Investigating HIV-1 Polypurine Tract Geometry via Targeted Insertion of Abasic Lesions in the (−)-DNA Template and (+)-RNA Primer*

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Hye Young Yi-Brunozzi and Stuart F. J. Le Grice‡

From the Reverse Transcriptase Biochemistry Section, Resistance Mechanisms Laboratory, HIV Drug Resistance Program, NCI, National Institutes of Health, Frederick, Maryland 21702

A variety of biochemical and structural studies indicate that two regions of the human immunodeficiency virus type 1 (HIV-1) polypurine tract (PPT)-containing RNA/DNA hybrid deviate from standard Watson-Crick geometry. However, it is unclear whether and how these regions cooperate to ensure PPT primer selection by reverse transcriptase-associated ribonuclease H and subsequent removal from nascent (+)-DNA. To address these issues, we synthesized oligonucleotides containing abasic lesions in either the PPT (+)-RNA primer or (−)-DNA template to locally remove nucleobases, although retaining the sugar-phosphate backbone. KMnO₄ footprinting indicates such lesions locally alter duplex structure, whereas thermal melting studies show significantly reduced stability when lesions are positioned around the scissile bond. Substituting the (−)-DNA template between positions −15 and −13 altered cleavage specificity, whereas equivalent substitutions of the (+)-RNA had almost no effect. The unpaired base of the DNA template observed crystallographically (−1C) could also be removed without significant loss of cleavage specificity. With respect to the scissile −1/+1 phosphodiester bond, template nucleobases could be removed without loss of cleavage specificity, whereas equivalent lesions in the RNA primer were inhibitory. Our data suggest an interaction between the p66 thumb subdomain of HIV-1 reverse transcriptase, and the DNA template in the “unzipped” portion of the RNA/DNA hybrid could aid in positioning the ribonuclease H catalytic center at the PPT/U3 junction and also provides insights into nucleic acid geometry around the scissile bond required for hydrolysis.

In retroviruses and long terminal repeat (LTR)³-containing retrotansposons, second or (−)-strand synthesis requires (a) specific cleavage between the 3′ end of the (−)-strand, polypurine tract (PPT), and the 3′ unique sequence (U3) of the LTR; (b) DNA-dependent DNA synthesis from the newly created primer; and (c) removal of the PPT primer from nascent (+)-DNA (1) (see Fig. 1A). Each of these steps can be accurately recapitulated in vitro when PPT sequences are embedded within a larger RNA/DNA hybrid (2–6), i.e. where the nucleic acid termini cannot influence enzyme orientation, implicating structural features of the PPT in both resistance to internal reverse transcriptase-associated ribonuclease H (RT/RNaseH) cleavage and specific processing at its 3′ terminus from (+)-RNA and (+)-DNA. Early NMR studies, using a short fragment of this hybrid, identified a 15° bend at the HIV-1 PPT/U3 junction (7), which may contribute to the accuracy of cleavage. Subsequent crystallographic studies with HIV-1 reverse transcriptase (RT) bound to a PPT-containing hybrid indicated a pattern of weakened base pairing centered −13 bp upstream of the PPT/U3 junction (8), a notion supported by chemical footprinting of the duplex in the absence of RT (9). Interestingly, the distance between the PPT/U3 junction and the upstream region of weakened base pairing is close to the spatial separation between the p66 thumb subdomain and RNase H catalytic center of HIV-1 RT (8, 10, 11). These combined observations suggested that an induced fit between the HIV-1 RT thumb subdomain and the upstream portion of the PPT might position the RNase H catalytic center over the PPT/U3 junction. As a consequence, this would render intervening regions inaccessible and RNase H-resistant. Implicating regions upstream of the PPT/U3 junction in enzyme positioning (9, 12) is supported by our studies of PPT recognition in the LTR-containing retrotansposon Ty3. Although the Ty3 PPT lacks the hallmark ra4dT and rc4G homopolymeric stretches characteristic of retroviral (−)-strand primers (13), altering nucleic acid geometry 10–12 bp upstream of the PPT/U3 junction also affects the precision of PPT cleavage (14).²

One approach to studying PPT cleavage specificity is by introducing structural changes in a manner preserving sequence and spatial context. As an example, we substituted the thymine analog 2,4-difluoro-5-methylbenzene and cytosine analog 2-fluoro-4-methylbenzene throughout the HIV-1 PPT (14), suggesting that alternative structural elements of the retrotansposon enzyme may participate in PPT recognition. The converse strategy, i.e. decreasing local flexibility, was evaluated by introducing locked nucleic

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‡ To whom correspondence should be addressed: Reverse Transcriptase Biochemistry Section, Resistance Mechanisms Laboratory, HIV Drug Resistance Program, NCI, National Institutes of Health, Frederick, MD, 21702. Tel.: 301-846-5256; Fax: 301-846-6013; E-mail: slegrice@ncifcrf.gov.

The abbreviations used are: LTR, long terminal repeat; DAb, abasic deoxyriboside linkage; HIV-1, human immunodeficiency virus, type 1; PPT, polypurine tract; RAb, abasic riboside linkage; RNase H, ribonuclease H; RT, reverse transcriptase; U3, unique 3′ sequence; nt, nucleotide.

² C. Dash, D. Lener, and S. Le Grice, unpublished observations.
acid analogs (16–18) into the HIV-1 PPT (−)-DNA template, indicating that regions at both the 5′ and 3′ end of the RNA/DNA hybrid are critical for correct enzyme processing. This study (6) also supports a finding of Schultz et al. (19), suggesting two PPT cleavage modes, namely the PPT/U3 junction and −5 bp into the U3 region, the latter of which might contribute to efficient (+)-strand synthesis. Related studies evaluating the interaction of protein subdomains with nucleic acid exiting DNA polymerase catalytic center (20–22) illustrate the value of nucleoside analogs in dissecting protein-nucleic acid interactions.

The “unzipped” portion of the HIV-1 PPT reported by Sarafianos et al. (8) was of particular interest to us, because template base −11 and primer base −13 (defining position −1 as the base pair 5′ to the PPT/U3 junction, Fig. 1B) are unpaired, thereby enhancing the flexibility of this region. At the same time, we and others have noted that template base +1 of the HIV-1 and murine leukemia virus PPT is surprisingly tolerant to substitution, accepting substitution with non-hydrogen-bonding pyrimidine isosteres and base mismatches with minimal effect on the accuracy and overall rate of hydrolysis (2, 9, 13). The latter observations might indicate that, as the RNA/DNA hybrid enters the RNase H catalytic center, the DNA strand is displaced, allowing “docking” of the scissile phosphodiester bond in the active site, a model consistent with the role proposed for the p66 RNase H primer grip (8). A detailed understanding of how duplex geometry influences the accuracy of PPT cleavage is important in understanding this critical step in the reverse transcription cycle.

In this communication, we examined the effect of introducing abasic lesions (23) into the unzipped region of both the HIV-1 (−)-DNA template and (+)-RNA primer between positions −15 and 11, as well as around the PPT/U3 cleavage junction. Although there is little information on abasic lesions within RNA/DNA hybrids, structural studies with duplex DNA suggest that local elimination of the base does not affect the sugar-phosphate backbone (24, 25) but, in general, increases flexibility (25). An abasic site can also affect whether its unpaired complement assumes an intra- or extrahelical configuration. Collectively, our data suggest positions −15, −14, and −13 of the HIV-1 PPT (−)-DNA template and (+)-RNA primer between positions −15 and −9 (defining −1 as the first base pair 5′ to the scissile phosphate) (8). For the present study, abasic lesions were introduced into the (−)-DNA template and (+)-RNA primer between positions −15 and −11. The second region selected for analysis was the −1/+1 scissile bond corresponding to the PPT/U3 junction, where lesions were introduced on either side of the junction and downstream at position +2. To determine how these substitutions affected duplex stability, the melting temperature (Tm) of each hybrid was determined, the results of which are presented in Table I.

For PPT variants containing substitutions between positions −15 and −11, most melted at temperatures similar to that of the wild type hybrid (67.1 °C), regardless of whether the nucleobase was eliminated from the (−)-DNA template or (+)-RNA primer. The maximum decrease in Tm was observed with duplexes containing the unpaired bases defined by Sarafianos et al. (8) i.e. template base −11 (Tm = 65.6 °C) and primer base −13 (Tm = 65.0 °C). From these results, we concluded that the stability of the unzipped portion of the HIV-1 PPT was not seriously affected by base elimination. In contrast, removing nucleobases around the PPT/U3 cleavage junction was significantly more destabilizing, depressing the Tm by as much as 12.3 °C (−1RAB). The destabilizing effect was evident as far as position +2, suggesting that abasic lesions inserted at the PPT/U3 junction influenced neighboring base pairs, possibly through the alteration of stacking interactions. For a duplex of
FIG. 1. A, generalized scheme of the reverse transcription cycle. DNA and RNA sequences are represented by closed and open boxes, respectively. i, \((-\) strand DNA synthesis is initiated from a host-encoded tRNA hybridized to the primer binding site (PBS). Concomitant with DNA synthesis, RNA of the resulting RNA/DNA hybrid is degraded by RT-associated RNase H activity. ii, nascent \((-\) DNA is relocated to the 3’ end of the viral RNA genome by a strand transfer event, promoted by homology between repeat (R) regions of \((-\) DNA and \((+\) RNA. iii, \((-\) strand DNA synthesis resumes, and the ensuing RNA/DNA hybrid is degraded, with the exception of the polypurine tract (PPT). iv, \((+\) strand DNA synthesis initiates from the PPT 3’-OH and proceeds to the 3’ end of the \((-\) DNA template. v, RNase H activity removes the PPT and tRNA primers, allowing a second- \((+\) strand jump, exploiting homology between PBS regions on the \((-\) and \((+\) strands. vi, bidirectional DNA synthesis generates a double-stranded proviral DNA flanked by long LTR sequences. The viral gene products for the structural genes (\(\text{gag}\)), enzymatic functions (\(\text{pol}\)), and the envelope (\(\text{env}\)) are indicated. 

B, structural features of the HIV-1 PPT and experimental rationale. Weakened base pairing defined by Sarafianos et al. (8) is indicated by open bars. Motifs of the p66 thumb adjacent to the polymerase (Pol) active site potentially contacting the DNA template and RNA primer when the RNase H catalytic center is positioned at the scissile bond (i.e. the PPT/U3 junction) have been indicated. Shaded pentamers indicate positions where nucleobases were removed from the template and primer. Base pair \(-1\) is defined as the first base pair 5’ to the PPT/U3 junction.
the length used in our studies (30 bp), a 10 °C reduction in \( T_m \) can be likened to introducing three base mismatches. Thus, if duplex geometry at the unmodified PPT/U3 junction is altered, as suggested by NMR (7) and chemical footprinting studies (9), this appears to be further destabilized as a consequence of nucleobase elimination.

**Susceptibility of Template Thymines to KMnO4 Oxidation**—To assess how targeted nucleobase elimination altered PPT structure, susceptibility of template thymines between positions -15 and +1 to KMnO4 oxidation was investigated (see Fig. 1B). This strategy can determine whether thymines are unpaired (28, 29) or are structurally distorted but exhibit weak hydrogen bonding (30). Template thymine reactivity in response to abasic insertions in both strands of the RNA/DNA hybrid was determined.

Fig. 2A presents thymine sensitivity when template nucleobases between positions -15 and -12 were removed. Clearly, removing any thymine will eliminate that particular hydrolysis product. Eliminating nucleobases -15, -14, and -13 slightly increased reactivity of adjacent thymines (Fig. 2A, lanes 1–3), which in conjunction with the \( T_m \) data of Table I, suggests local alteration in base stacking rather than extensive disruption of the duplex. Eliminating template base -12T had little effect on thymine reactivity at positions -13, -14, and -15 (Fig. 2A, lane 4). In contrast, -12T was highly susceptible to oxidation following removal of the unpaired nucleobase, -11C (Fig. 2A, lane 5), suggesting disruption of the original -12T/-11G mispair suggested by Sarafianos et al. (8) (Fig. 1B) when the stacking environment of the template is altered. The notion that eliminating base -11C is more destabilizing is supported by the observation that this substitution also affects -10T reactivity. With this exception, template substitutions between positions -15 and -12 did not alter KMnO4 sensitivity within the adjacent r(A)\(_3\)d(T)\(_4\) tract between -10T and -7T.

Reactivity of template nucleobase +1T in response to introducing abasic lesions at positions -1, +1, and +2 is shown in Fig. 2B. As expected, no product is evident for substrate +1DAb (Fig. 2B, lane 8). Also, altered migration of the +1 hydrolysis product on substrates +2DAb (Fig. 2B, lane 9) is accounted for by the fact that the DNA template was 5' end-labeled, thus the cleavage product lacks one base. Substitutions -1DAb and +2DAb resulted in enhanced +1T reactivity (Fig. 2B, lanes 7 and 9, respectively). Table I indicates that the \( T_m \) for substrates containing lesions at these positions was reduced by -10 °C, which is equivalent to loss of hydrogen bonding over three base pairs. The data of Fig. 2B and Table I thus indicate that abasic template lesions are more destabilizing when positioned at the PPT/U3 junction.

**Removal of Template Nucleobases between Positions -15 and -11 Affects PPT Cleavage**—Cleavage at the PPT/U3 junction in response to eliminating template nucleobases between positions -15 and -11 is shown qualitatively in Fig. 3A and quantitatively in Fig. 3B (for the former, lane d of each hydrolysis profile was selected). Using a 5’ end-labeled RNA primer, the wild type RNA/DNA hybrid (Fig. 3, A and B, panels i) was hydrolyzed predominantly at the -1/-1+1 junction and, to a lesser extent, between positions -2 and +6. No template substitution gave rise to aberrant cleavage within the PPT itself (data not shown). Removing template nucleobase -15 induced relaxed cleavage specificity, with the consequence that specific cleavage at the PPT/U3 junction was reduced (Fig. 3, A and B, panels ii). This effect was even more pronounced with substrate -14DAb, where the PPT/U3 junction was the least-preferred cleavage site (Fig. 3, A and B, panels iii). Although substrate -13DAb was hydrolyzed with improved specificity at the -1/-1+1 junction, positions +5 and +6 were still the favored sites (Fig. 3, A and B, panels iv). The data of Fig. 3, A and B, panels ii–iv, suggest alternative scenarios, namely that (a) specific contacts between the DNA strand between positions -15 and -13 and a structural motif of HIV-1 RT have been disturbed or (b) enhanced duplex flexibility upon nucleobase removal is incompatible with its trajectory between the catalytic centers of HIV-1 RT. Each of these possibilities will be discussed later.

| Table I | Melting temperatures of PPT substrates containing abasic lesions |
|---------|---------------------------------------------------------------|
|        | (-)-DNA \( T_m \) °C | (+)-RNA \( T_m \) °C |
| WT     | 67.1 ± 0.96         | 67.1 ± 0.96         |
| -15DAb| 68.0 ± 1.62         | -15RAb 68.2 ± 0.76  |
| -14DAb| 68.1 ± 0.74         | -14RAb 66.8 ± 1.25  |
| -13DAb| 69.0 ± 0.12         | -13RAb 65.0 ± 0.95  |
| -12DAb| 65.9 ± 0.95         | -12RAb 66.1 ± 1.39  |
| -11DAb| 65.6 ± 0.49         | -11RAb (mp) 65.3 ± 1.04 |
| -1DAb | 56.9 ± 0.95         | -1RAb 54.8 ± 0.99   |
| +1DAb | 58.9 ± 1.67         | +1RAb 57.6 ± 0.59   |
| -2DAb | 54.3 ± 0.64         | +2RAb 57.9 ± 0.12   |

sensitivity between bases -15T and -12T, the effect being more pronounced at thymines opposite the site of nucleobase removal (Fig. 2C, lanes 1–5). In an analogous manner to its template counterpart, primer substitution -11RAb increased -12T and -10T reactivity (Fig. 2C, lane 5), suggesting local disruption of the RNA/DNA hybrid. As might be expected, removing primer nucleobase +1 enhanced KMnO4 sensitivity of template thymine +1 (Fig. 2D, lane 8). Enhanced +1T reactivity was also evident following removal of primer nucleobase -1 (Fig. 2D, lane 7). Finally, +1T reactivity of substrate +2RAb was similar to that of the unsubstituted duplex (Fig. 2D, lanes 9 and 6, respectively), suggesting that stacking of template nucleobases at the scissile bond was unaffected by removing primer nucleobase +2.
these authors have suggested contributes to deformation of the duplex between positions −15 and −9 in the RT-RNA/DNA co-crystal (8). To examine whether altering the stacking environment of primer bases influences selection at the PPT/U3 junction, as well as to investigate a need for the unpaired ribonucleotide −13A (Fig. 1B), abasic lesions were likewise introduced into the RNA primer between positions −15 and −11. The results of this analysis are presented in Fig. 4.

Unlike their DNA complement, primer nucleobases −15, −14, and −13 could be eliminated with only a marginal impact on PPT cleavage specificity (Fig. 4, A and B, panels ii–iv). If nucleobase removal simply weakened duplex architecture, this would predict that lesions in the same position of the DNA template and RNA primer would have the equivalent effect on processing. However, the ability to alter PPT cleavage when template nucleobases −15, −14, and −13 are
removed, although this is unaffected by removing the equivalent primer nucleobases, suggests that the former are likely involved in specific protein/nucleic acid contacts. Furthermore, if the stacking pattern at the sequence 5'-A-G-A-3' described by Kopka et al. (31) contributed to duplex deformation, we had anticipated that PPT selection might be influenced by eliminating primer nucleobases positions −12 and −11. In contrast, we observed only a 2-fold decrease in cleavage at the PPT/U3 junction on substrates −12RAb (Fig. 4, A and B, panels vi) and −11RAb (Fig. 4, A and B, panels vi). At the same time, none of these primer substitutions resulted in enhanced cleavage within the PPT (data not shown), suggesting the 5'-A-G-A-3' sequence does not make a major contribution to PPT deformation and recognition by HIV-1 RT.

Targeted Removal of Nucleobases at the PPT/U3 Junction—

Studies with substrates containing mispaired bases (9) or non-hydrogen-bonding pyrimidine isosteres (12) in the (−)-DNA template have demonstrated that alterations in nucleic acid geometry are surprisingly well tolerated at the HIV-1 PPT/U3 junction without major implications for cleavage specificity. Relocating the RNase H catalytic center of HIV-1 RT over the PPT/U3 junction was evident in e.g. hydrolysis at position +1 was reduced on substrate −1DAb, and although this is restored on substrate +1DAb, the latter had very little +2 hydrolysis product. Significant cleavage of substrate +1DAb at position +5 is also evident. Finally, although correct cleavage is observed on substrate +2DAb, the major hydrolysis product now corresponds to hydrolysis between positions +1 and +2 (Fig. 5, A and B, panels iv).

Analysis of hybrids containing lesions in the RNA primer between positions −1 and +2 is shown in Fig. 6. In this case, although hydrolysis at positions +5 and +6 is unaffected, removing primer nucleobase −1G resulted in loss of cleavage at the PPT/U3 junction (substrate −1RAb; Fig. 6, A and B, panels iii). Note here that the −1 and −2 hydrolysis products migrate together, because the former lacks the nucleobase. Removing primer nucleobase +1A (substrate +1RAb) likewise eliminates −1/+1 cleavage, although permitting cleavage at +5 and +6 (Fig. 6, A and B, panels iv). In this case, the PPT/U3 hydrolysis product does not contain an abasic lesion and thus migrates with the expected mobility. The data of Fig. 6, A and B, panels ii and iii, therefore indicate that, although the sugar-phosphate backbone corresponding to the scissile −1/+1 phosphodiester bond of the PPT primer is preserved in the RNase H active center, critical contacts with residues of the active site are affected when the nucleobase is removed.

Potential sites of contact affected will be discussed in the following section. Finally, Fig. 6, A and B, panels iv, indicate normal cleavage at the PPT/U3 junction when nucleobase +2C was removed. The profiles of substrates +2RAb and +2DAb are particularly interesting, because the latter induced elevated cleavage at the +1/+2 junction (Fig. 5, A and B, panels iv) and increased KMnO4 sensitivity of template thymine +1, whereas the RNase H hydrolysis profile and KMnO4 reactivity of +1T of substrate +2RAb are similar to the unsubstituted hybrid.
DISCUSSION

Examining how nucleic acid geometry contributes to recognition and cleavage of the HIV-1 PPT to provide the (+)-strand primer has been addressed in this communication via targeted insertion of abasic lesions, which eliminate the nucleobase, although leaving the sugar-phosphate backbone intact. In the co-crystal of HIV-1 RT and a PPT-containing RNA/DNA hybrid (8), the RNase H active site was located several base pairs upstream of the PPT/U3 junction. Thus, interpretation of our results has required constructing a model that relocates protein/nucleic acid contacts to approximate this catalytic center positioned for hydrolysis at the scissile phosphodiester bond (Fig. 7). When the highly conserved His-539 of the RNase H active center is placed at this junction, extrapolating from co-crystals of RT with duplex DNA (10, 11) and RNA/DNA (8) suggests helix $\beta$-H of the p66 thumb subdomain would make multiple contacts with the sugar-phosphate backbone of the (+)-DNA template between positions $-16$ and $-13$, most likely involving residues Trp-266, Lys-263, Gly-262, Lys-259, Gln-258, and Asn-255. At the same time, helix $\beta$-I would be predicted to contact the (+)-RNA primer at positions $-12$ and $-10$ via Ser-280, Ala-284, and Gly-285 and Thr-286. This helix-turn-helix motif of the p66 thumb could therefore be envisaged as asymmetrically “grasping” the destabilized region of the RNA/DNA hybrid between positions $-15$ and $-9$. In addition, at the PPT/U3 junction, the model of Fig. 7 implicates p66 residues Arg-448, Asn-474, Gln-475, Gln-500, and His-539 in base, sugar, and phosphate contacts to (+)-RNA primer between positions $-2$ and $+2$. Using this model as a reference, the consequences of nucleobase removal at both regions of the RNA/DNA hybrid will be discussed. At the same time, it is important to take into consideration a 40° bend the hybrid will adopt over $\sim 5$ bp (between positions $-13$ to $-9$) as it undergoes a transition from A- to B-form geometry (8, 10, 11).

With respect to template substitutions $-15$DAb, $-14$DAb, and $-13$DAb, nucleobase removal will alter intrahelical stacking, which Fig. 7 suggests could affect multiple contacts with p66 helix $\alpha$.H. Experimentally, we observed that specific cleavage at the PPT/U3 junction diminishes with these three mutant substrates, and is accompanied by an increase in hydrolysis between positions $+1$ and $+6$, the latter observation suggesting relaxed specificity. The model of Fig. 7 also proposes fewer protein contacts with both the sugar-phosphate backbone and template nucleobases $-12$ and $-11$; in keeping with this notion, substrates $-12$DAb and $-11$DAb exhibit specific, and possibly enhanced, cleavage at the PPT/U3 junction. In addition to fewer protein contacts, the 40° bend the hybrid adopts in the presence of HIV-1 RT will be centered close to positions $-12$/-11. Removing these nucleobases might therefore be seen as a means of aiding the A- to B-form transition. In this respect, it is interesting to note that removing template nucleobase $-12$ has the most significant consequence for PPT architecture (Fig. 2A, lane 5) but is less deleterious with respect to PPT processing (Fig. 3, A and B, panels v).

With regard to the PPT (+)-RNA primer, nucleobase removal between positions $-15$ and $-11$ has very little affect on the specificity of PPT cleavage. Although a slight reduction in PPT/U3 cleavage was observed on substrate $-13$DAb, this was still the primary site of hydrolysis. If removing nucleobases between positions $-15$ and $-13$ nonspecifically destabilized the RNA/DNA hybrid, we might have expected the same result for PPT/U3 hydrolysis regardless of whether the nucleobase was removed from the template or primer. The sensitivity of
PPT processing to altering specifically the (−)-DNA template thus supports our contention that substitutions −15DAb, −14DAb, and −13DAb alter important protein/nucleic acid contacts with the p66 thumb.

Positioning the catalytic His-539 of the HIV-1 RNase H domain at the scissile −1/+1 phosphodiester bond allows contacts within the active site to be more accurately defined and compared with a model proposed for Escherichia coli RNase H. NMR analysis (32) and studies with nucleoside analogs (33) suggest Gln-72 of E. coli RNase H is hydrogen-bonded to the 2′-OH group of the nucleoside two bases 5′ to the scissile bond. In our model, the HIV-1 RT counterpart, Glu-500, could fulfill this role, contacting ribose of primer base −2G. Additionally, Cys-13 of E. coli RNase H has been implicated in an interaction with the 2′-OH of the ribonucleotide at position +1. For HIV-1 RT, Arg-448 could perform the same function. Building upon these reference points, Fig. 7 predicts Gln-475 of HIV-1 RT contacts nucleobase 2G and the ribose of 1G, whereas Asn-474 contacts the phosphate backbone of the −1/+1 phosphodiester bond, and Arg-448 would make additional contact with the ribose of base +2C. Surprisingly, although the RNase H primer grip of HIV-1 RT

![Diagram](image-url)
(8) contacts the DNA template several bases upstream, few (if any) template contacts are made opposite the scissile bond. Because previous studies have indicated that base pairing at position +1 is not absolutely required for hydrolysis (12), the necessity of template bases opposite the scissile bond was investigated here. Although we observed reduced hydrolysis, Fig. 7 indicates template nucleobases −1 and +1 can, in fact, be removed. Thus, once the RNA primer is correctly positioned in the RNase H active site through the contacts suggested above, transient opening of the duplex at positions −1 and +1 may occur to facilitate hydrolysis, which is formally analogous to a model forwarded by Nakamura et al. (32). Although the preference for cleavage at position +1 following removal of template nucleobase base +2 was unexpected, the data of Fig. 2 indicate this substitution greatly enhanced reactivity of +1T to KMnO4 oxidation, suggesting that loss of stacking induced by removing template nucleobase +2 allows both the −1/+1 and +1/+2 phosphodiester bonds of the primer access to the catalytic center. Although not shown experimentally, it is also conceivable that removing template nucleobase +2 induces its complement to assume an extra-helical configuration, which might also allow the +1/+2 phosphate junction to be accessed by the catalytic center.

For the RNA primer, removing nucleobases −1 and −2, although retaining the sugar-phosphate backbone, clearly inhibits hydrolysis at the PPT/U3 junction (Fig. 6, A and B, panels ii and iii). From studies with E. coli RNase H, Uchiyama et al. (34) suggest an outer sphere complex is formed between the catalytic Mg2+ ion and the 2′-OH group of the nucleoside 5′ to the scissile bond. We therefore interpret loss of PPT/U3 cleavage with substrate −1RAb as loss or alteration of this critical interaction. With respect to the nucleobase 3′ of the PPT/U3 junction, Haruki et al. (35) have exploited RNA/DNA hybrids containing phosphorothioate substitutions to propose that the pro-Rp-oxygen of the phosphate group 3′ to the scissile bond cooperates during catalysis with the catalytic His-124 of E. coli RNase H, analogous to the model of substrate-assisted catalysis proposed for type II restriction endonucleases (36). Fig. 7 suggests that eliminating primer nucleobase +1A could directly alter contacts with Arg-448 or indirectly influence backbone geometry to antagonize its cooperation with His-539. Finally, the wild type hydrolysis profile derived from substrate +2RAb indicates that critical primer contacts do not extend beyond the nucleobase 3′ to the scissile bond.

Although the model we have proposed here will require further validation, an induced fit would seem a plausible mechanism to correctly position HIV-1 RT on its cognate substrate for accurate cleavage at the PPT/U3 junction. Flexibility resulting from weakened base pairing centered −13 bp upstream of the −1/+1 phosphodiester bond would promote a primary interaction with helices h-H and a-I of the p66 thumb, which indirectly positions the RNase H catalytic center over the −1/+1 scissile bond. NMR studies are presently underway to validate this hypothesis.
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