Interaction of p55 Reverse Transcriptase from the Saccharomyces cerevisiae Retrotранспон Ty3 with Conformationally Distinct Nucleic Acid Duplexes*

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The 55-kDa reverse transcriptase (RT) domain of the Ty3 POL3 open reading frame was purified and evaluated on conformationally distinct nucleic acid duplexes. Purified enzyme migrated as a monomer by size exclusion chromatography. Enzymatic footprinting indicate Ty3 RT protects template nucleotides +7 through −21 and primer nucleotides −1 through −24. Contrary to previous data with retroviral enzymes, a 4-base pair region of the template-primer duplex remained nuclease accessible. The C-terminal portion of Ty3 RT encodes a functional RNase H domain, although the hydrolysis profile suggests an increased spatial separation between the catalytic centers. Despite conservation of catalytically important residues in the RNase H domain, Fe2+ fails to replace Mg2+ in the RNase H catalytic center for localized generation of hydroxyl radicals, again suggesting this domain may be structurally distinct from its retroviral counterparts. RNase H specificity was investigated using a model system challenging the enzyme to select the polypurine tract primer from within an RNA/DNA hybrid, extend this into (+) DNA, and excise the primer from nascent DNA. Purified RT catalyzed each of these three steps but was almost inactive on a non-polypurine tract RNA primer. Our studies provide the first detailed characterization of the enzymatic activities of a retrotранспон reverse transcriptase.

Following infection, retroviruses initiate their DNA synthesis program from a host-derived RNA hybrid to a specific region at the 5′ end of their (+) strand RNA genome, designated the primer binding site or PBS. However, tRNA use is somewhat heterogeneous, i.e. while avian viruses exploit tRNA Typ, Moloney murine leukemia virus uses tRNA Pro and d-type and human spumaretroviruses tRNA Lys+1,2 (1). In the case of HIV and related lentiviruses of simian, feline, and equine origin, tRNA Lys is selected as the replication primer. Early experimentation suggested complementarity between the PBS and sequences at the 3′ terminus of the replication primer as the sole specificity determinant during initiation of (−) strand synthesis (2). However, extensive analyses with Rous sarcoma virus (3–5) and HIV-1 (6–14) have provided a convincing argument that additional intermolecular base pairing between the replication primer and sequences of the viral genome 5′ to the PBS play a major role in controlling initiation. In the latter case, chemical footprinting data (11, 15) and kinetic analysis (12, 14) indicate a two-step initiation program. The first of these is characterized by slow addition of the first 5 dNTPs, during which DNA synthesis is highly distributive; subsequently, the replication machinery moves into a rapid and processive elongation mode. Avian viruses display a similar control mechanism, although the intermolecular interactions underlying this are subtly different, i.e. while the anticodon loop of tRNA Lys+3 is in intimate contact with U5-IR loop bases of the HIV genome, this occurs between the TΨC arm of tRNA Typ and U5-IR stem bases in Rous sarcoma virus (3–5).

Although restricted to an intracellular life cycle in the absence of an envelope gene, LTR-containing retrotранспонs Saccharomyces cerevisiae, representatives of which include Ty1 and Ty3, share many features of the reverse transcription cycle with their retroviral counterparts (16). Both are LTR-containing elements requiring a host-derived tRNA primer, in this case tRNA Met, to initiate (−) strand synthesis. In contrast, a distinguishing feature of these retrotранспонs is the limited complementarity between PBS sequences at the 5′ end of the genome and the tRNA primer, which in Ty3 is reduced from 18 to 5 nt. However, Keeney et al. (17) demonstrated that features of the TΨC arm are critical to transposition, and more recently Gabus et al. (18) provided experimental evidence that Ty3 compensates for this by exploiting a bipartite PBS. According to this model, a region with extensive complementarity (12 nucleotides) to the TΨC arm of the tRNA primer is located at the 3′ end of the genome. Although speculative, these authors have also suggested an initiation complex of two genomic RNAs could be stabilized through a short autocomplementary sequence in tRNA Met, which induces dimerization. A similar scenario prevails with Ty1, where reduced complementarity to the 3′ end of the tRNA primer (10 nt) is compensated by extended interactions with the D arm (19). This notion of co-operativity between distal cis-acting sequences on the genome may be not be unique to retrotранспонs. Brule et al.
Characterization of Ty3 Reverse Transcriptase

(20) have found that (−) strand transfer in HIV can benefit from complementary sequences in the tRNA anticodon stem and bases in the U3 region at the 3′ end of the genome. A better understanding of cis-acting sequences cooperating in (−) strand DNA synthesis in retrotransposons would therefore be beneficial. As in retroviruses, (−) strand synthesis in retrotransposons initiates from an RNase H-resistant, purine-rich sequence immediately adjacent to the U3 region at the 3′ end of the genome and designated the polypurine tract or PPT. This sequence must be (i) selected from the (−) RNA(−) DNA replication intermediate, (ii) extended at its 3′ terminus into (+) strand DNA, and (iii) excised from the nascent (+) strand to generate the appropriate 5′ LTR sequences for recognition by the integration machinery. Since imprecise removal of the PPT from (−) DNA may have consequences for integration, PPT selection and removal must by necessity be a highly accurate process. In this respect, Kirchner and Sandmeyer (21) and Wilhelm et al. (22) indicated that several ribonucleotides at the 3′ terminus of the Ty3 and Ty1 PPT could serve as (+) strand initiation sites. These studies have relied exclusively on analysis of DNA isolated from virus-like particles since, until recently, purified Ty3 RT and a reconstituted system recapitulating in vivo events have been unavailable. The goal of the present study was to prepare recombinant Ty3 RT and analyze both the nucleoprotein complexes and enzymatic activities (DNA polymerase and RNase H) mediating these events. DNase I footprinting of binary polymerization complexes indicates an organization unlike that demonstrated for several retroviral enzymes (23–25). A system of “PPT scanning” was also exploited to evaluate the precision with which Ty3 (+) strand synthesis is initiated. Surprisingly, this system indicated that the specificity of primer selection and removal was dependent on the nature of the PPT-containing RNA primer. Finally, alignment of amino acid sequences from the RNase H domains of several LTR-containing retrotransposons and plant caulimoviruses suggests an alternative distribution of catalytic residues.

Experimental Procedures

Cloning, Expression, and Purification of Ty3 RT

The 55-kDa RT open reading frame was amplified from the Ty3 POL3 gene (26) by the polymerase chain reaction as a BamHI/HindIII fragment and inserted between the equivalent sites of plasmid p6HT (27). This procedure generated plasmid p6HTy3RT, which allows IPTG-inducible expression of a polyhistidine extended enzyme. RT was purified from logarithmically grown and IPTG-induced cultures by a combination of metal chelate (nickel-nitrilotriacetic acid-Sepharose) and ion exchange chromatography (S-Sepharose). Purified enzyme was identified in the text and processed as described above. Ty3 RT was end-labeled with [32P]CTP and RNA ligase (New England Biolabs) under conditions recommended by the manufacturer.

Enzymatic Footprinting of Replication Complexes

DNase I—DNase I footprinting (24) was conducted on the 71-nt template/86-nt primer described above and whose template or primer was end-labeled with α-32P and polynucleotide kinase. End-labeling followed protocols specified by the manufacturer (Roche Molecular Biochemicals). 50 nt end-labeled template-primer was incubated with 85 nM Ty3 RT in 10 mM Tris/HCl, pH 8.0, 6 mM MgCl2, 80 nM NaCl for 10 min at room temperature. Two units of DNase I were added, and digestion allowed to proceed for 30 s. Hydrolysis was terminated by addition of an equal volume of phenol/chloroform/isomyl alcohol (25:24:1). DNA synthesis was initiated at 30 °C by addition of dATP, dGTP, dCTP, and dTTP to a final concentration of 100 μM. Reactions were removed at times indicated in the text and mixed with an equal volume of 7 M urea containing 0.1% bromphenol blue and xylene cyanol. Polymerization products were resolved by high voltage denaturing polyacrylamide gel electrophoresis and evaluated by autoradiography.

RNase H Activity

RNase H activity was initially evaluated on a 5′ end-labeled 90-nt RNA template (prepared by in vitro transcription) hybridized to the 36-nt DNA primer used to evaluate polymerase function (30). 10 nM enzyme was incubated with 20 nM template-primer in a buffer containing 10 mM Tris/HCl, pH 7.5, 25 mM MgCl2, 5 mM dithiothreitol. RNase H activity was initiated by addition of MgCl2 to a final concentration of 10 mM and allowed to continue at 30 °C. Aliquots were again removed at times indicated in the text and processed as described above. In addition to modification to this technique, RNase H activity was also examined on the same substrate whose 3′ terminus was end-labeled with [32P]CTP and RNA ligase (New England Biolabs) under conditions recommended by the manufacturer.

Polypurine Tract Utilization

Experiments evaluating Ty3 PPT utilization required a combination of both DNA polymerase and RNase H activities (31). A 65-nt, chemically synthesized (−) strand DNA template (Integrated DNA Technologies) containing the PPT complement was synthesized (−) strand RNA primers (Dr. Marion Reardon) spanning the PPT by heating to 90 °C and slow cooling in 10 mM Tris/HCl, pH 7.5, 25 mM MgCl2. The final concentration of all template-primer combinations following hybridization was 20 μM.

These substrates were incubated at room temperature for 45 min with Ty3 RT in buffer containing (final concentration) 10 mM Tris/HCl, pH 7.5, 25 mM NaCl, 6 mM MgCl2, 0.1 mM dithiothreitol, 0.25 mg/ml of TTP, and 0.25 mg/ml of Ty3 RT. Under these conditions, the replication complex remains stable over the digestion period. Control S1 digests of extended substrates in the absence of RT were also prepared.

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treated with 0.3 volumes of 1 N NaOH at 65 °C to hydrolyze all RNA primers, then neutralized by adding an equivalent volume of 1 N HCl. Nucleic acids were precipitated with ethanol; precipitated; dried; and resuspended in 7 M urea, 0.1% bromphenol blue, and 0.1% xylene cyanol. The remaining portion (i.e. containing RNA primers) was precipitated as described above and resuspended in the same gel loading buffer. DNA synthesis products were fractionated by high voltage denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

\[ Fe^{2+} \text{-mediated Cleavage of Duplex DNA by the RNase H Domain} \]

Replacement of Mg\(^{2+}\) in the RNase H domain with Fe\(^{2+}\) and hydroxyl radical-mediated cleavage of duplex DNA followed the protocol of Goett et al. (32). Substrate was the 71-nt template/36-nt primer used to evaluate DNA-dependent DNA polymerase activity, the template of which was 5' end-labeled with \(\gamma\)-\[^{32}\]P\(\text{ATP}\) and polynucleotide kinase according to standard protocols. Enzyme (1 \(\mu\)g) and template primer (50 nM) were incubated 5 min at room temperature in a buffer of 80 mM HEPES, pH 8.0, 50 mM NaCl. The following reactants were subsequently pipetted onto the wall of the reaction tube: 1 \(\mu\)l of 50 mM dithiothreitol, 1 \(\mu\)l of freshly prepared \(\text{H}_{2}\text{O}_{2}\), 2 \(\mu\)l of 2 mM Fe(NH\(_{4}\))\(_{2}\)(SO\(_{4}\))\(_{2}\) \(\cdot\) 6H\(_{2}\)O. Reaction vessels were carefully closed and centrifuged to initiate Fe\(^{2+}\)-mediated hydroxyl radical cleavage. After 5 min, the reaction was terminated by adding 40 \(\mu\)l of stop solution (0.1 M thiourea, 10.0 mM EDTA, 0.6% NaOAc, pH 6.2), and 1 \(\mu\)l of glycojen. Nucleic acids were precipitated with ethanol, collected by centrifugation, and resuspended in urea-based gel loading buffer. Hydrolysis products were fractionated by high voltage denaturing gel electrophoresis and visualized by autoradiography.

RESULTS

Purified p55 Ty3 RT Sediments as a Monomer—Although most lentiviral RTs studied to date exhibit a dimeric structure of asymmetrically organized subunits, the purified MLV enzyme is a monomer. Although surprising, the possibility of a monomeric RT organization is supported by recent data with recombinant enzyme from bovine leukemia virus (33), which was shown by rate sedimentation analysis to migrate as a monomer in both the absence and presence of duplex DNA. Following expression and purification of Ty3 RT (Fig. 1, A and B), its quaternary structure was evaluated by size exclusion chromatography. As indicated in Fig. 1B, the Ty3 enzyme migrated slightly faster than the monomeric polyhistidine-tagged p51 subunit of HIV-1 RT (mass 52 kDa) but behind bovine serum albumin (mass 67 kDa), which is consistent with a monomeric organization. However, these results do not rule out the possibility of other RT forms are required during Ty3 replication.

DNA-dependent DNA Polymerase Activity of Ty3 RT—The ability of Ty3 RT to support processive DNA synthesis in the absence of accessory factors such as the nucleocapsid protein was initially assessed. At the same time, we also wished to determine the extent to which processivity might be influenced by temperature, since yeast strains harboring Ty elements are temperature-sensitive (34). DNA synthesis was evaluated on a 71-nt DNA primer duplex. A complete picture of the nucleoprotein complex resulted in an altered enzymatic footprint on the same template-primer duplex. Since DNase I and S1 footprinting experiments have verified the presence of the stem-loop (24), it appears that Ty3 RT inefficiently resolves this structure at 37 °C. Lowering the incubation temperature to 30 °C conferred on Ty3 RT the capacity to polymerize through the hairpin, although the overall level of polymerase activity was lower than that obtained with the HIV-1 enzyme. In Fig. 2C, a time course of DNA-dependent DNA synthesis was performed with both the HIV-1 and Ty3 enzymes at 30 °C. Although it is again clear that Ty3 RT is less active than its HIV-1 counterpart, pausing between template nucleotides +10 and +15 is only observed with the latter, suggesting the Ty3 enzyme may have a more robust strand displacement activity.

Enzymatic Footprinting of Ty3 Replication Complexes—Enzymatic footprinting of HIV (24), EIAV (25), and MLV replication complexes (23) indicates that the retroviral polymerase is in close contact with DNA from template nucleotide +7 to −24/−27 of the template-primer duplex. Since the size of the Ty3 enzyme is considerably different from those we have previously evaluated, it was of interest to determine if this resulted in an altered enzymatic footprint on the same template-primer duplex. A complete picture of the nucleoprotein complex
can only be achieved by independent evaluation of resistance to the nucleases S1 and DNase I, which hydrolyze single-stranded and double-stranded DNA, respectively (Fig. 3A). For comparison, replication complexes containing p66/p51 HIV-1 RT were evaluated in parallel.

The results of S1 probing are illustrated in Fig. 3B. Since the single-stranded template of our substrate assumes an intramolecular base pairing, stalling of RT in this region is characterized by accumulation of P + 10 to P + 15 products. B, temperature sensitivity of DNA polymerase activity catalyzed by HIV-1 and Ty3 RT. For each temperature indicated, duplicate 10 min DNA synthesis profiles (a and b) are presented. C, time course of Ty3 and HIV-1 RT-catalyzed DNA synthesis at 30 °C. Lanes C, radiolabeled primer (36 nt). In both cases, DNA synthesis was evaluated after 1 min (lanes 1), 2 min (lanes 2), 5 min (lanes 3), 10 min (lanes 4), 20 min (lanes 5), and 45 min (lanes 6). Note that, while Ty3 RT is less active than the HIV-1 enzyme, stalled products in the range P + 10 to P + 15 are absent.

Combining the Ty3 RT-derived template and primer hydrolysis profiles suggests duplex DNA between positions −16 and −19 remains freely accessible to DNase I. Such data may indicate that the N-terminal DNA polymerase and C-terminal RNase H domains of Ty3 RT form independent domains separated by a small linker, as has been proposed for the murine enzyme (36). Alternatively, an interaction of Ty3 RT with the template-primer duplex may alter its structure sufficiently to render it locally hypersensitive to DNase I digestion.

**RNase H Hydrolysis Profiles Suggest an Altered Spatial Separation between the DNA Polymerase and RNase H Domains of Ty3 RT**—Currently available crystallographic (37–39), enzymatic (31, 40), and chemical probing data (41) suggest that the DNA polymerase and RNase H domains of Ty3 RT form independent domains separated by 17–18 bp. In order to determine whether the same holds for the Ty3 enzyme, its interaction with an RNA/DNA hybrid related to the DNA duplex of the previous section was evaluated. This substrate is depicted in Fig. 4A, comprising a 90-nt RNA template to which the identical 36-nt DNA primer is hybridized. The temporal sequence of RNase H-mediated hydrolysis is indicated in Fig. 4A and involves initial endonucleolytic cleavage at position −17, followed by a directional processing activity extending to position −8. Using the system illustrated in Fig. 4A, these activities are diagnosed by the accumulation of 71- and 62-nt hydrolysis fragments, respectively.

RNase H-mediated hydrolysis catalyzed by HIV-1 RT is illustrated in Fig. 4B. Initially, hydrolysis products of 71, 74, and, to a lesser extent, 76 nt accumulate, and are gradually replaced by fragments in the range 64–62 nt. A similar pattern emerges when Ty3 RNase H-mediated hydrolysis is evaluated, but differs significantly in the size of the hydrolysis products.
enzyme have been indicated by the stranded template and intramolecular hairpin have been indicated at display hyper-reactivity have been boxed. The positions of the single-nucleotides within each footprint that retain DNase I sensitivity or lane T template bases between positions intramolecular template base pairing has the consequence that only processing at this position. In the experiment of Fig. 3...ters (37–39), Ty3 is the first RT shown to terminate directional processing at template nucleotide 2. The final product of directional processing is a 65-nt fragment, indicating cleavage at template nucleotide 2. Accumulation of a 75-nt fragment at early stages represents a 54-nt fragment (Fig. 5A).

Hydroxyl radical cleavage by Fe²⁺-seeded p66/p51 HIV-1 RT, FIV RT, and the Ty3 enzyme was evaluated in parallel. As can be seen from the data of Fig. 5B, we were successful with both retroviral enzymes in Fe²⁺ substitution and hydroxyl radical cleavage at template nucleotide –17. In contrast, however, we failed to detect hydroxyl radical cleavage in the presence of Ty3 RT. The trivial possibility that Ty3 RT had lost activity during storage was eliminated in a parallel experiment in which RNase H activity was evaluated on an RNA/DNA hybrid in the presence of Mg²⁺. Under these conditions, full activity for each enzyme was achieved (data not shown). In light of this, we hypothesized that residues important for Mg²⁺ coordination in HIV-1 and E. coli RNase H are less conserved than the 54-nt fragment. In the Ty3 enzyme, thus affecting coordination geometry. The amino acid alignment presented in Fig. 7 indicates the counterparts of Asp⁴⁴³, Glu⁴⁷⁸, Asp⁴⁹⁸, and Asp⁵⁴⁹ in the HIV-1 enzyme are preserved, so the absence of these conserved carboxylates can be ruled out. However, according to the revised model of Kashiwagi et al. (43), Asn⁴⁷⁴ and Gly⁴⁴⁴ of HIV-1 RT are not in Mg²⁺ coordination at the RNase H domain, the counterparts of which are absent in the Ty3 enzyme. In addition, the monomeric nature of Ty3 RT may also influence the avidity with which Fe²⁺ is retained. Although these explanations are presently speculative, they indicate another significant difference between the retroviral and retrotransposon enzymes.

Initiation of Ty3 (+) Strand Synthesis—To determine whether the recombinant enzyme catalyzed more specialized activities required for accurate fulfillment of reverse transcription, we evaluated the capacity of Ty3 RNase H to excise the PPT primer from an RNA/DNA hybrid for extension into, and subsequent removal from, (+) strand DNA. Previous data from our laboratory (31, 44) have indicated that related retroviral enzymes will support each of these events in a single reaction. Our PPT “scanning” strategy is depicted in Fig. 6A, and investigates the recognition of both PPT- and non-PPT-containing RNA primers by the Ty3 enzyme. Primer P3 is complementary to (+) strand DNA sequences immediately 5’ to the sequence proposed to prime Ty3 (+) strand synthesis (20, 21). P3 thus evaluates the efficiency of non-PPT RNA-primed synthesis. P2 contains additional sequences 5’ to the PPT, and terminates within sites predicted to be most frequently used. Finally, P1 contains the Ty3 PPT and additional 3’ sequences, and must therefore be processed by Ty3 RNase H to reveal the authentic (+) strand primer. All experiments were performed in the presence of a dNTP mixture to reveal the steps of primer selection and extension. As an additional control, these primers...
were extended by DNA polymerase I Klenow fragment, which efficiently recognizes their 3' OH. The results of our investigation are presented in Fig. 6B.

While P1 and P2 were recognized by Ty3 RT, the data of Fig. 6B clearly indicate that the non-PPT RNA primer P3 does not support efficient (+) strand DNA synthesis, which is in keeping with reports on related systems (45–47). At this stage, we cannot determine whether this represents reduced affinity for the RNA-DNA hybrid or an inability of bound enzyme to catalyze DNA synthesis.

(+1) strand products of 40 and 27/26 nt accumulate when P1 is used as primer, the former of which is eliminated and the latter of which remains unchanged following NaOH treatment (to remove all ribonucleotides). Thus, the 40-nt species represents a (+) RNA/(-) DNA chimera, while the latter reflects RNase H-mediated primer removal following (+) strand DNA synthesis. That the "mature" DNA is a mixture of 27-nt and 26-nt species, rather than the predicted 25-nt species, indicates (a) that (+) strand synthesis initiation is slightly heterogeneous, and (b) the 3' residue of primer P2 does not belong to the PPT.

(+1) strand products of 50, 40, and 26/27 nt accumulate when P2 is used as primer. The largest corresponds to the intact primer (25 nt) covalently attached to (+) strand DNA, while the 40-nt product represents (+) strand DNA containing exclusively the PPT RNA primer. The shortest and principal products are 26/27 nt, resembling the "mature" DNA previously observed upon removal of primer derived from P1. In this case, however, these are shortened by a single nucleotide to 25/26 nt following alkaline hydrolysis. Hence, while initiation of (+) strand synthesis by Ty3 RT occurs without 3' processing of primer P2, the resulting chimera is not cleaved by the enzyme at the RNA-DNA junction as observed in experiments using primer P1, but rather at an inter-ribonucleotide bond.

FIG. 4. RNase H activity of purified Ty3 RT. A, schematic representation of the substrates used for analysis. The major HIV-1-derived cleavage sites on the 90-nt RNA template have been indicated, designating the first base pair of the RNA-DNA hybrid in the DNA polymerase catalytic center. +1. B, HIV-1 and Ty3 hydrolysis profiles derived from substrate whose RNA 5' terminus is 32P-labeled. For both enzymes, hydrolysis was evaluated after 30 s (lanes 1), 1 min (lanes 2), 2 min (lanes 3), 5 min (lanes 4), 10 min (lanes 5), 20 min (lanes 6), and 40 min (lanes 7). The major sites of hydrolysis and corresponding fragment sizes are indicated at the side of each panel. C, hydrolysis profiles generated from substrate whose RNA 3' terminus is 32P-labeled. Time points 1–7 are similar to those in B, while C represents the uncleaved RNA template.
the RNA component of each DNA-RNA chimera was removed. In both panels obtained with the Klenow fragment of DNA polymerase I, which is located within the following alkaline treatment. The PPT sequence most frequently used when P2 is used as primer. This ribonucleotide is subsequently removed that, while both (1) strand products generated by RT-associated RNase terminal base is a ribonucleotide (2) strand synthesis is interrupted and second strand transfer initiates within P1 and P2, italicized, lowercase ribonucleotides represent those of the PPT. P3 lies outside the PPT and thus serves as a control for RNase H and (+) strand initiation specificity. B, results of PPT selection/extension experiments. Left panels represent DNA synthesis profiles obtained with the Klenow fragment of DNA polymerase I, which efficiently recognizes RNA primers. Right panels represent the equivalent reactions catalyzed by Ty3 RT. In both panels denoted +NaOH, the RNA component of each DNA-RNA chimera was removed. C, summary of PPT selection and extension data with primers P1 and P2. Note that, while both (+) strand products generated by RT-associated RNase H activity are the same length, the 5’ terminal base is a ribonucleotide when P2 is used as primer. This ribonucleotide is subsequently removed following alkaline treatment. The PPT sequence most frequently used is located within the shaded area. 

suggests that the recognition signal for primer removal is not the RNA-DNA junction, but an intrinsic feature of the polypurine tract structure.

DISCUSSION

Despite many similarities between retroviruses and LTR-containing retrotransposons, several recent reports underscore a need for an in-depth study of retrotransposon RTs and accessory proteins with which they might interact. First, documentation of a bipartite PBS in Ty3 (18) suggests that sequences at both ends of the (+) strand Ty3 RNA genome constitute the PBS and contribute to efficient initiation of (−) strand synthesis. This situation effectively places a discontinuous A-form duplex in the nucleic acid binding site, which is unlike any retroviral system. Second, Kirchner and Sandmeyer (21) and Wilhelm et al. (22) have found additional bases at the 3’ ends of the Ty3 upstream LTR, implying that RNase H-mediated selection and release of the PPT primer is less precise than demonstrated for retroviruses. Finally, Nymark-McMahon and Sandmeyer (48) have noted that mutations in the integrase (IN) component of he Ty3 POL3 open reading frame have severe consequences for reverse transcription, implying an interaction between RT and IN, either as individual proteins or as the RT-IN polypeptide. In order to study reverse transcription in LTR-containing retrotransposons at the biochemical level, we have purified a 55-kDa protein representing the RT open reading frame of Ty3 and evaluated its interaction with nucleic acid duplexes encountered during replication, extending a recent in vitro evaluation of NC-mediated initiation of Ty3 reverse transcription (49) from the bipartite PBS.

The finding that purified enzyme migrated as a monomer by size exclusion chromatography, although surprising, is not unprecedented, since the same has been demonstrated for RT purified from bovine leukemia virus (33), MLV (36), and an active HIV-1/MLV chimera (50). In the case of MLV RT, it has been proposed that substrate binding induces dimerization, a feature we have not determined here. This was not the case for the bovine leukemia virus enzyme, which migrated to almost the same position in glycerol gradients in the absence and presence of substrate. Interestingly, the degree of similarity between the Ty3-gypsy family and certain retroviruses, in particular MLV, is sufficiently high that they have been speculated to comprise one large superfamily (16).

An unusual feature of our DNase I footprinting experiments is the 3’–4-bp region of duplex DNA remaining accessible, and in some cases rendered hypersensitive, to nuclease digestion (Fig. 2, C and D). This region is substantially removed from the “window” of hydroxyl radical accessibility between positions −7 and −11 noted for the HIV-1 enzyme (41). This could reflect either an altered DNA structure or that the retrotransposon enzyme has independent nucleic acid binding domains separated by a flexible linker, as suggested for the MLV RT (36). Such spatial separation of functional domains may leave the intervening nucleic acid susceptible to nucleolytic cleavage. Furthermore, although the increased size of the footprint relative to HIV-1 and EIAV RT was unexpected, it is in keeping with the RNase H hydrolysis profiles of Fig. 4, supporting the notion that the spatial separation of the catalytic centers of Ty3 RT exceeds the 18 bp observed with most retroviral enzymes (37–39). Although speculative, it is worth noting in retroviruses that (a) the length of the tRNA-PBS duplex is 18 bp and (b) 18 bases of the tRNA primer are copied before (+) strand synthesis is interrupted and second strand transfer initiates (51). Thus, retroviral enzymes may have evolved to accommodate 18 bp of duplex between their catalytic centers to efficiently mediate two critical RNA-mediated events in replication. In contrast, the equivalent events occur through entirely different mechanisms in Ty3. As indicated earlier, the Ty3 PBS is bipartite and is contributed from both ends of the genome. Moreover, nucleotides of tRNA\textsuperscript{Met} constituting the PBS complement are not inherited by the element from the primer prior to second strand transfer in Ty1, as is the case in retroviruses (52). Although this has not been demonstrated for Ty3, it is possible that events normally requiring recognition or copying of 3’ nucleotides of the tRNA primer are more relaxed in retrotransposons and reflected in altered spatial coupling of the catalytic centers of their polymerases.

Data from both a heteropolymeric RNA/DNA hybrid (Fig. 4B) and substrates recapitulating selection, extension and release of the PPT primer (Fig. 5B) confirms a bona fide Ty3
Experiments designed to evaluate these determinants are currently under way.

Our data may also have implications for the general mechanism of RNase H-mediated hydrolysis, which has remained in doubt. Highly conserved residues of the HIV-1 RNase H domain include Asp^{498}, Glu^{499}, Asp^{500}, and His^{539}. According to Kashiwagi et al. (43), His^{539} serves as a general base (precedents for which are the enzymes DNase I and ExoIII), while Asp^{549} is involved in appropriately positioning a water molecule activated by His^{539} for electrophilic attack. This mechanism assumes participation of a single metal ion, which is clearly defined in the crystal structure of the bacterial enzyme (53, 54), and a role for the highly conserved His^{539}. The sequence compilation of Fig. 7 indicates good conservation of appropriately spaced acidic residues constituting the -D-ED- motif within the RNase H domains of gypsy group of retrotransposons and plant caulimoviruses (55). However, two intriguing features in the former group, including Ty3 are: (a) substitution of this conserved histidine with an invariant tyrosine and (b) the emergence of a highly conserved histidine immediately adjacent to the counterpart of Asp^{500}. One possibility we have considered from this compilation is that the cluster of catalytically conserved residues of gypsy group of retrotransposons is not -D-ED-H-D-, but in fact -D-EDH-D-, i.e. His of the adjacent Asp/His pair serves to activate a water molecule in the same manner that has been suggested for His^{539} and His^{124} of the HIV-1 and E. coli RNases H, respectively. The high degree of conservation of tyrosine in the “His box” suggests this serves a more structural role, possibly through a direct contact with the nucleic acid substrate. Current efforts are aimed at evaluating this proposal by in vitro site-directed mutagenesis, as well as to determine whether the His/Tyr pair in Ty3 RT can be interchanged.

Finally, although we can accurately recapitulate events mimicking initiation of (-) (49) and (+) strand synthesis (this work), interactions between RT and IN should not be overlooked, since several IN mutations result in reverse transcription defects in Ty3 virus-like particles (48). Such data imply an interaction between these polypeptides, either individually or as the RT/IN polyprotein are presently under way.

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