Examining Ty3 Polypurine Tract Structure and Function by Nucleoside Analog Interference*

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Chandravanu Dash1, John P. Marino1,2, and Stuart F. J. Le Grice1,2

From the 1Resistance Mechanisms Laboratory, HIV Drug Resistance Program, NCI-Frederick, National Institutes of Health, Frederick, Maryland 21702 and the 2Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute and National Institute of Standards and Technology, Rockville, Maryland 20850

The abbreviations used are: LTR, long terminal repeat; PPT, polypurine tract; RTs, reverse transcriptases; U3, unique 3'-sequence; HIV-1, human immunodeficiency virus type 1; LNA, locked nucleic acid; Hfolate, tetrahydrofuran; RAbs, abasic ribosides; DAb, abasic deoxyribosides; Tm, melting temperature; DQF, double quantum filtered.

We have combined nucleoside analog interference with chemical footprinting, thermal denaturation, NMR spectroscopy, and biochemical studies to understand recognition of the polypurine tract (PPT) primer of the Saccharomyces cerevisiae long terminal repeat-containing retrotransposon Ty3 by its cognate reverse transcriptase. Locked nucleic acid analogs, which constrain sugar ring geometry, were introduced pairwise throughout the PPT (-)-DNA template, whereas abasic tetrahydrofuran linkages, which lack the nucleobase but preserve the sugar phosphate backbone, were introduced throughout the (-)-strand DNA template and (+)-strand RNA primer. Collectively, our data suggest that both the 5' and 3'-portions of the PPT-containing RNA/DNA hybrid are sensitive to nucleoside analog substitution, whereas the intervening region can be modified without altering cleavage specificity. These two regions most likely correspond to portions of the PPT that make close contact with the Ty3 reverse transcriptase thumb subdomain and RNase H catalytic center, respectively. Achieving a similar phenotype with nucleoside analogs that have different effects on duplex geometry reveals structural features that are important mediators of Ty3 PPT recognition. Finally, the results from introducing tetrahydrofuran lesions around the scissile PPT/unique 3'-sequence junction indicate that template nucleobase –1 is dispensable for catalysis, whereas a primer nucleobase on either side of the junction is necessary.

Although reverse transcription in long terminal repeat (LTR)3-containing retrotransposons of Saccharomyces cerevisiae (Ty1 and Ty3) and Saccharomyces paradoxus (Ty5) can be likened to that in retroviruses, notable differences have recently been documented. These include (a) initiation of (−)-strand DNA synthesis from an internal site of the host-coded tRNA primer as opposed to its 3' terminus (1), (b) initiation of (−)-strand DNA synthesis via self-priming (2), (c) use of a bipartite primer-binding site derived from each end of the viral RNA genome to initiate (−)-strand DNA synthesis (3), and (d) divergence in size and sequence of the (−)-strand polypurine tract (PPT) primers (see Fig. 1) (4, 5). A detailed biochemical study of these processes has been hampered by the lack of purified LTR-containing retrotransposon reverse transcriptases (RTs). However, the availability of active recombinant Ty1 and Ty3 enzymes (6, 7) now allows interactions with their cis-acting signals to be compared and contrasted with those of the retroviral counterparts.

A focus of our recent studies (8–13) has been understanding the mechanism that positions RT on the PPT-containing RNA/DNA hybrid for accurate cleavage at the PPT/unique 3'-sequence (U3) junction. Such specificity is required to create the (+)-strand primer and later to remove it from nascent (+)-DNA, thereby creating an integration-competent, double-stranded DNA provirus. Surprisingly, although a compilation of PPT sequences (14, 15) suggests a common "modular" make-up of homopolymeric A:dT and G:Gc blocks in retroviruses, this organization is absent in LTR-containing retrotransposon PPTs. Despite this, the Ty1 and Ty3 PPTs are accurately cleaved by their cognate RTs (7, 16). In the absence of any obvious sequence homology, we have considered the possibility that structural features of the Ty3 PPT upstream of the PPT/U3 junction facilitate enzyme positioning in an orientation placing the RNase H catalytic site over the scissile bond. At the same time, subdomains of the cognate RT would be expected to make specific contacts with both nucleobases and the sugar phosphate backbone. Crystallographic studies with human immunodeficiency virus type 1 (HIV-1) RT (17–19) showed that ~14 bp of duplex nucleic acid are accommodated between its RNase H catalytic center and the p66 thumb subdomain. Targeted nucleobase removal from the PPT (-)-DNA template showed that modifying positions –15, –14, and –13 (defining position –1 as the first base pair 5' to the PPT/U3 cleavage junction) altered cleavage specificity, suggesting that contacts mediated by the p66 thumb subdomain had been disrupted (13). Such a correlation indirectly suggests that protein/nucleic acid interactions involving either extremity of the PPT and defined by the spatial separation of the RNase H active site and thumb subdomain could be important modulators of cleavage specificity. The goal of this study was to test this hypothesis with a non-retroviral RT and "non-canonical" PPT.

One strategy to investigate nucleic acid geometry is to introduce nucleoside analogs into the duplex in a manner that does not disrupt sequence context, but that imparts increased or decreased flexibility. Using a scanning strategy with locked nucleic acid (LNA) analogs (20, 21) and abasic tetrahydrofuran (Hfolate) linkages (22, 23), which introduce rigidity and flexibility, respectively, into nucleic acids, we have defined two regions of the Ty3 PPT important for accurate cleavage of the scissile bond at the PPT/U3 junction. As might be predicted, one of these is the PPT/U3 junction, where LNA insertions and nucleobase removal as far as position –3 are capable of redirecting cleavage from the junction. The second region is the 5'-extremity of the PPT, where nucleoside analog insertion between positions –11 and –8 also induces altered cleavage specificity. However, the intervening region from posi-

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2 To whom correspondence should be addressed. Tel: 301-846-5256; Fax: 301-846-6013; E-mail: slgie@ncifcrf.gov.
3 The abbreviations used are: LTR, long terminal repeat; PPT, polypurine tract; RTs, reverse transcriptases; U3, unique 3'-sequence; HIV-1, human immunodeficiency virus type 1; LNA, locked nucleic acid; Hfolate, tetrahydrofuran; RAbs, abasic ribosides; DAb, abasic deoxyribosides; Tm, melting temperature; DQF, double quantum filtered.

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Ty3 Polypurine Tract Structure

| Virus     | Genus                  | Sequence                                 |
|-----------|------------------------|------------------------------------------|
| HIV-1     | (Lenti)                | UCUUAGCCACUUUAAAAAGTGGGAAAGGGCUAA        |
| HIV-2     | (Lenti)                | UACUUCAUUUAACAACACACCAAAAGGAGAAGGGCUAA   |
| SIV       | (Lenti)                | UUUUGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGU |
| CAEV      | (Lenti)                | CUGUGGAGAACACCCCAAAGAGGAGGUCUGUGA       |
| E1AV      | (Lenti)                | CCGACUGAGUUGUUUAGAACAACAGGAGGAGGUCUGUGA |
| FIV       | (Lenti)                | UUCUUGAGAACACCCCAAAGAGGAGGUCUGUGA       |
| BTV       | (Lenti)                | UCUCUUAUUAACCAACCAAAAGGAGAAGGGCUAA      |
| BLV       | (Coltia)               | UCGGCUCGUCCUUCUUUAAAGGAGGAGGAGGAGGAGGAC |
| PoLV      | (Gamma)                | UUCUACUUGACCUAACAGGGAGGAGGAGGAGGUCUGGAC |
| Mo-MLV     | (Gamma)                | UUUUUCUUGACCUAACAGGGAGGAGGAGGAGGUCUGGAC |
| Fr-MLV     | (Gamma)                | UUAAAGGAAUUGUUGUUGUUGGAAACAGGAGGAGGAGGAC |
| MMPV      | (Beta)                 | UGUGUCUCGUUUGUUGUUGUUGUUGAAACAGGAGGAGGAC |
| MMTV      | (Beta)                 | UUAAACGUGAAGCGAAACAGGAGGAGGAGGAGGAGGAC |
| AMV       | (Alpha)                | ACCUCGUGAACGUUUGUUGUUGUUGAAACAGGAGGAGGAC |
| RSV       | (Alpha)                | UAAAGGAAUUGUUGUUGUUGUUGUUGAAACAGGAGGAGGAC |
| HKRV-K10   | (Endogenous)           | UGGCGGGUUGGUGCAGAAAGGAGGAGGAGGAGGAGGAC |
| Ty1       | (LTR-retrotranspon)    | CAUUUGGGGCUCUUUAAAAAGGAGGAGGAGGAGGAGGAC |
| Ty3       | (LTR-retrotranspon)    | CCCUAGAGAGAAGAAGAAGAAGAAGAGGAGGAGGAGGAC |

**FIGURE 1.** Comparison of PPTs and flanking sequences from retroviruses and LTR-containing retrotransposons. Similarities among lentiviral PPTs (boldface and underlined bases) are particularly apparent, typically consisting of 4–12 adenines interrupted by a single nucleotide (usually a guanine), 3–7 contiguous guanines, and a 3′-terminal adenine. Adenine and guanine tracts are also common to other retroviruses and retroelements, except the PPT of the Saccharomyces cerevisiae LTR-containing retrotransposon Ty3. SIV, simian immunodeficiency virus; CAEV, caprine arthritis encephalitis virus; BIV, bovine immunodeficiency virus; FIV, feline immunodeficiency virus; FrELV, feline leukemia virus; FeLV, feline sarcoma virus; Rous sarcoma virus; FeLV, feline sarcoma virus; FR-MLV, Friend murine leukemia virus; Mo-MLV, Moloney murine leukemia virus; Fr-MLV, Friend murine leukemia virus; MPMV, Mason-Pfizer monkey-type virus; MMTV, mouse mammary tumor virus; AMV, avian myeloblastosis virus; RSV, Rous sarcoma virus; HERV, human endogenous retrovirus (adapted from Refs. 4 and 5).

**Experimental Procedures**

**Nucleic Acid Substrates**—r Spacer (introducing abasic H4 folate RNA lesions) and δ Spacer (introducing abasic H4 folate DNA lesions) were purchased from Glen Research Corp. (Sterling, VA). Hereafter, these are referred to as RAb and DAb, respectively. LNA phosphoramidites were purchased from Proligo Reagents. Substituted 39-nucleotide DNA and 29-nucleotide RNA oligonucleotides were synthesized at a 1:1 scale on a PE Biosystems Expedite 8909 nucleic acid synthesizer by a standard chemical synthesis method. Stepwise coupling yields for incorporation of the analogs were 98% as determined by trityl cation monitoring. Nucleic acid sequences and sites of substitution are illustrated in Fig. 2A and are described below. Deprotection and cleavage of the DNA oligonucleotides from the Controlled-Pore Glass support were carried out by incubation in 30% NH4OH for 36 h at 25 °C, whereas that of the RNA oligonucleotides were carried out following the instructions of Proligo Reagents. Oligonucleotides were purified by preparative PAGE and quantified spectrophotometrically (260 nm), assuming a molar extinction coefficient equal to the sum of the constituent deoxyribonucleotides. An unmodified 29-nucleotide RNA containing the PPT sequence and quaternary structure of its cognate polymerase, the results presented here with Ty3 RT suggest a common feature inasmuch as protein/nucleic acid interactions involving both extremities of the RNA/DNA hybrid cooperate to position the RNase H active site over the −1/+1 scissile bond.

**NMR Analysis**—20-Nucleotide oligodeoxyribonucleic acids (3′-dT dT dt dt dG dG dG dA dT dC dT dC dT dC dT dC dT dC dT dT dC dC dT dC dT dC dT dC dT dC dT-5′) (wild type), 3′-dT dT dG dG dG dA dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT-5′ (−2′/−1 LNA), 3′-dT dT dG dG dG dA dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC-5′ (−6′/−5 LNA), and 3′-dT dT dG dG dG dA dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC dT dC-5′ (−11′/−10 LNA), where X represents the LNA base) were hybridized to oligonucleotides containing 5′-r Ar Cr Cr Cu r Gr Ar Gr Ar Gr Ar Gr Ar Gr Ar r Ar Gr Ar Ar Gr Ar Gr Ar Gr Ar Gr Ar Gr Ar Gr Ar r Gr Ar Ar Gr Ar Ar Gr Ar-3′ (PPT primer) to prepare a 20-bp RNA/DNA hybrid. Individual RNA and DNA oligonucleotides were synthesized and purified as described above and desalted by dialysis against sterile double distilled H2O, followed by a buffer of 10 mM NaH2PO4/Na2HPO4 (pH 7.0) and 80 mM NaCl. Strand concentrations were determined by measuring the absorbance at 260 nm using extinction coefficients of 218 mM−1 cm−1 for the RNA strand and 162.2 mM−1 cm−1 for the DNA strand. The RNA/DNA hybrid was formed by mixing the RNA and DNA strands at a 1:1 stoichiometric ratio, heating the mixture to 90 °C, and slow cooling.

Double quantum filtered (DQF) COSY experiments were performed using samples of unmodified (1.2 mM) and LNA-substituted (−0.5 mM) 20-bp PPT-containing RNA/DNA hybrids in 10 mM phosphate (pH 7.0) and 80 mM NaCl dissolved in 90% H2O and 10% D2O at 30 °C. The spectrum of the unmodified Ty3 RNA/DNA hybrid was acquired in a Beckman DU 640 spectrophotometer. E260 was measured at 0.2 °C intervals from 30 to 80 °C. The Tm of each hybrid was calculated by the first derivative method described by the manufacturer.

**KMMnO4 Footprinting**—RNA/DNA hybrids containing analogs in the (−) strand were incubated at room temperature for 5 min in 20 mM NaCl. Annealed substrates were analyzed on a 10% nondenaturing polyacrylamide gel and visualized by ethidium bromide binding to determine that complete hybridization had been achieved. 10 μg/ml solutions of RNA/DNA hybrids were acquired in a Beckman DU 640 spectrophotometer. E260 was measured at 0.2 °C intervals from 30 to 80 °C. The Tm of each hybrid was calculated by the first derivative method described by the manufacturer.

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**Melting Temperature (Tm) Measurements**—For Tm measurements, equimolar concentrations of (+)-strand RNA primer were hybridized to (−)-strand DNA templates by heating to 90 °C and slow cooling in a degassed solution of 10 mM Na2HPO4/NaH2PO4 (pH 7.0) and 80 mM NaCl. Annealed substrates were analyzed on a 10% nondenaturing polyacrylamide gel and visualized by ethidium bromide binding to determine that complete hybridization had been achieved. 10 μg/ml solutions of RNA/DNA hybrids were acquired in a Beckman DU 640 spectrophotometer. E260 was measured at 0.2 °C intervals from 30 to 80 °C. The Tm of each hybrid was calculated by the first derivative method described by the manufacturer.

**KMMnO4 Footprinting**—RNA/DNA hybrids containing analogs in the (−) strand were incubated at room temperature for 5 min in 20
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**TABLE 1**

$T_m$ values for Ty3 PPT-containing duplexes with LNA doublet substitutions in the (–)-DNA template between positions –16 and +6

| Position | $T_m$ °C | Position | $T_m$ °C |
|----------|---------|----------|---------|
| –16/–15 | 71.9    | –5/+4   | 63.9    |
| –15/+4  | 73.8    | –4/+3   | 75.8    |
| –14/+3  | 70.9    | –3/+2   | 74.9    |
| –13/+2  | 75.8    | –2/+1   | 75.9    |
| –12/+1  | 64.0    | –1/+1   | 74.9    |
| –11/+0  | 65.0    | +1/+2   | 74.9    |
| –10/+9  | 64.0    | +2/+3   | 74.9    |
| –9/+8   | 63.0    | +3/+4   | 73.9    |
| –8/+7   | 65.0    | +4/+5   | 74.9    |
| –7/+6   | 63.0    | +5/+6   | 73.9    |
| –6/+5   | 64.0    |         |         |

**RNAse H-mediated PPT Selection**—Ty3 PPT selection was evaluated as described previously (10). 5’-End labeling of the PPT-containing RNA was performed with T4 polynucleotide kinase and [γ-$^32$P]ATP. Substrates were generated by annealing radiolabeled RNA to wild-type or substituted (–)-strand DNA. Hydrolysis was initiated by adding Ty3 RT to RNA/DNA hybrids in 10 mM Tris-HCl (pH 7.8), 80 mM NaCl, 5 mM dithiothreitol, and 9 mM MgCl$_2$ at 30 °C, with the enzyme and RNA/DNA hybrid present at final concentrations of 15 and 50 nM, respectively. Reactions were terminated after 10 min by adding an equal volume of formamide-based gel loading buffer (95% (v/v) formamide containing 0.1% (w/v) bromophenol blue and xylene cyanol), and the hydrolysis products were fractionated by high voltage electrophoresis on 15% (v/v) polyacrylamide gels containing 7 M urea. Products were visualized by autoradiography and/or phosphorimaging.

**DNA-dependent DNA Polymerase Activity**—DNA synthesis was measured on 39-nucleotide DNA templates annealed to a 5’-end-labeled 21-nucleotide DNA primer containing the PPT sequence. Polymerization was initiated by adding 10 nM enzyme to a mixture containing 50 mM template-primer, 200 μM dNTPs, 25 mM Tris-HCl (pH 7.8), 80 mM NaCl, 6 mM dithiothreitol, and 9 mM MgCl$_2$ at 25 °C and terminated after 10 min by adding an equal volume of gel loading buffer. DNA synthesis products were fractionated by high voltage electrophoresis on 15% (v/v) polyacrylamide gels containing 7 M urea in Tris borate/EDTA buffer. After drying, gels were subjected to autoradiography and/or phosphorimaging using a Personal Molecular Imager FX system (Bio-Rad).

**RESULTS**

**Experimental Strategy**—The strategy for our nucleoside analog interference studies with the Ty3 PPT is presented in Fig. 2. Position –1 is defined as the first base pair 5’ to the PPT/U3 junction, and duplex regions are defined as upstream or downstream of this junction (Fig. 2A). Overlapping LNA insertions were introduced pairwise throughout the (–)-DNA template between positions –16 and +6, whereas abasic H$_2$folate linkages were introduced singly into the (–)-DNA template and (–)-RNA primer between positions –12 and +2. Fig. 2B shows the structure of an LNA monomer, which induces a C-3’-endo configuration of the C-4’/C-2’ glycosylated sugar (20). In contrast, H$_2$folate linkages in the DNA or RNA strand (Fig. 2C) remove the nucleobase while preserving the sugar phosphate backbone (25). Prior to evaluating the effect of the analog insertion on cleavage specificity, the duplex stability and affinity of Ty3 RT for modified RNA/DNA hybrids were determined to eliminate the possibility that the altered enzyme activity was a consequence of gross conformational changes of the substrate.
FIGURE 3. Comparison of the pyrimidine H-5/H-6 correlated region from DQF-COSY experiments between the unmodified and LNA-substituted Ty3 RNA/DNA duplexes. A, the 20-bp Ty3 PPT-containing RNA/DNA duplex sequence. The PPT sequence is shaded, with the (+)-RNA strand in lowercase letters and the DNA strand in uppercase letters. The RNase H cleavage site is indicated by the arrow. B, overlay of the H-5/H-6 correlated regions of the unmodified (wild type (WT); black contours) and −2/−1 LNA-substituted (red contours) Ty3 RNA/DNA duplexes. C, overlay of the H-5/H-6 correlated regions of the unmodified (black contours) and −6/−5 LNA-substituted (red contours) Ty3 RNA/DNA duplexes. D, overlay of the H-5/H-6 correlated regions of the unmodified (black contours) and −10/−10 LNA-substituted (red contours) Ty3 RNA/DNA duplexes. Note that the positive and negative contours of the COSY cross-peaks are shown in the same color for each spectra. For the unmodified Ty3 PPT, the assigned RNA strand H-5/H-6 cross-peaks are indicated in lowercase letters, and the assigned DNA strand H-5/H-6 cross-peaks are indicated in uppercase letters.
Thermal Denaturation Profiles—Under our experimental conditions, a $T_m$ of 70 °C was determined for the unsubstituted RNA/DNA hybrid. Nucleobase removal from the RNA primer was more destabilizing, reducing the $T_m$ by between 8 °C ($^{11002}_{11002}$1RAb) and 10.2 °C ($^{11002}_{11002}$8RAb). This result likely reflects loss of both intrastrand stacking and interstrand hydrogen bonding. The destabilizing effect was slightly less pronounced for substitutions in the $^{11002}_{11002}$7-DNA template, where the $T_m$ was reduced by between 6.0 °C ($^{11002}_{11002}$12DAb) and 9.1 °C ($^{11002}_{11002}$6DAb). However, no individual position within the Ty3 PPT appeared particularly sensitive following nucleobase removal from either strand (data not shown).

In contrast, an unexpected pattern was obtained for RNA/DNA hybrids containing LNA insertions in the $^{11002}_{11002}$7-DNA template (Table 1). Consistent with a previous report (26), insertions between positions $^{11002}_{11002}$16 and $^{11002}_{11002}$12 and between positions $^{11002}_{11002}$4 and $^{11002}_{11002}$6 increased the $T_m$ by between 1.0 °C ($^{11002}_{11002}$14/$^{11002}_{11002}$13LNA) and 6 °C ($^{11002}_{11002}$4/$^{11002}_{11002}$3LNA). In contrast, LNA insertions between positions $^{11002}_{11002}$12 and $^{11002}_{11002}$4 uniformly decreased the $T_m$ by between 5 °C ($^{11002}_{11002}$11/$^{11002}_{11002}$10LNA) and 7 °C ($^{11002}_{11002}$5/$^{11002}_{11002}$4LNA). Resynthesis of representative $^{11002}_{11002}$7-DNA templates containing the $^{11002}_{11002}$7/$^{11002}_{11002}$6LNA and $^{11002}_{11002}$6/$^{11002}_{11002}$5LNA substitutions resulted in a similar reduction in the $T_m$ of the hybrid, excluding the simple possibility of experimental error during oligonucleotide synthesis or annealing (data not shown). The data of Table 1 thus suggest that constraining sugar ring geometry upstream of the Ty3 PPT/U3 junction has a destabilizing effect, possibly through disrupting stacking interactions at a particularly sensitive region of the RNA/DNA hybrid. Interestingly, the complementary sequence between positions $^{11002}_{11002}$12 and $^{11002}_{11002}$4 contains three adjacent and overlapping 5'-rArGrA-3' steps. Kopka et al. (27) noted that the ring-over-ring stacking of purines of such a sequence is not observed in an RNA/DNA hybrid modeled after the HIV-1 PPT, suggesting that it may contribute to deformability of this sequence. The presence of multiple steps of 5'-rArGrA-3' in the Ty3 PPT may therefore render the duplex highly malleable and, by inference, highly susceptible to LNA modification.

NMR Analysis of LNA-modified PPT Hybrids—To determine whether structural perturbations in the Ty3 PPT hybrid might explain the decreased thermal stability that results from LNA doublet substitution, a series of homonuclear NMR experiments were performed with the $^{11002}_{11002}$2/$^{11002}_{11002}$1LNA-, $^{11002}_{11002}$6/$^{11002}_{11002}$5LNA-, and $^{11002}_{11002}$11/$^{11002}_{11002}$10LNA-substituted PPTs, allowing an initial qualitative comparison with the unmodified hybrid. DQF-COSY spectra were acquired for the $^{11002}_{11002}$2/$^{11002}_{11002}$1LNA-substituted (Fig. 3B), $^{11002}_{11002}$6/$^{11002}_{11002}$5LNA-substituted (Fig. 3C), and $^{11002}_{11002}$11/$^{11002}_{11002}$10LNA-substituted (Fig. 3D) hybrids and provide a direct indicator of changes in base stacking geometry and dynamics in the

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**FIGURE 4. Determination of the dissociation constants for Ty3 RT with select Ty3 PPT variants by fluorescence anisotropy.** A, binding of Ty3 RT to unsubstituted RNA/DNA hybrids containing the fluorophore Cy3 on either the ($^{11002}_{11002}$)-DNA template ($^{f}_{o}$) or (+)-RNA primer ($^{F}_{i}$). B, dissociation constants for Ty3 RT with RNA/DNA hybrids containing abasic lesions and LNA insertions. The equivalent region of the PPT was examined for all substitutions.

**TABLE 1.**

| Substitution | $K_d$(nM) | Substitution | $K_d$(nM) | Substitution | $K_d$(nM) |
|--------------|-----------|--------------|-----------|--------------|-----------|
| -1RAb        | 2.0       | -1DAb        | 1.88      | -2/-1LNA     | 16.83     |
| -6RAb        | 1.79      | -6DAb        | 2.01      | -6/-5LNA     | 17.93     |
| -9RAb        | 1.64      | -9DAb        | 1.88      | -11/-10 LNA  | 19.01     |
| -12RAb       | 2.0       | -12DAb       | 2.43      | -13/-12LNA   | 13.85     |
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![Diagram of Ty3 Polypurine Tract Structure]

**Figure 5.** Effect of LNA doublet substitutions between positions −16 and +6 of the (−)-DNA template on cleavage at the PPT/U3 junction. Lane a, 16/−13LNA; lane b, 15/−14LNA; lane c, −14/−13LNA; lane d, −13/−12LNA; lane e, −12/−11LNA; lane f, −11/−10LNA; lane g, −10/−9LNA; lane h, −9/−8LNA; lane i, −8/−7LNA; lane j, −7/−6LNA; lane k, −6/−5LNA; lane l, −5/−4LNA; lane m, −4/−3LNA; lane n, −3/−2LNA; lane o, −2/−1LNA; lane p, −1/−1LNA; lane q, +1/−2LNA; lane r, +2/−3LNA; lane s, +3/−4LNA; lane t, +4/−5LNA; lane u, +5/−6LNA; lane W, unsubstituted hybrid. The sequence of the RNA/DNA hybrid and the positions of the LNA substitutions are indicated below, as is the PPT/U3 junction. The PPT region is shaded. For each hydrolysis profile, the PPT-containing RNA was 5′-end-labeled, and position −1 represents the PPT/U3 junction.

Hybrid. Strikingly, chemical shift perturbations were observed for almost all H-5/H-6 correlations in each of the LNA-substituted hybrids relative to the unmodified PPT, indicating that the LNA doublet insertion results in an altered structure over the entire PPT, with long-range effects observed for residues up to 16 bp distal to the site of the substitution (Fig. 3A), and not simply a local structural perturbation as has been observed in previous studies of LNA-substituted RNA/DNA hybrids (21, 26). The observed chemical shift perturbations for other proton resonances in the LNA-substituted PPTs also indicate that these insertions result in structural alterations over the entire PPT (data not shown). In addition, the structural perturbations appear to propagate more strongly upstream of the position of LNA substitution, with particularly large effects immediately adjacent to the PPT between positions −16 and −13. Significant line broadening was also observed for a number of H-5/H-6 resonances in each spectrum, with the most significant resonance line broadening resulting from the −10/−11LNA doublet substitution. Taken together, the NMR data demonstrate that the insertion of LNA doublets produces a structural perturbation that appears to significantly alter the hybrid conformation both within and immediately upstream of the Ty3 PPT. In this regard, the PPT-containing RNA/DNA hybrid appears unique in that a fixed altered local backbone geometry resulting from the LNA substitutions is propagated as long-range structural perturbations throughout the entire element. It is noteworthy to point out that LNA substitutions in RNA/DNA hybrids are known to alter the secondary structure of the nucleic acid locally, mostly restricted to the flanking 3′- and 5′-bases (28). However, there has been no report on the long-range global structural perturbations associated with local LNA substitutions in general RNA/DNA context as observed with Ty3 PPTs. The global nature of the structural effects could possibly explain the unusual observation of decreased Tm values for hybrids with LNA doublet substitutions between positions −12 and −4. However, more detailed structural analysis of the LNA-substituted hybrids will be required before specific structural changes can be correlated with altered thermal stability of the Ty3 PPT.

**Affinity of Ty3 RT for Modified RNA/DNA Hybrids**—To assess whether analog insertion affected the affinity for Ty3 RT for the modified PPT, equilibrium dissociation constants were determined for several substituted hybrids via fluorescence anisotropy (29, 30). For substrates containing RAb substitutions, the fluorophore Cy3 was introduced into the (−)-DNA template, whereas for substrates containing DAb and LNA substitutions, the fluorophore was introduced into the (±)-RNA primer. As shown in Fig. 4A, Kd values of 11.13 and 12.24 nM were determined for the unmodified hybrids containing Cy3 on the DNA and RNA strands, respectively. Although the affinity of Ty3 RT for the PPT-containing RNA/DNA hybrid was slightly reduced, these values are consistent with the Kd of ∼3.6 nM previously determined with duplex DNA (31). The dissociation constants for Ty3 RT with PPT variants containing LNA doublet substitutions at positions −2/−1, −6/−5, −11/−10, and −13/−12 were in general agreement with those for the unmodified RNA/DNA hybrids (Fig. 4B), ranging from 13.85 nM (−13/−12LNA) to 19.01 nM (−11/−10LNA). This result indicates that the modifications did not compromise recognition by Ty3 RT. Surprisingly, the affinity of Ty3 RT for substrates lacking template or primer nucleobases −1, −6, −9, and −12 increased ∼5-fold for duplexes substituted in either the RNA and DNA strand. This result might indicate that increased duplex flexibility, resulting from local alterations to base stacking and hydrogen bonding, stabilizes the nucleoprotein complex.

**Cleavage of LNA-subsitituted PPT Variants by Ty3 RT**—As shown in Fig. 2A, LNA substitutions were introduced pairwise into the (−)-DNA template throughout the PPT. In addition, the adjacent upstream sequences between positions −16 and −13 and downstream sequences between positions +2 and +6 were substituted to determine whether the LNA effect was restricted to the PPT-containing duplex or simply reflected a more general alteration of nucleic acid architecture. The results of this analysis are illustrated in Fig. 5.

For the wild-type RNA/DNA hybrid, the major hydrolysis sites were the PPT/U3 junction (defined as position −1) and positions +4 and +6 (Fig. 5, lane W). LNA substitutions between positions −16 and −13 had little effect on cleavage at the PPT/U3 junction (Fig. 5, lanes a−d), although the −14/−13LNA and −13/−12LNA substitutions eliminated cleavage at position +4 (Fig. 5, lanes e and d, respectively). In contrast, reduced cleavage at the PPT/U3 junction and enhanced cleavage immediately ahead of this were evident when LNA substitutions were relocated between positions −12 and −8 (Fig. 5, lanes e−h). The adjacent −8/−7LNA and −7/−6LNA substitutions appeared to selectively inhibit PPT/U3 cleavage (Fig. 5, lanes i and j, respectively),
whereas low level PPT/U3 cleavage was observed with hybrids substituted at positions −6/−5 and −5/−4 (Fig. 5, lanes k and l, respectively). LNA doublets between positions −4 and +1 had the most pronounced effect, severely reducing cleavage both at the PPT/U3 junction and ahead of this (Fig. 5, lanes m–p). For substitutions flanking the PPT/U3 junction (Fig. 5, lanes p–s), elimination or reduction of cleavage suggests that altering duplex geometry most likely influences its trajectory within the RNase H active site because the affinity of Ty3 RT for this hybrid was not seriously affected (Fig. 4B). PPT/U3 cleavage was gradually restored when the position of LNA substitution was relocated downstream of the scissile bond, although the +4 and +6 hydrolysis products were absent (Fig. 5, lanes t–u). Fig. 5 thus indicates that duplex geometry at either end of the PPT is sensitive to substitution, reflecting regions where either (a) altered geometry is incompatible with correct positioning of Ty3 RT or (b) enzyme contacts have been compromised, possibly through local alterations to sugar puckering (20, 21, 26). These possibilities will be discussed below.

Removal of PPT Template Nucleobases—Fig. 6 illustrates the effect of introducing H4folate lesions between positions −12 and +2 of the PPT (−)-strand DNA template. Removing nucleobase −12 did not affect specificity, but rather enhanced cleavage at the PPT/U3 junction (Fig. 6, lane a). In contrast, removing nucleobases between positions −11 and −8 reduced cleavage at the PPT/U3 junction while at the same time increasing cleavage in the U3 region downstream of this (Fig. 6, lanes b–e). Although subtle, a novel cleavage site, "tracking" (~12 bp from the position of analog insertion, was evident. As an example, substrate −11DAb exhibited enhanced cleavage at position +1, whereas substrate −9DAb rendered position +3 hypersensitive (Fig. 6, lanes b and d, respectively). Removing template nucleobases −7, −6, and −5 (Fig. 6, lanes f–l) was less deleterious in that, although cleavage ~12 bp from the site of analog insertion persisted (e.g. at position +5 on substrate −7DAb) (Fig. 6, lane f), the PPT/U3 junction was recognized. In contrast, Ty3 RT failed to recognize the PPT/U3 junction when template nucleobases −4, −3, and −2 were removed, but rather was redirected to cleave within the U3 region between positions +8 and +10 (Fig. 6, lanes i–k).

Template nucleobases −1 and +1 were removed to determine the importance of hydrogen bonding and/or base stacking opposite the scissile PPT/U3 junction or, in fact, the necessity for a template nucleobase because a previous study with HIV-1RT (9) demonstrated that this junction is surprisingly tolerant to substitution. For Ty3 PPT, our analysis showed that the template nucleobase 3’ to the PPT/U3 junction (position −1) could be removed without any major consequences (Fig. 6, lane l), whereas cleavage was eliminated when the nucleobase on the 5’-side of the scissile bond was removed (Fig. 6, lane m). Finally, a wild-type processing profile was observed when template nucleobase +2 was eliminated (Fig. 6, lane n).

Susceptibility of Template Thymines to KMnO₄ Oxidation—Thymine sensitivity to KMnO₄ oxidation can be exploited to determine whether such bases are unpaired (32, 33) or are structurally distorted but retain weak hydrogen bonding (34). Because thymines are evenly dispersed throughout the Ty3 PPT (−)-DNA template, chemical footprinting of the RNA/DNA hybrid was performed to gain insight into the structural consequences of targeted nucleobase removal (Fig. 7). Regardless of the position of nucleobase removal from the (−)-DNA template, only a single thymine in its immediate vicinity was KMnO₄-sensitive. This result suggests that the reduced Tₚₐₐₐ values accompanying nucleobase removal reflected local modification of duplex geometry. Also, as might be expected, removing any thymine eliminated that particular hydrolysis product. Eliminating nucleobase −10C (despite being 3’- and 5’-flanked by thymines) induced the reactivity of exclusively its 3’-neighbor, −11T (Fig. 7, lane c), suggesting that the 5’-thymine at position −9 retains a stably stacked and base-paired configuration. Selective reactivity of the 3’-thymine was also noted with substrates −8DAb and −6DAb, in which the modified nucleobase was likewise 3’- and 5’-flanked by thymine (Fig. 7, lanes e and g). Although supporting data with other RNA/DNA hybrids are unavailable, differential thymine reactivity has been noted for duplex DNA by McCarthy et al. (35). Although −4C is only 3’-flanked by a thymine, this base (−5T) still displayed KMnO₄ reactivity (Fig. 7, lane i). However, as the site of nucleobase removal approached the PPT/U3 junction, a different pattern emerged. Removing −3C enhanced the reactivity (although
weakly) of the adjacent 5’-thymine, −2T (Fig. 7, lane j), and likewise, removing −2T rendered the 5’-thymine (−1T), KMnO₄-sensitive (Fig. 7, lane k). At the same time, −2T was minimally KMnO₄-sensitive following removal of nucleobase −1T. These data imply that, in the absence of protein factors, the RNA/DNA hybrid likely adopts an unusual geometry near the PPT/U3 junction. This postulate is in keeping with our recent NMR studies showing an inversion of sugar pucker near the scissile bond (36). Finally, removing nucleobase +1C restored the original pattern in that the 3’-flanking thymine at position −1 was rendered KMnO₄-reactive.

Cleavage Specificity following Removal of PPT Primer Nucleobases—We next examined the targeted removal of PPT (+)-RNA primer nucleobases (Fig. 8). Because the RNA primer was 5’-end-labeled, hydrolysis products will lack the nucleobase and therefore migrate slightly faster than the unsubstituted RNA or its RNase H products in lane W. Moreover, we observed that hydrolysis products lacking guanine migrated slightly faster than those lacking adenine, producing the “staggered” appearance of the PPT/U3 hydrolysis product. Overall, removal of (+)-primer nucleobases had similar positional effects on PPT/U3 cleavage specificity as that of their (−)-DNA template counterparts. Substitutions between positions −11 and −8 (Fig. 8, lanes b−e) severely reduced PPT/U3 cleavage, whereas cleavage was less pronounced when nucleobases were removed from the intervening region between positions −7 and −4 (lanes f−j). Removing primer nucleobases −2, −1, and +1 completely eliminated PPT/U3 cleavage (Fig. 8, lanes k−m), suggesting that they mediate important contacts at the RNase H catalytic site. This result contrasts with results from substitutions in the same region of the (−)-DNA template, where nucleobase −1 could be eliminated without inhibiting PPT/U3 cleavage (Fig. 6, lane l). Relocation of the RNase H catalytic center a constant distance (~12 bp) relative to the site of analog insertion was most pronounced with the −8 and −4 substitutions, which promoted cleavage at positions +4 and +8, respectively (Fig. 8, lanes e and l). Also, in the vicinity of the PPT/U3 junction, the −2 and −1 substitutions induced hypersensitivity at positions +10 and +11, respectively (Fig. 8, lanes k and l).

DNA Polymerase Activity of Ty3 PPT Variants—Finally, we examined, F9 DNA-dependent DNA synthesis on duplexes containing LNA insertions (Fig. 9B) or H₂folate lesions (Fig. 9C) in the (−)-DNA template. For these experiments, a 5’-end-labeled DNA primer was employed to avoid cleavage at the PPT/U3 junction and removal of nascent DNA or the use of mutant enzyme lacking RNase H activity. A prominent feature of LNA insertions in the (−)-DNA template was stalling of the replication complex shortly after initiation of DNA synthesis on duplexes with substitutions between positions −4 and −1 (Fig. 9B, lanes m−p). As an example, a −1/+1 insertion led to stalling at positions P+4 and P+5 of the DNA template (Fig. 9B, lane p). This result suggests that, when this insertion occupies positions −5 and −6 of the duplex following translocation of the polymerizing machinery, the structural alteration induced by constrained sugar ring geometry impedes contact with important protein motifs of the enzyme. In a similar fashion, the −2/−1LNA substitution induced stalling at position P+4 of the template (Fig. 9B, lane o), i.e. when the LNA doublet would again be predicted to occupy positions −5 and −6 of the polymerizing complex. This effect continued as far as the −5/−4LNA sub-
stitution, after which subsequent positions of LNA substitution presumably already lie beyond this important protein motif. Current HIV literature (37–41) and our recent work with Ty3 RT (42) suggest that this would be the thumb of the DNA polymerase domain, where the H9251 helix of the HIV-1 enzyme may function as a sensor of nucleic acid geometry (37–41). Another feature, although small, was pausing at multiple positions throughout the template for substrates containing LNA insertions between positions 11002 and 11004 (Fig. 9B, lanes e–l), corresponding to the PPT/U3 junction. FIGURE 8. Effect of nucleobase removal from the (+)-RNA primer on cleavage at the PPT/U3 junction. The PPT-containing RNA mutant substrates were 5'-end-labeled. Lane a, -12RAb; lane b, -11RAb; lane c, -10RAb; lane d, -9RAb; lane e, -8RAb; lane f, -7RAb; lane g, -6RAb; lane h, -5RAb; lane i, -4RAb; lane j, -3RAb; lane k, -2RAb; lane l, -1RAb; lane m, +1RAb; lane n, +2RAb; lane W, unsubstituted hybrid.

FIGURE 9. Effect of LNA substitutions within and nucleobase removal from the PPT (−)-DNA template on DNA synthesis from the PPT 3' terminus. B, LNA substitutions. Lanes are as described in the legend to Fig. 5. C, nucleobase removal. Lanes are as described in the legend to Fig. 6. In B and C, the unextended and fully extended 5'-end-labeled primers are indicated as P and P + 18, respectively.
Ty3 Polypurine Tract Structure

to the region of the PPT-containing RNA/DNA hybrid destabilized by LNA insertion (Table 1).

DNA synthesis on PPT derivatives containing H4folate template lesions was unaffected in general, with the exception of the region between positions −4 and +1 (Fig. 9C, lanes i–l). Removing template nucleobase −4 induced pausing at positions P+1 and P+2 (Fig. 9C, lane i), suggesting again that, when this substitution (which alters base stacking) occupies positions −5 and −6 of the translocation complex, contacts with the Ty3 RT thumb subdomain are compromised. Likewise, removing template nucleobase −3 induced pausing at positions P+2 and P+3 of the translocation complex (Fig. 9C, lane j). In contrast, removing template nucleobase −2 did not induce aberrant stacking, indicating that, following addition of 2–3 nucleotides to the primer terminus, the polymerization machinery has acquired sufficient energy (possibly generated through dNTP hydrolysis) to bypass the lesion. As might be expected, removing template nucleobase −1 was most deleterious for DNA polymerase function because this would most likely affect positioning of the primer 3′-OH in the active site. These data again support our recent mutagenesis studies on the Ty3 thumb subdomain showing that alteration of the counterpart of α-helix H severely influences the stability of the replication complex (42). Thus, when Ty3 RT adopts the orientation with its RNase H catalytic center positioned for cleavage at the PPT/U3 junction, it is not unreasonable to assume that abnormal nucleic acid geometry upstream of this junction is also sensed by its thumb domain.

DISCUSSION

Although retroviral and LTR-containing retrotransposon PPTs differ significantly in both their length and sequence (5, 14), they are accurately removed from (+)-RNA and (+)-DNA by their cognate RTs, suggesting that common structural features of these RNA/DNA hybrids contribute to the sequestration and positioning of the viral polymerase such that its RNase H catalytic center is correctly located over the scissile bond at the PPT/U3 junction. For the HIV-1 RT, x-ray crystallography (19), chemical footprinting (8), and nucleoside analog interference (9, 11, 13) studies make a strong case for an interaction between the p66 thumb subdomain of RT and a destabilized ("unzipped") portion of the duplex 12–15 bp upstream of this junction (19). This region of the PPT has been implicated in an induced fit with HIV-1 RT as a potential mechanism to correctly locate its RNase H active site (8, 15). Whether a similar mechanism applies to PPT recognition by LTR-containing retrotransposon enzymes has been addressed here by scanning mutagenesis of both the DNA and RNA strands using analogs that increase or decrease the stability of the Ty3 PPT-containing duplex.

Although they might be expected to have different consequences for duplex architecture, LNA analogs and H4folate linkages at the 5′-end of the Ty3 PPT produce the same positional effect, implying that they influence duplex geometry rather than affect specific protein/nucleic acid contacts. Another consequence is the appearance of novel cleavage sites that appear to track a constant distance from the site of analog insertion. This is evident for duplexes with LNA substitutions between positions −11 and −8; incubation with Ty3 RT led to enhanced cleavage in the U3 region −12 bp from the site of insertion (Fig. 5, lanes f–i). In contrast, immediately adjacent LNA substitutions between positions −16 and −13 in duplexes, i.e. outside the PPT, had minimal effects (Fig. 5, lanes a–d), eliminating a general property of LNA insertion into RNA/DNA hybrids. Scenarios that might explain these novel cleavage sites include (a) a long-range effect of LNA substitution on nucleic acid geometry at and ahead of the PPT/U3 junction and (b) local alterations in duplex architecture promoting novel binding sites for Ty3 RT. LNA analogs in the (−)-DNA template will constrain the sugar ring in a C-3′-endo or N-type configuration, which is contrary to its normal orientation (20), whereas NMR studies have shown that LNA insertions can influence the sugar pucker of adjacent 5′- and 3′-residues (21, 28). Thus, the LNA doublet insertions evaluated here could locally alter sugar puckering over as many as 4 bases of the (−)-DNA template. Experimentally, we observed that PPT substitution between positions −11 and −8 induced novel cleavage at several sites ahead of the PPT/U3 junction. In keeping with our HIV-1 data, we therefore suggest that a structural element of Ty3 RT located 11–12 bp from its RNase H catalytic site "senses" an altered sugar pucker as a stable binding site. A logical protein motif would be the thumb of its DNA polymerase domain, which studies with HIV-1 RT (37–41) have shown is particularly sensitive to alterations in nucleic acid geometry. The DNA synthesis data of Fig. 9, combined with our recent mutagenesis studies on the Ty3 RT thumb subdomain (42), show that it, too, is responsive to subtle alterations to duplex architecture. Thus, the model we envisage proposes that each LNA-induced alteration to the sugar pucker between positions −11 and −8 can provide a transient interaction site for the Ty3 RT thumb subdomain, which would relocate the RNase H catalytic center over sites ahead of the PPT/U3 junction. Our recent NMR studies on the Ty3 PPT-containing RNA/DNA hybrid further support a contribution from sugar puckering to PPT selection (36).

The observation that H4folate linkages in the template and primer between positions −11 and −7 (Figs. 6 and 8, respectively) have a similar effect on PPT/U3 cleavage suggests that their effect might also involve alterations in sugar puckering. In this respect, molecular dynamics simulations of model duplexes have indicated that the abasic sugar adopts a variety of puckerings centered around the C-3′-endo pucker (43). Thus, for the wild-type PPT-containing duplex, our data suggest that malleability between positions −11 and −8 is an important mediator of interactions with the thumb subdomain of its cognate RT. Because most nucleic acid-polymerizing enzymes are known to bend the duplex in the vicinity of the catalytic center (44), flexibility inherent to this region of the Ty3 PPT could be envisaged as an important modulator of an induced fit with RT.

Ty3 hybrids with substitutions between positions −7 and −5 are cleaved at the PPT/U3 junction, albeit with reduced efficiency. The crystal structures of HIV-1 RT bound to duplex DNA (17, 18) and an RNA/DNA hybrid (19) indicate that considerably fewer contacts are made with nucleic acid in this region, which would occupy its connection subdomain. Thus, provided the appropriate contacts are made with these N- and C-terminal domains of Ty3 RT, manipulating the intervening nucleic acid structure appears to be less deleterious. A second and abrupt alteration in PPT cleavage specificity follows substitution in the adjacent region between positions −4 and −2, which most likely perturbs contacts with critical protein motifs close to the RNase H catalytic center. The RNase H primer grip motif of HIV-1 RT (19) has been shown to contact nucleic acid several bases upstream of the RNase H catalytic center, possibly as a means of guiding the trajectory of the RNA strand into the active site for cleavage. A recent crystal structure of Bacillus halodurans RNase H complexed with an RNA/DNA hybrid (45) has defined a similar region as the "phosphate-binding pocket," indicating that it mediates contact with the DNA strand 2–4 bp upstream of the scissile bond. Altering sugar pucker in the equivalent region of the Ty3 PPT (−)-DNA template might therefore be predicted to compromise interactions with its phosphate-binding pocket, thereby inhibiting RNase H activity.

Structural studies with RNases H from Escherichia coli (46–49) and B. halodurans (45) have implicated several active-site residues in con-
tacting the RNA strand in the catalytic center (46–49). For the E. coli enzyme, it has been proposed that Glu109 is hydrogen-bonded to the 2’-OH of the nucleoside 2 bases 5’ to the scissile bond, whereas Cys13 interacts with the 2’-OH of the ribonucleotide ahead of this. In addition, an outer sphere complex between the catalytic Mg2+ ion and the 2’-OH group of the nucleoside 5’ to the scissile bond has been predicted. Because a detailed structure for Ty3 RT is unavailable, assigning this function to equivalent residues becomes speculative. However, the recent structure of a co-crystal of B. halodurans RNase H with an RNA/DNA hybrid indicates that two of its catalytically critical carboxylate residues (Glu109 and Asp132) contact the ribose moiety of the RNA strand on either side of the scissile bond. For Ty3 RT, the equivalent residues are Glu401 and Asp426, respectively. Thus, constraining nucleic acid geometry or altering sugar pucker around the Ty3 PPT/U3 junction would be predicted to interfere with these critical contacts. Finally, the data of Fig. 6 show that template nucleobase –1 is dispensable for hydrolysis, possibly indicating separation of the DNA and RNA strands as the hybrid enters the catalytic center. This observation concurs with our recent studies of HIV-1 RT demonstrating that template nucleobases can also be removed without affecting cleavage specificity (13).

In the absence of crystallographic data, our studies with Ty3 RT have by necessity required comparison with and extrapolation from structures of the HIV-1, E. coli, and B. halodurans enzymes. In particular, we have proposed that the thumb subdomain of Ty3 RT interacts with a particularly malleable region of the RNA/DNA hybrid between positions –11 and –7 of the PPT and that nucleoside analogs alter duplex architecture in a manner that subtly changes these contacts. The thermal melting data of Table 1 support this notion and indicate that LNA insertions between positions –12 and –4 destabilize the hybrid, in contrast to published data. The same region exhibits unusual KmM4O4 reactivity in that thymines 5’ to H3folate lesions remain well stacked, whereas those 3’ to the lesion are oxidized (Fig. 7). The unusual KmM4O4 reactivity of template thymines immediately adjacent to the PPT/U3 junction (Fig. 7) suggests that the duplex geometry here also deviates from standard Watson-Crick base pairing. Taken together, these observations provide a strong argument that the Ty3 PPT has a pre-organized structure that facilitates RT binding in a productive hydrolytic mode. As an initial step in confirming this proposal, we have initiated NMR studies on both the wild-type and substituted duplexes, and preliminary data indeed indicate that a single substitution in the (−)−DNA template alters the sugar pucker of both its RNA complement and several neighboring RNA bases, indicating considerable malleability at the 5’−end of the Ty3 PPT (36).

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