Pre-existing Distortions in Nucleic Acid Structure Aid Polypurine Tract Selection by HIV-1 Reverse Transcriptase*

Received for publication, October 12, 2001, and in revised form, February 28, 2002
Published, JBC Papers in Press, March 1, 2002, DOI 10.1074/jbc.M109914200

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Precise cleavage at the polypurine tract (PPT)/U3 junction by human immunodeficiency virus type 1 (HIV-1) reverse transcriptase RNase H is critical for generating a correct viral DNA end for subsequent integration. Using potassium permanganate (KMnO₄) modification, we have identified a significant distortion in the nucleic acid structure at the HIV-1 PPT/U3 junction in the absence of trans-acting factors. Unusually high reactivity of template thymine +1 is detected when the PPT primer is extended by DNA or RNA at its 3' terminal. Chemical footprinting suggests that the extent of base unstacking in the wild-type species is comparable though the base unstacking in the wild-type species is comparable when the +1 A:T base pair is replaced by a C:T mismatch. However, reactivity of this template base is diminished after alterations to upstream (rA)₄:(dT)₄ or (rG)₆:(dC)₆ tracts. Importantly, there is a correlation between the structural deformation at base pair +1 and precise cleavage at the PPT/U3 junction by HIV-1 reverse transcriptase/RNase H. KMnO₄ modification also revealed unusually high reactivity for one of two (dT)₄:(rA)₄ stretches of the PPT/U3 junction, suggesting a significant structural distortion within the PPT itself in the absence of the retroviral polymerase. Structural abnormalities in this region are not only essential for resistance of the PPT to hydrolysis but also significantly impact the conformation of the PPT/U3 junction. Our data collectively suggest that the entire PPT sequence contributes to the structural distortion at the PPT/U3 junction, potentially providing a mechanism for its selective processing.

In retroviruses, the accuracy with which the (+) strand, polypurine tract (PPT) primer is selected from the RNA-DNA replication intermediate and excised from nascent (+) strand DNA defines the 5' long terminal repeat terminus and is critical for production of an integration-competent provirus (1). Because the bulk of the replication intermediate is non-specifically hydrolyzed, structural features of the PPT (a) render it RNase H-insensitive and (b) control precise cleavage at the junction with adjacent U3 DNA or RNA sequences (1). Although several reports have studied PPT processing relative to alterations in its sequence (2–6) or that of the cognate retroviral polymerase (7–9), the structural basis for this remains elusive. The recent structure of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) bound to a PPT-containing RNA/DNA duplex has provided important mechanistic insights regarding the PPT resistance to hydrolysis (10). These authors identified an unusual distortion within the (rA)₄:(dT)₄ stretches of the PPT, i.e. misaligned base pairing between the template and primer nucleotides, suggesting that extension of this deformation into the RNase H active site may confer resistance to hydrolysis. The crystal structure also revealed extensive contacts between the RNase H “primer grip” and the RNA/DNA hybrid. However, because a structure of the complete RNA/DNA duplex PPT in the absence of RT is unavailable, it was unclear whether structural distortions were introduced upon binding of RT or inherent to the PPT sequence. Moreover, Sarafianos et al. (10) could not refine the structure of the conserved (rG)₆:(dC)₆ tract at the PPT 3' terminus. Therefore, the mechanism of the precise cleavage at the PPT/U3 junction by RNase H has remained obscure. A crucial role of the guanine-rich segment in the specific hydrolysis of the primer was highlighted by biochemical studies (3, 4), which showed that mutations in this region result in imprecise cleavage of the PPT. Furthermore, the NMR structure of an 8-bp RNA/DNA oligonucleotide containing the last four 3'-terminal residues of the PPT and the first 4 bp of U3 shows that the width and shape of the major groove are unusual (11), with a bend of ~13° between the two halves of this hybrid. Taken together, it is clear that structural studies should encompass the entire PPT rather than its separate constituents when attempting to define the specificity of cleavage at the PPT/U3 junction.

Here, we have exploited chemical footprinting of duplex and chimeric RNA/DNA hybrids, mimicking the steps of PPT selection and removal from (+) RNA and DNA (Fig. 2A). For over two decades, potassium permanganate (KMnO₄) modification (12, 13) has been employed for identification of unstacked thymine bases in the context of duplex DNA. When supported with mechanistic data, for example, DNA unwinding (14–17) or formation of a “transcription bubble” by eukaryotic and prokaryotic RNA polymerases (18–21), increased thymine reactivity was ascribed to bases being unpaired. In related studies, this method revealed structural distortions where weak hydrogen bonding between complementary bases was preserved (22).

Because the PPT/(−) DNA hybrid comprises the T:A base pair at the PPT/U3 junction as well as two (T)₄):(A)₄ blocks within the PPT sequence, KMnO₄ modification of template thymines allowed us to probe nucleic acid structure at the specific site of RNase H cleavage. To date, KMnO₄ modification has been restricted to a study of duplex DNA. Here we show for the first time that this approach can also be successfully employed to reveal structural distortions in the context of RNA/
DNA hybrids. We report here that the first template thymine in the U3 DNA duplex immediately adjacent to the 3’ end of PPT is readily susceptible to KMnO₄ oxidation. Reactivity is preserved when the PPT(+)/DNA primer is substituted with its all-RNA counterpart, indicating an RNA-DNA junction is not a major determinant of this distortion. Moreover, mutations in the conserved (rG)₆(dC)₆ and (rA)₄(dT)₄ tracts severely diminish KMnO₄ accessibility to position +1. Interestingly, there is a good correlation between reactivity at this position and selective RNase H cleavage, suggesting that the structural distortion at the PPT/U3 junction is induced by the distinct PPT sequence. A second consequence of KMnO₄ footprinting is unusually high reactivity for one of two upstream (dT)₄(rA)₄ tracts, revealing a significant degree of structural distortion within the PPT itself in the absence of the retroviral polymerase. We demonstrate that these distortions play a role in the selective RNase H cleavage at the PPT/U3 junction and resistance of the PPT to hydrolysis.

EXPERIMENTAL PROCEDURES

Preparation of RNA/DNA Hybrids—DNA, RNA, or chimeric oligonucleotides were purchased from Oligos etc. or Integrated DNA Technologies, Inc. Based on observations with simian immunodeficiency virus (SIV), we retained the (U)₅ sequence at the HIV PPT 5’ terminus. Oligonucleotides were purified by 15% denaturing polyacrylamide gel electrophoresis. DNA templates for KMnO₄ modification and RNA or RNA-DNA chimeras for evaluating RNase H activity were 5’-end-labeled in 20-µl reactions using T4 polynucleotide kinase and 20–30 µCi of [γ-³²P]ATP (Amersham Biosciences). After annealing to the complementary strand at 90°C and slow cooling, hybrids were subjected to nondenaturing electrophoresis through 10% polyacrylamide gels in 1× Tris-borate EDTA buffer (Bio-Rad) at 100 V for 10 h at 4°C. Radiolabeled hybrids were visualized by autoradiography, excised, and electroeluted at 100 V for 1 h at 4°C. Purified hybrids were precipitated and vacuum desiccated. Nucleic acids were finally dissolved in 10 mM Tris-HCl, pH 8.0, buffer containing 50 mM NaCl.

Modification of RNA/DNA Hybrids with KMnO₄—The present protocol was adopted from that of Kvaratskhelia et al. The present protocol was adopted from that of Kvaratskhelia et al. (23). PPT/U3 hybrids containing 5'-³²P-labeled DNA templates were incubated at room temperature for 5 min in a buffer comprising 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 100 µM MgCl₂. The total reaction volume was 20 µl. Reactions were initiated by adding 2 µl of freshly prepared 25 mM KMnO₄ solution and terminated after 30 s with 2 µl of 14 M ß-mercaptoethanol. After ethanol precipitation, samples were treated with 100 µl of 1 M piperidine for 30 min at 90°C. Piperidine was removed by vacuum desiccation. Nucleic acids were washed three times with 50 µl of water and vacuum-dried after each resuspension. Samples were finally resuspended in formamide loading mix and analyzed by electrophoresis through 15% denaturing polyacrylamide gels. Modification products were analyzed on a Bio-Rad Molecular Imager FX and quantified using Bio-Rad Quantity One software.

PPT/U3 Hydrolysis—Reactions were carried out in 20 mM Tris-HCl, pH 7.5, 80 mM NaCl, and 5 mM dithiothreitol. Unless otherwise stated, prior to hydrolysis, 100 nM PPT/U3 hybrid containing 5’-end-labeled primer and 27 nM recombinant p66/p51 (24) HIV-1 RT were mixed with buffer and incubated at 37°C for 60 s. Hydrolysis was initiated by adding MgCl₂, to a final concentration of 6 mM. At the times indicated, aliquots were removed and mixed with an equal volume of 7 M urea in 1× Tris-borate EDTA. Hydrolysis products were fractionated by electrophoresis through 15% denaturing polyacrylamide gels containing 7 M urea and evaluated by autoradiography. Quantitation was as described above.

RESULTS

KMnO₄ Footprinting of the HIV-1 3’ PPT—KMnO₄ preferentially oxidizes C₅-C₆ double bonds in unstacked thymine bases in DNA (12). Electrophilic attack on the double bond proceeds with an out-of-plane trajectory onto the pi system, rendering the phosphodiester backbone susceptible to piperidine cleavage. However, thymines present in the context of a fully stacked structure are shielded from oxidation. Because the application of this methodology to date has been restricted to studies of duplex DNAs, we first examined the utility of KMnO₄ modification for structural studies of RNA/DNA hybrids. The data depicted in Fig. 1 clearly demonstrate that whereas thymines in single-stranded DNA exhibit hyper-reactivity to KMnO₄, such reactivity is severely diminished upon formation of conventional RNA/DNA or DNA/DNA hybrids. In contrast, thymines located in the single-stranded overhang region in the hybrid duplexes remain hyper-reactive to KMnO₄.

We next used KMnO₄ footprinting to reveal structural distortions in DNA/RNA hybrids containing the (−) strand DNA template annealed to PPT primers extended at their 3’ terminus by 5, 10, or 15 deoxynucleotides. A homogeneous preparation of duplex was imperative for accurate footprinting because thymines present in unhybridized template DNA would result in deceptive KMnO₄ reactivity. Therefore, all nucleic acid du-
plexes were purified via nondenaturing polyacrylamide gel electrophoresis after annealing. A representative gel is provided in Fig. 2C, indicating no unhybridized tritium-labeled template.

Two unusual features of the PPT-containing duplexes were immediately revealed. First, template thymine +1 was readily susceptible to KMnO4 attack (Fig. 3B, lane 1). This base is located in the duplex DNA part of the hybrid and therefore should be insensitive to oxidation. Clear differences in KMnO4 reactivity between fully stacked and unpaired thymines were revealed with PPT-DNA chimeras of different lengths of +1 DNA. Thus, in +5 DNA species, thymines +6, +7, +13, and +14 were readily modified by KMnO4 because they were located in the single-stranded region of the template. However, constraining thymines +6 and +7 within the duplex DNA structure by extending the primer with 10 deoxynucleotides sharply reduced their KMnO4 reactivity (Fig. 3B, lane 2). Quantitation of the results is presented in Fig 3C. Similarly, reactivity at positions +6, +7, +13, and +14 was diminished on a substrate whose primer was extended by 15 deoxynucleotides (Fig. 3B, lane 3). Despite this, enhanced reactivity of template nucleotide +1 position persisted in all species.

Secondly, several template thymines in the PPT RNA/DNA hybrid were KMnO4-sensitive. Quantitation revealed that reactivity of thymines −7 and −8 was comparable to that of fully stacked bases in the duplex DNA, whereas the reactivity of those between −10 and −15 increased about 10-fold (Fig 3C). Differing reactivities of DNA template thymines in an RNA/DNA duplex also indicated that KMnO4 modification could be used to probe base stacking in such hybrids. In fact, the crystal structure of the PPT/(-) DNA hybrid complexed with HIV-1 RT indicates similar biased distortion in the (rA)3(dT)3 tract. In this structure (10), the two adenines immediately upstream of the G:C tract (equivalent to −7 and −8 in our experiments) are properly paired with complementary DNA sequence, whereas the remaining 7 bp of the A:T tracts are distorted. The same authors (10) demonstrated that these irregularities consist of mismatches, weakly paired bases, and unpaired bases. Whereas the crystal structure embodies the conformation of the PPT in the complex with RT, our data indicate that such distortion is a feature of free PPT/(-) DNA hybrid.
The Entire PPT Sequence Contributes to Structural Distortion at the PPT/U3 Junction—To obtain additional information regarding the extent of distortion at +1 position, we designed a mismatch substituting primer dA with dC while preserving the template dT (Fig. 4A). KMnO$_4$ reactivity of the mismatched thymine was comparable to that in the wild-type structure (Fig. 4B), supporting the notion the first base pair in the (+) U3 DNA sequence is significantly distorted. We next addressed features of the substrate underlying such a distortion. Because position +1 in (+) DNA-containing hybrids is located immediately at the RNA/DNA junction, we examined whether the chimeric nature of the substrate influenced this structural anomaly. We therefore substituted the U3 DNA sequence with its RNA counterpart (Fig. 4A), mimicking the DNA/RNA hybrid formed during (-) strand synthesis that is selectively resolved by RNase H to generate the (+) strand primer. Fig. 4B, lane 3 shows that reactivity of +1 thymine persisted, suggesting that the RNA/DNA junction is not a major determinant.

We next examined the contributions to this distortion by different regions of the PPT duplex. The conserved (rG)$_6$(dC)$_6$ and (rA)$_4$(dT)$_4$ tracts were mutated separately, and their impact on (+1) distortion was evaluated by KMnO$_4$ modification. In the (rG)$_6$(dC)$_6$ tract, two G:C base pairs were substituted with C:G. Despite this relatively mild mutation, a significant reduction in the reactivity at template thymine +1 occurred (Fig. 4B, lane 4), whereas reactivity in the upstream A:T tract was unaffected, suggesting that this mutation only altered the structure locally around to the PPT/U3 junction, with the conformation of the (rA)$_4$(dT)$_4$ tracts remaining unchanged.

Another mutation included substitution of dT:dA base pairs with dC:rG at positions −9, −10, −14, and −15 to reduce breathing in this region that might have accounted for increased KMnO$_4$ sensitivity. Indeed, the reactivity of thymines −12 and −13 was significantly reduced. More importantly, however, was a dramatic reduction in the reactivity of template nucleotide +1 (Fig. 4B, lane 5). The latter effect is striking because the closest of the A:T mutations is introduced 9 bp upstream of position +1, whereas the adjacent guanine tract remained intact. These data indicate that the mutations in the (rA)$_4$(dT)$_4$ tracts significantly altered the entire hybrid structure, effectively yielding a distortion-free conventional RNA/DNA duplex. This notion is strongly supported by the RNase H activity studies of the following section. Whereas the reactivity of single-stranded thymines +6, +7, −22, and −26 serves as a control in the footprinting experiments (Fig. 3B), the observation that thymines −12 and −13 exhibit very different reactivities in AT mutant hybrid and wild-type PPT structures (see lanes 2, 3, and 5 of Fig. 4B) further supports the notion that KMnO$_4$ footprinting can be successfully employed for identification of the unstacked thymines in the context of an RNA/DNA hybrid.

Cleavage Specificity of PPT Mutants—In view of reduced KMnO$_4$ accessibility at position +1 as a function of PPT alterations, we investigated whether this was paralleled by altered cleavage specificity. The substrates of Fig. 5B were incubated with HIV-1 RT, and their hydrolysis profiles are depicted in Fig. 5A. Wild-type substrate was preferentially cleaved at the junction. However, this precision was lost when mutations were introduced into the (rG)$_6$(dC)$_6$ tract. Additional hydrolysis at positions −1 and −2 was detected at approximately the same frequency as that at the PPT/U3 junction (Fig. 5B). Substitutions within the G:C stretch affected the accessibility of only template nucleotide +1 to KMnO$_4$ oxidation, whereas the footprint pattern for the adenine stretch remained unchanged (Fig. 4B), suggesting a local structural perturbation around the PPT/U3 junction. Consistently, the RNase H cleavage profile was changed only at the small segment adjacent to the junction, whereas the rest of the PPT retained its resistance to cleavage.

The most dramatic alterations in RNase H cleavage were observed with substitutions in the two upstream (rA)$_4$(dT)$_4$ tracts. The preferential cleavage was detected at position −7 of the PPT primer. This could be explained by enzyme binding with its polymerase catalytic center over the primer 3′ OH and RNase H domain located 17 bp downstream at −7, i.e. in the manner expected from a conventional RNA/DNA hybrid. Again, this activity profile can be explained from the footprinting data of Fig. 4B. Mutations in the (rA)$_4$(dT)$_4$ tracts significantly diminished KMnO$_4$ accessibility at both −12/−13 and the PPT/U3 junction, effectively eliminating distortions throughout the entire RNA/DNA hybrid. Consequently, resistance of the PPT to RNase H cleavage and the selective hydrol-
The RT Positioning on the 3′ or 5′ Primer Terminus Is Not Critical for Selective RNase H Cleavage at the PPT/U3 Junction—To examine a role of the RT/3′ primer terminus contacts in the precise cleavage at the PPT/U3 junction, we have analyzed RNase H cleavage rates for PPT/(−) DNA hybrids containing 5-, 10-, 12-, and 15-nt extensions on the PPT 3′ end. The hydrolysis profiles of all these species were very similar to that presented in Figs. 6 and 8 for the 10-nt primer, except that the PPT/+ 5 nt U3 sequence was cleaved at about a 1.5-fold higher rate than other 3′ extensions tested (data not shown). Interestingly, the activity data agree well with that of chemical footprinting depicted in Fig 3B, where slightly increased reactivity was also detected for thymine +1 in 5 DNA chimeric primer. These results reconcile well with those of Gottke et al. (25), which also indicated that the 3′ primer end/RT contacts are not critical for the precise cleavage. These authors have shown that during (+) strand DNA synthesis, RT pauses after addition of the 12 deoxynucleotides to the PPT, and at this stage, another molecule of the enzyme binds to the PPT in the reverse orientation, with polymerase domain located toward its 5′ terminus (25). However, all the PPT primers examined by Gottke et al. contained 18-bp RNA (25). Therefore, it is impossible to conclude whether the specific cleavage at the PPT/U3 junction is a result of RT positioning at the 5′ primer end or whether other structural features of the PPT contribute to the selectivity. To address this question, we tested substrates that lacked the (U)5′ sequence or contained a 10-ribonucleotide extension at the PPT 5′ terminus. The data in Fig 7 conclusively show that the 5′ end does not have to be precisely 18–19 nt long for RNase H to preferentially cleave at the PPT/U3 junction. Thus, if the 18–
19-nt length was critical, then RT positioned at the 5′ end of the shortened PPT RNA (15 nt) should yield enhanced hydrolysis rates at positions +3 or +4, which represent conventional RNase H cleavage sites (see Fig. 7). Instead, the primary cleavage was observed at the PPT/U3 junction (Fig. 7, lane 2). The extension of the PPT primer by 10 RNAs on its 5′ terminus yielded at least two cleavage products (Fig. 7, lane 4). One is clearly within the PPT and is 18 nt removed from the RNA 5′ terminus. This is likely the result of the “polymerase-independent” mode of RNase H cleavage. However, the preferential cleavage is still detected at the PPT/U3 junction, 25 nt removed from the RNA 5′ terminus. Clearly, cleavage at this site cannot be explained solely by binding of RT to the RNA 3′ or 5′ terminus. Therefore, a plausible explanation for these (Fig. 7) and other similar results (Fig. 6) is that RT is recognizing some feature(s) of the PPT sequence itself that directs cleavage to the “appropriate” location. Interestingly, these results are in agreement with a previous study of longer transcripts (~80 bp) containing PPT sequences that also indicated that the cleavage at the PPT/U3 junction is not created as a result of RT binding to the 5′ end of the PPT oligoribonucleotide (26).

The Extent of Structural Distortion at the PPT/U3 Junction Significantly Affects Specific Cleavage—Hydrolysis was evaluated in the context of single and double base pair mismatches at the PPT/U3 junction (Fig. 8A). Surprisingly, introducing a single mismatch at position +1 enhances cleavage at this position (Fig. 8B, ii). Similar data were reported for RT of Moloney murine leukemia virus on its cognate PPT. Whereas Pullen et al. (3) did not provide a structural explanation for this observation, our chemical footprinting data indicate that the extent of base unstacking at position +1 in the T:C mismatch and the wild-type structure is similar (Fig. 4A, lanes 1 and 2). However, the base-pairing pattern at the +1 position in these two species may still differ. For example, the T:C mismatch implies a complete disruption of hydrogen bonding, whereas the A:T base pair in the wild-type species may only be subtly distorted. As a result, the two species yield different reactivities for RNase H. Introducing a two-base, +1/–1 mismatch had serious ramifications for PPT selection. Although mutant CA retains the T:C mismatch at position +1, there is a dramatic reduction in PPT cleavage efficiency when an additional A:G mismatch is introduced at position −1 (Fig. 8B, iii). KMnO₄ footprinting of the CA double mismatch indicated higher reactivity for the +1 thymine when compared with the wild-type or a single mismatch, consistent with more significant base unstacking (data not shown). Finally, a +1 G:A/–1 A:G mismatch eliminates cleavage at the PPT/U3 junction (Fig. 8B, iv). Due to the absence of thymine base at the +1 position, KMnO₄ modification did not allow us to probe the structure. However, the sequence would imply greater distortion at the PPT/U3 junction. As an internal control for activity (Fig. 8B), low level cleavage was consistently observed at position −7 of the PPT. Quantitation (Fig. 8C) shows that these effects range from a 2-fold increase in PPT cleavage for mutant MM, relative to the wild-type sequence, to a 20-fold decrease with the double +1 G:A/–1 A:G mismatch.

In conclusion, our data indicate that HIV-1 RT/RNase H activity is sensitive to distortions in the nucleic acid structure. Presumably, a single base pair distortion at position +1 is important for selective RNase H cleavage. Larger nucleic acid abnormalities at the PPT/U3 junction or distortion of the upstream (rA)₆:(dT)₆ tracts segment of the PPT adversely affects hydrolysis, possibly influencing correct positioning of RT on the PPT or accurate placement of the scissile bond in the RNase H catalytic center.

**DISCUSSION**

We show here that the nucleic acid conformation at the HIV-1 PPT/U3 junction is significantly distorted in the absence of trans-acting factors. In the recent co-crystal of HIV-1 RT harboring a PPT RNA/– DNA hybrid, the structures of the (rG)₆:(dC)₆ tracts at the 3′ end of PPT and the adjacent U3 sequence were not refined (10). Therefore, our KMnO₄ modification data provide novel structural information on the selectivity of RNase H hydrolysis adjacent to this site. In fact, we show a correlation between the structural deformation at base pair +1 and precise cleavage at the PPT/U3 junction. Previous biochemical studies indicated that primary RNase H cleavage occurs about 17–19 bp from the polymerase active site (27). Whereas this holds true for ordinary RNA/DNA hybrids, our data indicate that a significant increase in the cleavage rate at the PPT/U3 junction does not necessarily coincide with this
was hydrolyzed within 600 s. The cleavage data represent the results of four independent experiments. Lowercase letters

...and the (rA)4:(dT)4 tract is induced by the unique PPT sequence. The crystallographic analysis. We suggest that distortion in the adenine-rich segment of the PPT, complementing the our data reveal the origin of the structural distortion detected at the PPT/U3 junction during reverse transcription.

Our chemical footprinting experiments have revealed that the distal of two (rA)4:(dT)4 tracts upstream of the junction is also distorted in the absence of RT. Structural anomalies in the same region in the context of HIV-1 RT covalently linked to a PPT-containing RNA/DNA hybrid have been identified crystallographically (10). Whereas many nucleic acids perturb base pairings locally at the scissile bond during catalysis (28, 29), the structure of the RT-PPT complex indicated an unusually long (7 bp) distortion. This observation raised a question as to what might cause such an anomaly. We find that in the absence of enzyme, several template thymines of the distal (rA)4:(dT)4 tract are readily susceptible to K_MnO_4 oxidation, consistent with their unstacking in the PPT-containing duplex. Crystallographic analysis has also shown that residues constituting the RNase H primer grip make extensive contacts with the nucleic acid (10). However, these contacts are mostly restricted to the phosphate backbone, and there are limited direct interactions with nucleotide bases to account for disruption over such a long stretch of the RNA/DNA hybrid. Taken together, our data reveal the origin of the structural distortion detected in the adenine-rich segment of the PPT, complementing the crystallographic analysis. We suggest that distortion in the (rA)4:(dT)4 tract is induced by the unique PPT sequence. The crystal structure of the A-rich RNA/DNA hybrid r(caaagaaaag):d(CTTTTCTTTG) revealed A-like molecule (30), whereas the NMR structure of the G-rich hybrid r(gaggacug):d(CAGTCTC) indicated that the dimensions of the major groove are reminiscent of B-type DNA duplexes (11). Combining these diverse PPT-based structures within one molecule may conceivably lead to the distortion we observe. A contribution by the neighboring (rU)5:(dA)5 region should also be considered because this segment is critical for retroviral replication (5, 6).

Finally, upon binding of HIV-1 RT to the PPT, RNase H primer grip contacts may stabilize and/or further distort the structure to confer resistance to hydrolysis.

The nucleic acid structural distortions fulfill two different tasks, namely: (a) they create a RNase H-selective cleavage site, and (b) they render the PPT primer resistant to hydrolysis. Distortion at the PPT/U3 junction may have very different structural characteristics from that of the upstream (rA)4:(dT)4 tract. The former may be a relatively local distortion, whereas the irregularities in the (rA)4:(dT)4 tract expand over a long stretch of RNA/DNA hybrid. Nevertheless, these two unique features appear to be directly connected within the overall PPT architecture. Thus, mutations in the (rA)4:(dT)4 tract not only diminish structural breathing in this segment but also affect the conformation of the PPT/U3 junction by probably altering the entire RNA/DNA hybrid structure (Fig. 4). These structural changes directly influence RNase H cleavage, impacting both the resistance of the whole PPT to hydrolysis and the selectivity of cleavage at the PPT/U3 junction (Fig. 5).

Identification of amino acids in RT responsible for recognition of distorted structures in the PPT-containing RNA/DNA hybrids is now an attractive challenge. Gotte et al. (25) have shown that RT pauses after addition of the 12 deoxyribonucleotides to the PPT, and at this stage, another molecule of the enzyme binds to the PPT in the reverse orientation with the polymerase domain located toward its 5’ terminus. Using this location of RT over the PPT and the recently published crystal structure data of the PPT-RT complex (10), we modeled the enzyme with RNase H active site located at the PPT/U3 junction. We found that in this complex, the polymerase domain would make extensive contacts with the 7-bp distorted region of the adenine-rich segment. In particular, ~10 amino acids of the p66 thumb subdomain could contact this structurally anomalous region of the PPT. Thus, both distortions may play a pivotal role in accurate positioning of RT for precise cleavage at the PPT/U3 junction. It is also noteworthy that the RNase H domain of HIV-1 RT exhibits structural similarity with retroviral integrases and Holliday junction endonucleases (31, 32). These enzymes have been shown to introduce local distortions in the nucleic acid structure at the scissile bond (17, 23, 33). It is therefore intriguing to explore whether the same mechanism is employed by the retroviral polymerase for nonspecific hydrolysis of RNA/DNA hybrids. Another unusual structure requiring a high degree of RNase H specificity is the 3’-DNA-tRNA junction, at which cleavage is a prerequisite to second or (+)
strand transfer (34, 35). Whether structural anomalies at this junction control tRNA release becomes an important issue. Finally, whereas PPT sequences are conserved among retroviral genomes, there is significant divergence among their counterparts from long terminal repeat-containing retrotransposons of Saccharomyces cerevisiae (36). Despite this, we demonstrated that Ty3 RT recognizes its PPT sequence (5'-GAGAGAGAGAGA-3') with a high degree of precision (37). Experiments to better understand these systems are presently under way.

Acknowledgments—We thank D. Lilley (Dundee University, Dundee, UK) and G. Klarmann, J. Miller, and J. Rausch (National Cancer Institute–Frederick) for useful suggestions and critical reading of the manuscript.

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