Interaction of the Ty3 Reverse Transcriptase Thumb Subdomain with Template-Primer
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Amino acid sequence alignment was used to identify the putative thumb subdomain of reverse transcriptase (RT) from the Saccharomyces cerevisiae LTR-retrotransposon Ty3. The counterpart to helix αH of HIV-1 RT, which mediates important interactions with duplex nucleic acid ~3-6 base pairs behind the DNA polymerase catalytic center, was identified between amino acids 290 and 298 of the Ty3 enzyme. The consequences of substituting Gln290, Phe292, Gly294, Asn297 and Tyr298 of Ty3 RT (the counterparts of HIV-1 RT residues Gln258, Leu260, Gly262, Asn265 and Trp266, respectively) for both DNA polymerase and RNase H activity was examined. DNA-dependent DNA synthesis was evaluated on unmodified substrates and on duplexes containing targeted insertion of locked nucleic acid analogs and abasic lesions in either the template or primer. Using this combined strategy, our data suggests an interaction of Ty3 RT residues Tyr298 with primer nucleotide -3, Gly294 with primer nucleotide -4 and Asn297 with template nucleotide -6. Substitution of Ala for Gln290 was well tolerated, despite the high degree of conservation at this position. Mutations in the thumb subdomain of Ty3 also affected RNase H activity, suggesting a closer spatial relationship between its N- and C-terminal catalytic centers compared to HIV-1 RT.

Crystallographic studies of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) with duplex DNA (1,2) and an RNA/DNA hybrid (3) have identified several protein motifs essential for efficient nucleic acid polymerization and hydrolysis, as well as translocation of the replication machinery over a variety of conformationally-distinct nucleic acid duplexes. In addition to the DNA polymerase and ribonuclease H (RNase H) catalytic centers, the β12 – β13 hairpin, or “primer grip”, of the p66 DNA polymerase domain maintains the primer terminus in an orientation appropriate for nucleophilic attack on the incoming deoxynucleoside triphosphate (4-7). Contacts to the template are mediated via elements of the p66 fingers and palm subdomains collectively designated the “template grip” (8-10). Participation of the “RNase H primer grip” in imposing the correct trajectory on the RNA strand of RNA/DNA hybrid for hydrolysis at the RNase H catalytic center has been suggested (11-13). Finally, a network of contacts between residues of helices αH and αI at the base of the p66 thumb with the primer backbone 3-6 bp behind the polymerase active center may comprise a “translocation track” and also assist the transition in nucleic acid geometry from A- to B-form downstream of the DNA polymerase catalytic center, an event accompanied by considerable duplex bending (14-20). The combined biochemical and biological data available from mutagenesis studies lends strong support to the proposed functions of these protein motifs.

Information gleaned from such studies has accrued almost exclusively from analysis of retroviral enzymes, in particular those of human, avian and murine origin. In contrast, there is a paucity of detailed biochemical information on equivalent events mediated by RT from long-terminal repeat (LTR)-containing retrotransposons such as Ty1 and Ty3 of Saccharomyces cerevisiae and Ty5 of Saccharomyces paradoxus. Such an analysis is particularly important in view of differences at several steps of the LTR-retrotransposon reverse
transcription cycle. Examples of such differences include (a) initiation of (-) strand DNA synthesis from an internal site of the cognate tRNA primer in Ty5 (21), (b) use of a bipartite primer binding site to initiate (-) strand DNA synthesis in Ty3 (22) (c) a self-primed initiation of (-) strand DNA synthesis (23), (d) a role for RNA branching and debranching in Ty1 (-) strand DNA transfer (24,25), and (e) divergence in both size and sequence of the (+) strand, polypurine tract (PPT) primers of Ty1 and Ty3 (26,27). The recent availability of active recombinant Ty1 (28,29) and Ty3 RT (30-32) now allows cis-acting signals of LTR-retrotransposons and protein motifs of their cognate RTs to be compared and contrasted with their retroviral counterparts.

In this communication, secondary structure prediction programs and amino acid sequence alignments were used to identify the Ty3 RT equivalent of HIV-1 RT helix αH. Crystallographic analysis of HIV-1 RT indicates that Gln258, Gly262 and Trp266 of this α helix are in close contact with the sugar-phosphate backbone of the primer, playing an important role in translocation and as a “sensor” of nucleic acid configuration (1-3,33,34). These studies also implicated p66 residue Asn265, in this case via an interaction with the sugar-phosphate backbone of the template. Mutations at these positions of the HIV-1 enzyme have pleiotropic effects, including alterations in DNA binding, processivity of DNA synthesis, frameshift fidelity (18-20) and PPT usage (15). Selected residues within this putative α helix of Ty3 RT were mutated and evaluated with respect to the DNA polymerase and RNase H functions of the purified enzymes. In addition to studying duplex DNA and RNA/DNA hybrids, polymerization efficiency on duplex DNA containing targeted insertion of locked nucleic acid analogs or abasic lesions were purchased from Integrated DNA Technologies, Coralville, IA. RNA oligonucleotides for analysis of RNase H activity were purchased from Dharmacon, Boulder, CO.

EXPERIMENTAL PROCEDURES

Oligonucleotides – Standard DNA oligonucleotides and those containing locked nucleic acid analogs or abasic lesions were purchased from Integrated DNA Technologies, Coralville, IA. RNA oligonucleotides for analysis of RNase H activity were purchased from Dharmacon, Boulder, CO.

Preparation and Purification of Ty3 RT Mutants - Point mutations in the DNA polymerase domain of Ty3 RT expressed on plasmid p6HTy3RT (30) were introduced using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). Briefly, sense and antisense mutagenic oligos were purchased for each point mutation and annealed to the denatured p6HTy3RT plasmid. Extension and incorporation of the mutagenic primers, during temperature cycling was performed with PfTurbo DNA polymerase. The reaction was treated with Dpn I endonuclease to digest the parental DNA template and then transformed into XL10-Gold ultracompetent E.coli cells. Ty3 RT mutants were purified by a combination of metal chelate and size exclusion chromatography according to Lener et al. (31) and stored in a 50% glycerol-containing buffer at -20°C. Protein concentration was determined using the Quick Start™ Bradford Protein Assay (Bio-Rad, Hercules, CA).

DNA Polymerase Activity - RNA-dependent DNA polymerase activity was evaluated on a 152-nt RNA template (prepared by in vitro transcription), corresponding to nucleotides 2225-2363 of Ty3 genome plus 14 additional nucleotides hybridized to a 21 nt DNA primer. Template-primer was annealed by incubation at 95°C in 10 mM Tris/HCl (pH 7.8), 100 mM NaCl and slow cooling to room temperature. A reaction mixture containing 50 nM template-primer and each dNTP at a final concentration of 250 µM was prepared in a buffer comprising 10 mM Tris/HCl (pH 7.8), 80 mM NaCl, 5 mM dithiothreitol, 0.01% (v/v) Triton X-100 and 9mM MgCl₂. DNA
synthesis was initiated at 30˚C by addition of wild type or mutant RT to a final concentration of 100 nM in a final reaction volume of 10 µl and terminated after 10 min by mixing with an equal volume 89 mM Tris-borate, pH 8.3, 2 mM EDTA, and 95% (v/v) formamide containing 0.1% (w/v) bromophenol blue and xylene cyanol. Polymerization products were resolved by high-voltage, denaturing 10% polyacrylamide gel electrophoresis and visualized by phosphor imaging. Processivity during RNA-dependent DNA polymerase activity was evaluated using the substrate indicated above, but increasing the RT concentration to 200 nM. After incubating RT and template/primer for 5 min at 30˚C, DNA synthesis was initiated by addition of dNTPs and heparin trap to final concentrations of 250µM and 2mg/ml, respectively. The efficiency of the competitor was determined by adding this prior to Ty3 RT and template/primer, which resulted in complete inhibition of DNA synthesis (data not shown). DNA-dependent DNA polymerase activity was evaluated on HhaI-digested, single-stranded M13 mp18 DNA (USB) to which the [32P]-end-labeled M13 universal primer was hybridized (HhaI, unlike the majority of restriction enzymes, cleaves single stranded DNA). Assay conditions were similar to those described above, with the exception that 1.5 nM template-primer was used.

**Determination of Dissociation Rate Constants by Nucleotide Incorporation** - 100 nM wild type and mutant Ty3 RT were preincubated for 5 min at 30˚C with 8 nM template-primer (40mer DNA, hybridized to a 5’ [32P] end-labeled 30mer DNA primer) in 10 mM Tris/HCl (pH 7.8), 80 mM NaCl, 5 mM dithiothreitol, 0.01% (v/v) Triton X-100 and 9mM MgCl2. Heparin was subsequently added to a final concentration of 2mg/ml, and incubation was continued for varying times before 250 µM dNTP was added. One minute following dNTP addition, the reaction was stopped with an equal volume 89 mM Tris borate, pH 8.3, 2 mM EDTA, and 95% (v/v) formamide containing 0.1% (w/v) bromophenol blue and xylene cyanol. Starting polymerization with the dNTP mixture at varying times following heparin addition reveals the fraction of enzyme bound to the template at particular time point. The fraction of enzyme bound to the template as a function of time was plotted and fit using KaleidaGraph with single exponential function:

\[ f(t) = \exp(-k_{off} \times t) \]

where \( k_{off} \) is dissociation rate constant and \( t \) is the incubation time with the trap.

**DNA Polymerase Activity on Substrate Analog** - A 40nt DNA template containing an abasic lesion was hybridized to a [32P] end-labeled 18nt DNA primer such that the lesion was positioned 3, 4, 5 or 7 nucleotides from the primer 3’ OH (defining position -1 as the first template/primer base pair in the catalytic center). 2.5 nM template-primer was pre-incubated at 30˚C with 15nM Ty3 RT in the reaction mixture described above. Synthesis was initiated by the addition of dATP to a final concentration of 8µM, which permitted addition of a single nucleotide. Single nucleotide extension on duplexes containing LNA substitutions of the primer or template was evaluated in a similar manner, or in the presence of all four dNTPs.

**RNase H Activity** – Non-specific RNase H activity was determined on a 5’ end-labeled 40 nt RNA template annealed to a 30 nt DNA primer (31). A reaction mixture containing 50 nM template-primer was prepared in a buffer of 10 mM Tris/HCl (pH 7.8), 80 mM NaCl, 5 mM dithiothreitol and 9mM MgCl2. Hydrolysis was initiated by addition of enzyme to a final concentration of 100 nM in a final volume of 10 µl and terminated after 30 min by mixing with an equal volume 89 mM Tris-borate, pH 8.3, 2 mM EDTA, and 95% (v/v) formamide containing 0.1% (w/v) bromophenol blue and xylene cyanol. Hydrolysis products were resolved by high-voltage, denaturing 15% polyacrylamide gel electrophoresis and visualized by phosphor imaging. For Ty3 PPT selection, a 46-nt, (-) strand, DNA template (corresponding to nucleotides 4780-4809 of the Ty3 genome) was hybridized to a 29 nt PPT-containing RNA, containing 13 nt 3’ to the PPT/U3 cleavage site. The duplex was annealed as described above and a reaction mixture containing 50 nM template-primer was prepared in 25 mM Tris/HCl (pH 7.8), 9 mM MgCl2, 80 mM NaCl, 5 mM dithiothreitol and 0.01% (v/v) Triton X-100.
Hydrolysis was initiated by addition of RT to a final concentration of 150 nM in 80 µl volume. The reaction mixture was incubated at 30°C. Hydrolysis products were evaluated by high voltage electrophoresis and autoradiography.

**Sequence Alignment and Structure Modeling** - The RT sequences of chosen retroviruses and LTR-retrotransposons were multi-aligned using ClustalW function of MacVector. The alignment was done in respect to well defined amino acids motifs 1 to 7 from the RT fingers and palm subdomains (38). The secondary and tertiary structure prediction was accomplished using Metha-Server (http://bioinfo.pl). Two templates with the highest 3D-Jury score (Protein Data Bank code 1HMV and 1MU2 for HIV-1 and HIV-2 RT, respectively) were chosen to predict the tertiary structure of Ty3 RT, using the MODELLER program of the same server.

**RESULTS**

**Mutagenesis Strategy** - The lack of detailed structural information for Ty3 RT necessitated applying amino acid sequence alignment and structural prediction programs in order to locate the counterpart of HIV-1 RT helix αH. Within the DNA polymerase active center of Ty3 RT, the proposed -Tyr-Leu-Asp-Asp- active site motif between residues 211 and 214 was recently verified biochemically by site-directed mutagenesis (39). Multiple alignments, involving amino acid sequences of both retroviral and LTR–retrotransposon RTs, were conducted (Fig.1 A). Using this strategy, seven conserved segments defined previously by Xiong and Eickbush (38) were identified within Ty3 RT. As revealed by crystallographic data for retroviral RTs (1,2,40-42) these conserved segments are located within the fingers and palm subdomains, with the last segment (motif 7, or the β12 - β13 hairpin of HIV-1 RT) connecting the palm and thumb subdomains. C-terminal to this segment in Ty3 RT (amino acids 288 - 300) is a region with homology to HIV-1 RT helix αH (residues 256-268). Secondary structure prediction analysis was also used to confirm that this sequence of Ty3 RT could assume a helical configuration. Based on these combined approaches, we have tentatively designated Ty3 RT residues 290 - 298 the structural counterpart to HIV-1 RT helix αH. The same structural prediction analysis was used to identify the Ty3 counterpart of helices αI and αJ in the thumb of HIV-1 RT (Fig. 1[C]). Since Gln258, Gly262 and Trp266 of HIV-1 RT occupy the same face of αH and are involved in multiple contacts to the sugar-phosphate backbone of the primer (Fig. 1[B]) (1-3), their Ty3 counterparts Gln290, Gly294 and Tyr298, respectively, were selected for mutagenesis. In addition, Ty3 residue Asn297 was selected since its HIV-1 equivalent (Asn265) contacts the sugar-phosphate backbone of the template. Finally, Ty3 residue F292 was altered to alanine based on the high degree of conservation at this position of LTR-retrotransposon RTs.

**DNA Polymerase Activity of Thumb Mutants** - Mutations in the Ty3 RT thumb were initially characterized with respect to their DNA- and RNA-dependent DNA polymerase activities. Activity was determined in the context of multiple rounds of DNA synthesis, or by inclusion of the competitor heparin, restricting this to a single binding event, the results of which are presented in Figure 2. Under conditions allowing multiple rounds of synthesis, DNA-dependent DNA synthesis activity and pausing patterns of mutants Q290A, N297A, Y298F and Y298W (Fig. 2 [A], Lanes 1, 6, 8 and 9, respectively) were similar to wild type Ty3 RT (Fig. 2 [A], Lane w). All F292 substitutions, as well as mutant Y298A, caused a slight diminution in DNA polymerase activity (Fig. 2 [A], Lanes 2 - 4, and 7 respectively). In contrast, the relatively modest G294A substitution resulted in enhanced pausing at multiple positions on the DNA template, some of which were unique to this mutant (Fig. 2 [A], Lane 5). In the presence of heparin, only mutants Q290A and Y298F displayed activity similar to that of wild type Ty3 RT (Fig. 2 [B], Lanes 1, 8 and w, respectively), while other mutants showed varying levels of activity. A significant reduction in polymerase activity was observed with mutants F292A, G294A, Y298A and Y298W. Such a reduction may reflect decreased affinity for template-primer or, alternatively, template specific features such as secondary structure. A second processivity assay with a shorter DNA template was performed and likewise demonstrated that
processivity of these mutants was still affected (data not shown).

RNA-dependent DNA polymerase activity in the absence of heparin (Fig. 2 [C]) was essentially the same pattern for all mutants, with the exception that enhanced pausing was not evident with mutant G294A on an RNA template (Fig. 2 [C], Lane 5). Under conditions permitting a single binding event, the activity of mutants Q290A, Y298F and Y298W was equivalent to, or slightly less than Ty3 RT (Fig. 2 [D], Lanes 1, 8, 9 and w, respectively). All remaining mutants displayed severe polymerization and processivity defects under these conditions.

**Dissociation Rate Constants** - As a more quantitative measure of interactions involving Ty3 RT and duplex DNA, dissociation rate constants were determined for mutants carrying alanine substitutions (Fig. 3). For each mutant, a time course is presented, and dissociation rate constants have been tabulated in the insert. The value for mutant Q290A (0.098sec\(^{-1}\)) is close to that of wild type Ty3 RT (0.055sec\(^{-1}\)), which is in keeping with the data of Fig. 2. Such values are within the general range we (43) and others (44) previously described for p66/p51 HIV-1 RT. The most significant effect was observed for mutant G294A, which dissociates ~8-fold more rapidly from template-primer (0.425sec\(^{-1}\)), followed by F292A (0.247sec\(^{-1}\)), Y298A (0.224sec\(^{-1}\)) and N297A (0.150sec\(^{-1}\)).

**Single Nucleotide Extension on Duplex DNA Containing Locked Nucleic Acid Substitutions.** - In the absence of crystallographic data, an indirect approach was taken to investigate specific contacts between duplex DNA and the Ty3 thumb subdomain. DNA duplexes containing locked nucleic acid (LNA) substitutions were synthesized and used in a single nucleotide extension assay. These 2'-O, 4'-C-methylene-linked bicyclic analogs (Fig 4 [A]) have the property of locking the deoxyribose ring in the C3'-endo, configuration, thereby increasing the local organization of the phosphate backbone (45). LNA modifications were localized within the region of the primer and template predicted to be involved in contacts with the Ty3 RT thumb subdomain (positions -3 through -7, defining -1 as the first base pair in the catalytic center, Fig. 4 [B]). In the context of duplex DNA, the LNA methylene linker is oriented into the minor groove (46) which could potentially disrupt contacts between the Ty3 RT and the sugar-phosphate backbone. Fig. 4 [C] indicates that wild-type Ty3 RT was significantly inhibited when the LNA primer substitution was positioned close to the DNA polymerase catalytic center, i.e., nucleotides -3 and -4. When compared to the unsubstituted duplex, the inhibitory effect decreased when primer nucleotide -5 was substituted, and modest stimulation was noted when nucleotides -6 and -7 were replaced. Such a result suggests that primer nucleotides -3 to -5 comprise a region of close contact with the enzyme. In contrast, LNA template substitutions at positions -3, -4 and -5 did not inhibit primer extension, but induced the opposite effect, with the efficiency of nucleotide addition increasing as the substitution was relocated from position -3 to -5. However, replacement of template nucleotides -6 and -7 inhibited polymerization, indicating potential involvement of the sugar-phosphate backbone of template bases -6 and -7 in contacting Ty3 RT. This asymmetric LNA-induced pattern of inhibition for both primer and template co-localize with interactions defined crystallographically for HIV-1 RT (2,34).

While the inhibitory effect of primer between positions -3 and -5 and template modification at positions -6 and -7 was consistent with predicted LNA-induced structural interference, stimulation of DNA synthesis when template nucleotides positions -3, -4 and -5 were replaced was unexpected. One possible explanation for this might be local deformation of the DNA duplex. Incorporation of the LNA analog into the duplex has been demonstrated to induce minor groove narrowing and possibly alter enzyme contacts (47,48). Incorporation of an LNA analog into the DNA duplex would therefore be predicted to alleviate this effect, as observed experimentally in Fig. 4[C].

**Activity of Ty3 Mutants on DNA Containing LNA Primer Substitutions** - One way to exploit
targeted LNA insertions is whether the structural interference they induce is relieved in the context of a particular RT mutant, which would suggest a point of contact between the wild type residue and the LNA analog. The histograms of Fig. 5 compare the efficiency of single nucleotide addition of each Ty3 mutant on duplexes containing LNA primer substitutions with the activity of the same mutant on unsubstituted DNA. The method of evaluation is analogous to that recently adopted by Zang et al. (49) to study nucleotide addition opposite guanine analogs containing N2 adducts. The inhibitory effect of LNA substitution at positions -3 and -4 was not observed with Ty3 RT mutants F292A, F292W, F292Y and G294A (Fig. 5, Panels [iii] - [vi], respectively) which previously showed decreased processivity and template-primer affinity (Fig. 2). We attribute such tolerance to LNA modification to relaxed interactions between the thumb subdomain and the sugar-phosphate backbone of the primer. For mutants Q290A, N297A, Y298A, Y298W and Y298F (Fig. 5. Panels [ii] and [vii] - [x], respectively), primer modifications cause more diverse effects. As with wild-type RT, an LNA analog at position -3 and -4 decreased primer extension efficiency for most of the mutants. The exception was mutant Y298A (Panel [viii]), where substrate containing a -3 substitution was more efficiently extended than both the -4-substituted and unmodified duplex (Fig. 4 [C] and Fig. 5, Panel [i]). This phenotype clearly differs from that of mutants at Y298W and Y298F, demonstrating that removing the bulky aromatic side chain at position 298 compensates for LNA-induced interference, implicating Y298 in contacting the DNA primer at position -3. Furthermore, mutant Q290A is stimulated on a -5-substituted primer (Panel [iii]), suggesting this may be a point of contact with Q290. With the exception of wild type Ty3RT and mutant Y298F, priming efficiency on duplexes containing -6 and -7 substitutions was enhanced, in particular with mutants which showed a more pronounced defect in processivity or template-primer binding (see Fig. 2[B] and [D]) This result suggests that the LNA-induced deformation introduced into the DNA duplex partially compensates for the inability of mutant enzymes to correctly bind template-primer (for example, by increasing accessibility to the minor groove).

**Activity of Ty3 Mutants on DNA Containing LNA Template Substitutions** - As shown in Fig. 6, LNA substitution at position -3, -4 and -5 of the template increases priming efficiency for wild type Ty3 RT and its variants compared to the unmodified template. Conversely modification at position -6 and -7 decreases priming, with the former being slightly more inhibitory. Elimination of the inhibitory effect on substrates containing -6 and -7 substitutions with F292 mutants, in particular mutant F292A, suggests a more global effect, possibly through destabilizing thumb architecture (see Fig. 1[C]). In contrast, inhibition of DNA synthesis is relieved for N297A RT on specifically the -6-substituted template, suggesting the wild type residue contacts the sugar-phosphate backbone of this nucleotide, i.e., while introducing an LNA analog interferes with this interaction, decreasing the size of the side chain of N297 suppresses the inhibition.

Relatively low stimulation of priming was noticed for mutant F292A on a -4-substituted substrate (Fig. 6, Panel [iii]). In our structural model of Fig. 1 [C], the side chain of F292 is oriented towards the hydrophobic core of the thumb and may participate in stabilizing the local structure (Fig 1[C]). Replacing this residue with Ala could introduce a structural deformation to induce nonspecific interference with the DNA template containing the LNA analog at position -4.

**Single Nucleotide Extension on Duplex DNA Containing Abasic Primer Lesions** – Modification of duplex DNA with LNA analogs was designed to probe Ty3 RT interactions with the sugar-phosphate backbone of the minor groove. An alternative assay, using abasic lesions (50-55), was applied to examine potential interactions with nucleobases of the duplex. Single nucleotide extension was evaluated on the substrate of Fig. 4[B] whose primer in this case contained an abasic lesion at positions -3, -4, -5 or -7. As with LNA insertions, the activity of each mutant on the substituted duplex was compared to the same mutant on the wild type duplex. For wild type RT, primer extension was
reduced on substrates P-3Ab and P-4Ab, while near-wild type levels were observed for substrate P-5Ab and full activity was achieved with substrate P-7Ab. (Fig. 7, Panel [i]). Thus, while data with wild type Ty3 RT in Fig. 7, Panel [i] does not directly indicate which thumb residues contact nucleic acid, we predict that productive primer extension requires an interaction with primer bases -3 and -4. Similar results were obtained with mutant Q290A (Fig. 7, Panel [ii]). Also, while priming efficiency of mutants F292A and N297A was lower than wild-type RT (Fig. 7, Panels [iii] and [v], respectively), the profiles on each substituted duplex follow a similar pattern to wild Type Ty3 RT, supporting the notion that these amino acids do not mediate critical primer nucleobase contacts between positions -3 and -7.

In contrast, a different pattern was observed for mutant G294A, where activity comparable to that on the unsubstituted duplex was restored with substrates P-4Ab and P-5Ab (Fig. 7, Panel [iv]). To understand this result, modeling of HIV-1 RT complexed with duplex DNA was performed, where G262 (the counterpart of Ty3 RT residue G294) was substituted with Ala. This exercise indicated that the methyl group introduced into the side chain was pointed towards the stacking interface between nucleobases -4 and -5 (data not shown). Removing the primer nucleobase might then be considered a means of suppressing steric interference introduced by a Gly -> Ala substitution, arguing that the G294 main chain of Ty3 RT mediates contacts to primer nucleobase -4.

The most difficult effect of nucleobase elimination to explain was the priming efficiency of mutant Y298A, where removing the aromatic side chain inhibits priming on substrate P-4Ab and P-5Ab (Fig. 7, Panel [vi]). Clearly, this phenotype cannot be explained in terms of suppression of steric interference. From studies on LNA-substituted primers, Y298 was proposed to contact the sugar-phosphate backbone at position -3 (Fig. 5, Panel [vii]), and potentially stabilize the local A-like conformation of the DNA duplex. However, in the experiment of Fig. 7, removing a nucleobase will alter the stacking environment in its immediate vicinity. Since our proposal of Fig. 1[B] implicates G294 in contacting the sugar-phosphate backbone at position -4, a Y298A substitution might indirectly affect contacts involving neighboring nucleobases by changing stacking interactions.

**Mutations in the Thumb Subdomain Affect RNase H activity** - Altering protein motifs contacting nucleic acid near the DNA polymerase catalytic center would be predicted in the first instance to inhibit this enzymatic function. However, nucleic acid positioning within the C-terminal RNase H domain and RNase H function might be indirectly affected, a notion supported by previous our studies of mutants of the HIV-1 RT thumb (15). Amino acid sequence alignment of retroviral and LTR-retrotransposon RT (56) also indicates the latter lack a connection subdomain, which would suggest a closer spatial relationship between the two catalytic centers.

We therefore evaluated RNase H activity of our Ty3 RT mutants on a non-specific 40-nt RNA/30-nt DNA hybrid, and a second substrate mimicking the junction between the Ty3 polypurine tract (PPT) and downstream U3 RNA. Since accurate cleavage at the PPT/U3 junction is necessary for subsequent integration of the double stranded DNA element, PPT processing determines whether RNase H cleavage specificity is altered. The results of our RNase H analysis are presented in Fig. 8.

Under conditions allowing multiple binding events, wild type, Q290A, N297A, Y298A and Y298F RT displayed comparable activity (Fig. 8[A], Lanes w and 1, 6, 7 and 8 respectively). Interestingly, while the relatively modest Y298F substitution had negligible effect, a F292Y substitution severely impaired RNase H activity (Fig. 8[A] Lanes 8 and 4, respectively). Also, while Ala could be substituted for Tyr298 without loss of RNase H activity (Fig. 8[A], Lane 7), the equivalent substitution of F292 severely affected activity (Fig. 7[A], Lane 3). Based on our model of Fig. 1[C], such results are consistent with the notion that Ty3 RT residue F292 is involved in maintaining overall thumb architecture. Finally, although mutant G294A retained DNA- and RNA-dependent DNA polymerase activity (Fig. 2 [A] and [C], Lanes 5), the RNase H activity of this mutant was more severely affected (Fig. 8[A], Lane 5).

In Fig. 8[B], RNase H cleavage specificity was evaluated via hydrolysis of an RNA/DNA hybrid mimicking the Ty3 PPT/U3
junction. Since the RNA strand of this hybrid contains an additional 13 nt downstream the junction, this substrate allows simultaneous evaluation of both specific and non-specific hydrolysis (32). As an example, while low-level hydrolysis occurs between positions +1 and +10, wild type RT and mutants Q290A and Y298A cleave preferentially at the PPT/U3 junction (Fig. 8[B], Lanes w, 1 and 7, respectively). A minor, but reproducible effect of the Y298A mutation was elevated cleavage within the PPT at position -2. Although the significance of this novel cleavage is not immediately apparent, it is noteworthy that Ty3 proviral DNA, unlike other retro-elements, has a 2 bp terminal extension. In general, the efficiency of PPT/U3 cleavage by the remaining mutants was similar to that on the non-specific RNA/DNA hybrid. The exception was mutant Y298F (Fig. 8[B], Lane 8), which was less efficient at PPT/U3 cleavage although (a) hydrolysis of the same RNA/DNA hybrid between positions +1 and +10 was unaffected and (b), this mutant efficiently cleaved a non-specific RNA/DNA hybrid (Fig. 8[A], Lane 8). A similar result was obtained with HIV-1 RT carrying a mutation at Trp266, which we have proposed here is the counterpart to Ty3 RT residue Tyr298 (15).

DISCUSSION

There is general consensus that the thumb subdomain of several DNA polymerases makes numerous contacts with the duplex product of DNA synthesis approximately 3-8 bp behind the catalytic site (57). Studies with the Klenow fragment of Escherichia coli (58) and HIV-1 RT (14-20), have suggested that a helix-turn-helix motif of the thumb tracks the minor groove of the nascent duplex, serving as an important modulator of both the processivity and fidelity of DNA synthesis. Using a combination of amino acid sequence alignment and homology modeling programs, Q290, G294, N297 and Y298 of Ty3 RT were tentatively identified here as counterparts of HIV-1 helix αH residues Q258, G262, N265 and W266, respectively (Fig. 1[A]). The predicted function of this helix-turn-helix motif in stabilizing the Ty3 ternary complex was evaluated via changes in processivity and the dissociation rate constants for alanine scanning mutants. Subsequently, the effect of targeted placement of either conformationally-restrained LNA analogs or abasic lesions into template and primer was employed to localize contacts between these residues of Ty3 RT and duplex DNA.

Primer extension in the presence of heparin (Fig. 2[B] and [D]) highlighted a severe defect in processivity for mutants F292A, G294A and Y298A, a moderate effect for mutants F292W, F292Y, N297A and Y298W and a wild type phenotype for mutants Q290A and Y298F. The same reaction without heparin (Fig. 2, [A] and [C]) highlighted differences between Ty3 RT mutants most seriously affected in the processivity assay, indicating that the substitution G294A most dramatically affected ternary complex stability on a DNA duplex. Consistent with this trend, the largest increase in the dissociation rate was observed for mutant G294A (~8-fold), followed by F292A and Y298A (~5-fold). The effect of Ty3 RT substitutions G294A and Y298A demonstrated here are consistent with analogous mutations in HIV-1 RT (G262A and W266A, respectively), which likewise exhibited elevated dissociation rate constants, together with reduced processivity and frameshift fidelity (19).

Comparing processivity and dissociation rate constants suggests an important function for Ty3 RT residues F292, G294 and Y298, and to lesser extent N297 in stabilizing the ternary complex. However, these approaches do not discriminate between direct contacts with duplex DNA and their alternative function of stabilizing local protein structure. To address this question, nucleoside analog interference mapping was exploited to probe potential contacts between the predicted Ty3 RT helix-turn-helix motif and duplex DNA immediately adjacent to the DNA polymerase catalytic center. For wild type Ty3 RT single LNA substitutions induced a different pattern of priming inhibition, depending on whether the template or primer was substituted (Fig. 4 [C]). LNA interference was noted at primer positions -3, -4 (and to a lesser extent -5) and positions -6, -7 of the template. Moreover, duplexes containing dual LNA insertions indicated that template interactions do not extend beyond position -7 (data not shown). The interference patterns obtained with our analog interference strategy co-localize with interactions defined for HIV-1 reverse transcriptase.
interaction with the sugar-phosphate backbone of duplex DNA (2,34), illustrating the value of this approach when crystallographic data is unavailable.

While the analog-induced inhibition pattern highlights a general region of interaction with the enzyme, suppression of LNA interference by Ty3 thumb subdomain mutants aids in defining specific interactions. For example, mutant Y298A efficiently extends a DNA primer containing the LNA analog at position -3 (Fig. 5, Panel [viii]). In contrast, wild type RT and mutants Y298W and Y298F are significantly inhibited by the same primer substitutions (Fig. 4 [C] and Fig. 5, Panels [i], [ix] and [x]). This result suggests interference between a bulky substituent at position 298 and the methylene-linked sugar at position -3 of the primer (Fig. 4 [A]). Modeling studies (not shown) suggest that substituting alanine for Y298 compensates for such steric hindrance. Single nucleotide extension by mutant N297A uncovered a second example of specific suppression of LNA-induced interference (Fig. 6, Panel [vii]). In this case the inhibitory effect of LNA insertion into position -6 of the DNA template was eliminated.

Interference suppression was also observed with F292A, F292W and F292Y (Fig. 5, Panels [iii] - [v]). However in this case, the priming inhibition was relieved equally with primers containing -3, -4 and -5 LNA substitutions, potentially revealing a long-range effect of substituting F292. In our homology modeling Fig. 1[C], the side-chain of F292, like its HIV-1 equivalent, L260, projects into the thumb rather than towards nucleic acid. It is therefore reasonable to assume that these residues may mediate hydrophobic interactions with other helical elements of the thumb subdomain. Thus, the "long-range" suppression observed when F292 is replaced may be an indirect consequence of altering thumb architecture on contacts between neighboring residues and duplex DNA. An alteration in thumb architecture would also be consistent with inhibition of RNase H activity with mutants F292A, F292Y and F292W (Fig. 8).

Since this alteration is not expected to be limited to the interaction of a single residue with duplex DNA but rather affect the local environment (for example destabilization of a structural element), the trajectory of the heteroduplex within the binding cleft of Ty3 RT may be distorted. Such changes and their implications for RNase H activity have been previously documented (3,15).

Single nucleotide extension reactions with substrates containing strategically-placed abasic analogs in the template-primer duplex supported our contention that base pairs -3, -4, and to a lesser extent -5 participate in important contacts within the Ty3 RT nucleic acid binding cleft. These results are consistent with crystallographic data determined for the HIV-1 RT/dsDNA complex (2). The inhibitory effect of nucleobase removal at position -4 and -5 of the primer noted for most mutants was suppressed with mutant G294A (Fig. 7, Panel [iv]), where modeling (data not shown) suggests that the extra methyl group introduced by Ala would most likely be oriented towards the stacking interface between nucleobases -4 and -5, which would cause steric interference. Thus, removing one of these nucleobases might be predicted to compensate for this interference. With respect to the unmodified duplex, structural hindrance hypothesized here may alter duplex trajectory within the RT binging cleft resulting in loss of RNase H activity with this mutant (Fig. 8), although an increased dissociation rate is an alternative explanation. Although a role for Ty3 residue Q290 was not apparent answered our results, mutagenesis data at positions F292, G294, N297 and Y298 is consistent with our amino acid sequence alignment and homology modeling, revealing a thumb architecture similar to that previously defined from HIV-1 RT-dsDNA complex (Fig. 1[C]).

Finally, crystallization of monomeric Moloney murine leukemia virus RT (42) has suggested a lack of conservation with residues of HIV-1 RT proposed from biochemical and structural studies to mediate translocation (14,15,18,33,34,59), in particular Q258, G262 and W266, whose Ty3 counterparts are the subject of this communication. Although it is conceivable that the thumb subdomain of the murine enzyme has a unique architecture, the data of this communication suggest that the monomeric Ty3 (30) and heterodimeric HIV-1 enzymes exploit common structural motifs for translocation of the polymerization machinery.
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Figure Legends

**Figure 1** - [A], Alignment of helix αH from the thumb subdomain of HIV-1 RT with the equivalent region from related retroviruses and LTR-retrotransposons. Amino acid numbering at the bottom of the panel is that of p66 HIV-1 RT, and that at the top from Ty3 RT. [B], HIV-1 RT contacts to template (blue) and primer nucleotides (red) in the vicinity of the DNA polymerase catalytic center, and proposed contacts made by their Ty3 RT thumb counterparts (magenta). The nucleotides contacting helix αH are shaded. The model is based on that of Ding et al. (34), where thick lines represent interactions with distances shorter than 3.3A, thin lines interactions between 3.3 - 3.6A and dashed lines interactions between 3.6 and 3.8A. Note that in the original structure, Cys280 was replaced by Ser. [C], Model for helices of the HIV-1 (left, amino acids 243 - 382) and Ty3 thumb subdomains (right, amino acids 271 - 338). Hydrophobic amino acids potentially involved in maintaining HIV-1 and Ty3 RT thumb architecture are indicated in red. Amino acids in yellow are those either demonstrated (HIV-1 RT) or proposed (Ty3 RT) to interact with template/primer. Additional residues of HIV-1 RT helix αH which interact with the DNA template (Tyr286, Arg284, Cys280), and their Ty3 counterpart (Gly314, Cys318, Asp319 and Lys320), are indicated in green.

**Figure 2** - [A], [B], DNA-dependent DNA polymerase activity of Ty3 RT mutants. Activity was determined in either the absence (Panel [A]) or presence of the competitor heparin (Panel [B]). Amino acids selected for mutagenesis are indicated above each panel, below which is the residue to which they were altered. Lane w, wild type Ty3 RT. The reaction was performed with a HhaI-digested single-stranded M13 DNA template to which a 5'-radiolabeled M13 universal primer was annealed. [C], [D], RNA-dependent DNA polymerase activity, evaluated in the absence (Panel [C]) and presence of heparin (Panel [D]). Lane notations are as in Panels [A] and [B]. Nucleic acid substrate was an in vitro transcribed 152-nt RNA template (comprising a sequence 2225-2363 of Ty3 genome), to which a 5’-radiolabeled DNA primer was annealed.

**Figure 3** - Dissociation rate constants for selected Ty3 RT thumb mutants. Dissociation rates were determined by measuring the fraction of extended primer as described under "EXPERIMENTAL PROCEDURES". Duplex DNA substrate was a 40-nt template to which a 5’-radiolabeled 30-nt primer was annealed.

**Figure 4** - [A], Sugar pucker of LNA analogs. For comparison, the C2'-endo pucker of DNA sugars and C3'-endo pucker of RNA sugars is presented. [B], Location of LNA analog insertions (filled pentagons) in the primer and template of a model DNA duplex. Position -1 is defined as the first base-pair in the DNA polymerase catalytic center. [C], Single nucleotide extension analysis with wild type Ty3 RT on duplex DNA containing single LNA-substitutions of the primer (left) or template (right). The position of LNA insertion is indicated above each panel. P, unextended primer.

**Figure 5** - Quantitation of single nucleotide extension analysis with Ty3 RT mutants on duplex DNA containing single LNA-substitutions between primer positions -3 and -7. The mutant enzyme and position of LNA insertion are indicated above and below each panel, respectively. For each mutant, the results are presented as a fraction of P+1 extension product obtained with the LNA-substituted template relative to the same mutant on the unmodified duplex. For ease of interpretation, values have been expressed in a logarithmic scale. A similar trend by LNA-substitution was obtained in experiments where the incubation time was extended (data not shown).

**Figure 6** - Quantitation of single nucleotide extension analysis with Ty3 RT mutants on duplex DNA containing single LNA-substitutions between positions -3 and -7 of the template. Lane notations are as in the legend to Fig. 5. A similar trend by LNA-substitution was obtained in experiments where the incubation time was extended (data not shown).
**Figure 7** - Single nucleotide extension analysis with Ty3 RT alanine scanning mutants on duplex DNA containing single abasic lesions between positions -3, -4, -5 and -7 of the primer. Lanes w, unsubstituted duplex. P, unextended primer.

**Figure 8** - RNase H activity of Ty3 RT mutants. [A], Activity determined on a non-specific RNA/DNA hybrid, where Ty3 RT cleaves at positions corresponding to 13 and 21 nt behind the DNA polymerase catalytic center (defined as -13 and -21, respectively). [B], activity determined on a Ty3 polypurine tract-containing RNA/DNA hybrid. The position of specific cleavage at the PPT/U3 junction, is indicated. Lane notations are as in the legend to Fig. 2. Lanes c, no enzyme, lanes w, WT Ty3 RT.
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**FOOTNOTES**

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1Abbreviations used: HIV, human immunodeficiency virus; LTR, long terminal repeat; nt, nucleotides; PPT, polypurine tract; RNase H, ribonuclease H; RT, reverse transcriptase.
Fig. 1

### A

| Ty3 | QAQKRLCHQSRVYTR | -- | FIPNCSKIA | -- | QPI |
|-----|------------------|----|------------|----|-----|
| Cerr | ELSRLGFLQCVYR | -- | KFLHPGQA | -- | JAS |
| gprv | KVRSLGFLASYR | -- | VIKDFAAJARPITD |
| T/F | ELQCSFLGCSVYR | -- | KFLPKNQ | -- | LTH |
| Nap | ELQCSFLGFLFYR | -- | KFLQDLSAP | -- | LSP |
| Dymo | ELSRLGFLQSVR | -- | KFLKHRFAEIVSPDL |
| SFV | QLSILGSLNNR | -- | NFIHPFAELV | -- | QTL |
| NLV | QLSILGNSLNNR | -- | NFIHPFAELV | -- | QTL |
| FIV | ELSRLGFLQSVR | -- | KFLKHRFAEIVSPDL |
| ELV | DLSNLGQSN | -- | NFIHPFAELV | -- | QTL |
| RV | DLSNLGQSN | -- | NFIHPFAELV | -- | QTL |
| CAEV | KLQKLVEVLVMA | -- | QSIISGESINE | -- | ILK |
| HIV | DIQKVLGKLNMA | -- | QIYPGIKVQ | -- | LCK |

α Helix H

### B

### C
Fig. 2

[A] - Heparin

[B] + Heparin

DNA-Dependent DNA Polymerase

[C] - Heparin

[D] + Heparin

RNA-Dependent DNA Polymerase
Fig. 3
Fig. 6
Fig. 7

[i] WT  
[ii] Q290A  
[iii] F292A  

[iv] G294A  
[v] N297A  
[vi] Y298A
Fig. 8
Interaction of the Ty3 reverse transcriptase thumb subdomain with template-primer
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