Nonpolar Thymine Isosteres in the Ty3 Polypurine Tract DNA Template Modulate Processing and Provide a Model for Its Recognition by Ty3 Reverse Transcriptase*

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Despite diverging in sequence and size, the polypurine tract (PPT) primers of retroviruses and long terminal repeat-containing retrotransposons are accurately processed from (+) U3 RNA and DNA by their cognate reverse transcriptases (RTs). In this paper, we demonstrate that misalignment of the Ty3 retrotransposon RT on the human immunodeficiency virus-1 PPT induces imprecise removal of adjacent (+)-RNA and failure to release (+)-DNA from the primers. Based on these observations, we explored the structural basis of Ty3 PPT recognition by chemically synthesizing RNA/DNA hybrids whose (−)-DNA template was substituted with the non-hydrogen-bonding thymine isostere 2,4-difluoro-5-methylbenzene (F). We observed a consistent spatial correlation between the site of T → F substitution and enhanced ribonuclease H (RNase H) activity −12–13 bp downstream. In the most pronounced case, dual T → F substitution at PPT positions −1/−2 redirects RNase H cleavage almost exclusively to the novel site. The structural features of this unusual base suggest that its insertion into the Ty3 PPT (−)-DNA template weakens the duplex, inducing a destabilization that is recognized by a structural element of Ty3 RT −12–13 bp from its RNase H catalytic center. A likely candidate for this interaction is the thumb subdomain, whose minor groove binding tract most likely contacts the duplex. The spatial relationship derived from T → F substitution also infers that Ty3 PPT processing requires recognition of sequences in its immediate 5′ vicinity, thereby locating the RNase H catalytic center over the PPT-U3 junction, a notion strengthened by additional mutagenesis studies of this paper.

Although reverse transcriptase (RT)'-associated ribonuclease H (RNase H) activity degrades RNA of the RNA/DNA replication intermediate with little sequence specificity, it must precisely remove the RNA and polypurine tract (PPT) primers of (−)-strand (1) and (+)-strand DNA synthesis (2), respectively, to generate sequences at the 5′ and 3′ termini of the double-stranded DNA recognized by the integration machinery (3–8). Since the PPT is most likely embedded in a considerably larger RNA/DNA hybrid, precise hydrolysis at the PPT-U3 junction observed in vitro (9) suggests unique structural features may participate by correctly positioning this junction in the RNase H catalytic center. Our recent chemical footprinting of HIV-1 PPT-containing RNA/DNA hybrids (10) and a comparison with the crystal structure of HIV-1 RT bound to a related duplex (11) support this notion. Nucleic acid in the RT-RNA/DNA co-crystal is distorted 8–14 bp upstream of the PPT-U3 junction, comprising weakly paired, unpaired, and mispaired bases (Fig. 1A). Subsequent chemical footprinting studies (10) revealed that template thymines of this region and thymine +1 (i.e. immediately 3′ to the PPT) deviate from standard Watson-Crick base pairing in the absence of the retroviral enzyme. The finding that these naturally occurring HIV-1 PPT distortions are 10–14 bp apart was particularly intriguing, since this approximates the distance between the thumb subdomain and RNase H catalytic center of the heterodimer-associated p66 subunit (11–14). Therefore, it is possible that the A-tract-induced HIV-1 PPT distortion plays a role in sequestering RT via an interaction with structural elements at the base of the p66 thumb, thus positioning the RNase H catalytic center over the PPT-U3 junction for correct processing.

To examine whether this hypothesis may account for the precision of PPT processing in related long terminal repeat-containing elements, we focused here on the Saccharomyces cerevisiae retrotransposon Ty3. Differences in the primary structure of the HIV-1 and Ty3 PPTs (the former is a p66/p51 heterodimer, whereas Ty3 RT is a 55-kDa monomer) as well as in the sequence of their PPTs make the comparison between these two systems very interesting. Our preliminary analysis of Ty3 RT has indicated that its DNA polymerase and RNase H catalytic centers are separated by −21 bp (15) rather than the 17 bp observed in HIV-1 RT, suggesting a different spatial arrangement of thumb subdomain and RNase H catalytic center. Moreover, the Ty3 PPT differs in both size (12 bp) and sequence (Fig. 1B) from the HIV-1 counterpart. Whereas Ty3 RT processes its PPT with the appropriate precision in vitro (15, 16), we show here that it fails to remove (+)-DNA and imprecisely processes (+)-RNA 3′ to the HIV-1 PPT, suggesting co-evolution of enzyme and substrate.

RNA/DNA hybrids, whose (−)-DNA template was both individually and dually substituted with 2,4-difluoro-5-methylbenzene (F) for thymine, were used here to investigate structural features of the Ty3 PPT, mediating its recognition and processing. 2,4-Difluoro-5-methylbenzene is isosteric with thymine but has severely reduced hydrogen bonding capacity (17). Thus, F is a particularly useful tool to study the role of hydrogen bond-
ing and base structure and has been extensively used to evaluate the fidelity of DNA synthesis (18–21). However, to date there have been no studies on the recognition of F-substituted RNA/DNA hybrids. In the present study, T → F substitutions were designed to introduce flexibility and, possibly, structural changes at different positions of Ty3 PPT-containing RNA/ DNA hybrids, without changing the sequence of the primer. We show that subtle alterations to the structure of the Ty3 PPT (+)-RNA/(−)-DNA hybrid reposition the RNase H domain, inducing a novel but highly specific cleavage within the U3 region, 2 nt downstream from the site of F insertion. This suggests that correct processing of the (+)-strand primer may proceed through interaction of a structural subdomain of Ty3 RT with sequences immediately 5′ to the PPT and −12 bp from the PPT-U3 junction.

**EXPERIMENTAL PROCEDURES**

### Materials—Ty3 RT was expressed and purified as described (16). Unsubstituted DNA oligonucleotides were obtained from Integrated DNA Technologies (Gaithersburg, MD). 2,4-Difluoro-5-methylbenzene was purchased as the phosphoramidite from Glen Research. The DNA oligonucleotides containing single and pairwise F substitutions were synthesized using the Expedite 8809 automated synthesizer (PerkinElmer Life Sciences). RNA oligonucleotides were purchased from Dharmacon (Boulder, CO). All other reagents were of the highest purity and purchased from Sigma.

**PPT Selection—**To evaluate HIV-1 PPT selection a 55-nt, (−)-strand DNA template (corresponding to nucleotides 9048–9103 of the HIV-1, for which a considerable body of literature is available (5, 7–9, 22–24). Fig. 1C indicated that the Ty3 PPT is accurately released from (+) U3 RNA or DNA by Ty3 RT. We next evaluated HIV-1 PPTs extended at the 3′ terminus by either (+)-RNA or (+)-DNA (HIV-1 PPT-R or HIV-1 PPT-D, respectively. Fig. 2A). The hydrolysis pattern obtained with HIV-1 RT on a duplex extended with (+)-RNA (Fig. 2B, i) is similar to the one that we (9) and others (7, 8, 22) have reported, namely preferential cleavage at the PPT-U3 RNA junction and minor cleavage on either side (we define positions −1 and +1 as the bases on each side of the processing site). However, Ty3 RT cleaved this HIV-1 substrate with significantly altered specificity. Hydrolysis occurred at three positions of the adjacent RNA/DNA hybrid, centered around a 2,4-difluoro-5-methylbenzene (F; Fig. 3). Because RNase H cleaves RNA at the PPT(−)-DNA junction, if specific cleavage occurs, or within the PPT itself. The data of Fig. 2B, ii, again show minimal hydrolysis at the PPT-U3 DNA junction. In Fig. 2C, dNTPs were included to examine hydrolysis of the HIV-1 PPT(+)-DNA duplex in the context of DNA synthesis. The results indicated that, despite efficient polymerization from the HIV-1 PPT-D substrate, the removal of U3 DNA was again impaired, even after a 1-h incubation with Ty3 RT, eliminating the possibility that structural features of the HIV-1 substrate prevented binding of Ty3 RT. Following DNA synthesis, the 3′-OH of the extended primer is located 20 bp from the PPT-U3 junction, which would be ideally situated for Ty3 RNase H-mediated hydrolysis (Ty3 RNase H cleaves RNA substrates around 11 and 21 nt from the extremity that directs binding (15)). Similarly, the 5′-end of the HIV-1 PPT-R does not direct hydrolysis, since the same cleavage pattern was observed using a substrate extended by 15 nt at the 5′-end of the PPT. Therefore, altered processing of HIV-1 PPT-R (Fig. 2B, i) and lack of cleavage of HIV-1 PPT-D by Ty3 RT (Fig. 2B, ii, iii) must reflect recognition of a structural feature assumed by the HIV-1 PPT. In a similar experiment, Ty3 PPT variants were completely and nonspecifically hydrolyzed by HIV-1 RT (data not shown).

**RESULTS**

**Altered Processing of the HIV-1 PPT by Ty3 RT—**A clear difference between the PPTs of Ty3 and more extensively studied retroviruses is the presence of contiguous (rA:dT) and (rG: dC) tracts in the latter, which might provide a structural basis for recognition. This difference prompted us to investigate the manner in which Ty3 RT processes its cognate PPT and that of HIV-1, for which a considerable body of literature is available (5, 7–9, 22–24). hydrolysis of an HIV-1 PPT extended by (+)-DNA at its 3′ terminus. In this case, we can only observe cleavage at the PPT-U3 DNA junction, if specific cleavage occurs, or within the PPT itself. The data of Fig. 2B, iii, again show minimal hydrolysis at the PPT-U3 DNA junction. In Fig. 2C, dNTPs were included to examine hydrolysis of the HIV-1 PPT(+)-DNA duplex in the context of DNA synthesis. The results indicated that, despite efficient polymerization from the HIV-1 PPT-D substrate, the removal of U3 DNA was again impaired, even after a 1-h incubation with Ty3 RT, eliminating the possibility that structural features of the HIV-1 substrate prevented binding of Ty3 RT. Following DNA synthesis, the 3′-OH of the extended primer is located 20 bp from the PPT-U3 junction, which would be ideally situated for Ty3 RNase H-mediated hydrolysis (Ty3 RNase H cleaves RNA substrates around 11 and 21 nt from the extremity that directs binding (15)). Similarly, the 5′-end of the HIV-1 PPT-R does not direct hydrolysis, since the same cleavage pattern was observed using a substrate extended by 15 nt at the 5′-end of the PPT. Therefore, altered processing of HIV-1 PPT-R (Fig. 2B, i) and lack of cleavage of HIV-1 PPT-D by Ty3 RT (Fig. 2B, ii, iii) must reflect recognition of a structural feature assumed by the HIV-1 PPT. In a similar experiment, Ty3 PPT variants were completely and nonspecifically hydrolyzed by HIV-1 RT (data not shown).

**Dual T → F Substitutions of the Ty3 PPT DNA Template Modulate Cleavage Specificity—**The results of Fig. 2 suggest that structural features of the Ty3 PPT may contribute to the specificity of processing. Therefore, we used the base analog 2,4-difluoro-5-methylbenzene (F; Fig. 3A), which is isosteric
with thymine but fails to hydrogen-bond with adenine (25). F was substituted for several thymines of the DNA template complementary to the PPT (3'-C-C-T-C-T-C-T-C-T-C-T-T-T-5'). Such a strategy subtly alters the stability of the PPT-containing heteroduplex, at the site of substitution, and allowed us to determine the impact on both the structure of the duplex and cleavage specificity.

Initially, a series of doubly F-substituted Ty3 PPT RNA/DNA hybrids (Fig. 3A) was examined to determine whether localized destabilization of the nucleic acid duplex affected either the kinetics or specificity of processing. Indeed, adjacent F insertion at template positions −1/−2 had a profound effect, redirecting the RNase H catalytic center primarily over position +10/+11 of the non-PPT RNA/DNA hybrid (Fig. 3C, ii). This repositioning of Ty3 RT was also observed with substrates containing dual −5/-7 (Fig. 3C, iii) or −9/-11 substitutions (Fig. 3C, iv), which enhance cleavage at positions +6 and +3 of the RNA/DNA hybrid, respectively. Although less dramatic than the effect observed by a −1/−2 substitution, phosphor imaging and quantification (Fig. 4D) indicated that F-induced +6 and +3 cleavage is equivalent to or exceeds that at the PPT-U3 junction (Fig. 4C, i). Therefore, cleavage at the PPT-U3 junction is affected differently by adjacent or interrupted T → F substitutions. Adjacent substitutions create a more pronounced local destabilization that will sequester the majority of the enzyme at the new recognition site. Alternatively, the distortion induced by the presence of two adjacent T → F substitutions might render the PPT-U3 junction un-cleavable. Notably, the combined data of Fig. 4, B and C, also show a constant spatial correlation of 12–13 bp between the site of F insertion and that of enhanced RNase H activity.

Characterization of F-substituted Ty3 PPT RNA/DNA Hybrids—Three independent experiments were performed to evaluate if F insertion affected the Ty3 PPT structure (Fig. 4). Since the lack of hydrogen bonding has been correlated with a substantial drop in the Tm of shorter nucleic acid duplexes (18, 20), we determined the melting temperature of the F-substituted RNA/DNA hybrids. Wild type Ty3 RNA/DNA hybrid had a Tm of 69 °C (Fig. 4A). A single T → F substitution at position +2 (i.e. outside the PPT-containing duplex) reduced this to 65.6 °C, whereas that of substrates harboring dual substitutions varied from 63.0 to 60.5 °C. Thus, whereas T → F substitutions had the expected consequences on decreasing duplex stability, the Tm of all RNA/DNA hybrids was considerably
Although minor differences were noted in the peak and trough heights at 277 and 210 nm, respectively, mutant substrates differed minimally from the wild type PPT. Finally, in Fig. 4C, we examined the sensitivity of template thymines to chemical modification by KMnO_4 following T → F substitution. Previously, we successfully applied this strategy to the HIV-1 PPT, illustrating that template thymines +1 and −10 to −15 adopted a distorted structure (10). In contrast, very little KMnO_4 sensitivity is observed in the wild type Ty3 PPT (lane C), suggesting the absence of preexisting structural perturbations. However, the possibility that these might be induced following enzyme binding could not be excluded. Furthermore, the single +2 F (Fig. 4C, lane 1) or dual −1/−2, −5/−7, and −9/−11 T → F substitutions (Fig. 4C, lanes 2–4, respectively) were accommodated without altering the structure of neighboring A:T base pairs. Since F is insensitive to KMnO_4 oxidation, this prohibited any direct evaluation on the structure of the dF:rA pair. The combined data of Fig. 4 therefore provide a strong argument that T → F substitution within or adjacent to the Ty3 PPT is not accompanied by global changes in structure but rather a subtle and localized alteration in hydrogen bonding.

Single T → F Substitutions of the Ty3 PPT RNA/DNA Hybrid—To conduct a more detailed analysis of Ty3 PPT architecture, a second series of RNA/DNA hybrids was prepared containing single T → F substitutions from positions −1 to −11 of the (−) DNA template (Fig. 5A). In each case, processing at the PPT-U3 RNA junction was measured. In these experiments, RT partitions between the correct recognition site and a second binding site occurred at 44°C. The mutant substrates were accommodated without altering the structure of neighboring A:T base pairs. Since F is insensitive to KMnO_4 oxidation, this prohibited any direct evaluation on the structure of the dF:rA pair. The combined data of Fig. 4 therefore provide a strong argument that T → F substitution within or adjacent to the Ty3 PPT is not accompanied by global changes in structure but rather a subtle and localized alteration in hydrogen bonding.

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higher than the temperature at which PPT processing was evaluated (30°C). Fig. 4B compares the CD spectrum of each doubly substituted RNA/DNA hybrid to the wild type. The spectra were in good agreement with published data on polypurine-containing RNA/DNA hybrids, which assume an intermediate configuration between A-like and B-like (8, 26, 27).
Fig. 5. Processing of Ty3 PPT substrates harboring single T → F substitutions. A, Ty3 PPT-R substrate, indicating the sites of substitution. B, evaluation of F-substituted PPT substrates. The position of the T → F substitution is indicated below each panel. Lane notations and incubation times are as in the legend to Fig. 4. For each T → F substitution of the (−)-DNA template, the position of enhanced RNase H activity is indicated by the open arrow. C, quantification of PPT processing data. For all substrates, hydrolysis at each base pair of the RNA/DNA hybrid from position −1 to +11 was calculated as a percentage of total starting material. The 2-min time point was selected for quantitation.
had been obscured, we also radiolabeled the RNA substrates at their 3′-end. RNase H hydrolysis products obtained after 30 s were quantified. The results (Fig. 6C) indicated that cleavage at the PPT-U3 junction of mutant 3C → 3U represented only ~30% of total cleavage at the PPT termini, compared with ~80% for the WT. This significant change in cleavage specificity suggests that the accuracy and extent of Ty3 PPT processing are influenced by sequences adjacent to its 5′-end.

**DISCUSSION**

The molecular mechanism through which the PPT is recognized and specifically processed remains elusive, despite numerous studies describing the effects of altering either nucleic acid sequence (4, 7, 8, 22) or the structural motifs of RT (23, 24, 28). This is of particular importance, since the fidelity with which the PPT is processed has significant bearing on subsequent steps in replication. Previous studies with HIV-1 and equine infectious anemia virus (9) showed their PPTs were faithfully selected by RT when embedded within a considerably larger DNA/RNA hybrid (~120 bp) (i.e. where nucleic acid termini cannot influence positioning of RT). These and other observations (7, 8) implicate the PPT as an active participant in its recognition and processing. Furthermore, despite differences in nucleic acid sequence and RT architecture, a universal mechanism for PPT selection by the cognate RT might be expected. In this paper, the local flexibility of the Ty3 PPT was varied to determine its effect on (+)-strand primer removal. Introducing the non-hydrogen-bonding thymine isostere, F, consistently induces novel RNase H-mediated cleavage 12–13 bp from the site of substitution. For example, the dual T → F substitution redirects the RNase H domain almost exclusively to the novel cleavage site (Fig. 3D, ii). Additional mutagenesis studies indicate that altering sequences immediately 5′ to the Ty3 PPT (and ~13 bp from the PPT-U3 junction) significantly alters the balance of 5′ and 3′ processing (Fig. 6).

Finally, Ty3 RT hydrolyzes the HIV-1 PPT-R substrate ~13 bp downstream from a region demonstrated by chemical footprinting and x-ray crystallography to contain weakly paired, mispaired, and unpaired bases (10, 11). These independent lines of evidence suggest the coordinated action of two regions of Ty3 RT during PPT selection: the RNase H domain, which must be positioned at the PPT-U3 junction, and a structural motif within or adjacent to the DNA polymerase domain, which interacts with nucleic acid ~13 bp upstream of the processing site.

Although supporting structural evidence for this Ty3 RT motif is presently unavailable, it is possible to extrapolate from crystallographic data for the HIV-1 enzyme (11–13) to infer the region of the retrotransposon polymerase that interacts with nucleic acid ~13 bp from its RNase H catalytic center. In all nucleic acid-containing structures of HIV-1 RT, extensive contacts are made between the base of the p66 thumb subdomain and the substrate 3–7 bp behind from the DNA polymerase catalytic center. The RNase H catalytic center of HIV-1 RT must therefore be 10–14 bp downstream from the base of the thumb subdomain. In particular, helix eH of the thumb is partially embedded within the minor groove of double-stranded DNA. Gly206, Lys208, and Trp206 of helix eH are part of the minor groove binding track, a motif implicated in correct tracking of the enzyme over nucleic acid (29). Mutagenesis studies suggest that this motif could function as a “sensor” of duplex configuration, detecting base pair alterations introduced by lesions (30). Since crystallographic (11) and chemical footprinting data (10) have both identified distortions within the HIV-1 PPT, recognition of this structure by the minor groove binding track is a plausible mechanism that helps position the RNase H catalytic center directly over the PPT-U3 junction. Indeed, mutations in the minor groove binding track have been correlated with altered specificity of HIV-1 RT processing (31). It is very likely that Ty3 RT also has a thumb subdomain and a minor groove binding track. This may be the structural element of Ty3 RT that interacts with the F-induced structural perturbation. Indeed, secondary structure analysis has identified a putative thumb subdomain for Ty3 RT and a motif related to the HIV-1 minor groove binding tract. In fact, the data of Figs. 3 and 5 indicate that whereas T → F substitutions are not associated with major structural distortions, the local perturbations they introduce are sufficient to sequester a Ty3 enzyme “scanning” the PPT-containing RNA/DNA hybrid and induce cleavage 12–13 bp downstream. This hypothesis also explains the hydrolysis profile obtained by Ty3 RT on the HIV-1 PPT.

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*D. Lener and S. Le Grice, unpublished observations.*
Positioning of the Ty3 RNase H catalytic active site over positions +2 to +4 (Fig. 2B) locates the thumb subdomain 12–13 bp upstream, around positions −10 to −11. Our chemical footprinting analysis (10) identified distortions between positions −10 and −15 (Fig. 1A). Recognition of such distortion by the putative thumb subdomain of Ty3 RT is consistent with recognition of the T → F-induced local structural destabilization.

Data of Fig. 6 implicate a cis-acting element (i.e. the short dGrC block immediately upstream of the (+) strand primer) in Ty3 PPT recognition. Regions 5’ of the PPTs of murine leukemia (31) and simian immunodeficiency virus (32) have also been shown to control the efficiency and accuracy with which they are processed. Likewise, in vivo studies with another long terminal repeat-containing retrotransposon, Ty1, have shown that an A:T-rich region immediately upstream of the PPT participates in its selection (33). Thus, despite the absence of sequence homology between these long terminal repeat-containing elements, features of the RNA/DNA hybrid 5’ to the Ty3 PPT appear to influence its selection. Although we show here that alteration of this upstream region is associated with changes in PPT processing, the underlying structural basis is not clear. However, since G:C tracts are associated with major groove compression (34), it is possible that subtle differences in groove width immediately preceding the Ty3 PPT serve to "lock" the RT thumb in position, thus ensuring that the RNase H domain is correctly positioned over the biologically relevant processing site. Initial NMR studies with a Ty3 PPT-containing RNA/DNA hybrid in the absence of Ty3 RT have suggested that it may adopt an unusual configuration,3 which would support our postulation. Although our work has exploited the hydrogen-bonding isostere F, several alternative modified nucleosides are now available to better understand the molecular basis of PPT and tRNA primer processing and selection in HIV-1 and Ty3, including 2-aminopurine, 2,6-diaminopurine, purine riboside, and the non-hydrogen-bonding cytosine analog 2-fluoro-4-methylbenzene (35). The latter analog, in combination with F, has been used to probe the structure of the HIV-1 PPT and elucidate the molecular basis of its selection.4 These studies show that introducing F or 2-fluoro-4-methylbenzene into the HIV-1 PPT induces novel cleavage 3–4 nt downstream the insertion site instead of the 12 nt observed for Ty3, suggesting that the molecular bases for Ty3 and HIV-1 PPT selection are different.

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