is which Ty3 RT subunit contributes RNase H activity and what conformational changes are necessary to support this. Supplementary Figure 5b depicts a catalytic interaction of the RNase H domain with the substrate. This was prepared using the human RNase H1 complex structure\(^2\) and assumes that the Ty3 RNase H active site interacts with nucleotide −13 or −12, the preferred cleavage sites in a 3′→5′ end-directed cleavage mode. Bringing the active site of the RNase H domain from subunit A or B into the proximity of the RNA backbone would necessitate a substantial conformational change. Such large changes of the palm–fingers arrangement relative to the thumb–RNase H fragment are possible, as evidenced by major conformational differences between subunits A and B. The subunit B RNase H domain is located closer to the scissile phosphate, and its movement (probably together with its thumb subdomain) could be accommodated by a translation of ~40 Å without invoking severe clashes, while preserving dimerization contacts of the palm and fingers subdomains. A corresponding rearrangement of subunit A RNase H would disrupt the dimer structure and eliminate critical contacts between the substrate and thumb subdomain of subunit A. This implies that subunit B’s RNase H domain contributes activity, an idea that is supported by biochemical data presented below. Conformational changes of the protein could induce substrate deformation similar to that observed with HIV-1 RT\(^1\), although the exact nature of these changes is difficult to predict and a similar issue for HIV-1 RT has been elucidated only recently with crystallography\(^1\).

The requirement for conformational changes conducive to substrate cleavage implies that RNA hydrolysis would be infrequent, in agreement with published experiments examining RNase H activity concurrent with DNA synthesis\(^9\). Hydrolysis is rare during DNA synthesis, occurring primarily after the enzyme reaches the end of the substrate, possibly providing sufficient time for rearrangement into an RNase H–competent mode. Infrequent and transient interactions of the RNase H domain with the substrate emerge as a common element of the mechanism of RTs. For HIV-1 RT, the RNA-DNA sub- 

![Figure 1](image1.png)

**Figure 1** Overall structure of Ty3 RT and the dimer interface. (a) Cartoon representation of Ty3 RT in complex with an RNA-DNA hybrid substrate. Protein subdomains are colored blue for fingers, red for palm and green for thumb, and the RNase H domain is in yellow. Lighter shades of the same colors are used for subunit B. Secondary structure elements are labeled (numbers for strands and letters for helices) using the same scheme as in our previous work on XMRV RT\(^2\). Residues forming the DNA polymerase and RNase H active sites are shown as spheres. (b) Comparison of the structures of subunits A and B, colored as in a. Arrows indicate the movements of the thumb and RNase H domains that transform their conformation in subunit A to that of subunit B. (c,d) Residues involved in dimer formation. Protein structure is colored as in a.

![Figure 2](image2.png)

**Figure 2** Comparison of RNase H and connection subdomains. (a–e) Cartoon representations of Ty3 RNase H domain (a), HIV-1 connection subdomain (b), human RNase H1 protein (c; protein chain from PDB 2QK9 (ref. 28)), human RNase H1 with bound RNA-DNA substrate (d; PDB 2QK9 (ref. 28)) and HIV-1 RNase H domain (e; PDB 1RTD (ref. 16)). Strands of the central β-sheet are labeled, and residues forming the active site and phosphate-binding pocket are shown as sticks. The C-terminal region of the first β-strand, which differs in length between Ty3 RNase H and cellular enzymes, is indicated with a dotted box.
flexible linker and thus is very mobile. Only in the presence of RNA-DNA does it become transiently organized on the substrate25. This feature of RNase H domains may regulate their function in specialized cleavage events during primer generation and removal.

Substrate binding

The PPT RNA-DNA hybrid in our structure adopts a conformation intermediate between A- and B-form duplex, and the width of the minor groove is between 9 and 10.4 Å. The substrate comprises the entire PPT sequence along with four residues from the U3 region and should therefore provide an accurate model of the PPT structure. Its orientation in the structure would correspond to minus-strand DNA extension with possible simultaneous generation of the 3′ end of the PPT primer. We detected no major structural deformations of the hybrid, which superimposes well with the random RNA-DNA hybrids in structures recently reported for HIV-1 and XMRV RT17,23. At the resolution of our structure (3.1 Å), subtle deformations of the hybrid, which superimposes well with the random RNA-DNA hybrids in structures recently reported for HIV-1 and XMRV RT17,23, are observed. This bipartite substrate interface is consistent with biochemical data supporting our structure is the fact that nucleotides between these regions are more tolerant to modification, showing they do not form contacts with the protein.

Template nucleotide +1, which would base-pair with the incoming dNTP, is stabilized by interactions of its 2′-OH group with the backbone of Gly186(A) (Fig. 3b). Nucleotide +1 is also stabilized by a ‘pin’ structure comprising the side chains of Arg118(A) and Asp116(A), which has been characterized previously for monomeric gammaretroviral RTs23,29 and the heterodimeric HIV-1 enzyme30,31. 2′-OH groups of the RNA also form interactions with thumb residues Asn297(A) and Arg300(A) and the backbone of fingers residue Leu187(A).

The 3′-OH on the DNA strand is located at the DNA polymerase active site of subunit A, whose configuration resembles that of RTs from retroviruses16,23 (Supplementary Fig. 6), with key carboxylate residues coordinating two divalent metal ions5. The fact that the polymerase domain and active site of Ty3 RT subunit A are superimposable with HIV-1 RT demonstrates that this subunit probably contributes polymerase activity. However, one difference in the Ty3 polymerase active site is the residue stabilizing the base of the incoming dNTP (which is absent in our structure). This is well conserved among retroviral RTs23,29, but is replaced by Phe185(A) in Ty3 RT (Supplementary Fig. 3).

Upstream of the active site, the DNA strand forms extensive interactions with helix F of the subunit A thumb (Fig. 1a), which for retroviral RTs is inserted into the minor groove of the hybrid16,23,32.

Figure 3 Substrate binding. (a) Ty3 RT structure colored according to surface potential (red, negative; blue, positive; ±15 kTe). Nucleic acid is shown in cyan (DNA) and yellow (RNA). (b) Diagram of protein–nucleic acid interactions. Arrow indicates the PPT-U3 junction (the preferred site of RNase H cleavage). The 5′ RNA nucleotide not observed in the structure is shown in gray. Ovals are colored by protein domain as in Figure 1; solid ovals denote subunit A and empty ovals subunit B. Parallel horizontal lines indicate van der Waals interactions. Diagonal and vertical lines indicate hydrogen bonds (red) or side chains (black). (c) Interactions between RNase H domains (yellow) and the DNA strand (blue). Interacting protein residues are shown as sticks.

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Tyr298(A) and Gly294(A) form van der Waals interactions with the sugar-phosphate backbone of DNA nucleotides −3 and −4, respectively, while Lys287(A) interacts with the phosphate group of DNA nucleotide −5, and Asn297(A) forms an additional hydrogen bond with the 2′-OH of RNA nucleotide −5. An important structural residue is Phe292(A), located on the side opposite the substrate interface and stabilizing helix F. Interactions mediated by the thumb subdomain support previous biochemical studies showing the importance of Phe292, Gly294 and Tyr298 (ref 8). Among several substitutions, G294A RT is the most affected in the absence of a heparin trap, indicating its critical contribution to this component of the interface. Experiments with LNA-substituted nucleic acids have also predicted novel substrate contacts mediated by Arg60 and Gln65. These residues close to the DNA backbone and Arg441(A) participate in substrate interactions with the Ty3 RT–hybrid 3 complex also indicated formation of a 2:1 protein–nucleic acid complex (Supplementary Fig. 7).

We next prepared three Ty3 RT mutants. The first contained dual alanine substitutions in the region involved in dimer formation, at Arg140 and Arg203 (Fig. 1c), and the second had alanine substitutions of Arg441 and Arg442. Arg441(B) and Arg442(B) participate in dimer formation (Fig. 1d), whereas Arg441(A) and Arg442(A) are located close to the DNA backbone andArg441(A) participates in substrate binding (Fig. 3c). The third variant contained dual substitutions in novel substrate contacts mediated by Arg60 and Gln65. These residues in subunit B participate in the substrate interface, whereas in subunit A they are located distal from the RNA–DNA binding cleft. Therefore, our experiments should assess only their role in subunit B.

We first examined the oligomeric state of the Ty3 RT variants with substitutions in the dimer interface. Although mutant R140A R203A was unstable in GF experiments, AUC indicated it failed to form dimers in the presence of hybrid 3 (Supplementary Fig. 7). When R441A R442A RT was mixed with hybrid 3, GF–MALS (Fig. 4a; measured molecular weight of 105 kDa) and AUC (Supplementary Fig. 7) indicated a mild defect in dimer formation. Enzymatic activity of Ty3 RT variants was next examined. RNA-dependent DNA polymerase activity was evaluated with a template derived from the 5′-terminal region of the HIV genome that forms extensive secondary structures (Fig. 4b). Wild-type Ty3 RT (lane W) and mutant R60A Q65A were less processive than HIV-1 RT (lane H), as evidenced by transient pausing at the base of the poly(A) hairpin and an inability to resolve the TAR hairpin (Fig. 4c). Despite this, both enzymes displayed similar activity, indicating that substrate contacts mediated by Arg60(B) and Gln65(B) are not essential for processivity and strand-displacement.

**Biochemical characterization**

To confirm substrate-induced dimerization, we coupled high-resolution gel filtration (GF) with multi-angle light scattering (MALS) to determine the molecular weight of nucleoprotein complexes (Fig. 4a). As shown previously, Ty3 RT eluted as monomer in the absence of substrate, with a molecular weight of 53.1 kDa, compared with the expected 54.6 kDa for a monomer (Fig. 4a). When mixed with a 27-bp RNA–DNA hybrid containing a 2-nt RNA 5′ overhang (hybrid 3), the nucleoprotein complex eluted much earlier than either the protein monomer or the RNA–DNA hybrid alone (Fig. 4a). The molecular weight of this complex was 119 kDa, compared with a calculated value of 126 kDa for a dimer interacting with hybrid 3. Finally, analytical ultracentrifugation (AUC) sedimentation velocity experiments with the Ty3 RT–hybrid 3 complex also indicated formation of a 2:1 protein–nucleic acid complex (Supplementary Fig. 7).
activity. In contrast, mutant R140A R203A showed a strong processivity defect, with polymerization products accumulating at the base of the poly(A) hairpin. Lastly, for R441A R442A Ty3 RT the major product was a single-nucleotide extension of the primer, possibly indicating an inability to release pyrophosphate after formation of the initial phosphodiester bond. In conclusion, Ty3 RT dimerization and substrate contacts identified in our crystal structure are required for efficient polymerization.

RNase H activity was evaluated on a hybrid with a recessed 3'-DNA terminus to monitor 3' end-directed cleavage when the DNA 3' OH occupies the polymerase active site. As previously reported, we observed products of cleavage 13 nt downstream of the DNA 3' end and less prominent products resulting from an internal cleavage mode ~19 nt from the DNA 3' end (Fig. 4d). R60A Q65A RT showed reduced RNase H activity (Fig. 4d), supporting our notion that subunit B residues contacting the substrate are important for RNase H activity. This can be explained with the assumption that subunit B RNase H undergoes a conformational change to allow substrate cleavage. Arg60(B) and Gln65(B) would not change position and would contribute to substrate stabilization during and after the conformational change. RNase H activity of R140A R203A and R441A R442A RTs was also severely affected. Therefore, residues identified as participating in dimer formation and substrate binding are important for RNase H activity.

Our structure, moreover, implies that enzymatic activities of Ty3 RT reside in different subunits of the dimer. Although the homodimeric nature of Ty3 RT complex makes it challenging to verify this notion biochemically, we exploited the dimerization defect of mutant R140A R203A. Both Arg140 and Arg203 are critical to the dimer interface of subunit A, whereas in B they are distal from the dimer or substrate interface. Therefore, when R140A R203A is mixed with RNase H–deficient protein (D426N, which we used for crystallization), only two out of four possible dimer combinations should form, namely a D426N homodimer, lacking RNase H activity, and a mixed dimer with subunit B RNase H undergoing a conformational change to allow substrate cleavage. Arg60(B) and Gln65(B) would not change position and would contribute to substrate stabilization during and after the conformational change. RNase H activity of R140A R203A and R441A R442A RTs was also severely affected. Therefore, residues identified as participating in dimer formation and substrate binding are important for RNase H activity.

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Comparison of retroviral and LTR retrotransposon RTs
We document here key differences between Ty3 and HIV-1 RT. First, for the LTR retrotransposon enzyme, substrate binding is a prerequisite to dimerization, whereas the lentiviral enzyme is a stable dimer in the absence of substrate. Second, only the p66 subunit of the HIV-1 RT heterodimer contains a copy of the RNase H domain, so both enzyme activities reside in one subunit.

Topologically, however, the two enzymes are surprisingly similar. Supplementary Figure 3 aligns Ty3 RT subunit A and HIV-1 RT p66 (fingers-palm-thumb-connection). Their structures, as well as that of monomeric XMRV RT, are quite similar (Fig. 5a,b). The fingers, palm and thumb, together with the connection or RNase H domains of these subunits or proteins can be superimposed with an r.m.s. deviation of 2.1 Å (320 Cα atom pairs) for Ty3 compared with XMRV, and 2.9 Å (237 Cα atom pairs) for Ty3 compared with HIV-1 (PDB 1RTD (ref. 16)). Differences are relatively minor, including (i) an N-terminal extension in Ty3 RT, (ii) altered trajectory of the protein backbone between Thr201(A) and Arg206(A) of the Ty3 RT palm owing to deletion between helix C and strand 6, and (iii) the absence of thumb helix E in the HIV-1.

When Ty3 RT subunit B is compared with the HIV-1 p51 subunit, structures of individual subdomains are also similar. Moreover, their arrangement is strikingly analogous (Fig. 5c,d). p51's connection is rotated and placed between its palm and fingers, analogous to the Ty3 subunit B RNase H domain. As the dimeric organization of HIV-1 RT is well-documented, this further supports the notion that our structure represents the physiological architecture of Ty3 RT and that DNA polymerase activity is the property of subunit A. There are, however, several notable differences between HIV-1 RT p51 and Ty3 RT subunit B. The p51 palm subdomain has a different position, and the palm-fingers module cannot be superimposed between the two subunits of the dimer as well as for Ty3 RT. Moreover, the p51 thumb is further from its palm in order to accommodate the larger p66 subunit and, in particular, its RNase H domain. This larger separation of palm and thumb requires that short β-sheets 8, 9 and 10 at the C terminus of the palm subdomain are unfolded in HIV-1, whereas their structure is maintained in Ty3 RT.

When Ty3 RT subunit A, HIV-1 p66 and XMRV RT are superimposed, the trajectories of the nucleic acid substrates are very similar for XMRV and Ty3 enzymes. The substrate of HIV-1 RT passes further away from the connection subdomain owing to the presence of its RNase H domain, as described previously. Overall, substrate interactions around the DNA polymerase active site are conserved between the three enzymes, and equivalent residues can be identified in each protein. However, toward the RNase H domains the substrate interfaces involve different sets of residues.

DISCUSSION
We present the first crystal structure of a retrotransposon RT, revealing an unanticipated architecture featuring an asymmetric homodimer induced by substrate binding. A separation of ~13 nt between the 3’ end of the DNA and the RNase H active site, which was observed for Ty3 RT in biochemical experiments, was difficult to rationalize using retroviral RT structures because the active site of the RNase H modeled on the HIV-1 p66 or XMRV connection subdomains would be facing away from the substrate. Dimerization thus offers an
elegant explanation for the shorter distance between the polymerase and RNase H active sites.

Previous studies have proposed that structural deformations protect the PPT from RNase H cleavage. Although we observed no major distortion of the RNA-DNA substrate in our structure, subtle alterations may be responsible for its special features. Further structures of Ty3 RT with random-sequence RNA-DNA and PPT bound at different registers should shed light on this issue. Another interesting question is the role of the described Ty3 RT–integrationase fusion that has been detected in virus-like particles, which may facilitate folding of RT.

Although the overall conformations of Ty3 and HIV-1 RT are comparable, a critical difference is the homodimeric nature of the former. Homodimers with the high degree of asymmetry observed here are rare. Important questions to be addressed are the conformation of substrate-free monomeric Ty3 RT and the mechanism of substrate-induced dimerization. It is likely that the more compact, subunit B–like conformation is preferred in the absence of the nucleic acid.

Substrate binding could then stabilize a more open, subunit A–like conformation, allowing dimerization. Another distinctive and, to our knowledge, unprecedented feature of the Ty3 RT structure is that its asymmetry allows for the separation of enzymatic activities between subunits and brings the RNase H domain into position to mediate hydrolysis. Whether this applies to LTR retrotransposon enzymes remains to be determined.

METHODS

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

Accession codes. Protein Data Bank: Coordinates have been deposited with accession code 4OL8.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

E.N. obtained crystals of Ty3 RT–substrate complex; E.N. and M.N. solved and analyzed the structure; J.T.M., M.K.B. and I.S. performed biochemical experiments; E.N. and R.H.S. performed biophysical protein characterization; J.J. prepared the expression construct and conducted initial crystallization experiments; S.F.J.L.G. and M.N. designed the research and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

**Protein purification.** Ty3 RT was cloned into an expression vector with a 3C protease cleavage site between the His-tag and the protein. His-tagged RT with the RNase H–inactivating substitution D262N was expressed in E. coli and purified by immobilized metal affinity, ion exchange, and gel exclusion chromatography. The His-tag was removed by overnight incubation with 3C protease. Purified protein eluted from the gel filtration column at a volume expected for the monomeric form. Protein for SAD phasing was expressed in selenomethionine-containing media in E. coli BL21(DE3) Magic cells. Cells were induced with 0.4 M IPTG, grown overnight at 18 °C, harvested by centrifugation and lyzed by sonication in buffer containing 20 mM HEPES (pH 7.0), 50 mM NaCl, 10 mM imidazole, 50 mM N-β-mercaptoethanol (buffer A). An overnight incubation with buffer A containing 40 mM imidazole, protein was eluted with buffer A containing 300 mM imidazole. Following ammonium sulfate precipitation, protein was dissolved in 20 mM HEPES (pH 7.0), 50 mM NaCl, 10% glycerol and 1 mM DTT (buffer B), applied to a Mono S column (GE Healthcare) and eluted with a linear gradient of NaCl from 0.1 to 0.5 M. RT-containing fractions were precipitated with ammonium sulfate, centrifuged in buffer containing 20 mM HEPES (pH 7.0), 100 mM KCl, 1 mM DTT and 10% glycerol, and applied to a Superdex 200 size exclusion column (GE Healthcare). Peak fractions were concentrated using a 10 kDa cut-off centrifuge (Millipore) to 15 mg/ml.

**Crystallography.** Crystalization trials were prepared for protein alone, as well as in the presence of RNA-DNA hybrids ranging from 14 to 26 bp. HPLC-purified oligonucleotides were purchased from Metabion International AG. Before crystallization, protein was mixed with RNA-DNA hybrid in a 1:1:2 molar ratio and a final protein concentration of 7 mg/ml. Hybrids were produced by annealing either an RNA oligonucleotide, 5′-AACAGAGUGCCGACACCUG-3′, with a DNA oligonucleotide, 5′-CAGGTTGCGACTCTG-3′ (hybrid 1), or an RNA oligonucleotide, 5′-CUGGAGAGGAGAAGUG-3′, with a DNA oligonucleotide, 5′-CAGTTCTCTTCCTTC-3′ (hybrid 2). Hybrid 2 corresponds to the Ty3 PPT sequence with the PPT-U3 junction located 12 nt from the 3′ end of the DNA strand and is efficiently and specifically cleaved at PPT-U3 by Ty3 RT (not shown). No crystals were obtained with substrates corresponding to the first hybrids, in which the PPT-U3 junction was located 13 nt from the 3′ end of the DNA strand.

The first crystals were obtained in the presence of hybrid 1, with a 16 bp duplex portion and a 2 nt 5′ RNA overhang in 1.7 M sodium citrate, and diffractions X-rays to only 7 Å resolution. Substituting the random RNA-DNA sequence with the Ty3 PPT sequence (hybrid 2) yielded better quality crystals. The best crystals were obtained by the hanging drop vapor diffusion method and the optimal crystallization condition contained 0.1 M Tris (pH 8.5), 0.2 M ammonium sulfate and 17% PEG 3350. Before data collection, crystals were cryoprotected by addition of 30% glycerol to the crystallization drop to a final concentration of 25% and flash frozen in liquid N2.

X-ray diffraction data for the selenomethionine crystal (at Se peak wavelength 0.979Å) were collected at beamline 14.1 at BESSY on a MAR 225CCD detector at 100 K. Data were processed and scaled by XDS42. Data collection statistics are given in Table 1. The structure was solved by the single anomalous diffraction (SAD) method using the AutoSol module of Phenix43. Iterative building with COOT44 was performed, and refinement was performed in Phenix with TLS (Translation-Libration-Screw). Rfree was calculated with 5% of unique reflections. In the final model, 99.1% of the residues are within the allowed regions of the Ramachandran plot. Structural analyses, including superpositions and structural figures, were prepared in PyMol (http://www.pymol.org/). Coordinates of the structure have been deposited in the Protein Data Bank under the accession code 4OL8.

**Substrate binding assays.** For substrate-binding assays we used hybrid 3, with a 27 bp double-stranded region (RNA: 5′-AACAGAGUGCCGACACCUGAUUCC AUGACU and DNA: 5′-AGTCATGGAATCAGGTGTCGACTCTG). Ty3 RT was mixed with RNA-DNA hybrids at 2:1 molar ratio and applied to a Superdex 200 column (GE Healthcare) purified in 20 mM HEPES (pH 7.0), 150 mM KCl, 5% glycerol and 1 mM DTT. The eluted species were monitored by E260 and E290. Molecular weight of those species was determined using the multi-angle light scattering method on Optilab T-rEX and Dawn Heleos II (Wyatt Technology Corporation, USA).

**RNA-dependent DNA polymerase assays.** HIV-1 RNA template, prepared by in vitro transcription, was purified by denaturing polyacrylamide electrophoresis, followed by electroelution and precipitation. Purified RNA was mixed with an equimolar amount of a 5′ Cy5 labeled DNA oligonucleotide complementary to nt 98–113 of the HIV-1 genome (5′-Cy5-CAGACGGCCACACACTACGTCC Coralville, IA) in 10 mM Tris, pH 7.8, 25 mM KCl and annealed by heating to 95 °C for 2 min followed by slow cooling to 4 °C. The polymerization reaction contained 200 nM template-primer, 200 µM dNTPs, 10 µM Tris (pH 7.8), 130 mM NaCl, 9 mM MgCl2, 5 mM DTT and 10% glycerol. DNA synthesis was initiated by adding enzyme to a final concentration of 400 nM and was allowed to proceed at 30 °C for the indicated times. Aliquots were quenched with an equal volume of 7 M urea and 1× TBE, heated to 95 °C for 2 min, and polymerization products were fractionated by denaturing PAGE. The gel was imaged on a Typhoon Trio + Imaging system with Image Quant Total Lab software (GE Healthcare, Piscataway, NJ).

**RiNase H assays.** RNA and DNA oligonucleotides were purchased from IRT DNA (40-mer: 5′-Cy5- UCAUGUGCCUGAUUGCAUUGGCAUAAAGUC UCNA) was hybridized to DNA (28-mer: 5′-TGGAGTTTATGCGTATCG AGTAGCT in 10 mM Tris (pH 7.8), 25 mM KCl and annealed by heating to 85 °C for 3 min followed by cooling to 4 °C at 0.2 °C per second. The reactions contained 750 nM RNA-DNA, 10 mM Tris (pH 7.8), 150 mM NaCl, 9 mM MgCl2, 5 mM DTT and 10% glycerol. Hydrolysis was initiated by adding enzyme to a final concentration of 675 nM (or for the mixture of two variants, 337.5 nM each of R140A R203A and D426N) and proceeded at 30 °C for the indicated times. Samples were processed and visualized as described above. Original images of gels used in this study can be found in Supplementary Figure 8.

**Sedimentation velocity.** Sedimentation velocity experiments were performed in a Beckman-Coulter ProteomeLab XL-I Analytical ultracentrifuge, equipped with AN-50Ti rotor (8-holes) and 12 mm path length, double-sector- optical-Epon cells, loaded with 400 µL of samples and 410 µL of buffer (20 mM HEPES-KOH, pH 7.0, 100 mM KCl and 0.5 mM EDTA). WT protein and variants R140A R203A or R441A R442A were mixed with RNA-DNA hybrid at 1:2.5 molar ratio. The experiments were carried out at 4 °C and 48,000 r.p.m. using continuous scan mode and radial spacing of 0.003 cm. Scans were collected in 6 min intervals at 260 nm. The fitting of absorbance versus cell radius data was performed using SEDFIT software, version 14.3e45 and continuous sedimentation coefficient distribution c(s) model, covering range of 0.1–10 S.

Biophysical parameters of the buffer: density ρ = 1.00639 g/cm 3 (4 °C), viscosity η = 0.01567 poise (4 °C), and proteins: partial specific volume V-bar = 0.7418 cm 3/g (20 °C), and V-bar = 0.7352 cm 3/g (4 °C), were calculated using SEDNTERP software (version 1.09, http://www.jphilo.mailway.com/download.htm).

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