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Uncovering the Complexities of Retroviral Ribonuclease H Reveals its Potential as a Therapeutic Target

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Abstract

Successful long-term management of HIV infection will require targeted inhibition of multiple steps essential for virus replication. Currently, both nucleoside- and nonnucleoside-based inhibitors of DNA polymerase function, in combination with antagonists of HIV protease, have been shown to be clinically beneficial. However, it is clear that RNase H activity of the multifunctional HIV-1 reverse transcriptase (RT) is absolutely required for completion of retroviral DNA synthesis, thereby rendering this function an attractive target for drug development. Although generally viewed as a sequence-independent activity, highly precise RNase H cleavage is required in order to remove the RNA primers of (-) and (+) strand DNA synthesis (a host-derived tRNA and the polypurine tract, respectively), thereby preserving the ends of linear DNA and facilitating integration. The availability of highly purified, recombinant RT/RNase H has allowed a thorough dissection of these multiple events and their potential for therapeutic intervention. Our current understanding of retroviral RNase H function and the status of small molecule inhibitors are the focus of this review.

Key words

HIV. Reverse transcriptase. RNase H. PPT. Antiviral therapy.

Introduction

Reverse transcription converts single-stranded retroviral RNA to double-stranded DNA. The resultant DNA is then integrated into chromosomes of the host, where it persists and in many cases causes disease[1]. This essential step in the life cycle of all retroviruses is catalyzed by reverse transcriptase (RT), a multifunctional enzyme that has RNA-directed DNA synthesis, DNA-directed DNA synthesis and ribonuclease activities[2]. Numerous drugs that target RT have been developed in an attempt to interfere with replication of human immunodeficiency virus type 1 (HIV-1)[3]. These drugs are primarily nucleoside analogs lacking a 3´ OH, and disrupt completion of DNA synthesis by terminating nascent DNA polymerization. However, the propensity of RT to make errors at a rate of approximately 3.4×10^{-5} per base pair/replication cycle, coupled with the large number of viral replication cycles that occur, leads to the rapid emergence of drug-resistant variants[4]. These variants encode altered RTs that exhibit decreased susceptibility to inhibitor and in some cases, increased removal of the chain-

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terminator from the DNA relative to the wild-type enzyme[5]. Selection of drug-resistant viruses is a severe limitation to the efficacy of anti-retroviral therapy. Thus, successful management of HIV infection will require targeted inhibition of multiple steps essential for virus replication.

HIV-1 RT is a heterodimer comprised of 66-kDa and 51-kDa subunits[6]. The latter is a proteolytically processed fragment of p66 that lacks a 15 kDa carboxyl-terminal domain. However, all enzyme activities reside in the p66 subunit. The structure of HIV-1 RT has been determined crystallographically and is likened to a right hand[7-10]. The fingers, palm and thumb subdomains of p66 form a cleft that accommodates the duplex nucleic acid at the polymerase active site. This active site catalyzes both RNA- and DNA-directed DNA synthesis while a second active site interacts with the duplex approximately 18 nt behind the polymerase and is responsible for degradation of RNA within RNA/DNA hybrids. This RNase H activity is absolutely required for retroviral DNA synthesis, and is therefore an attractive target for drug development[11]. Surprisingly, there has been little progress reported on the development of RNase H-specific chemotherapeutic compounds. Several biochemical assays that accurately mimic RNase H function at different steps of reverse transcription are available[12] and should allow for detailed evaluation of potential inhibitors.

RNase H-mediated events in HIV-1 replication

Multiple events are required to convert (+)-stranded retroviral RNA to double stranded DNA (Fig. 1; reviewed in[13]). First or (-) strand DNA synthesis begins from a host-derived tRNA hybridized to the primer binding site (PBS) near the 5´ end of the viral genome. RNA-dependent DNA synthesis proceeds until the 5´ end of the genome is encountered, generating (-) strong stop DNA. Concurrent with DNA synthesis, the RNA strand of this nascent RNA/DNA duplex is non-specifically degraded by RNase H, exposing the repeat (R) sequence at the 3´ end of the nascent (-) strand DNA. R sequences, present at each end of the genomic RNA, facilitate translocation of (-) strand DNA to the 3´ end of the viral (+) strand RNA. Such strand transfer can occur either inter- or intramolecularly and occasionally occurs before the 5´ end of R is polymerized[13]. RNase H is absolutely required for strand transfer; mutations that selectively abolish RNase H activity do not promote strand transfer and accumulate (-) strand strong stop DNA. After strand transfer, polymerization of (-) strand DNA continues until completion.

During RNA-dependent DNA synthesis, portions of the RNA in the nascent duplex are degraded non-specifically by RNase H, leaving (+)-strand RNA frag-

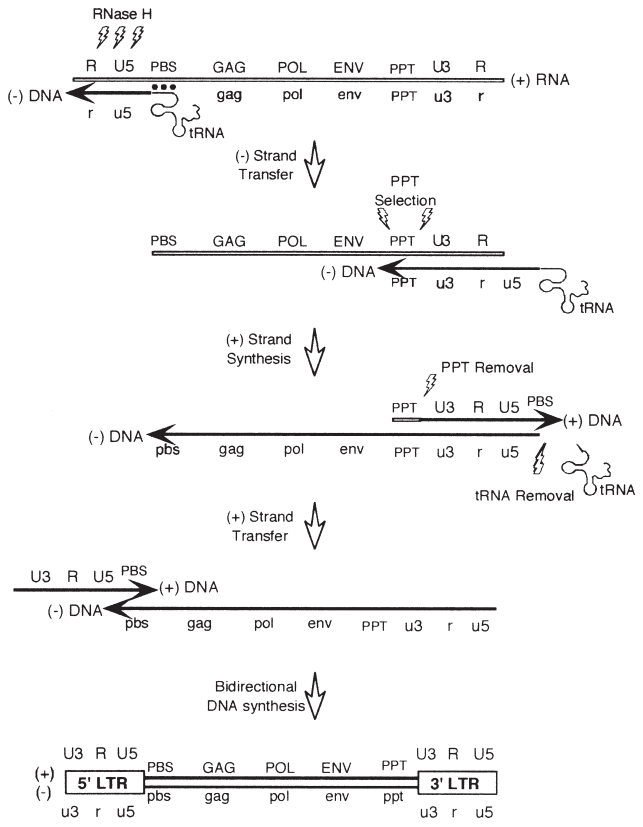

Figure 1. *RNase H-mediated events in retroviral replication. Steps are described in detail within the text. The lightning bolt indicates RNase H cleavage locations. The cPPT is omitted for clarity.*

ments hybridized to (-) strand DNA. These fragments have a free 3´-OH that potentially can be extended by the polymerase activity of RT. However a purine-rich RNA fragment known as the polypurine tract (PPT) is resistant to RNase H degradation, and used almost exclusively to prime (+) strand DNA synthesis in all retroviruses. The element near the 3´ end of the genome (3´ PPT) is a requisite primer for the initiation of second or (+) strand DNA synthesis in all retroviruses. 3´ PPT-initiated (+) strand DNA is elongated through the U3, R and U5 sequences, and also through a portion of the tRNA until synthesis terminates upon encountering the first or second modified base. This allows RNase H to selectively remove tRNA from the (-) strand DNA, thereby exposing the (+) strand PBS DNA sequence[14,15]. A second strand transfer occurs, and the nascent (+) strand DNA is translocated to the 3´ end of the (-) DNA strand via PBS complementarity. At this point it is assumed that the replication intermediate is circularized and bi-directional DNA synthesis continues, during which a strand displacement function of RT is required.

In HIV-1, other lentiviruses, spumaviruses and the Ty1 retrotransposon, a second PPT is found near the center of the genome (cPPT) and is also used as a primer for (+) strand synthesis[16-22]. Mutations within the cPPT prevent or severely slow HIV-1 replication in culture, and changing the location of the cPPT established a new (+) strand initiation site[17]. Other studies detected additional non-PPT (+) strand initiation sites within HIV-1, although these sites are used at a lower frequency compared to the PPTs[23,24]. Synthesis of (+) strand DNA in avian and murine retroviruses is also initiated from multiple sites[25].

RNase H removal of RNA primers

RNase H activity removes RNA primers used to initiate (-) and (+) strand DNA synthesis. The PPT is removed largely intact at the RNA-DNA junction after it has been extended a minimum of 12 nucleotides (reviewed in reference[11]). In contrast, the tRNA primer is not removed until later in replication, so that it's pbs can be used as a template to make (+) strand PBS DNA (Fig. 1). Using model substrates and an endogenous RT reaction, Wu, et al.[26] demonstrated that HIV-1 RT copies into the tRNAlys3 until the first or second modified base is encountered 18 or 21 nucleotides from the 5´-end of the tRNA. The tRNA is then cleaved at the penultimate residue leaving a single rA attached to the (-) strand DNA[15,26]. It is unclear whether polymerase-dependent RNase H activity makes the cleavage. However it was shown that tRNA removal can be accomplished in a polymerase-independent manner, both with model substrates[15] and an RT mutant lacking the DNA polymerase domain[27]. This suggests that a specific sequence and/or structure is recognized by RNase H and dictates cleavage specificity. Mutational analysis revealed that several base pairs 5´ to the scissile phosphate are important specificity determinants[27]. These bases can potentially interact with a portion of the RNase H primer grip, a structural feature of HIV-1 RT that contributes to RNase H specificity (see below). After the initial cleavage event, subsequent cleavages or the activity of nucleocapsid (NC) protein are required to dissociate the tRNA and allow strand transfer[26].

The RNase H-mediated events described above are critical for retrovirus survival, and failure to perform them prevents synthesis of integration-competent double-stranded DNA. While RNase H nonspecifically hydrolyzes the RNA genome during RNA-dependent DNA synthesis, PPT processing (i.e. selection and removal) and tRNA removal are critical reactions that by necessity require extreme specificity. Failure to initiate (+) strand DNA synthesis at the 3´PPT/U3 junction, or to precisely remove the PPT primer alters the U3 terminus and the resulting attachment site for viral integrase (IN). Likewise, tRNA removal must occur at the PBS junction with high precision in order to generate the correct U5 IN attachment site. It is almost paradoxical that an enzyme that performs non-specific degradation of the RNA genome must also demonstrate exquisite precision at several crucial steps in the life cycle. Just how this balance is achieved has been the subject of intense research. It is becoming increasingly clear that the key to such precision lies in the structures and sequence of substrates utilized by RT and it's associated RNase H.

Mechanism of RNA hydrolysis

The structure of the isolated RNase H domain was the first of the HIV-1 proteins to be solved crystallographically[28]. In addition, the structures of the HIV-1 RT apoenzyme and complexes containing duplex DNA and RNA/DNA hybrids are available[7-9,29,30]. All structures are quite similar with respect to the RNase H domain, and are remarkably similar to *E. coli* RNase HI despite sharing only 24% sequence identity[31-34]. The RNase H domain is folded into a 5-stranded mixed β-sheet flanked by four asymmetric α-helices. The strong conservation of structure suggests this domain is very stable and predicts that the catalytic mechanisms are similar. The active site contains four acidic amino acids, Asp443, Glu478, Asp498 and Asp549, that are highly conserved both at the sequence and structural levels in retroviral and *E. coli* RNase HI. In addition, His539, which is found in a six-residue loop connecting β5 and αE, also constitutes part of the active site. This *His-loop* appears to be flexible or disordered in the structure of the isolated RNase H domain[28], but is better defined in the HIV-1 RT 7 heterodimer[29,30]. Mutation of any of these residues severely disrupts RNase H activity[35-37] and HIV-1 replication[38,39].

Extrapolating the revised *E. coli* RNase HI catalytic mechanism put forth by Haruki, et al.[40] to HIV-1 RT (Fig. 2), suggests a single divalent metal would be bound by Asp443, Asp498 and Gly444 and stabilize the negatively-charged transition state intermediate of RNA hydrolysis. His539 acts as a general base by abstracting a proton from Water A which is held in place via hydrogen bonding to Asp549. This generates a nucleophilic hydroxyl ion that attacks the scissile phosphate. His539 is properly positioned

through a hydrogen bond to an oxygen of the phosphate group 3´ to the scissile bond. The essential components of this mechanism are supported by extensive mutagenesis and the use of phosphorothioate substrate analogs with the *E. coli* RNase HI and HIV-1 enzymes[33,40,41 and references therein]. Although this model assumes participation of a single divalent metal in the reaction, there remains some debate as to whether or not a second metal ion is required for catalysis. The number of metals is relevant to the mechanism, as the second metal is proposed to activate Water molecule A to serve as the attacking nucleophile[28], analogous to the exonuclease mechanism of Klenow fragment of DNA polymerase 1[42].

RNase H requires either Mg^{2+} or Mn^{2+} for activity, though Mn^{2+} is inhibitory at high concentrations[41]. The structure of *E. coli* RNase HI reveals a single Mg^{2+} in the active site[33] while the structure of the isolated RNase H domain of HIV-1 RT reveals two Mn^{2+} [28]. Crystallographic analysis shows that Asp443 and Asp549 bind the second metal. To further complicate the picture, mutations that eliminate RNase H activity in the presence of Mg^{2+} can sometimes be rescued by Mn^{2+} [43,44], suggesting alternative mechanisms for metal binding may exist. Recently, a quantitative assessment of metal ion binding stoichiometry revealed that only a single Mg^{2+} is bound in the RNase H active site by wild-type HIV-1 RT[45]. An elegant new model potentially solves this dilemma. The 'activation/attenuation' model states that the first metal is required to activate hydrolysis and is coordinated as described above[41]. At higher concentrations of Mn^{2+}, a second metal ion binds to Asp549 and the RNA substrate. This alters the position of His539 (and likely the *His loop*) and prevents it from abstracting a proton from Water molecule A, thereby inhibiting the reaction. It remains to be determined if the highly specific cuts made by the RNase H activity of HIV-1 RT are altered by the number of metal ions at the active site.

Using model RNA/DNA hybrids, data from several laboratories has shown a two-step hydrolytic mechanism for retroviral RNase H. Following location of the DNA polymerase domain over the 3´ OH of a recessed DNA end, initial cleavage (defined as polymerization-dependent[46]) occurs ~17 bp downstream, defining the spatial separation of the DNA polymerase and RNase H active centers. Subsequent polymerization-independent processing from the site of endonucleolytic cleavage proceeds to within 8 nt of the polymerase catalytic center, after which the remaining RNA/DNA presumably dissociates.

Mutations affecting RNase H function

As described above, highly conserved residues of the RNase H domain include the triad of acidic residues Asp443, Glu478, Asp498, together with His539 and Asp549, which cumulatively are implicated in catalysis (Fig. 2). Although mutation of any of these residues might therefore abolish enzymatic function, current data is somewhat contradictory. Both Glu478Gln[35] and Asp443Ala[36,37] substitutions selectively destroy RNase H function, consistent with a role in binding the divalent metal. Asp498 is proposed to fulfill a similar function, but its replacement in HIV-1 RT

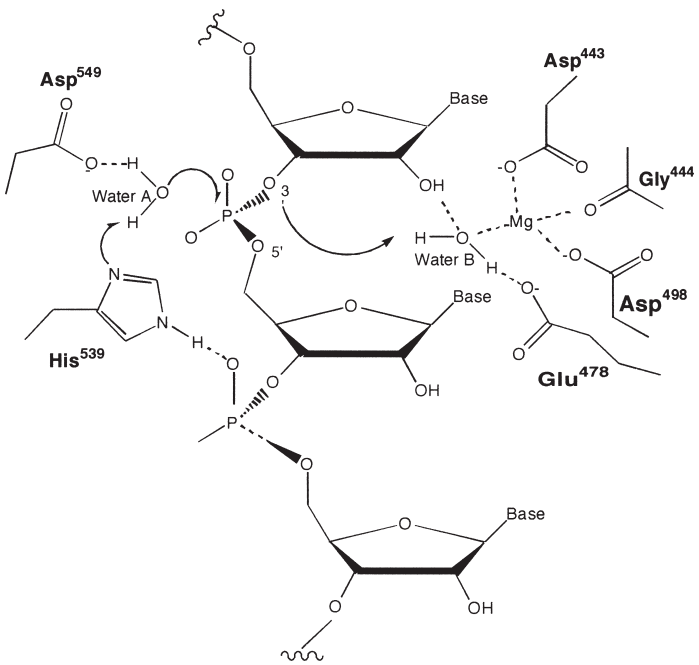

Figure 2. *Proposed catalytic mechanism for HIV-1 RNase H. The model is based on studies of Haruki, et al.[40] with E. coli RNase H. HIV-1 counterparts of E. coli catalytic residues are indicated. Although a single divalent cation is presented, the possibility of a two-metal catalyzed reaction can not been excluded (see text).*

generates an unstable enzyme that is only stabilized by compensatory alteration of Asp443[36,37]. However, activity is reduced but not eliminated following alteration of His539 or Asp549, a characteristic also noted when their counterparts in *E. coli* RNase H and RT of the *Saccharomyces cerevisiae* retrotransposon Ty3 are substituted[47]. Since the imidazole ring of His539 is believed to activate the attacking molecule, and the orientation of this ring is stabilized through hydrogen bonding to the phosphate group 3´ to the scissile bond[40], it is surprising that it can tolerate a variety of substitutions. Asp549, the other hydrogen- 9 bonding partner of the attacking water molecule behaves similarly, namely that its replacement with Asn or Ala reduces activity rather than eliminating it. HIV-1 RT harboring the mutation Asp549Asn retains high levels of polymerization-dependent RNase H activity but is substantially impaired in polymerization independent function. However, the same mutant was incapable of processing the HIV (+) strand PPT primer from adjacent (+) DNA, suggesting precise positioning of structurally distinct substrates in the RNase H catalytic center may be critically dependent on the geometry of the conserved carboxylate cluster.

Functional coupling of the DNA polymerase and RNase H activities of HIV-1 RT predicts that mutations in one domain might impact on the activity of the other[48]. However, two mutations affecting RNase H activity are particularly interesting. A 13-residue truncation of the catalytically-inert p51 subunit (p66/p51Δ13 RT) eliminates polymerization-dependent RNase H activity despite the fact that the p66 RNase H domain is intact[49]. This phenotype can be explained if subtle alterations to p51 architecture affect positioning of its thumb subdomain and thus its interaction with the RNase H domain of p66. As expected for loss of polymerization-dependent function, p66/p51Δ13 RT fails to support DNA strand transfer. Interestingly strand transfer activity and polymerization-dependent RNase H activity were restored when mutant enzyme was supplemented with nucleocapsid protein (NC)[50]. One speculative interpretation of this data is that a discrete binding site exists on HIV-1 RT for NC, perhaps the cavity resulting from proteolytic processing of the p66/p66 homodimer during heterodimer maturation. While retaining RNA-dependent DNA polymerase function, this mutant fails to initiate (-) strand DNA synthesis from the cognate tRNA-primer, providing additional evidence for the close association of these two RT-associated functions. A second notable alteration is the RT mutant Tyr232Ala, which harbors an alteration in the N-terminal DNA polymerase domain of p66. Tyr232 comprises a residue of the DNA polymerase "primer grip", a motif proposed by Jacobo-Molina, et al.,[8] to hold the primer terminus in an orientation appropriate for nucleophilic attack on an incoming dNTP (see below). This enzyme only catalyzes polymerization-independent RNase H activity, i.e., it cleaves 10 the RNA template at position –8 without the requirement for –17 cleavage. Since several studies indicate that polymerization-dependent and-independent RNase H activities are temporally coupled, the ability to hydrolyze exclusively at template nucleotide –8 is difficult to rationalize, although relocation of the RNA/DNA hybrid in the nucleic acid binding cleft following loss of primer contacts is one possibility.

Structure of RNA/DNA duplex bound by HIV-1 RT

The structural determination of HIV-1 RT complexed with various primer-template combinations provides a wealth of detail on interaction of the enzyme and nucleic acid[8,9,29,30,51]. One of the most interesting findings was that the primer-template is bent in the vicinity of the p66 thumb subdomain[8]. The bend may be a consequence of the unusual structure characteristics of the duplex. Five base pairs of the nucleic acid substrate assume an A-like form in the polymerase active site, and the last nine base pairs assume an A/B hybrid-like configuration near the RNase H domain. The transition from A-like to B-like occurs smoothly over 4-5 bp and is associated with a 40-Å bend in the nucleic acid, a feature observed in other nucleic acid polymerizing enzymes[52]. The sugar-phosphate backbone of the nucleic acid interacts predominately with the palm, thumb and RNase H domains of p66. Most contacts are nucleotide sequence-independent, and are primarily Van der Waals interactions[29]. The structure of RT bound to a PPT-containing RNA/DNA hybrid reveals very similar interactions, particularly with the DNA primer[30]. However, in this case RT makes significant contacts with 2´OH groups of the RNA template. In addition, several lysine residues of p51 hydrogen bond exclusively with the RNA strand.

The minor groove in both the duplex DNA and the RNA/DNA hybrid structures exhibits a pronounced variability, and is nearly 11 Å wide near the polymerase active site, slowly decreasing to about 9 Å across the bend. Over the next 3 bp, this dramatically narrows to 6 Å, the typical value for B-DNA, then widens slightly to 8 Å for ~ 4 bp, and then again narrows to 6 Å 15 nt behind the templating nucleotide. In the vicinity of the would-be scissile phosphate bond of the RNA/DNA hybrid, the minor groove widens again to 7-8 Å. In the PPT-containing RNA/DNA hybrid, the RNase H active site is positioned over a sequence that is refractory to cleavage; thus the minor groove may need to be wider to facilitate cleavage. Indeed substitution of dU for dT residues in the PPT-containing hybrid (which widens the minor groove[53]), increases susceptibility to RNase H cleavage. It is interesting that the overall structure of the nucleic acid substrate complexed with HIV-1 RT is extremely similar in both the duplex DNA and RNA/DNA hybrid. Thus it appears that the structure observed is sequence-independent and likely results from constraints imposed by RT binding.

Perhaps the most unexpected structural feature of HIV-1 RT complexed with the PPT-containing RNA/DNA hybrid is the disruption of Watson-Crick base pairing at the 5´end of the duplex. RT was knowingly positioned with the 3´ end of the DNA primer in the polymerase active site such that the RNase H domain contacts a portion in the center of the PPT, in order to determine why this element is resistant to

cleavage[30]. The unusual structural distortion begins with weak base pairing (larger than normal separation between bases) encompassing the position of the presumptive scissile bond (Fig. 3). An unpaired primer base, a G-T mispair and a frame-shifted A-T base pair (in the direction of the polymerase active site) then follow, after which the register is restored by an unpaired template base followed by two more weak base pairs at the 5´ end of the PPT. The unusual nucleic acid structure positions the scissile bond approximately 2 Å from the RNase H active site residues, thus preventing cleavage. Extensive protein contacts are made with the primer strand near this abnormal structure at the 5´ end of the PPT. These contacting residues are termed the RNase H primer grip and will be discussed later.

PPT Structure

It is tempting to speculate that the unzipped portion of the PPT is imparted by RT binding, and is likely to be observed in other non-PPT RNA/DNA duplexes. However, it is also possible that the interaction of RT with a unique PPT conformation leads to unzipping, due to incompatibility with the structural constraints imposed by RT. The sequence of the HIV-1 PPT (in bold) is 5´- UUUU**AAAAGAAAAAGGGGGG**A-3´. It is well documented that A and G tracts within nucleic acids are associated with bends and unusual groove widths[54,55]. Previous analysis by NMR and CD spectroscopy suggests that RNA/DNA hybrids rich in ribopurine residues have characteristics of A or A/B hybrid form[56-58]. Furthermore, the NMR structure of an 8-bp RNA/DNA approximating the 3´ end of the PPT and the adjacent U3 sequence reveals a bent duplex and an unusually wide major groove[59]. Recently, chemical footprinting was applied to probe the entire structure of a PPT RNA/DNA hybrid[60], using a technique that identifies unstacked thymine bases within duplex nucleic acid. Interestingly, thymines found opposite of the rA immediately adjacent to the PPT and the A tract at the 5´end displayed increased susceptibility to footprinting, indicating that a structural deformation exists in these areas of the PPT *in the absence of HIV-1 RT* (Fig. 3). Moreover, altering the degree of deformation changed the specificity of RNase H activity at the 3´ end of the PPT, suggesting that precise PPT cutting requires a specific duplex structure (see below). Collectively, these data suggest that the PPT contains a pre-existing unusual structure[60].

DNA polymerase and RNase H primer grips of HIV-1 RT

In the structures of HIV-1 RT complexed with an RNA/DNA hybrid and duplex DNA, an extensive collection of amino acids contacts the primer strand in two separate locations. The first area, designated the DNA polymerase primer grip, involves the β12-β13 hairpin and helices αH and αI of the p66 thumb[8,29,51]. Residues of the β hairpin contact the primer strand near its 3´ terminus, helping to position it near the polymerase active site. The residues of αH and αI of the thumb contact the primer strand 3 to 6 nt behind the incoming dNTP. Helix αH lies partially within the minor groove of duplex substrates and has been denoted the "minor groove binding track"[61] and a "translocation track"[51]. These amino acids are highly conserved among RTs[62], and their substitution affects both DNA polymerase and RNase H activi-

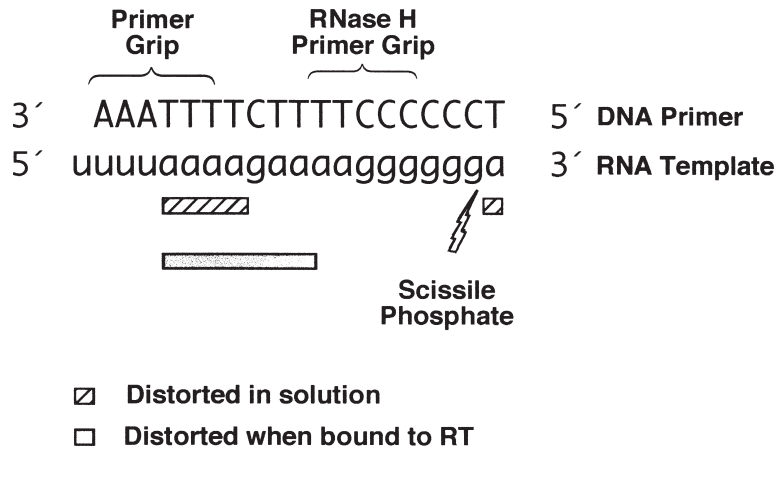

Figure 3. *Model of PPT cleavage specificity. Distorted regions of PPT containing duplexes in the absence (hatched boxes) and presence of HIV-1 RT (filled box) are indicated. Nucleic acid distortions in the absence of RT were determined via sensitivity of thymines to KMnO4 oxidation[60], while distortions of the PPT-containing duplex were derived from crystallization of HIV-1 RT in the presence of an RNA/DNA hybrid[30]. Note that the figure presents PPT RNA as the template, i.e., during (-) strand synthesis following first strand transfer (Fig. 1). The scissile phosphate provides a 3´OH for initiation of (+) strand synthesis, where the PPT is now exploited as a primer and subsequently removed from (-) DNA. Portions of RT that interact with the primer strand and regulate RNase H activity are shown.*

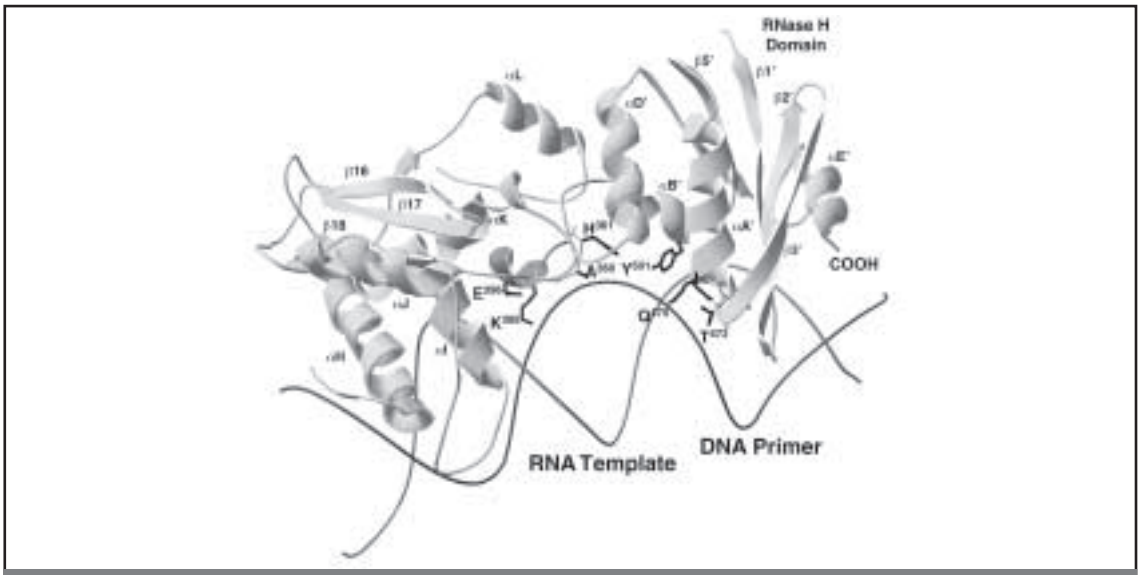

Figure 4. *Amino acids of p51 and p66 HIV-1 RT constituting the RNase H primer grip. The DNA primer is represented in blue and RNA template in red. Only structural elements of p66 and p51 in the vicinity of the RNase H primer grip are highlighted, of which p51 residues are indicated in cyan. The extreme Cterminus of the RNase H domain (Leu560) is indicated in orange. Amino acid notations are E = Glu; H = His; K = Lys; Q = Gln; T = Thr; Y = Tyr. Helices aH and aI of the p66 thumb are shown and constitute part of the DNA polymerase primer grip. The RNase H domain is indicated.*

ty[61,63-67]. This implies that contacts provided by these side chains are important for positioning nucleic acid in both active sites within HIV-1 RT.

Another region with extensive primer strand contacts is termed the RNase H primer grip (Fig. 4)[30]. These residues position the DNA primer strand near the RNase H active site and likely help set the trajectory with which the RNA/DNA hybrid enters the RNase H domain. Amino acid residues comprising this region are: Gly359, Ala360, His361, Thr473, Asn474, Gln475, Lys475, Tyr501, Ile505 of p66, and Lys359 and Glu396 of p51. This structural element interacts with the DNA primer 4 to 9 nt upstream relative to the scissile bond of the RNA/DNA hybrid (and 11 to 15 bp behind the incoming dNTP; Fig. 3). These residues are conserved among retroviral RTs and *E. coli* RNase HI[68]. Mutational analysis of some of these positions both *in vitro* and *in vivo* reveal their importance to RNase H specificity at the PPT[69-71]. The RNase H primer grip provides strong interactions with the primer strand in a sequence-independent manner[30], thus cleavage specificity cannot be controlled solely by these interactions. Sarafianos, et al.[30] suggest that the minor groove of the hybrid near the RNase H active site, coupled with intrinsic curvatures of certain sequences, are also important specificity determinants.

Model for PPT selectivity

How the specificity of PPT cleavage and extension is conferred is an important issue. It is clear that the PPT has an unusual structure that prevents internal hydrolysis but directs precise cleavage at the 3´ end. Mutations to either the DNA polymerase domain of RT or the RNase H domain can change the specificity of PPT cleavage[63,67,71-73]. Specificity is also modulated by base changes that alter the PPT structure[60,74]. Thus, both RT and the sequence and structure of the substrate contribute to cleavage specificity.

As described earlier, the HIV-1 PPT adopts an unusual conformation near the 5´ end. When the scissile bond at the 3´ end of the PPT is positioned in the RNase H active site, the distorted section of the PPT contacts the p66 thumb portion of the DNA polymerase primer grip (Fig. 3), implying that interaction of the thumb with an unusual conformation may trigger RNA hydrolysis. Whether the thumb specifically "senses" the distortion and activates cleavage, or forces the RNA into the active site as a consequence has yet to be determined. However, the thumb subdomain appears to be relatively fixed in all of the RT structures that contain duplex nucleic acid[9,30] (superposing the structures on the polymerase active site yields a rms. value of ~0.7 Å for helix αH). Thus it is possible that this interaction causes the nucleic acid to change its trajectory slightly, and be forced closer toward the RNase H active site residues. Powell, et al.[72] showed that Trp266 mutants of the p66 thumb lose the ability to specifically process the PPT from a (+) strand DNA-RNA chimera, despite retaining non-PPT hydrolysis activity. These data strongly support the critical nature of the thumb/hybrid interaction. In addition, RNase H primer grip residues are also important PPT cleavage specificity determinants. Since these residues contact the primer nearly opposite the scissile bond, they may impart subtle effects on positioning. In particular, Tyr501Ala substitutions alter the pattern of PPT cleavage both *in vitro*[71] and *in vivo*[70], while mutation of the Mo-MLV RT equivalent of Tyr501 decreases fidelity of A-tract polymerization[75], demonstrating that this residue is important for positioning the duplex nucleic acid. Furthermore, the G6

tract near the 3´ end of the PPT is critical for proper cleavage[74] and should stabilize the hybrid, something that is lacking in the uncleavable portion of the PPT[30]. Thus HIV-1 RT has evolved an elegant mechanism to control the specificity of PPT selection, relying on interactions between the upstream distortion and p66 thumb, and also residues of the RNase H primer grip to ensure proper positioning of the RNase H active site.

Development of RNase H inhibitors

The observation that inhibiting HIV-1 RNase H function is lethal for virus spread[35,76] suggested this RT-associated function as a *bona fide* antiviral target. Despite this, only several RNase H inhibitors have been described[77-81] (Fig. 5). In most cases, these inhibitors work at unacceptably high concentrations *in vitro* or have significant cytotoxicity to be of clinical relevance. A recent study of Min, et al.,[82] highlighted naturally-occurring napthoquinones as potential inhibitors, of which 1,4- napthoquinine inhibited RNase H function at concentration ~7-fold lower than required for inhibiting RNA-dependent DNA polymerase activity (9.5 µM *vs* 67 µM, respectively). Unfortunately, for many of these compounds, no antiviral activity was reported. However, two interesting candidates have recently emerged that merit further study. Borkow, et al.[83] found that N-(4-tert-Butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) inhibited DNA polymerase and RNase H activities of HIV-1 RT, but was more selective for RNase H function. The notion that both enzymatic functions are targeted by BBNH is not surprising, since this may reflect inhibitor binding to the nucleic acid binding cleft and competing with the nucleic acid substrate. More importantly, BBNH inhibited HIV-1 replication in cord blood mono-

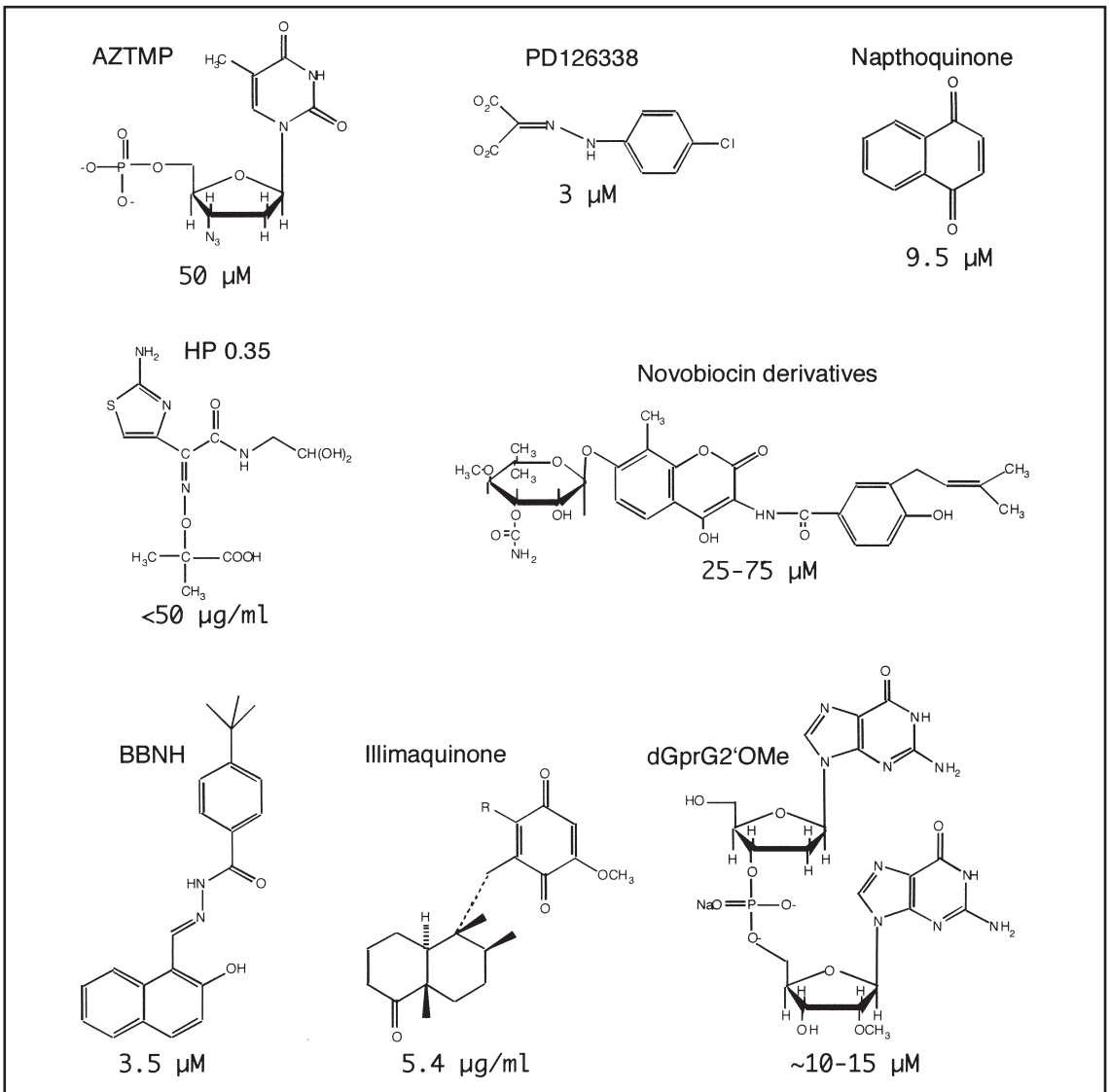

Figure 5. *Structures of RNase H antagonists. The IC50 for each compound, determined with recombinant HIV-1 RT/RNase H, is indicated. These values were obtained from the literature (see text for references). Note: IC50 values can only be compared when obtained under similar assay conditions.*

nuclear cells with an IC50 of 1.5 μM. The target specificity of BBNH will be revealed after isolation of resistant variants of HIV-1 and determining where the mutations occur, although *in vitro* data provides a strong argument that the RNase H domain is targeted by this antagonist. Arion, et al.[69] showed that HIV-1 RT harboring the mutation Tyr501Trp was 6-fold resistant to inhibition by BBNH, while the mutant Tyr501Arg was completely resistant. In addition, replication of HIV-1 molecular clones containing either mutation was significantly compromised. As described earlier, Tyr501 of the RNase H primer grip aids in conferring the appropriate trajectory on the RNA strand of a hybrid into the catalytic center for hydrolysis.

PD126338, the chlorophenylhydrazone of mesoxalic acid[84] is another interesting RNase H inhibitor that appears to be more specific for DNA strand transfer, a step that requires RNase H-mediated degradation of the donor template to facilitate transfer of nascent DNA to an acceptor. Discovery of this class of inhibitor is exciting as it opens the possibility of targeting a *specific* event in the replication cycle. As previously noted, DNA strand transfer, PPT processing and tRNA removal represent processes mandating a high degree of precision of the C-terminal RNase H domain. Thus, implementation of model substrates mimicking different steps in HIV replication might uncover multiple classes of RNase H inhibitors. To this end, we have developed a high-throughput, fluorescence-based RNase H assay mimicking the step of PPT removal from nascent (+) strand DNA (depicted schematically in Fig. 6A). The DNA template contains two inverted repeat sequences, IRA and IRB, the latter of which contains an extra, fluorescent base analog, 3-methyl-isoxanthopterin (3-MI)[85,86]. The stacking environment of neighboring bases within the uncleaved substrate quenches 3-MI fluorescence within IRB. Following hydrolysis at the PPT-U3 DNA junction, spontaneous dissociation of the short (+) DNA oligonucleotide permits hybridization of IRA and IRB. The absence of a base-pairing partner allows the fluorophore to assume an "activated" configuration when free of the quenching environment. In addition to avoiding the use of radioactive materials, this system has the advantage that the fluorophore 3-MI is not positioned directly at the site of hydrolysis, and thus will not interfere with enzyme binding or hydrolysis.

Finally, efforts to identify HIV-1 RNase inhibitors H might benefit from ongoing screening strategies directed against another key HIV-1 enzyme, integrase. Crystallographic analysis has shown that RNase H and IN comprise a superfamily of polynucleotidyl transferases sharing a –D-D-(35aa)-E-motif in their catalytic center[87]. Thus, it is possible that small molecule antagonists of HIV-1 IN may also target the RNase H domain of RT. Alternatively, because the active center motifs of RNase H and integrase are similar but not identical, inhibitors that are poorly active against one target may be more effective against another. For example, we demonstrated that several derivatives of coumarin, originally identified as antagonists of HIV-1 IN[88], are effective against RNase H in the 2-10 mM range *in vitro*. Conversely, a diketo acid which was extremely potent against HIV-1 IN[89] had no effect on HIV-1 RNase H function. Therefore, high throughput screening for RNase H inhibitors should be implemented in parallel with ongoing screening for HIV-1 IN inhibitors.

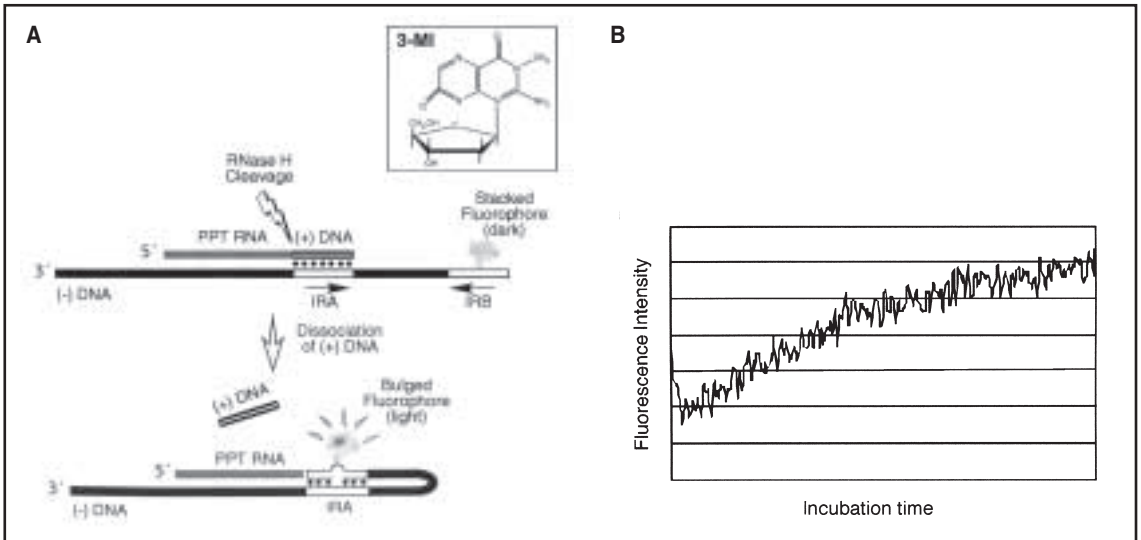

Figure 6. *A) Development of a non-radioactive, high-throughput assay to monitor RNase H-mediated cleavage of PPT RNA from nascent (+) DNA. The PPT-DNA chimera contains 5 nt of (+) DNA linked to 15 PPT RNA nucleotides, and the arrow represents the site of cleavage. IRA and IRB are inverted repeat sequences, differing in that IRB contains the guanosine analog 3-MI as an extra base. In the absence of hydrolysis, the stacking of 3-MI with neighboring bases quenches fluorescence. Following hydrolysis, hybridization of IRA and IRB displaces 3-MI, which, in its bulged configuration, displays increased fluorescence intensity. B) Evaluation of PPT cleavage. Fluorescence intensity is plotted as a function of enzyme concentration.*

Comparison of retroviral and retrotransposon RNase H

The recent availability of active, recombinant RT/RNase H from the *Saccharomyces cerevisiae* retrotransposons Ty1[90] and Ty3[68,91] facilitates comparison to their retroviral counterparts in order to understand the evolution of a multi-functional RT/RNase H. Using phylogenetic analysis, Malik and Eickbush[68] documented that while the DNA polymerase domain of RTs from retroviruses, Ty3/*gypsy* elements and caulimoviruses are similar, their C-terminal RNase H domains are only distally related. More importantly, after alignment of conserved active site carboxylates, the flexible *His-loop* of bacterial and retroviral RNases H is absent from LTR-containing retrotransposon enzymes. In fact, the histidine of the conserved retroviral His-loop aligns with tyrosine in Ty3 (Fig. 7) and other related retrotransposons, suggesting the possibility of an alternative catalytic mechanism. This tyrosine is absolutely essential for Ty3, as we demonstrated that its alteration is lethal for transposition[47]. Since both tyrosine and histidine have been identified at or near the equivalent position in Ty1 RT, the role of these residues remains to be elucidated.

Related studies in our laboratory have uncovered several notable differences between retroviral and retrotransposon RNase H. While the two-step hydrolytic mechanism of HIV-1 RT cuts at –17 and –8 as described above, and these two modes of hydrolysis were demonstrated for purified Ty3 RT/RNaseH, initial cleavage occurs at position –21, and proceeds only as far as 13 bp of the polymerase catalytic center[47,91]. Since the remaining 13 bp DNA/RNA hydrolysis product generated by Ty3 RNase H would be significantly more stable, simple dissociation of the substrate may not define the limits of RNase H-mediated hydrolysis. Interestingly, the sites of initial and final cleavage in both cases are separated by approximately one helical turn (–17 and –8 for HIV-1 RT vs –21 and –13 for Ty3 RT). These two observations can perhaps be rationalized with a highly speculative scenario: while the polymerase domain remains fixed on the primer 3´ OH, other domains of the enzyme may breath, allowing the RNaseH domain to move approximately one helical turn and cleave the RNA, after which the strained complex reverts to its original conformation.

Finally, an intriguing feature of retroviral and retrotransposon RNases H has resulted from studies on processing of their PPT primers. While we have demonstrated that both HIV-1 and Ty3 RTs will correctly process their cognate PPTs[73,91] from (+) DNA and (+) RNA sequences, Ty3 RT fails to process (+) DNA from the HIV-1 PPT, and incorrectly processes (+) RNA from the same sequence. In contrast, HIV-1 RT hydrolyzes model Ty3 PPTs with an apparent lack of specificity. Based on sequence divergence of the respective PPTs (5´-AAAAGAAAAGGGGGG-3´ for HIV-1 vs 5´- GAGAGAGAGGAAG-3´ for Ty3), these observations provide a strong argument for co-evolution of enzyme and substrate, and merit further evaluation.

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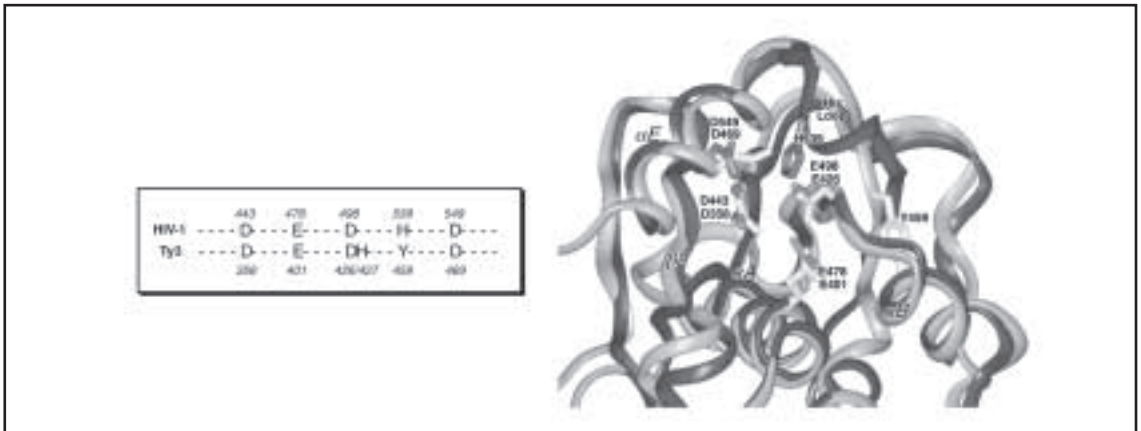

Figure 7. *Spatial distribution of conserved residues constituting the active centers the HIV-1 and Ty3 RTRNase H. Alignment of catalytic residues between HIV-1 and Ty3 RTs is shown at the top[91]. A partial superimposition of the peptide backbones of each domain is illustrated. HIV structural elements and amino acid numbering is dark grey, while those of Ty3 are in light gray. Note the absence of the flexible "His-loop" in the Ty3 enzyme. Reprinted with permission from reference[47].*

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