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RNase H Activity: Structure, Specificity, and Function in Reverse Transcription

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Abstract

This review compares the well-studied RNase H activities of human immunodeficiency virus, type 1 (HIV-1) and Moloney murine leukemia virus (MoMLV) reverse transcriptases. The RNase H domains of HIV-1 and MoMLV are structurally very similar, with functions assigned to conserved subregions like the RNase H primer grip and the connection subdomain, as well as to distinct features like the C-helix and loop in MoMLV RNase H. Like cellular RNases H, catalysis by the retroviral enzymes appears to involve a two-metal ion mechanism. Unlike cellular RNases H, the retroviral RNases H display three different modes of cleavage: internal, DNA 3' end-directed, and RNA 5' end-directed. All three modes of cleavage appear to have roles in reverse transcription. Nucleotide sequence is an important determinant of cleavage specificity with both enzymes exhibiting a preference for specific nucleotides at discrete positions flanking an internal cleavage site as well as during tRNA primer removal and plus-strand primer generation. RNA 5' end-directed and DNA 3' end-directed cleavages show similar sequence preferences at the positions closest to a cleavage site. A model for how RNase H selects cleavage sites is presented that incorporates both sequence preferences and the concept of a defined window for allowable cleavage from a recessed end. Finally, the RNase H activity of HIV-1 is considered as a target for anti-virals as well as a participant in drug resistance.

Keywords

RNase H; reverse transcriptase; human immunodeficiency virus; type 1 (HIV - 1); Moloney murine leukemia virus (MoMLV); reverse transcription; polypurine tract (PPT)

1. Introduction

Reverse transcription is performed by the retroviral enzyme called reverse transcriptase. This multifunctional enzyme carries out RNA-dependent DNA polymerization, DNA-dependent DNA polymerization, strand displacement synthesis, strand transfers, and degrades the RNA strand in RNA/DNA hybrids. To perform these diverse functions, reverse transcriptase uniquely combines two distinct enzymatic activities, a DNA polymerase activity that uses RNA or DNA as a template, and an RNase H activity that cleaves the RNA strand of an RNA/DNA hybrid (Gilboa et al., 1979). These activities are localized in two separate protein domains. The polymerase domain comprises the N-terminal two-thirds of reverse transcriptase, while the

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RNase H domain is the C-terminal one-third [reviewed in (Champoux, 1993; Telesnitsky and Goff, 1993a)]. Mutations that inactivate the functions of either domain result in a retrovirus incapable of replication (Tanese and Goff, 1988; Telesnitsky and Goff, 1993b).

The utilization of an RNase H activity during retroviral replication represents a unique strategy to copy a single-stranded RNA genome into a double-stranded DNA, since the minus-strand DNA remains base-paired to the retrovirus genome in the first cycle of DNA synthesis. The RNase H activity is essential in several aspects of reverse transcription [reviewed in (Champoux, 1993; Arts and LeGrice, 1998; Rausch and Le Grice, 2004)]. This review examines and compares the structures, activities, and functions of the human immunodeficiency virus, type 1 (HIV-1) and Moloney murine leukemia virus (MoMLV) RNases H, and considers the prospects of targeting the RNase H activity of HIV-1 by anti-viral drugs, as well as the role of RNase H in drug resistance.

2. RNase H in Reverse Transcription

Polymerization-dependent RNase H activity occurs during minus-strand synthesis and initiates the degradation of the RNA genome. Secondary structures in the template such as hairpins can cause pausing by reverse transcriptase, and such pauses promote RNase H cleavages and facilitate strand transfers (Luo and Taylor, 1990; DeStefano et al., 1992; Peliska and Benkovic, 1992; DeStefano et al., 1994a; Lanciault and Champoux, 2006). However, the polymerization rate of reverse transcriptase is greater than the hydrolysis rate of the enzyme and the polymerization-dependent RNase H activity is insufficient to completely degrade the genomic template (DeStefano et al., 1991b; Dudding et al., 1991; Kati et al., 1992; DeStefano et al., 1994b; Gotte et al., 1995; Kelleher and Champoux, 2000).

The polymerization-independent RNase H activity participates in removal of genomic RNA that remains annealed to the minus-strand DNA. This synthesis-independent degradation of the RNA genome, along with displacement synthesis, enables efficient synthesis of the plus-strand DNA (DeStefano et al., 1991b; DeStefano et al., 1992; Gopalakrishnan et al., 1992; DeStefano et al., 1994b; Fuentes et al., 1996; Smith et al., 1999; Kelleher and Champoux, 2000; Schultz et al., 2004). Polymerization-independent cleavages by RNase H can also generate the polypurine tract (PPT) primer and remove the extended tRNA and PPT primers. Numerous studies have shown that these cleavages require recognition of specific sequences by the enzyme [for example, see (Finston and Champoux, 1984; Rattray and Champoux, 1987; Huber and Richardson, 1990; Luo et al., 1990; Pullen and Champoux, 1990; Furfine and Reardon, 1991a; Pullen et al., 1992; Smith and Roth, 1992; Fuentes et al., 1995; Schultz et al., 1995; Powell and Levin, 1996)]. In addition, the RNase H activity assists the initiation of plus-strand synthesis, since the PPT primer is not extended efficiently unless a gap is generated by cleavage at several sites that are located downstream of the PPT (Schultz et al., 2003).

The activity and specificity of RNase H must be finely tuned during reverse transcription [for example, see (Kotewicz et al., 1988; Julias et al., 2002; Rausch et al., 2002; Purohit et al., 2005)]. Excessive uncontrolled degradation of the template RNA could cause the primer strand to dissociate from the template strand and terminate synthesis. Insufficient RNase H activity could allow remaining RNA fragments to slow synthesis of the plus-strand DNA, or interfere with specific cleavages like PPT primer generation or primer removal to generate improper LTR ends. Because of its multiple essential roles in reverse transcription, the RNase H activity of HIV-1 reverse transcriptase is an excellent target for anti-virals.

3. Structure of HIV-1 and MoMLV RNase H domains

While the first reverse transcriptase to be purified and biochemically characterized was avian (Molling et al., 1971), the majority of structure-function studies have focused on the reverse

transcriptases of mammalian retroviruses. Of these, the two most prominently studied enzymes are the reverse transcriptases of HIV-1 and MoMLV. HIV-1 is heterodimer of p66 and p51 (di Marzo Veronese et al., 1986). The p51 subunit is proteolytically derived from the p66 subunit and lacks the C-terminus containing the RNase H domain. MoMLV reverse transcriptase is found as a 76 kDa monomer (Moelling, 1974; Roth et al., 1985; Das and Georgiadis, 2004). Co-crystal structures of HIV-1 reverse transcriptase with duplex substrate have indicated that the distance between the polymerase and RNase H active sites is 17 nucleotides for the DNA/DNA substrate (Jacobo-Molina et al., 1993; Huang et al., 1998) and 18 nucleotides for the RNA/DNA hybrid (Sarafianos et al., 2001). The substrate-dependent difference in the distances between the active sites of reverse transcriptase is likely nucleic acid-dependent, which matches with biochemical observations (Gotte et al., 1998). Figure 1 shows the co-crystal structure of HIV-1 reverse transcriptase with a PPT-containing RNA/DNA primer template (Sarafianos et al., 2001).

The RNases H of HIV-1 and MoMLV, both alone as a domain and in the holoenzyme of reverse transcriptase, exhibit very comparable tertiary folding (Davies et al., 1991; Kohlstaedt et al., 1992; Lim et al., 2006). An excellent comparison of the three dimensional structures of these two RNase H domains is presented in an accompanying article of this special issue [see panel B, Figure 6, in (Coté and Roth, 2008)]. Also, comparisons to the structurally similar *E. coli*, *B. halodurans*, and most recently, human RNases H have proven highly informative regarding the structures and functions of the HIV-1 and MoMLV RNase H domains (Yang et al., 1990; Jacobo-Molina et al., 1993; Huang et al., 1998; Sarafianos et al., 2001; Das and Georgiadis, 2004; Nowotny et al., 2005; Lim et al., 2006; Nowotny et al., 2007). The MoMLV RNase H domain consists of 5 β -strands and 5 α -helices, whereas the HIV-1 RNase H domain lacks one of the α -helices (the positively charged C-helix; see section 3.2), but is otherwise similar. Notably, the MoMLV RNase H has a network of hydrophobic residues that interact in the N-terminal portion of the enzyme while the RNases H of HIV-1 and *E. coli* have less hydrophobic residues in this region and are more comparable to each other in this regard (Lim et al., 2002). Recent linker-scanning analyses of the MoMLV connection and RNase H domains have found that only the very C-terminus contains non-essential regions not required for viral replication (Puglia et al., 2006), indicating that the intact RNase H structure is important for function.

Crystallographic studies with isolated RNase H domains have revealed the orientation of the four conserved acidic amino acid residues in the active sites of the HIV-1 and MoMLV RNases H (indicated in Figure 1) (Davies et al., 1991; Sarafianos et al., 2001; Lim et al., 2006). One to two divalent cations are bound in the RNase H active site region, and the coordination of these cations participates in primer-template binding by reverse transcriptase and catalysis [see section 4; (Cristofaro et al., 2002)]. In the co-crystal structure of the HIV-1 holoenzyme, the RNase H domain binds the minor groove of the RNA/DNA substrate and directly contacts both the DNA and RNA strands (Sarafianos et al., 2001). In the only co-crystal structure of MoMLV reverse transcriptase published to date, the DNA is poorly ordered (Das and Georgiadis, 2004), so residues proposed to contact the substrate have been derived by modeling and comparisons to the bacterial and HIV-1 co-crystal structures (Yang et al., 1990; Jacobo-Molina et al., 1993; Huang et al., 1998; Sarafianos et al., 2001; Nowotny et al., 2005; Lim et al., 2006). Future modeling studies will benefit from the recently reported co-crystal structures of the human RNase H with 14, 18, and 20-mer RNA/DNA hybrids (Nowotny et al., 2007).

There are two subregions of the retroviral RNase H domain, the RNase H primer grip, and the C-helix and loop, which have distinct structural features and make significant contributions to the RNase H activity. In addition, the connection domain that joins the RNase H and polymerase domains has a demonstrable influence on RNase H activity. Analysis of these regions by

structure-based mutagenesis combined with biochemical or in vivo studies has helped to elucidate their functions.

3.1 RNase H primer grip

Based on the co-crystal structure of HIV-1 reverse transcriptase, the RNase H primer grip is defined as a region adjacent to the RNase H active site that contacts the nucleotides in the DNA primer strand which are base paired with RNA positions -4 to -9 relative to the scissile phosphate (designated as between the -1 and +1 nucleotides in the RNA strand) (Sarafianos et al., 2001) (indicated in Figure 1). The RNase H primer grip region is found in both the MoMLV and HIV-1 RNases H (Sarafianos et al., 2001). In the heterodimeric HIV-1 enzyme, these residues are Lys395 and Glu396 in p51, Gly359 and Ala360 in the p66 polymerase domain, His361 in the p66 connection domain, and Thr473, Asn474, Gln475, Lys476, Tyr501, and Ile505 in the p66 RNase H domain. In monomeric MoMLV, a similar region of the protein forms the primer grip and several of the residues found in the HIV-1 RNase H are conserved in MoMLV (Sarafianos et al., 2001; Lim et al., 2006). Superposition of the MoMLV RNase H structure on the HIV-1 reverse transcriptase co-crystal has revealed that residues Arg534, Ser557, Gln559, Arg560, Tyr586, Lys612, and Asn613 would contact the DNA primer strand at the -4 through -6 positions relative to the RNA strand scissile phosphate (Lim et al., 2006).

The RNase H primer grip contributes to proper binding and positioning of the substrate at both the DNA polymerase and RNase H active sites; consequently this region of the RNase H domain can influence both DNA polymerization and RNA degradation (Arion et al., 2002; Julias et al., 2002; Rausch et al., 2002; Zhang et al., 2002; Julias et al., 2003; McWilliams et al., 2006). Point mutations in the RNase H primer grip of HIV-1 reverse transcriptase decrease RNase H activity, alter RNase H cleavage specificity (including recognition of the PPT), and reduce the efficiency of strand transfer (Arion et al., 2002; Julias et al., 2002; Rausch et al., 2002; Julias et al., 2003; McWilliams et al., 2006). RNase H primer grip mutations also alter the efficiency of initiation of viral DNA synthesis (Julias et al., 2002).

Two contact residues in the RNase H primer grip have proven especially interesting. The first of these is Tyr501 in HIV-1 RNase H which contacts the phosphate backbone of the DNA nucleotides that are base paired with the -6 and -5 RNA nucleotides (Sarafianos et al., 2001; McWilliams et al., 2006). HIV-1 Tyr501 influences RNase H cleavage efficiency and specificity (Arion et al., 2002; Rausch et al., 2002; McWilliams et al., 2006), as substitutions at this position either eliminate RNase H activity completely, or significantly reduce activity and deleteriously affect PPT processing and strand transfer. Mutation of the equivalent residue, Tyr586, in MoMLV reverse transcriptase decreases RNase H activity substantially, increases substitution mutations near A tracts and also decreases the efficiency of the second strand transfer reaction during reverse transcription as compared to the wild enzyme (Zhang et al., 2002; Mbisa et al., 2005). In addition to emphasizing the importance of the RNase H primer grip in substrate binding, these latter observations suggest that this region additionally influences the fidelity of polymerization.

The second important contact residue in the RNase H primer grip is residue Gln475 in HIV-1 RNase H. Gln475 contacts the deoxyribose rings of the DNA nucleotides that are base paired with RNA positions -5 and -4, interacts with the RNA strand at the +1 position on the ribose ring, and additionally makes a base-specific contact at the -2 RNA position (Sarafianos et al., 2001). Mutagenesis studies have shown that Gln475 is important in RNase H cleavage specificity (Rausch et al., 2002). When mutations in Gln475 and/or Tyr501 are combined with mutations in the PPT sequence, both the specificity and rate of PPT cleavage are affected to a greater extent than when only the protein or the PPT are altered individually (McWilliams et

al., 2006), indicating that these mutations not only disrupt individual contacts between enzyme and substrate but also affect the structure of the enzyme-substrate complex.

Close to the RNase H primer grip and the active site, a region termed the phosphate binding pocket has been identified in the co-crystal structures of the *B. halodurans* and human RNases H (Nowotny et al., 2005; Nowotny et al., 2007). This pocket is formed by three (human) or four (*B. halodurans*) residues that contact the DNA strand on the phosphate between the -3 and -2 nucleotides relative to the scissile phosphate. Like the RNase H primer grip, the phosphate binding pocket is postulated to function in substrate recognition. Comparative modeling studies have tentatively identified a phosphate binding pocket in the RNase H domain of HIV-1 reverse transcriptase, but only two residues, Thr473 and Lys476, are involved (Nowotny et al., 2007). Notably in the co-crystal structure of HIV-1 reverse transcriptase, these two residues interact with the phosphate between the -5 and -4 nucleotides of the DNA strand, and there are no residues contacting the phosphate between the -3 and -2 nucleotides of the DNA strand, (Sarafianos et al., 2001). The equivalent residues in the MoMLV RNase H domain, Ser557 and Arg560, are conserved, and along with a third residue, Glu559, are modeled to interact with the phosphate between the -5 and -4 nucleotides of the DNA strand (Lim et al., 2006). Further experiments are required to determine whether the phosphate binding pocket exists in the retroviral RNases H, and to understand whether the phosphate binding pocket and the RNase H primer grip are overlapping or distinct regions.

3.2 C-helix and Loop

Comparative sequence alignments and crystal structures have shown that a positively charged α -helix (termed the C-helix) and loop are present in the MoMLV, human, and *E. coli* RNases H but are missing in HIV-1, *B. Halodurans*, and avian sarcoma-leukosis virus RNases H (Johnson et al., 1986; Jacobo-Molina, 1993 #248; Yang et al., 1990; Davies et al., 1991; Katayanagi et al., 1993; Nowotny et al., 2005; Nowotny et al., 2007). Functional studies with *E. coli* RNase H first suggested that the C-helix contributes to efficient substrate binding (Kanaya et al., 1991).

Several observations argue that the C-helix in the MoMLV reverse transcriptase has structurally important roles in the activity and specificity of RNase H. Elimination of the C-helix in MoMLV reverse transcriptase yields a form of reverse transcriptase that has little RNase H activity in vitro and a virus that is replication defective (Telesnitsky et al., 1992; Lim et al., 2002). Mutation of individual residues in the C-helix that are predicted to be solvent exposed and contact the RNA/DNA substrate results in defects in minus-strand and plus-strand priming as well as primer removal (Lim et al., 2002).

3.3 Connection domain

Residues in the connection domain (of p66 and p51) located between the RNase H and polymerase domains of HIV-1 (see Figure 1) make specific contacts with the substrate (Sarafianos et al., 2001) and have subtle but distinct roles in the activity of RNase H. Most of these contacts are with the DNA strand (Lys395 and Glu396 in p51; Ala360, His361 and Thr362 in p66) but one is with the RNA strand (Lys390 in p51). Interestingly, His361 contacts the phosphate backbone of the DNA primer strand close to Tyr501 of the RNase H primer grip, and substitution of alanine for His361 significantly decreases HIV-1 RNase H activity in vitro and viral titers in vivo (Julias et al., 2003). Mutagenesis of other residues in the connection domain that contact the substrate appear to decrease but not eliminate the RNase H activity of HIV-1 reverse transcriptase (Julias et al., 2003). Modeling studies comparing the C-helix of the MoMLV and *E. coli* RNases H with the connection domain of the p66 subunit of HIV-1 reverse transcriptase indicate that the p66 connection domain has positively charged residues that are more widely spaced, closer to the substrate, and may partially substitute for the substrate

binding properties conferred by the C-helix in the MoMLV and *E. coli* enzymes (Lim et al., 2006).

Modeling studies that superimpose the connection domain of MoMLV reverse transcriptase on the corresponding domain in the p66 subunit of HIV-1 reverse transcriptase reveal that the MoMLV connection domain has a similar overall architecture to the HIV-1 polypeptide but there are significant structural differences (Das and Georgiadis, 2004). Most notably, the polypeptide region in MoMLV that joins the connection domain to the RNase H domain extends away on opposite ends of the connection domain. Also, MoMLV reverse transcriptase has an additional 32 residues that join the connection and RNase H domains which are absent in HIV-1 reverse transcriptase. It has been suggested that this linking region might provide conformational flexibility to the RNase H domain in the MoMLV enzyme (Das and Georgiadis, 2004). Linker-scanning analysis of the MoMLV connection and RNase H domains found no region within the connection domain that can tolerate insertions as measured by viability of virus, indicating that the connection domain has an essential role in viral replication (Puglia et al., 2006). However, mutations of specific residues in the MoMLV connection domain remain to be tested.

3.4 Future crystal structures

The structure of the HIV-1 RNase H domain has been determined from crystals of the intact enzyme, from the isolated RNase H domain, and from co-crystals of HIV-1 reverse transcriptase with substrate (Arnold et al., 1992; Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993; Huang et al., 1998; Sarafianos et al., 2001). While the HIV-1 reverse transcriptase is complexed with a PPT-containing hybrid in a co-crystal, the substrate is not bound for proper cleavage of the PPT to generate the plus-strand primer, and there are unusual features in the base-pairing, including mismatches and unpaired bases, and thus it seems unlikely that this reflects the typical interaction of HIV-1 reverse transcriptase with an RNA/DNA substrate (Sarafianos et al., 2001). RNA/DNA hybrids containing the PPT apparently assume a unique structure that differs from other RNA/DNA hybrids (Fedoroff et al., 1993; Salazar et al., 1993; Powell and Levin, 1996; Fedoroff et al., 1997). Moreover, chemical footprinting analyses with the HIV-1 PPT-U3 junction have shown that the structural distortions observed in the PPT in the co-crystal structure mentioned above do not require the binding of enzyme (Kvaratskhelia et al., 2002). Finally, superimposing the HIV-1 reverse transcriptase on the recent co-crystal structure of human RNase H containing an RNA/DNA hybrid indicates that the substrate does not simultaneously associate with the active sites of the HIV-1 polymerase and RNase H domains (see section 5) (Nowotny et al., 2007). The determination of additional structures of HIV-1 reverse transcriptase with non-PPT-containing as well as PPT-containing substrates will facilitate a better understanding of how the HIV-1 RNase H domain interacts with hybrid duplexes.

Crystal structures that include the RNase H domain of MoMLV have proven challenging to resolve, likely in part due to the C-helix. In the co-crystal structure of the full length MoMLV reverse transcriptase, the RNase H domain exhibited a high degree of disorder and the DNA substrate was evident but poorly ordered, so no detailed determination of the interaction between the substrate and the murine enzyme has yet been achieved (Das and Georgiadis, 2004). Moreover, the successful structure of the isolated MoMLV RNase H domain was obtained only when the C-helix was removed (Lim et al., 2006). Thus, there remain portions of the MoMLV RNase H domain without structural definition. While much has been learned from structure-based mutagenesis studies that rely on models and comparisons to predict how the MoMLV RNase H domain would interact with the substrate, co-crystal structures of MoMLV reverse transcriptase that clearly resolve the primer-template strands will be invaluable. For example, attempts to model the primer-template into the MoMLV RNase H

structure based on HIV-1 and Taq polymerase have been unsuccessful, suggesting that the trajectories of the substrates are different in these enzymes (Das and Georgiadis, 2004). Also, it appears that the RNase H domain of MoMLV has additional conformational flexibility within the connection domain that may impact substrate interactions and activity (Das and Georgiadis, 2004).

4. Enzyme activity

The RNase H activity of reverse transcriptase acts as an endonuclease that hydrolyzes the RNA strand in an RNA/DNA hybrid to generate 5' phosphate and 3' hydroxyl ends (Krug and Berger, 1989; DeStefano et al., 1991a; Champoux, 1993). The isolated MoMLV RNase H domain retains enzymatic activity (Tanese and Goff, 1988), but is unable to carry out specific cleavages such as removal of the tRNA or PPT primers in vitro (Schultz and Champoux, 1996; Zhan and Crouch, 1997). The specificity of the isolated murine RNase H domain can be restored in *trans* by adding an isolated MoMLV polymerase domain (Schultz and Champoux, 1996). The isolated RNase H domain of HIV-1 is inactive, but nuclease activity is reconstituted by introducing the p51 subunit, by adding the thumb and connection subdomains, or by various N-terminal fusions on the RNase H domain (Evans et al., 1991; Hostomsky et al., 1991; Smith and Roth, 1992; Smith and Roth, 1993; Smith et al., 1994; Smith et al., 1997; Smith et al., 1998), including the C-helix of the *E. coli* RNase H (Stahl et al., 1994; Keck and Marqusee, 1995).

Several studies have shown that the polymerase domain of reverse transcriptase is required for the proper substrate interactions and specificity of RNase H in the intact enzyme (Evans et al., 1991; Hostomsky et al., 1991; Smith and Roth, 1993; Smith et al., 1994; Schultz and Champoux, 1996). Multiple individual residues in the polymerase domain contribute to proper positioning of the substrate and influence RNase H activity and specificity. For example, substitution of alanine or threonine for Try266 in the thumb subdomain of HIV-1 reverse transcriptase yields an enzyme unable to generate the PPT primer or remove an extended PPT primer (Gao et al., 1998; Powell et al., 1999). Also, some substitutions at residue Phe61 of the HIV-1 fingers subdomain that contacts the template strand downstream of the primer terminus (Huang et al., 1998) are defective in specific generation and removal of the PPT primer (Mandal et al., 2006). However, not all polymerase domain residues that contact the substrate influence the RNase H activity, as mutagenesis of a different putative downstream-contacting residue, Tyr64, in the fingers subdomain of MoMLV reverse transcriptase has no impact on RNase H activity (Paulson et al., 2007).

Divalent metal ions are required for RNase H activity (Cirino et al., 1995). The active sites of the MoMLV and HIV-1 RNases H contain four highly conserved acidic residues (three aspartate, one glutamate) that likely coordinate the binding of two divalent metal cations that are essential for catalysis (Davies et al., 1991; Huang et al., 1998; Lim et al., 2006). The coordination of the divalent cations in the RNase H active site has an important role in stabilizing primer-template binding (Cristofaro et al., 2002). In a co-crystal structure of full length HIV-1 reverse transcriptase in a ternary complex containing substrate and the incoming dNTP, a single Mg^{2+} ion was observed in the RNase H active site (Huang et al., 1998). The similar RNase H domain of *E. coli* binds one (Katayanagi et al., 1993) or two (Goedken and Marqusee, 2001) divalent cations. Numerous studies have considered one-metal ion and two-metal ion mechanisms for catalysis by RNase H [for example, see (Davies et al., 1991; Katayanagi et al., 1993; Oda et al., 1993; Cowan et al., 2000; Goedken and Marqusee, 2001; Cristofaro et al., 2002; Klumpp et al., 2003; Pari et al., 2003)]. However, the most recent co-crystal structures of the *B. halodurans* and human RNases H with bound substrate strongly suggest that RNases H, including the retroviral enzymes, utilize a two-metal ion mechanism of catalysis (Figure 2) (Nowotny et al., 2005; Nowotny and Yang, 2006; Yang et al., 2006;

Nowotny et al., 2007). In this mechanism, the first metal ion A activates the nucleophilic water molecule and the second metal ion B, possibly in conjunction with metal ion A, stabilizes the transition state intermediate.

In the absence of other specificity determinants on an RNA/DNA hybrid (see below), the RNase H activities of human and murine retroviral reverse transcriptases preferentially cleave between two ribonucleotide residues in an RNA chain, and between the penultimate and last ribonucleotide of an extended RNA primer rather than precisely at the RNA-DNA junction (Schultz et al., 2000). This preference is reflected in incomplete removal of the extended tRNA primer for HIV-1, where a ribo A is left at the 5' end of the minus strand, and for MoMLV, where the 5' ends of minus strands are found both with and without a ribo A (Furfine and Reardon, 1991a; Pullen et al., 1992; Smith and Roth, 1992; Schultz et al., 1995). A single ribonucleotide is also left by MoMLV RNase H after cleavage of extended non-PPT RNA primers (Schultz et al., 2000). However, precise cleavage occurs at the RNA-DNA junction of the extended PPT primer for both the murine and human retroviral RNases H [for example, see (Rattray and Champoux, 1989; Huber and Richardson, 1990; Randolph and Champoux, 1994; Gotte et al., 1999; Schultz et al., 2000)]. In this case the preference for the RNA-DNA junction likely reflects the high specificity directing the cleavage event responsible for creating the PPT primer. Thus, although the enzyme is not prohibited from cleaving at an RNA-DNA junction, with the exception of the extended PPT, reverse transcriptase exhibits a strong preference for cleaving one nucleotide away from the junction.

5. Substrate Interactions

Nuclease footprinting studies have shown that HIV-1 reverse transcriptase associates with template nucleotides from +7 to -23 and primer nucleotides from -1 to -25 (Wohrl et al., 1995a). The MoMLV reverse transcriptase makes contacts from -26 to +9 nucleotides on the non-template strand and from -27 to +7 nucleotides on the template strand (Wohrl et al., 1995b; Winshell and Champoux, 2001). Reverse transcriptase appears to make more extensive contacts with an RNA/DNA substrate than with a DNA/DNA substrate (Jacobo-Molina et al., 1993; Huang et al., 1998; Sarafianos et al., 2001). In the co-crystal structure with RNA/DNA, the majority of contacts are near the polymerase active site of reverse transcriptase and most of these involve the sugar-phosphate backbone of the substrate, with some base-specific contacts observed as well (Sarafianos et al., 2001).

The interactions between reverse transcriptase and its substrate determine the positioning of the RNase H domain for cleavage. In addition to the RNase H primer grip, primer-template binding models based on crystal structures describe a primer-template binding cleft (with fingers, palm, thumb, and connection subdomains) and a primer grip in the polymerase domain (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993; Ding et al., 1998; Sarafianos et al., 2001). A conformational change in the position of the thumb subdomain occurs when the enzyme binds the primer-template, and a second conformational change occurs upon dNTP binding to form the ternary complex in which the fingers subdomain moves closer to the active site and the thumb subdomain more tightly clamps around the DNA (Huang et al., 1998). Whether this latter conformational change affects RNase H activity and/or specificity, or whether the RNase H domain undergoes conformational changes when associating with substrate remains unknown. Based on the recent co-crystal structure of human RNase H with an RNA/DNA hybrid, modeling studies reveal that the hybrid substrate from this structure would have to be bent by a minimum of ~35° to be accommodated in the substrate-binding cleft of HIV-1 reverse transcriptase (Nowotny et al., 2007). Moreover, it has been suggested that this bent substrate cannot engage both the polymerase and RNase H active sites at the same time. According to this intriguing proposal, the bound substrate must "toggle" between the two

active sites, perhaps by a shift in the position of the substrate in the binding cleft or by a change in substrate conformation (Nowotny et al., 2007).

Three distinct types or modes of RNase H cleavages have been described based on how reverse transcriptase interacts with RNA/DNA hybrids. These cleavage modes are referred to as DNA 3' end-directed, RNA 5' end-directed, and internal (Figure 3).

5.1 DNA 3' end-directed cleavage

To carry out DNA 3' end-directed cleavage, the polymerase domain of reverse transcriptase preferentially associates with a recessed DNA 3' primer terminus annealed to a longer RNA strand, positioning the RNase H domain approximately 15 to 20 nucleotides away from the recessed end for cleavage on the RNA strand (Furfine and Reardon, 1991b; Gopalakrishnan et al., 1992; Kati et al., 1992; DeStefano et al., 1994b) (Figure 3, part A). This type of cleavage can occur during polymerization on an RNA template, as well as in the absence of DNA synthesis by the polymerization-independent RNase H activity. The frequency of RNase H cleavages during polymerization as measured in the presence of a trap varies depending on the source of the enzyme. For avian myeloblastosis virus (AMV) reverse transcriptase, it appears that the RNA strand is cleaved only once for every 100–200 nucleotides synthesized, whereas for HIV-1 and MoMLV the cleavage frequency centers on 100–120 nucleotides (DeStefano et al., 1991b). For HIV-1 reverse transcriptase, but not the AMV enzyme, smaller products with average lengths of 7 nucleotides have also been detected in the presence of a trap (DeStefano et al., 1994b). Together, these results suggest that during reverse transcription, RNase H cleavage concomitant with synthesis of the minus strand is insufficient by itself to completely degrade the genome RNA to fragments small enough to spontaneously dissociate from the nascent DNA strand (Kelleher and Champoux, 2000). As expected, DNA 3' end-directed cleavages correlate with pause sites during polymerization (Suo and Johnson, 1997a; Suo and Johnson, 1997b). The two important determinants of DNA 3' end-directed cleavages are distance from the recessed DNA 3' end and the nucleotide sequence in the vicinity of the cleavage site (see section 6.1).

5.2 RNA 5' end-directed cleavage

The polymerase domain of reverse transcriptase will also preferentially bind the 5' end of a recessed RNA annealed to a DNA strand (DeStefano et al., 2001). The resulting form of cleavage is termed RNA 5' end-directed because the recessed RNA 5' end serves to position the polymerase active site on the DNA strand near the RNA 5' end (Figure 3, part B). This positioning places the RNase H domain on the RNA strand such that cleavages occur approximately 13 to 19 nucleotides from the RNA 5' end (for example, see (Schatz et al., 1990; Wohrl and Moelling, 1990; Gopalakrishnan et al., 1992; DeStefano et al., 1993; Gotte et al., 1995; Palaniappan et al., 1996; Gao et al., 1997; Gao and Goff, 1998). RNA 5' end-directed cleavages represent a polymerization-independent form of RNase H activity. Notably, RNA 5' end-directed cleavages have been reported as close as 7 nucleotides and as far as 21 nucleotides from the RNA 5' end (Fu and Taylor, 1992; Ben-Artzi et al., 1993; DeStefano et al., 1993; DeStefano, 1995; Wisniewski et al., 2000b; Wisniewski et al., 2000a; Wisniewski et al., 2002), possibly reflecting a tendency for the enzyme to slide after the initial binding event (see below).

Three determinants have been identified for RNA 5' end-directed cleavages. Similar to DNA 3' end-directed cleavages, two of these determinants are distance from the recessed end (DeStefano et al., 1993), and the nucleotide sequence in the vicinity of the cleavage site [see section 6.1; (Furfine and Reardon, 1991b; Fu and Taylor, 1992; DeStefano et al., 1993; Schultz et al., 2006)]. The third determinant is the accessibility of the end. Thus, while the 5' end at a nick is not recognized by reverse transcriptase for RNA 5' end-directed cleavages, a gap of 2

or more bases is sufficient for such recognition and cleavage by the HIV-1 and MoMLV enzymes (Schultz et al., 2004; Schultz et al., 2006).

In other studies, RNA 5' end-directed and associated cleavages have been examined with regard to their temporal relationships (Wisniewski et al., 2000b; Wisniewski et al., 2000a; Wisniewski et al., 2002). In these kinetic studies based primarily on one RNA sequence, a primary 5' end-directed cleavage occurred 18 nucleotides from the RNA 5' end and was followed by independent, secondary cleavages that were either 7 to 10 nucleotides from the RNA 5' end or by additional cuts that occurred 4 to 7 nucleotides from the RNA 3' end. The observed secondary cleavages were proposed to result from an initial binding followed by extensive sliding of the enzyme on a hybrid substrate. These kinetic observations would explain the stepwise degradation of an RNA strand in a hybrid that begins from a recessed RNA 5' end, but do not address the influence of sequence on RNA 5' end-directed cleavages.

5.3 Internal cleavage

Reverse transcriptase can also cleave internally on an RNA/DNA hybrid (Figure 3, part C). Internal cleavage is synthesis-independent and does not involve positioning by either a DNA 3' end or an RNA 5' end (Finston and Champoux, 1984; Fuentes et al., 1995; Schultz et al., 2000; Schultz et al., 2003; Schultz et al., 2004). The most important determinant of internal cleavage appears to be the RNA sequence in the vicinity of the cleavage site [(Schultz et al., 2004); see section 6.1].

5.4 Use of different binding modes in reverse transcription

It is important to consider when during reverse transcription the RNase H activity of reverse transcriptase would carry out each of the three modes of cleavages depicted in Figure 3. Such information would be valuable in choosing anti-virals for RNase H and for designing assays for drug screening (see section 7).

During minus-strand DNA synthesis, polymerization-dependent RNase H activity would initiate genome degradation through DNA 3' end-directed cleavages that produce nicks and small gaps in the RNA template but as mentioned previously this is probably insufficient to completely degrade the genome RNA (DeStefano et al., 1991b; Kati et al., 1992; DeStefano et al., 1994b; Kelleher and Champoux, 2000). Polymerization-dependent RNase H cleavages correlate with pause sites during RNA-dependent polymerization (Suo and Johnson, 1997a; Suo and Johnson, 1997b). Pause sites occur when polymerization by reverse transcriptase encounters a secondary structure such as a hairpin on the template strand, and remains associated but stalled on the template. This pausing by the polymerase activity can increase RNase H cleavages and promote template switching during synthesis (Roda et al., 2002; Roda et al., 2003; Lanciault and Champoux, 2006; Purohit et al., 2007). However, there are no dramatic pause sites in the vicinity of the MoMLV PPT (Randolph and Champoux, 1994; Wu et al., 1996) and thus it appears unlikely that DNA 3' end-directed cleavages concomitant with minus-strand synthesis generate the murine virus PPT primer. It remains to be determined whether HIV-1 reverse transcriptase generates the PPT primer by a DNA 3' end-directed cleavage at a pause site during synthesis or by a polymerization-independent internal RNase H cleavage event.

To facilitate plus-strand DNA synthesis and strand transfers, internal and RNA 5' end-directed cleavages are most likely responsible for additional degradation of the viral genome (DeStefano et al., 1991b; Gopalakrishnan et al., 1992; DeStefano et al., 1994b; Kelleher and Champoux, 2000). Internal RNase H cleavages most likely occur initially because reverse transcriptase can bind and cleave the genome without nucleic acid end-directed positioning. Multiple internal cleavage sites situated close together would generate the 2–3 base gaps that are sufficient to

allow the kinetically favored RNA 5' end-directed cleavages to proceed (Schultz et al., 1999; Schultz et al., 2006).

Cleavages to generate the PPT primer are sequence specific (Pullen et al., 1993; Powell and Levin, 1996; Klarmann et al., 1997) and can occur internally on a RNA/DNA hybrid without DNA synthesis (Huber and Richardson, 1990; Luo and Taylor, 1990; Wohrl and Moelling, 1991) or end-directed binding (Finston and Champoux, 1984; Fuentes et al., 1995; Schultz et al., 2000). While the MoMLV reverse transcriptase readily generates the PPT primer by internal cleavages (Schultz et al., 2000), the HIV-1 enzyme is less efficient in this mode (see section 6.3; (Wohrl and Moelling, 1990; Fuentes et al., 1995; Powell et al., 1999; Schultz et al., 1999); Schultz, Zhang and Champoux, unpublished). The PPT primer is not extended efficiently by displacement synthesis at a nick, but is extended if there is a gap immediately downstream of the primer. Therefore, it appears that RNase H cleavage at the several internal cleavages sites located just downstream of the PPT facilitate extension of the plus-strand primer (Schultz et al., 2003).

Most likely, polymerization-independent RNase H activity is also responsible for removal of the extended tRNA and PPT primers (Furfine and Reardon, 1991a; Pullen et al., 1992; Smith and Roth, 1992; Pullen et al., 1993; Schultz et al., 1995; Powell and Levin, 1996; Klarmann et al., 1997; Schultz et al., 1999; Schultz et al., 2000). After extending the PPT primer, plus-strand synthesis continues until the first 18 bases of the tRNA primer are copied. Further extension is temporarily blocked by a methylated base 18 nucleotides into the tRNA template. At this point, the predominant RNase H cleavage site for tRNA removal which is located one nucleotide away from the tRNA-DNA junction is positioned appropriately for DNA 3' end-directed cleavage (i.e. 17 nucleotides from the nascent DNA 3' end) and importantly the sequences of the extended HIV-1 and MoMLV tRNA primers contain preferred nucleotides at the correct position for cleavage (see section 6.2 and Figure 5). With regard to the length of the extended DNA required for PPT primer removal, cleavage at the RNA-DNA junction of the extended PPT primer requires at least 2 to 3 deoxynucleotides on the PPT primer 3' end (Gotte et al., 1999; Schultz et al., 2000). PPT primer removal could occur by an internal or possibly an RNA 5' end-directed cleavage after extension and is likely directed by the same sequence features responsible for generating the PPT primer (Rattray and Champoux, 1989; Huber and Richardson, 1990; Randolph and Champoux, 1994; Fuentes et al., 1995; Powell and Levin, 1996; Schultz et al., 2000). The sequence specificities relating to the tRNA and PPT primers are considered more extensively below in section 6.

6. Cleavage specificity of RNase H

6.1 Nucleotide preferences

Statistical analyses of nucleotide frequencies in the sequence surrounding mapped internal cleavage sites recognized by HIV-1 and MoMLV RNases H reveal that preferred nucleotides are located at certain positions both upstream and downstream of the scissile phosphate (located between the -1 and +1 nucleotides) (Figure 4, blue) (Schultz et al., 2004). All of the preferred nucleotides that comprise an RNase H cleavage site fall within the extensive enzyme-substrate contacts that were observed in the co-crystal structure of HIV-1 reverse transcriptase complexed with a PPT-containing hybrid (Sarafianos et al., 2001) and by nuclease footprinting studies with both enzymes (Wohrl et al., 1995a; Wohrl et al., 1995b; Winshell and Champoux, 2001). For HIV-1, the base preferences span 15 nucleotides, and are located at six positions, -14, -12, -7, -4, -2, and +1, relative to the cleavage site. For MoMLV, the preferences cover 12 nucleotides, and are located at four positions, -11, -6, -2, and +1. While both enzymes exhibit similar preferences at positions +1 and -2, other position preferences appear distinct.

For RNA 5' end-directed cleavages, similar statistical analyses of sequences surrounding cleavage sites have shown essentially the same preference at the +1 and -2 positions for the MoMLV and HIV RNases H, and at the -4 position for HIV-1 as was observed for internal cleavage (note that in this case a C is also allowed at +1) [Figure 4, violet; (Schultz et al., 2006)]. Unlike internal RNase H cleavages, there are no preferred positions further upstream of the cleavage sites in the RNA 5' end-directed mode. This likely emphasizes the importance of the RNA 5' end itself as a specificity determinant, which may substitute for additional upstream nucleotides. DNA 3' end-directed cleavage sites appear to share the same nucleotide preferences as RNA 5' end-directed cleavage sites (Schultz, Zhang and Champoux, manuscript in preparation). Also, mutagenesis of individual nucleotide positions confirms the preferred sequences identified by statistical analyses (Schultz, Zhang and Champoux, manuscript in preparation).

An interesting question is how the preferred nucleotides at specific positions reflect the interactions between the enzyme and substrate that promote cleavage. The preferred nucleotides surrounding an internal RNase H cleavage site may facilitate hydrolysis through base-specific contacts or by influencing the hybrid substrate structure in features such as the trajectory of the helical axis, or the width of the major or minor grooves (Sarafianos et al., 2001; Han et al., 2003; Kopka et al., 2003). For HIV-1 RNase H, one example of an interaction that likely contributes to sequence specificity is the base-specific contact between Gln475 and the -2 guanine base. It is also possible that the preferred nucleotides reveal preferences in the DNA strand instead of or in addition to the RNA strand. It would be intriguing to evaluate why a particular site is not cleaved, and to consider whether such a site associates with the RNase H domain in reverse transcriptase in a structurally interesting way. Some nucleotides at preferred positions correlate with an absence of internal cleavage (Figure 4, red).

6.2 Removal of tRNA primer

Many studies have demonstrated that removal of the extended tRNA primer by the HIV-1 and MoMLV reverse transcriptases requires sequence specificity (Furfine and Reardon, 1991a; Pullen et al., 1992; Smith and Roth, 1992; Schultz et al., 1995; Zhan and Crouch, 1997). The tRNA primer of HIV-1 is incompletely removed, and a ribo A is left at the 5' end of the minus-strand DNA (Furfine and Reardon, 1991a; Pullen et al., 1992; Smith and Roth, 1992). In the case of MoMLV, tRNA primer removal typically leaves a ribo A at the 5' end of minus-strand DNA, but complete primer removal is also observed (Schultz et al., 1995; Zhan and Crouch, 1997).

Figure 5 compares how the sequences surrounding the extended tRNA primers of HIV-1 and MoMLV match with the nucleotide preferences identified for an internal cleavage site. Although removal of the extended tRNA primer would appear to occur in the DNA 3' end-directed mode by a stalled reverse transcriptase (see section 5.1), the sequences for these cleavage sites are consistent with the preferred nucleotides identified for internal cleavage sites (Schultz et al., 2004). For HIV-1, the observed cleavage that leaves a ribo A at the 5' end of the minus-strand DNA (Furfine and Reardon, 1991a; Smith and Roth, 1992; Pullen et al., 1993) matches well in sequence with the preferred nucleotides for an internal cleavage, except that there is a disfavored C at distal position -14 (Figure 5, part A). The absence of cleavage at the RNA-DNA junction for HIV-1 may be explained by the presence of a disfavored deoxy C at the +1 position instead of the preferred A or U. For MoMLV where both junctional and non-junctional cleavages are observed, preferred nucleotides are present at the correct positions for both the cleavage at the RNA-DNA junction and the cleavage that leaves a ribo A [(Schultz et al., 1995; Zhan and Crouch, 1997); Figure 5, part B].

6.3 Generation of PPT primer

The PPT consists of a 5' rA:dT-rich block and a 3' rG:dC tract that is typically followed by one to three A's, with cleavage occurring between the G-stretch and the next A [(Sorge and Hughes, 1982); reviewed in (Rausch and Le Grice, 2004)]. Interestingly, the HIV-1 PPT sequence itself contains a structural distortion in the absence of reverse transcriptase (Kvaratskhelia et al., 2002) although such a distortion was not detected in the MoMLV PPT (Winshell and Champoux, unpublished). The reverse transcriptase of MoMLV can cleave the 3' end of the polypurine tract by an internal cleavage to generate the PPT primer (Finston and Champoux, 1984; Schultz et al., 2000), and the MoMLV PPT sequence contains preferred nucleotides at all of the positions associated with an internal cleavage at this site (Figure 5, part B). Although generation of the HIV-1 PPT primer by an internal cleavage event is relatively inefficient compared to the MoMLV case, it can occur by this mode of cleavage [(Fuentes et al., 1995); Schultz, Zhang and Champoux, unpublished results]. Consistent with this observation, the HIV-1 PPT sequence has an A at the -7 position instead of the preferred bases, C or G. This might render the HIV-1 PPT sequence somewhat less than ideal for internal cleavage, but preferred nucleotides are found at all other positions (Figure 5, part A). Whether the HIV-1 PPT primer can be more efficiently generated in a polymerization-dependent mode at a pause site remains to be evaluated.

Several studies examining generation of the PPT primer have elucidated specific positions important for cleavage, and some of these positions overlap with those identified for the more general internal cleavage mode (see section 6.1 and Figure 4). For MoMLV, early *in vitro* experiments with purified enzyme and PPT-containing substrates indicated that mutations at positions -1, -2, -4, and -7 disrupted proper PPT cleavages (Ratray and Champoux, 1989). When optimal PPT sequences were selected from randomized sequences during MoMLV replication *in vivo*, the wild type sequence was preferred at positions -2, -5, -6, -10, and -11 and preferences at other positions were significantly lower (Robson and Telesnitsky, 2000). The importance of positions -2, -6, and -11 match with three of the preferred positions identified by statistical analyses for internal cleavage sites, while positions -1, -4, -5, -7, and -10 may reflect specific cleavage of the PPT by the MoMLV reverse transcriptase.

Consistent with preferred nucleotides spanning positions +1 through -14 for HIV-1 RNase H, both the 5' and 3' ends of the PPT are important for viral replication *in vivo* (McWilliams et al., 2003; Miles et al., 2005). In the co-crystal structure of HIV-1 reverse transcriptase with a PPT RNA/DNA (Sarafianos et al., 2001), the +1 RNA base makes hydrogen-bonding contacts with Arg448, and the -2 RNA guanine makes hydrogen-bonding contacts with Gln475. There are no base contacts with positions -4 or -7, but there are phosphate contacts from -4 to -9 in the DNA strand with the RNase H primer grip region. Using model substrates and purified enzyme, positions -2 and -4 are essential for correct cleavage of the PPT (Pullen et al., 1993) and substitutions at positions +1, -2, and -4 of the PPT substantially decrease generation of the PPT primer (Rausch and Le Grice, 2007). In other studies examining the recognition and cleavage of the PPT by HIV-1 RNase H, the effects of mutations in the PPT and in the primer grip region of the RNase H domain have been assessed both *in vitro* and *in vivo* (Julias et al., 2002; Julias et al., 2004; McWilliams et al., 2006; Jones and Hughes, 2007). These studies indicate that changes at positions -2 and -5 reduce viral replication and disrupt PPT cleavage *in vivo*, and while less dramatic, mutation of position -4 affects cleavage specificity. These data are consistent with positions +1, -2, -4, and -7 being important for internal cleavage by HIV-1 RNase H, but do not address positions -12 or -14 (see section 6.1). Interestingly, the -5G appears to enable the PPT to assume an appropriate structure for cleavage by RNase H rather than providing a base specific contact for reverse transcriptase (Jones and Hughes, 2007). This result may indicate that the -5 position is specifically important for the PPT structure and resulting cleavage, and would explain why this position is not represented in the

preferred nucleotide positions observed for general RNase H cleavage by HIV-1 (Schultz et al., 2004; Schultz et al., 2006).

6.4 Model for how RNase H selects cleavage sites

Several general observations about the locations of RNase H cleavage sites have defined the principles underlying site selection by retroviral RNases H (Schultz et al., 2004; Schultz et al., 2006). First, internal cleavages occur at sites that contain specific nucleotides at preferred positions surrounding the cleavage site. Second, internal cleavage sites located within a defined set of distances from a recessed RNA 5' end are recognized as RNA 5' end-directed cleavages. This window of distances from the RNA 5' end is approximately 13–19 nucleotides for HIV-1 and MoMLV RNases H (Schultz et al., 2006). Third, there is also a window for sequence-dependent DNA 3' end-directed cleavage; this window overlaps that of the RNA 5' end-directed cleavage window, but extends 15–20 nucleotides from the DNA 3' recessed end (Schultz, Zhang and Champoux, manuscript in preparation).

In a model for how RNase H selects a cleavage site, the first specificity determinant is the surrounding sequence, which defines the eligibility of a site for cleavage. The most important positions are at –2 and +1 for both HIV-1 and MoMLV RNases H. Other positions upstream of the cleavage site appear distinct for these two enzymes. If a site contains preferred nucleotides at all of the optimal positions, then the sequence is a strong internal cleavage site. Deviations from the preferred sequence would present a weaker cleavage site. If an eligible site lies within the cleavage window for DNA 3' end or RNA 5' end-direct cleavage, then cleavage is favored. If multiple sites are available on the same substrate within the cleavage window, independent end-directed cleavages can occur. Figure 6 illustrates how five cleavages sites (A-E) with eligible sequences would be cleaved if presented to reverse transcriptase in a hybrid as internal sites or as both types of end-directed sites.

The above observations prompt two questions: Why do RNA 5' end-directed cleavages have a broader cleavage window than DNA 3' end-directed cleavages? Why is the window for DNA 3' end-directed cleavage further from the recessed end than the window for RNA 5' end-directed cleavage? Since the spatial distance between the polymerase and RNase H active sites is slightly greater for binding to an RNA/DNA hybrid as compared to duplex DNA (18 versus 17 nucleotides) (Jacobo-Molina et al., 1993; Huang et al., 1998; Sarafianos et al., 2001), the nature of the substrate itself is unlikely to account for the different distances. Perhaps a recessed DNA 3' end which is recognized as a primer terminus by the polymerase domain is rigidly fixed in the primer-template binding cleft and this accounts for the narrower window for this mode of RNase H cleavage. In this case, the distance for the allowable cleavage may actually reflect the spatial distance between the active sites, with the width of the window depending on the internal flexibility of both the enzyme and the duplex substrate. On the other hand, a recessed RNA 5' end may not be tightly bound to the primer binding site or laterally locked in relation to the primer-template binding cleft. It seems more likely that some feature of a recessed 5' end is recognized by the polymerase domain such that the point of discontinuity where the duplex region gives way to a single strand is more loosely bound as compared to the primer terminus that locates cleavage for a recessed DNA 3' end. This hypothesis could account for the greater distance of the DNA 3' end-directed as compared to RNA 5' end-directed cleavages and also could explain the broader window for RNA 5' end-directed cleavage. Additional flexibility with respect to width of the window for RNA 5' end-directed cleavage may also be afforded by the ability of the enzyme to slide forward or backward slightly on the substrate before carrying out RNase H cleavage [see section 5.2; (Wisniewski et al., 2000b; Wisniewski et al., 2000a; Wisniewski et al., 2002)].

7. RNase H and Antiretroviral Therapy

Reverse transcriptase is a primary target for antiretroviral therapy of HIV-1 infected patients. Both the improvement of existing drug regimens and the development of novel drugs against HIV-1 reverse transcriptase are necessary. While the vast majority of anti-virals are specific for the polymerase domain, the RNase H activity of reverse transcriptase represents an excellent target for antiretroviral drugs as well.

7.1 Nucleotide Reverse Transcriptase Inhibitors, Non-Nucleotide Reverse Transcriptase Inhibitors, and RNase H activity

There are two major classes of drugs that inhibit reverse transcriptase, and both of these target the polymerase domain. The first class is the nucleotide analogues called nucleotide or nucleoside reverse transcriptase inhibitors (NRTIs). NRTIs inhibit replication by competing with cellular dNTPs for incorporation into the nascent DNA chain; upon incorporation, the absence of a 3' hydroxyl group on the NRTI prevents additional synthesis and causes premature chain termination (Goldschmidt and Marquet, 2004). The second class of inhibitors is the non-nucleotide or non-nucleoside reverse transcriptase inhibitors (NNRTIs), which consist of several families of structurally different types of small molecules. NNRTIs are non-competitive inhibitors that bind a hydrophobic pocket near the polymerase active site of the p66 subunit in HIV-1 reverse transcriptase [reviewed in (Domaal and Demeter, 2004)]. The association of an NNRTI with reverse transcriptase not only forms the NNRTI pocket, but also alters the relative positions of the RNase H and polymerase domains [reviewed in (Hang et al., 2007)]. Different NNRTIs may cause differential effects on the positioning of these two domains and, consequently, may influence RNase H activity; notably some NNRTIs appear to increase RNase H activity (Gopalakrishnan and Benkovic, 1994; Palaniappan et al., 1995; Shaw-Reid et al., 2005).

While both NRTIs and NNRTIs target the DNA polymerase activity of reverse transcriptase, the occurrence of drug resistance mutations in the RNase H and connection domains may have clinical significance and underscores the importance of thoroughly evaluating the effects of anti-viral drugs on both the polymerase and RNase H activities of reverse transcriptase (see section 7.3).

7.2 RNase H as an anti-viral target

RNase H represents an exciting possibility for developing new inhibitors of HIV-1 reverse transcriptase that target novel binding sites [reviewed in (Klumpp and Mirzadegan, 2006)]. One potential class of RNase H inhibitors involves drugs that alter the interactions between the RNase H domain and substrate or that alter the alignment of substrate in the RNase H active site. For example, drugs that nonspecifically associate with the minor groove of DNA/DNA and RNA/RNA substrates expand the width of the minor groove while shortening that of the major groove (White et al., 1998). Such alterations in substrate structure could impair the ability of RNase H to perform general cleavages, specific PPT primer generation or primer removal after extension. Since the RNase H domain can have distal effects on the polymerase domain, it may be possible to identify drugs that interact with the RNase H domain and also deleteriously affect polymerization.

Another class of potential agents could interfere with the retroviral RNase H activity by increasing, decreasing, or altering RNase H cleavages. Inhibitory agents that decrease RNase H activity would block retroviral replication through multiple mechanisms, potentially including an overall reduction in genome degradation, a decrease in the ability to carry out template switches and plus-strand initiation, or removal of the PPT or tRNA primers. However inhibitory drugs might also increase analogue excision (see section 7.4). Stimulatory agents

that increase RNase H activity could negatively impact reverse transcription by causing premature dissociation of the enzyme that leads to lowered polymerase processivity and in turn increases strand transfers. An important question is whether there are specific types of RNase H cleavage that might be especially suitable or selectively targeted by such drugs (see section 7.3). It is also important to consider the metabolic stability and cell penetration of a candidate drug as well as which type of drug might have a lower chance of promoting drug resistance. Notably, oligodeoxynucleotides targeting the PPT have been used to direct RNase H cleavage of RNA in intact virions, but cellular uptake of such substrates is inefficient (Matskevich et al., 2006).

Some drug candidates that specifically inhibit RNase H activity are effective at low micromolar concentrations. Diketo acids, N-hydroxyimides, N-acyl hydrazones, and hydroxylated tropolones are promising as agents that selectively inhibit the RNase H activity of HIV-1 [reviewed in (Klumpp and Mirzadegan, 2006); see also (Borkow et al., 1997; Klumpp et al., 2003; Shaw-Reid et al., 2003; Budihas et al., 2005)]. Diketo acids and N-hydroxyimides are metal chelators that may sequester the metal cofactors in the RNase H active site (Klumpp et al., 2003; Shaw-Reid et al., 2003). While the inhibition mechanism of hydroxylated tropolones is unknown, metal chelation is also a possibility (Budihas et al., 2005). A recent crystal structure of HIV-1 reverse transcriptase complexed with a N-acyl hydrazone analogue shows that this RNase H inhibitor binds distal from the RNase H active site at a novel position near the NNRTI binding pocket and the polymerase active site (Himmel et al., 2006), and suggests that this class of compounds may cause structural alterations in the polymerase domain which interfere with RNase H activity by altering the trajectory of the primer-template, but co-crystal structures that include substrate will be important to resolve this issue. Also, RNase H inhibitors could potentially be used in combination with drugs that target polymerase activity, as in vitro studies pairing several DNA polymerase inhibitors with a diketo acid suggest that such combinations are synergistic in inhibiting reverse transcription by HIV-1 reverse transcriptase (Shaw-Reid et al., 2005). Clearly, further biochemical and crystallographic studies are required to resolve how these compounds inhibit RNase H activity.

7.3 Assays for RNase H inhibitors

During reverse transcription, the relative contributions of internal, DNA 3' end-directed, and RNA 5' end-directed RNase H cleavages remain unknown (see section 5.4). Consequently, it is important to utilize a variety of assays that measure different RNase H activities to screen candidate RNase H inhibitors. In addition, any candidate drugs must be screened to selectively target the retroviral activity and not impair the human RNase H, which is likely essential as disruption of the RNase H1 gene in mice causes embryonic lethality (Cerritelli et al., 2003). One promising example is a hydroxylated tropolone, which exhibits ~30-fold selectivity for inhibiting the HIV-1 enzyme as compared to the human enzyme (Budihas et al., 2005).

The types of assays used to screen for drugs specifically targeting RNase H must be carefully considered. In general, in vitro assays provide rapid results and allow analysis of specific substrates or cleavages (e.g. comparing polymerization-dependent versus polymerization-independent RNase H activity). Specific assays to screen for inhibitors of RNase H activity might include screening for specific cleavages like PPT generation or primer removal, internal cleavage on a hybrid without recessed ends, RNA 5' versus DNA 3' end-directed cleavages, and general cleavage activity with homopolymeric hybrids (e.g. polyA/polydT). Also, a judicious selection of substrate sequence is an important consideration in assay design. Recently, several different NNRTIs were found to partially inhibit RNA 5' end-directed activity and at the same time stimulated DNA 3' end-directed cleavage, indicating that choice of substrate is an important parameter in evaluating drug effects on RNase H activity (Hang et al., 2007).

A straightforward in vitro assay has been described that uses fluorescence-based methodology to test for selective inhibition of retroviral RNase H activity (Parniak et al., 2003). In this assay, the blunt-ended quenched substrate is an 18 nucleotide 3'-fluorescein-labeled RNA annealed to a complementary 18 nucleotide 5'-Dabcyl-modified DNA. RNase H cleaves the RNA strand near the 3' end to release a fluorescent tetranucleotide. Interestingly, the primary cleavage site between the 14th and 15th nucleotides from the heteropolymeric RNA 5' end matches well with the preferred nucleotides surrounding an RNA 5' end-directed site (see section 6.1). This fluorescence resonance energy transfer (FRET) assay allows for high-throughput screening of RNase H inhibitors.

In vivo assays offer a complete and perhaps more thorough analysis of RNase H inhibition because all the components involved in reverse transcription (such as nucleocapsid protein and different substrates) are present. However, in vivo assays are typically more lengthy than in vitro assays. In addition, analysis typically involves only the final products of reverse transcription (such as generation of circle junctions), so these types of assays do not necessarily reveal specific stages where RNase H activity might be impaired.

7.4 RNase H in retroviral resistance

One of the greatest challenges in anti-viral therapy is the rapid emergence of drug resistant strains of HIV-1. To overcome drug resistant strains, it is important to determine the molecular basis of resistance. In sequencing analysis of HIV-1 for clinical drug resistance, most studies analyze the polymerase domain of reverse transcriptase and do not sequence the connection or RNase H domains [reviewed in (Jones et al., 2007)]. However, recent evidence suggests that mutations in reverse transcriptase outside of the polymerase domain may have clinical significance in resistance to NRTIs.

Mutations in the RNase H domain that decrease RNase H activity can increase the resistance of reverse transcriptase to the NRTI 3'-azido-3'-deoxythymidine (AZT) [reviewed in (Jones et al., 2007); also see the accompanying article of this special issue by L. Menéndez-Arias (Menéndez-Arias, 2008)]. For example, mutations in the RNase H domain that decrease RNase H activity may allow more time for excision of the incorporated NRTI (Nikolenko et al., 2005). Consistent with this possibility, AZT treatment of HIV-1 containing thymidine analog mutations (TAMs) in the polymerase domain has selected for mutations in the connection and RNase H domains that significantly increase resistance to AZT (Brehm et al., 2007). When TAMs in the polymerase domain were combined with mutations in the connection domain of HIV-1 reverse transcriptase derived from patients treated with antiviral drugs, substantial increases in resistance to the NRTI 3'-azido-3'-deoxythymidine (AZT) were observed (Nikolenko et al., 2007). Other mutations in the RNase H primer grip region, when combined with a polymerase domain containing TAMs, elicit a similar increase in AZT resistance (Delviks-Frankenberry et al., 2007). Together, these data strongly argue that C-terminal mutations in the reverse transcriptase can increase clinical NRTI resistance. It remains to be determined whether these mutations decrease RNase H activity, alter substrate binding, increase NRTI excision, and/or decrease template switching, but these observations indicate that C-terminal mutations in the reverse transcriptase that affect RNase H activity could be clinically significant.

Interestingly, selected resistance to NNRTIs in HIV-1 reverse transcriptase has been localized to amino acid changes in the polymerase domain that impact RNase H activity and specificity. Mutations that cause NNRTI resistance typically alter interactions between the NNRTI pocket and the inhibitor. During NNRTI therapy, two very common resistance mutations (K103N, Y181C) and two less common resistance mutations (P236L, V106A) affect RNase H activity. These four mutations generate versions of reverse transcriptase that have no apparent gross defects in DNA polymerization yet exhibit alterations in the rates of DNA 3' end-directed and

RNA 5' end-directed RNase H activity as compared to wild type reverse transcriptase (Gerondelis et al., 1999; Archer et al., 2000; Archer et al., 2001). Since the observed defects in RNase H activity do not interfere with the selection of these mutations by NNRTI therapy in patients, these resistance mutations offer insights into acceptable alterations of RNase H function during reverse transcription in vivo.

7.5. Future prospects

Recent studies indicate that the HIV-1 RNase H activity of reverse transcriptase is a viable target for anti-virals as well as an under-appreciated location for mutations that contribute to anti-viral resistance. The determination of additional crystal structures that reveal binding modes of inhibitors and mechanisms for resistance will facilitate structure-based inhibitor design. Co-crystal structures containing MoMLV or HIV-1 reverse transcriptase and inhibitors both with and without substrate will offer comparisons that improve drug design and hopefully lead to the development of additional drugs to treat HIV-1 infected individuals.

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Figure 1.

Co-crystal structure of HIV-1 reverse transcriptase and an RNA/DNA substrate. A ribbon diagram of the HIV-1 reverse transcriptase associated with a PPT RNA/DNA hybrid, based on the report by Sarafianos et al. (pdb entry 1HYS) (Sarafianos et al., 2001). The polymerase, connection, and RNase H domains of p66 are shown in blue, yellow, and red, respectively; p51 is shown in gray. The RNA template and DNA primer strands are shown in green and purple, respectively. The location of the RNase H active site is indicated with the four key catalytic residues shown as ball and stick structures (Asp443, Glu478, Asp498 and Asp549). The approximate locations of the primer terminus and the RNase H primer grip are also indicated, as is the approximate distance in base pairs between the polymerase and RNase H active sites on the hybrid substrate. Diagram generated with Swiss PDB Viewer.

Figure 2.

Schematic representation of the two-metal ion mechanism of catalysis for the RNase H activity of HIV-1 reverse transcriptase [adapted from (Nowotny and Yang, 2006)]. The four acidic residues, Asp443, Glu478, Asp498, and Asp549, in the RNase H active site are drawn in green with the RNA substrate shown in red. The two metal ions are indicated in yellow and labeled as A and B, and metal ion coordination and hydrogen bonds are indicated with dashed lines. The attacking nucleophile is shown in black with an arrow drawn to the scissile phosphate. A key water molecule mediating an interaction between Asp498 and metal ion A is indicated as a blue oval.

Figure 3.

The three modes of cleavage for retroviral RNase H. Reverse transcriptase is shown schematically in blue, with the active sites of the RNase H (RnH) and polymerase (Pol) domains indicated. The hybrid substrate contains RNA (red) and DNA (black) strands, with the 3' ends indicated by an arrowhead. A. DNA 3' end-directed cleavage. B. RNA 5' end-directed cleavage. C. Internal cleavage.

Figure 4.

The positions and base preferences flanking internal and RNA 5' end-directed RNase H cleavage sites for HIV-1 and MoMLV reverse transcriptases. The locations of preferred positions relative to a cleavage site at the scissile phosphate (arrow) located between nucleotides -1 and +1 are indicated on an RNA strand (thick black line). The preferences for or against nucleotides for HIV-1 RNase H are shown above the line and for MoMLV RNase H are shown below the line. The preferred nucleotides for each position are indicated in uppercase (strongly preferred) or lowercase (preferred) for internal (blue) and RNA 5' end-directed (violet) cleavage sites. Disfavored nucleotides for internal cleavage sites are indicated in red.

Figure 5.

Comparison of sequences surrounding cleavage sites for the tRNA primer and PPT region of HIV-1 and MoMLV with the positions and preferred nucleotides for internal cleavage. The relevant sequences flanking the PPT and tRNA regions are indicated for HIV-1 (A) or MoMLV (B). The $-1/+1$ site for cleavage is indicated by an arrow and a vertical line. The match between the preferred positions and an internal cleavage site is indicated as preferred (blue box) or disfavored (red box). The DNA portion of the extended tRNA is underlined. Alignment of the tRNA is shown for the cleavage at the RNA-DNA junction (RNA-DNA) and for cleavage between two ribonucleotides that leaves a ribo A at the end of the minus strand (RNA-riboA).

Figure 6.

Selection of cleavage sites by retroviral RNase H. Five different cleavage sites are shown on an RNA strand as A - E with various nucleotide positions numbered as indicated. Four hybrid substrates (thick black lines) are indicated below where a filled circle represents the position of an RNA 5' or a DNA 3' recessed end on substrates 1–3 and the blunt end on substrate 4. All sites are eligible for internal cleavage (blue) in substrate 4, where the RNA/DNA hybrid has blunt ends. Otherwise sites on a given substrate are only cleaved if they fall within the window of cleavage for RNA 5' end-directed (violet) or DNA 3' end-directed (green) cleavage. For substrate 1 where the recessed end begins at position 1, sites B and C are cleaved by either form of end-directed cleavage and site A is present but not cleaved. For substrate 2 where the recessed end begins at position 6, sites C and D are cleaved if the recessed end is RNA, only site D is cleaved if the recessed is DNA, and sites A and B are present but not cleaved. For substrate 3 where the recessed end begins at position 8, site D is cleaved if the end is RNA, sites D and E are cleaved if the end is DNA, and sites A, B, and C are present but not located within an end-directed window.