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An Unusual Mechanism of Self-Primed Reverse Transcription Requires the RNase H Domain of Reverse Transcriptase To Cleave an RNA Duplex

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The reverse transcription of retroviruses and long terminal repeat-containing retrotransposons requires that tRNA species serve as primers. We recently reported that the long terminal repeat-containing retrotransposon Tf1 is a unique exception in that reverse transcription is independent of tRNA and is instead initiated by a self-priming mechanism. The first 11 bases of the Tf1 transcript fold back and anneal to the primer binding site in a process that results in the priming of minus-strand strong-stop DNA. Data presented here demonstrate that a cleavage occurs between the 11th and 12th bases of the transcript, resulting in the generation of the primer. Mutagenesis experiments presented here indicate that the RNase H domain of the Tf1 reverse transcriptase is required for the cleavage reaction, suggesting that this RNase H may have the novel ability to cleave double-stranded RNA at the end of a duplexed region.

The propagation of retroviruses and retrotransposons depends on the reverse transcription of their mRNA into the double-stranded DNA molecules that are inserted into the genome of host cells. The complex series of mechanisms that occur during reverse transcription is highly conserved among a broad class of retroelements that include retroviruses and long terminal repeat (LTR)-containing retrotransposons (9, 34). For example, the initiation mechanism of reverse transcription is universal among these elements and includes the hybridization of a specific tRNA to the retroelement mRNA at the primer binding site (PBS) located just downstream of the 5′ LTR (9, 22). As demonstrated for both retroviruses (35) and retrotransposons (4, 16), a tRNA serves as primer for the synthesis of minus-strand strong-stop DNA, the first product of reverse transcription. The requirement of a tRNA primer for reverse transcription is thought to be common to all LTR-containing retroelements because the retroviruses and retrotransposons that have been sequenced have a PBS with strong complementarity to specific tRNA molecules.

An unusual exception to the broad class of LTR-containing retroelements that possess complementarity to tRNA species is the LTR-containing retrotransposon Tf1, isolated from the fission yeast *Schizosaccharomyces pombe* (25). In addition to LTRs, Tf1 possesses coding sequences for Gag, protease (PR), reverse transcriptase (RT), and integrase proteins (25). On the basis of phylogenetic analysis, Tf1 is a member of the Gypsy family of retrotransposons and retroviruses (33, 39). The Tf1 PBS has no complementarity to any known tRNA or to the highly conserved CCA sequence found at the 3′ end of all tRNAs (25). The copy of Tf1 that was sequenced was found to possess high levels of transposition activity, suggesting that reverse transcription was being primed by a tRNA-independent mechanism (24, 26).

In a recent report, data supporting the model that Tf1 undergoes a novel mechanism of self-primed reverse transcription were presented (Fig. 1A) (23). The observation was made that the first 11 bases of the transcript are complementary to the PBS as identified by previous primer extension studies (23, 26). The formation of a looped structure containing this RNA duplex was proposed to lead to the priming of minus-strand strong-stop DNA. Mutations made in the PBS and the 5′ end of the Tf1 transcript greatly reduce the in vivo transposition activity and the accumulation of minus-strand strong-stop DNA in Tf1 particles (23). The combination of single base mutations in the 5′ end of the transcript with individual mutations in the PBS that result in restored complementarity rescues the transposition defects of the individual mutations and therefore demonstrates that the 5′ end of the transcript does anneal to the PBS. Because the formation of the loop structure does not directly result in the presentation of a 3′ OH that could be used by RT for priming, we speculated that a nuclease may cleave within the RNA duplex and thus create a 3′ OH. An alternative possibility is that Tf1 RT may prime minus strong-stop DNA from an internal base at a 2′ OH, as is the case for the retron RTs of bacteria (14, 19, 20). Little evidence that favors one over the other of these two possibilities exists, except that primer extension reactions used to map the 5′ end of Tf1 transcripts never show any evidence of a species of Tf1 mRNA that is cleaved within the first 11 bases (24, 26). As a result, the function of the folded RNA loop in the mechanism of reverse transcription remains unresolved. The importance of this issue is enhanced by the evidence that indicates that the self-priming mechanism of reverse transcription defines a new family of retrotransposons that includes two other elements, CfT-I and Tf2 (23).

The work reported here was initiated to identify the function of the RNA loop structure in the priming of Tf1 reverse transcription. Additional methods to detect cleavage of the Tf1 mRNA were developed, resulting in the firm identification of a specific nuclease activity that cuts the Tf1 mRNA between the 11th and 12th bases. The detection of cleaved Tf1 mRNA required that its subsequent degradation by RNase H be inhibited. In addition, genetic evidence that indicated that the cleavage of the Tf1 transcript at the RNA duplex required the Tf1 RNase H is presented.

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TABLE 1. Yeast strains used

Strain	Genotype	Source or reference	Plasmid description
YHL912	*h⁻ ura4-294 leu1-32*	J. Boeke, 21X5A	
YHL1282	YHL912/pHL449-1	23	Wild-type Tf1-*neo*AI
YHL1032	YHL912/pFL20	26	*S. pombe* vector with ARS[a] and stabilization fragment
YHL1836	YHL912/pHL490-80	1	Tf1-*neo*AI with frameshift in PR
YHL4763	YHL912/pHL915-4	This paper	Tf1-*neo*AI with RNase H mutation D710N
YHL4765	YHL912/pHL916-1	This paper	Tf1-*neo*AI with RNase H mutation D710N[b]
YHL4771	YHL912/pHL919-3	This paper	Tf1-*neo*AI with RNase H mutation D779N
YHL4773	YHL912/pHL920-1	This paper	Tf1-*neo*AI with RNase H mutation D779N[b]
YHL4950	YHL912/pHL940-1	This paper	Tf1-*neo*AI with RT polymerase mutation D563N
YHL4954	YHL912/pHL942-2	This paper	Tf1-*neo*AI with RT polymerase mutation D563L
YHL4959	YHL912/pHL944-1	This paper	Tf1-*neo*AI with RT polymerase mutation D564N
YHL4960	YHL912/pHL945-1	This paper	Tf1-*neo*AI with RT polymerase mutation D564N[b]
YHL5482	YHL912/pHL1237-1	This paper	Tf1-*neo*AI with RT polymerase mutation D564N + RNase H D710N
YHL5484	YHL912/pHL1238-1	This paper	Tf1-*neo*AI with RT polymerase mutation D564N + RNase H D710N[b]
YHL5486	YHL912/pHL1239-1	This paper	Tf1-*neo*AI with RT polymerase mutation D564N + RNase H D779N
YHL5488	YHL912/pHL1240-1	This paper	Tf1-*neo*AI with RT polymerase mutation D564N + RNase H D779N[b]

[a] ARS, autonomously replicating sequence.
[b] These strains are identical to those listed just above, except that the plasmids were generated from independent PCRs.

MATERIALS AND METHODS

Media. The *S. pombe* minimal liquid and plate media were composed of EMM (30). Selective plates contained EMM and 2 g of dropout mix per liter, a powder that contained adenine, uracil, and all amino acids except for nutrients that are absent as required for selection (32). Ten micromolars vitamin B_1 (thiamine) was added to EMM when indicated to repress the *nmt1* promoter. 5-Fluoroorotic acid (FOA) (PRC Inc., Gainesville, Fla.) plates were made by adding 1 g of FOA per liter to EMM supplemented with 100 μg of uracil per ml. YES 5-FOA–G418 plates contained 5 g of yeast extract (Difco), 2 g of complete dropout mix, 1 g of 5-FOA, and 500 mg (corrected for purity) of Geneticin (Gibco) per liter.

Strains and plasmid constructions. The yeast strains used in this paper are listed in Table 1. The oligonucleotides used are listed in Table 2. Plasmid pHL449-1 contained the *nmt1* promoter fused to Tf1-*neo* at the start of the transposon transcript (23). Plasmid pHL476-3 was identical to pHL449-1 except that a reading frameshift in the beginning of integrase was created (23). The construct pHL490-80 also was identical to pHL449-1, except for a frameshift mutation inserted at the *Sac*I site found in the beginning of PR (1). The plasmids with single point mutations in RT were constructed by fusion PCR, with two overlapping 30-base oligonucleotides with the mutant sequence and two PCR oligonucleotides that contained unique restriction sites located on either end of the final PCR product. The fusion PCR products were produced by mixing oligonucleotides that hybridize to the 5′ (HL87) or 3′ (HL88) end of the fusion product with template that consisted of two half-PCR fragments that were each made with one overlapping mutation oligonucleotide and one of the two flanking oligonucleotides (23). The RT point mutations were generated on a 2,261-bp fragment that was cloned into pHL449-1 at the unique *Avr*II and *Bsr*GI sites. All PCRs described were performed with the native *Pfu* enzyme (Stratagene). The specific oligonucleotides used in the construction of each mutation are shown in

TABLE 2. Oligonucleotides

Oligonucleotide	Sequence (5′ to 3′)	Use
HL87	CGATAATTGAACGCTACACC	PCR oligonucleotide used as 5′ flank for all *Avr*II-*Bsr*GI fragments
HL88	GTTTATTTGACATGTATGGC	PCR oligonucleotide used as 3′ flank for all *Avr*II-*Bsr*GI fragments
HL115	GATTCTACTAGAAACTAATGCTTCAGATGTTGC	Top-strand fusion oligonucleotide of overlapping pair with RNase H mutation D710N
HL116	GCAACATCTGAAGCATTAGTTTCTAGTAGAATC	Bottom-strand fusion oligonucleotide of overlapping pair with RNase H mutation D710N
HL119	TTCAAAATTTTAACAAACATCGAAACTTAATTGG	Top-strand fusion oligonucleotide of overlapping pair with RNase H mutation D779N
HL120	CCAATTAAGTTTCGATGGTTTGTTAAAATTTTGAA	Bottom-strand fusion oligonucleotide of overlapping pair with RNase H mutation D779N
HL153	GTAGTATGTTATATGAATGATATTTTAATTC	Top-strand fusion oligonucleotide of overlapping pair with polymerase mutation D563N
HL154	GAATTAAAATATCATTCATATAACATACTAC	Bottom-strand fusion oligonucleotide of overlapping pair with polymerase mutation D563N
HL155	GTAGTATGTTATATGCTTGATATTTTAATTC	Top-strand fusion oligonucleotide of overlapping pair with polymerase mutation D563L
HL156	GAATTAAAATATCAAGCATATAACATACTAC	Bottom-strand fusion oligonucleotide of overlapping pair with polymerase mutation D563L
HL157	GTATGTTATATGGATAATATTTTAATTCAT	Top-strand fusion oligonucleotide of overlapping pair with polymerase mutation D564N
HL158	ATGAATTAAAATATTATCCATATAACATAC	Bottom-strand fusion oligonucleotide of overlapping pair with polymerase mutation D564N
HL193	CATTTATATAGCTCATAACTGATCGAATCCTC	Splint oligonucleotide used in ligation reaction
HL194	GAGGATTCGATCAGT	Substrate oligonucleotide for ligation reaction
HL195	GAGGATTCGATCAGTT	Substrate oligonucleotide for ligation reaction
HL196	GAGGATTCGATCAGTTA	Substrate oligonucleotide for ligation reaction
HL197	GAGGATTCGATCAGTTAT	Substrate oligonucleotide for ligation reaction
HL198	GAGGATTCGATCAGTTATG	Substrate oligonucleotide for ligation reaction

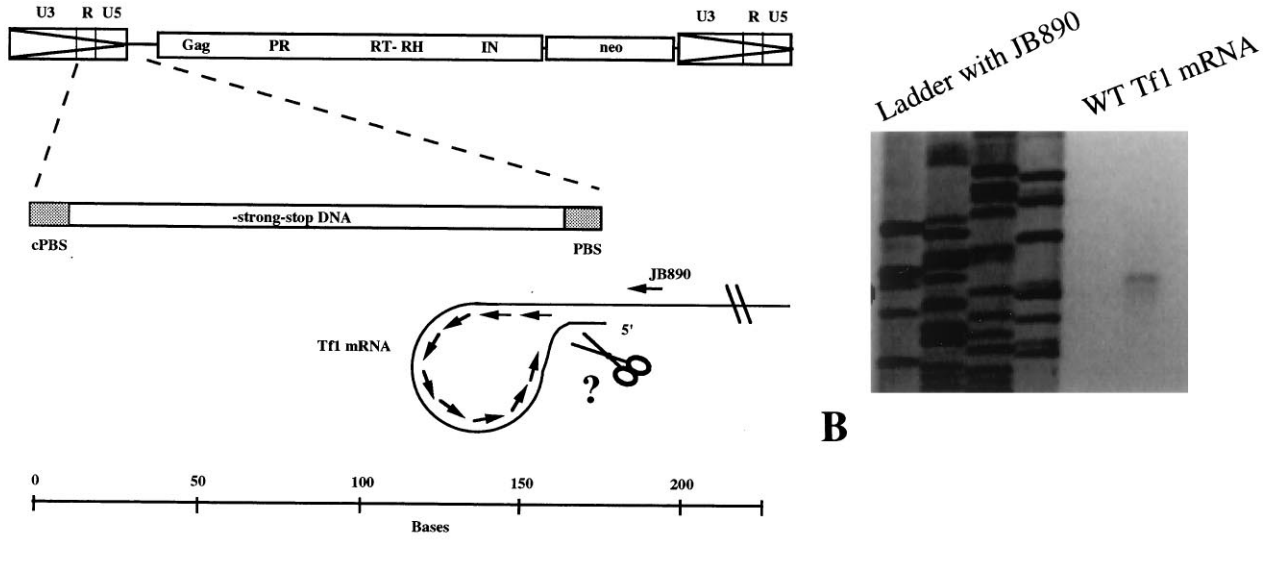


FIG. 1. Model for the minus-strand priming of Tf1 reverse transcription. (A) The mechanism for self-primed reverse transcription of Tf1. The structure of Tf1 is shown at the top of this diagram to indicate the relative positions of strong-stop DNA and the RNA loop that forms in the Tf1 mRNA. Priming begins when the Tf1 mRNA folds into a loop because of the annealing of the first 11 bases to the PBS. Although the 11 bases from the 5′ end of the mRNA serve as primer for strong-stop DNA, no 3′ OH is available. One possible mechanism that could produce the 3′ OH required for priming is the activity of a nuclease that would introduce a cleavage within the first 12 bases of the mRNA. The LTRs are depicted by the triangles, and the open reading frame is indicated by a rectangle that is labeled with the names of the proteins that the bases encode. The position in Tf1 that corresponds to the minus-strand strong-stop sequence is indicated by the dashed lines. The PBS and the first 11 bases are labeled with shading. The 5′ end of the Tf1 mRNA is shown in the looped structure, and the circle of arrows indicates the direction of strong-stop DNA synthesis. The scissors and the question mark indicate the possibility that a nuclease could create a 3′ OH by cleaving the mRNA. The oligonucleotide used in the primer extension assays, JB890, is shown at the position at which it anneals to the mRNA. IN, integrase. (B) Primer extension analysis of Tf1 mRNA. This sequence gel contained the products of a primer extension reaction that included a ^{32}P-labeled oligonucleotide, JB890, and wild-type (WT) Tf1 mRNA extracted from virus-like particles.

Table 2. The double mutant plasmids pHL1237-1, 1238-1, 1239-1, and 1240-1 were created by using DNA from the D564N mutant plasmid, pHL944-1, as template in fusion PCRs that contained the appropriate mutated oligonucleotides required to generate either the D710N or the D779N substitution in RNase H. The mutation site of each plasmid generated by fusion PCR was sequenced to verify the nature of the base changes. In addition, each PCR-generated construct was made in duplicate by an independent PCR to guard against the possibility of untemplated PCR-generated mutations. Each duplicated plasmid gave results in transposition and primer extension assays equivalent to that of its partner.

Transposition assay. Strains were first grown as patches on EMM to induce the *nmt1* promoter fused to the *neo*-marked Tf1. The presence of the bacterial *neo* gene allows *S. pombe* to grow in 500 μg of G418 per ml. After 4 days of 32°C incubation, the plate was replica printed to EMM containing 5-FOA to select against cells containing the Tf1-*neo* plasmid (3). This plate was then replica printed to YES medium (30) containing G418 as well as 5-FOA and incubated at 32°C for 2 days to determine the frequency at which Tf1-*neo* inserts into the genome (24, 26).

Particle preparation and extraction of nucleic acid. The preparations of large-scale yeast extracts and the subsequent analysis on sucrose gradients were based on previously published protocols (7, 8, 10, 23). A total of 500 ml of EMM medium minus uracil was inoculated at an optical density at 600 nm of 0.05 and grown to an optical density at 600 nm of 1.0. The cells were harvested, washed, and broken as previously described (26). Five milliliters of a supernatant of the cell extract recovered from a 3,000-rpm spin (SS34 rotor) lasting 5 min was loaded onto a 20 to 70% linear gradient of sucrose in buffer B-EDTA (15 mM KCl, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] · KOH [pH 7.8], 5 mM EDTA, 1 μg of aprotinin per ml, 0.5 μg of leupeptin per ml, 0.7 μg of pepstatin per ml, 3 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride) and spun for 24 h at 25,000 rpm in a Beckman SW28 rotor. To isolate nucleic acids from particles, 1.2-ml fractions were collected and 0.8 ml each of fractions 26 to 30 was pooled and diluted in buffer B up to a volume of 11.5 ml. This diluted sample was spun at 35,000 rpm in a Beckman SW41 rotor for 1 h to pellet the particles. Pellets were resuspended in 0.4 ml of 25 mM EDTA–0.1% sodium dodecyl sulfate (SDS)–50 μg of proteinase K per ml (Boehringer Mannheim) and incubated at room temperature for 2 h. This solution was then phenol extracted and ethanol precipitated. One-tenth of the material from one 500-ml culture was then used for each primer extension reaction.

Isolation of RNA and DNA from whole-cell extracts. About 10^9 cells (100 units of optical density at 600 nm) were harvested. The cells were resuspended in a glass tube (13 by 100 mm) with 200 μl of EB (0.5 M NaCl, 0.2 M Tris-Cl [pH 7.5], 10 mM EDTA, and 1% SDS), and 200 μl of PICA (1:1 mixture of EB-equilibrated phenol and chloroform that contained 1/24 isoamyl alcohol) was added to enough acid-washed glass beads to fill over the meniscus. The mixture was vortexed vigorously for 30 min in a multivortexer (Baxter Scientific Products), and an additional 400 μl each of EB and PICA was added before a final 30-s vortex. The samples were centrifuged, and the supernatants were PICA extracted again and phenol extracted twice. After ethanol precipitation, 1/10 of the sample was used for either DNA blot analysis (1) or RNA templated primer extension.

Primer extension. The ethanol-precipitated RNA template was resuspended in 15 μl of 1× avian myeloblastosis virus reverse transcription buffer (Boehringer Mannheim) that contained 1 pM γ-^{32}P-labeled oligonucleotide JB890. This mixture was allowed to anneal at 42°C for 1 h. Next, 10 μl of an extension cocktail that was 25 mM $MgCl_2$–2.5 mM dithiothreitol–0.5 mM deoxynucleoside triphosphates–0.625 U of avian myeloblastosis virus RT per μl (Boehringer Mannheim) was added. The elongation reaction mixture was incubated at 37°C for 1.5 h before it was ethanol precipitated and loaded onto a sequencing gel.

Splinted RNA ligation. The conditions required to ligate a DNA oligonucleotide onto the 5′ end of an RNA molecule by using a splint oligonucleotide were previously reported (29). One-tenth of the RNA from the whole-cell nucleic acid extract (about 10 μg) was resuspended in 12 μl of annealing buffer (5.0 mM NaCl, 2.0 mM Tris, pH 7.5), and 2 μl each of the splint and the 5′ substrate oligonucleotide was added from 20 μM stock solutions. This mixture was heated to 92°C for 2 min and cooled to room temperature over 20 min. Two microliters of 10× ligase buffer was added (USB; Amersham Life Sciences) along with 2 μl of T4 DNA ligase (USB; Amersham Life Sciences), and the solution was incubated for specified times at 25°C.

RESULTS

Mapping the 5′ end of Tf1 mRNA by primer extension. The first 11 bases of the Tf1 transcript anneal to the PBS to form a structure that is required for the priming of minus-strand strong-stop cDNA (23). One mechanism that would allow priming to proceed from the formation of the RNA loop and duplex requires that the first 11 bases of the transcript be cleaved by an endonuclease, resulting in the production of a 3′ OH that RT could use to prime DNA synthesis (Fig. 1A). To test this model, cells induced for Tf1 expression from the plasmid pHL449-1 were lysed and cell extract was loaded onto

a sucrose gradient so that Tf1 mRNA could be isolated from particles. The Tf1 mRNA was examined for evidence of cleavage within the first 11 bases. The results of in vitro primer extension assays designed to map the 5′ end of the mRNA with the ^{32}P-labeled oligonucleotide JB890 are shown in Fig. 1B. The extension products consisted of a single band that comigrated with Tf1 base 187 of the adjacent sequence ladder that was made with 5′-phosphorylated JB890. Base 187 is the start of the Tf1 transcript expressed from the *nmt1* promoter, and this differed by one nucleotide from the native start of Tf1 mRNA, the A at residue 188, as reported earlier (24). The lack of extension products shorter than the full-length species ostensibly suggested that no cleavage of the first 11 bases from the Tf1 transcript occurred.

One explanation for the inability to detect a cleaved Tf1 transcript was that the putative cleavage event could be immediately coupled to the extension of the 11-base primer by RT. If true, the RNase H domain of RT would immediately degrade any Tf1 mRNA that had been cleaved, resulting in no accumulation of cleaved RNA. It was decided to generate several mutations in active site residues of the DNA polymerase and RNase H domains of Tf1 RT, with the goal of inhibiting degradation of any cleaved mRNA. The mutations in the RNase H domain would directly inhibit RNA degradation, while the base changes in the DNA polymerase domain would inhibit extension of the primer and in turn block degradation of the RNA because RNase H degrades only RNA present in RNA:DNA heteroduplexes. Several sequence alignments of RNases H that indicate which residues are conserved among all the enzymes have been published (6, 12, 31, 42). The biochemical analysis of mutant RNases H purified from human immunodeficiency virus type 1 (HIV-1) and Moloney murine leukemia virus has demonstrated that the conserved residues are essential for activity (28, 31, 36). Crystallographic studies of RNase H from *Escherichia coli* and HIV-1 have identified that three of the essential residues, two aspartic acids and one glutamic acid, form a catalytic center (5, 15, 42). To identify which amino acid residues in the RNase H of Tf1 form this catalytic center, the sequence of Tf1 was added to the previously published alignments of RNases H and, as shown in Fig. 2A, the secondary structure information from the HIV-1 RNase H was included (12, 42). The three carboxylic residues that make up the catalytic center are marked with triangles in Fig. 2A. By analyzing this alignment, it was concluded that two catalytic aspartic acids in Tf1 RNase H are D-719 and D-779 (because the protease cleavage sites that define the N and C termini of Tf1 RT have not been precisely mapped, the residue numbers refer to the position within the primary translation product). Both of these were individually changed to asparagine. Figure 2A also shows an alignment of the critical amino acid residues of RT at the conserved YXDD motif of the DNA polymerase. Previously published alignments of RT sequences and the mutagenesis of the YXDD motif in HIV-1 demonstrated that the twin aspartic acids were essential residues (21, 40, 41). The two aspartic acids in the polymerase domain of Tf1 RT are numbers 563 and 564 and were mutated to asparagine to minimize the possibility of structural perturbations.

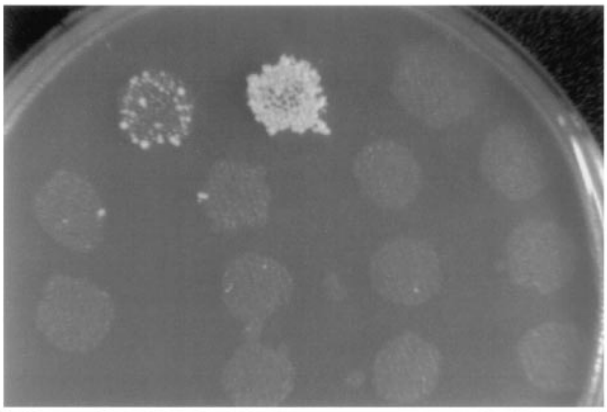
The effects of the point mutations generated in RT were first assessed by measuring the transposition frequencies of *S. pombe* strains bearing mutant versions of Tf1 as expressed from the *nmt1* promoter on the plasmid pHL449-1. As described previously, cells are monitored for transposition by using a *neo* gene inserted into the Tf1 in pHL449-1 (23). After patches of cells are induced for transcription on plates lacking thiamine and subsequently grown on 5-FOA to select against the presence of pHL449-1, cells that undergo transposition can be detected on G418–5-FOA agar because genomic copies of *neo* accumulate as the result of transposition. Figure 2B shows that each of the point mutations in Tf1 RT caused a dramatic reduction in transposition frequency, suggesting that the conserved aspartic acid residues in Tf1 are critical for RT activity. The wild-type Tf1 caused confluent growth on the G418–5-FOA plates, while the negative control strains that contained integrase and PR frameshift mutants showed low levels of resistance to G418.

To assess directly the impact of the mutations on reverse transcription, the levels of reverse transcripts in cells were measured by DNA blot analysis. Cultures were induced for transposition in liquid medium, and total DNA was isolated. As reported previously, levels of mature-sized double-stranded cDNA can be determined by digesting total DNA with *Bst*XI and then performing DNA blot analysis (1). Figure 2C shows that the 2.1-kb cDNA fragment derived from the right end of Tf1-*neo* was produced by the wild-type Tf1 but was absent in all of the strains that carried mutations in RT and the control strain that contained a frameshift in PR. These data indicate that the RT mutations inhibited transposition by directly reducing the levels of reverse transcript.

The ability of the RT mutations to allow the accumulation of Tf1 mRNA cleaved near the 11th base was tested by primer extension analysis of RNA extracted from particles and annealed to ^{32}P-labeled oligonucleotide JB890. The products of the in vitro primer extension assays are shown in Fig. 3A. The RNA isolated from strains with the mutations in residues 710 and 779 of RNase H showed no evidence of cleavage, while all three mutations in the polymerase domain, D563N, D563L, and D564N, resulted in primer extension products that were approximately 11 bases shorter than full length. The presence of the shorter extension products suggested that the Tf1 RNA may be cleaved near the 11th base. To determine exactly the size of the short extension product, a sample of the extension reaction was run adjacent to the sequence ladder produced with the same oligonucleotide, JB890 (Fig. 3B). The shorter product comigrated with the G at position 199 of Tf1, which is the 12th base of the native mRNA and corresponds with a cleavage between the 11th and 12th bases of the native Tf1 mRNA.

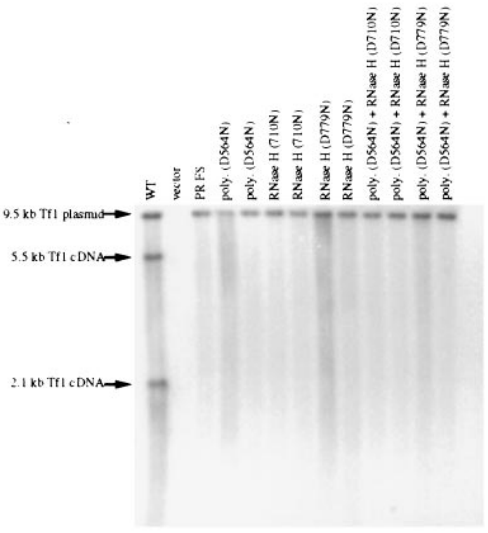
Splinted ligation assay for cleaved Tf1 mRNA. The primer extension assays suggested that Tf1 RNA was being cleaved, but because of the indirect nature of the assay, the same shortened extension product could also be the result of a base-specific modification that could cause premature termination of reverse transcription. Because an alternative mechanism for the priming of Tf1 reverse transcription includes initiating DNA synthesis from a 2′ OH, it was important to determine whether the shorter extension product was the result of cleaved RNA or of the presence of a 2′ OH branch point on the 11th base. If the Tf1 RNA is cleaved and the first 11 bases serve as primer, a 3′ OH must be present on the primer, leaving the phosphate attached to the 5′ end of the remaining RNA. Therefore, a cleavage could be specifically detected by the ligation of the 3′ OH of an oligonucleotide to the 5′ end of the large RNA product of the cleavage reaction. The resulting product would be a Tf1 DNA-RNA chimera that could be longer than the full-length message. The ligation of a DNA oligonucleotide to the 5′ end of an RNA with a 5′ phosphate can be catalyzed by T4 DNA ligase if an additional oligonucleotide is used as a splint to position together the 3′ and 5′ ends of the ligation substrates (29). Figure 4A is a diagram of the components of the splinted ligation reaction. Because the 3′ end of the DNA oligonucleotide and the 5′ end of the RNA must be splinted without a gap, a set of 5′ oligonucleotides

A



IN frameshift	WT Tf1	no Tf1	
RNase H (D710N)	polymerase (D564N)	polymerase (D564N)	PR frameshift
poly.+RNase H (D564N D710N)	RNase H (D779N)	RNase H (D779N)	RNase H (D710N)
	poly.+RNase H (D564N D779N)	poly.+RNase H (D564N D779N)	poly.+RNase H (D564N D710N)

B



C

FIG. 2. The effect of mutations in RT on reverse transcription and transposition. (A) Partial alignment of RTs. The shaded residues indicate positions conserved among three of the five elements. In addition, the shaded positions are highly conserved among RTs. The retroviruses listed are HIV-1, Moloney murine leukemia virus, Rous sarcoma virus, and Gypsy (GYP). The triangles indicate which residues are known to have catalytic function. The locations of the beta strands and the alpha helices are shown as observed in the HIV-1 crystal structure. Residues labeled 563 and 564 of Tf1 are the two aspartic acids that form the YXDD motif. The aspartic acids 710 and 779 are two of the three conserved catalytic residues in Tf1 RNase H. (B) Transposition frequency assay. The strains and their position on the plate are indicated. These strains were first grown on EMM plates that lacked vitamin B_1 to induce Tf1 transcription. The resulting patches of cells were then replica printed first to 5-FOA-containing medium and then to plates that contained 5-FOA and G418 to measure transposition frequencies. (C) DNA blot of reverse transcripts from all the strains included in panel B. Cells were grown to stationary phase in liquid EMM that lacked vitamin B_1. The extracted DNA was digested with *Bst*XI and loaded onto an agarose gel. The DNA was transferred to a membrane that was hybridized with a ^{32}P-labeled *neo* restriction fragment. WT, wild type.

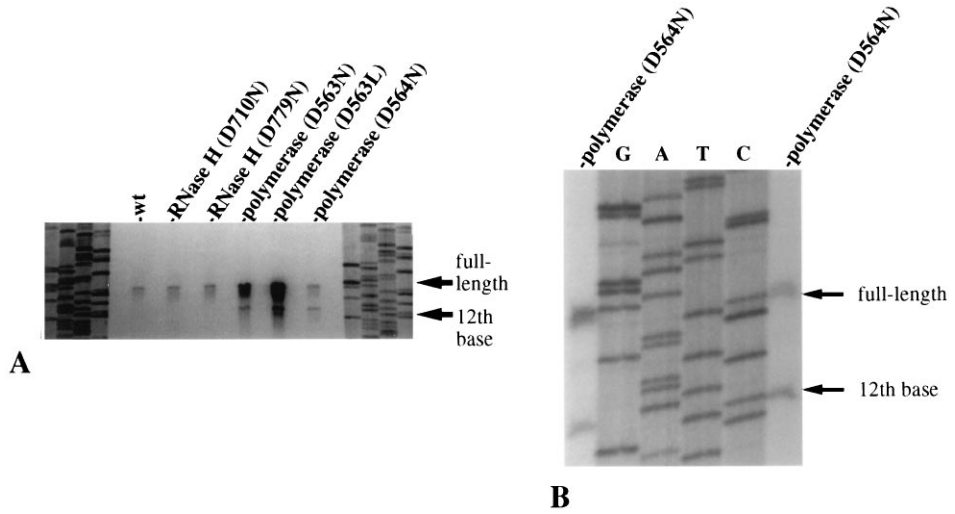


FIG. 3. Primer extension analysis of RNA extracted from Tf1 VLPs. (A) Extracts made from six strains were subjected to sucrose gradient analysis. Fractions that contained VLPs were extracted for Tf1 mRNA that was subsequently analyzed by primer extension. The RNA analyzed came from strains that contained wild type (wt) or one of five mutant versions of Tf1 RT. The flanking sequencing ladders were generated with kinase-treated JB890, the same oligonucleotide that was used in the primer extension reactions. The arrows indicate the positions of the 12th base of the native Tf1 transcript and the full-length extension product. (B) Measurement of the exact length of the primer extension products. A primer extension sample that was derived from the D564N mutant was loaded alongside a sequence ladder created with kinase-treated JB890, the same oligonucleotide used in the primer extension reaction. The arrows indicate the positions of the 12th base of the native Tf1 transcript and the full-length extension product.

were designed to map the exact position of a cleavage if one were detected. This strategy is also diagrammed in Fig. 4A. Total RNA from YHL4959, a strain carrying the D564N mutation in the YXDD motif, was mixed with T4 DNA ligase, the splint oligonucleotide (HL193), and one of each of the 5′ oligonucleotides. To characterize the results of the ligations, oligonucleotide JB890 was used in in vitro primer extension reactions of the ligation products to identify DNA-RNA molecules that were 6 bases longer than the full-length Tf1 transcript as expressed by the *nmt1* promoter. Figure 4B contains the results of primer extension reactions of ligations that were stopped after 1 or 3 h of incubation. Only a single 1-h ligation reaction resulted in a larger extension product than the full-length band, indicating that the 5′ oligonucleotide HL197 did ligate to the 5′ end of the RNA. The exact size of the extension product templated by the oligonucleotide HL197 ligated to the RNA was, as predicted, 6 bases larger than the full-length product (Fig. 4C). The 3-h reaction that contained HL197 also produced this ligated product, but in this case, no shorter extension product remained, demonstrating that the entire population of RNA that templated the short extension product had been quantitatively converted into the ligated product. These results indicated that the shorter extension product templated by Tf1 mRNA was the result of cleavage and not the presence of a modification such as a 2′ OH linkage. The oligonucleotide specificity was maintained in the 3-h reaction with HL197, showing far more reactivity than any other 5′ oligonucleotide. The selective ligation of HL197 to the RNA provided confirmation that the cleavage occurred between the 11th and 12th bases of the native Tf1 mRNA. The low level of ligation to HL196 was likely the result of inefficient ligation across a single base gap and not the result of heterogeneous cleavages of the mRNA, since the extension product templated by ligated HL196 was 1 base shorter than that produced by HL197 (data not shown). We know of no other report of this type of side reaction.

Testing the role of RNase H activity in the cleavage of Tf1 mRNA. One hypothesis about the source of the nuclease activity that catalyzed the cleavage between the 11th and 12th bases of the Tf1 mRNA is that the RNase H domain of Tf1 RT was responsible. One argument against this possibility is that RNase H activity is thought only to cleave RNA when annealed to DNA and, in the case of Tf1, the cleavage likely occurred in RNA that was annealed to RNA (Fig. 1A). Nevertheless, recent in vitro results show that RNase H from HIV-1 RT can cleave RNA in an RNA:RNA duplex (11). Evidence that suggested that Tf1 RNase H might be required for RNA cleavage was provided by the result (Fig. 3A) that the primer extension of RNA from Tf1 elements with mutations in the conserved sites of RNase H did not reveal cleaved RNA, while mutations in the YXDD motif did. The mutations in RNase H might have blocked cleavage altogether. To ask whether Tf1 RNase H participates in the cleavage of the first 11 bases, the level of cleaved RNA produced by Tf1 with the D564N mutation was compared with levels made by Tf1 elements that carried mutations in RNase H active site residues and the D564N change. The mutations D710N and D779N in the RNase H domain of RT were chosen because the results in Fig. 2 indicated that these two residues were essential for RNase H function. Figure 2B also indicates that versions of Tf1 that carried both the D564N and an RNase H mutation were defective for transposition. Figure 5 shows primer extension results of total RNA preparations made from strains carrying Tf1 with the D564N mutation and either D710N or D779N. Although the strains with the single D564N mutation showed significant levels of cleaved RNA, all of the strains with mutations in RNase H lost the ability to accumulate cleaved RNA. These results showed that conservative mutations in amino acids thought to be essential for Tf1 RNase H blocked cleavage, indicating that RNase H itself is required for cleavage activity. Although no RT antibodies are available to test the effect of the RNase H mutations on the levels of RT protein, the results of in vitro RT assays with artificial primer and template indicated that the D710N and the D779N mutations resulted in Tf1 VLPs that had wild-type activity (data not shown). We have attempted to measure the levels of RNase H

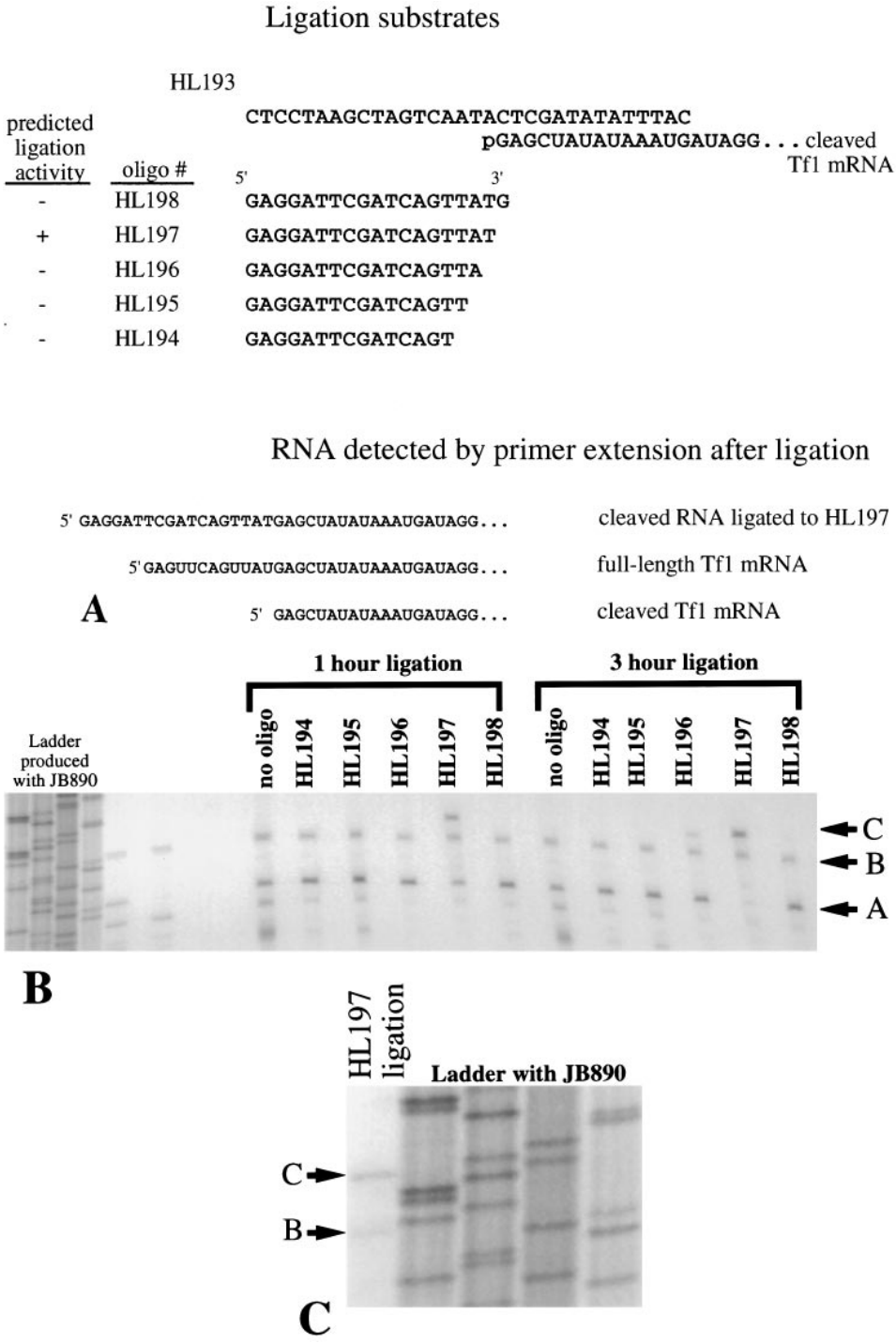


FIG. 4. Splinted ligation assay for cleaved Tf1 mRNA. (A) Substrates of the ligation reactions. To test for the presence of cleaved Tf1 mRNA, total RNA extracted from the strain with the D564N mutation was annealed to the splint oligonucleotide, HL193, and each of the oligonucleotides HL194 through HL198. This assay results in ligation of the RNA to one of the substrate oligonucleotides if the 5′ phosphate of the RNA anneals adjacent to the 3′ OH of one of the substrate oligonucleotides when constrained by the splint oligonucleotide. The ligation results predicted to occur if Tf1 mRNA is cleaved between the 11th and 12th bases are shown. The major products detected by the primer extension assay are shown. These include the full-length Tf1 mRNA, the cleaved mRNA, and the cleaved mRNA ligated to HL197. The full-length mRNA is 12 bases longer than the cleaved RNA because the Tf1 transcript as expressed from the *nmt1* promoter has one more base at its 5′ end than does the native. (B) The results of the splinted ligation reaction. Total RNA was extracted from cells carrying the D564N mutation in Tf1 RT. The RNA was annealed simultaneously to the splint oligonucleotide HL193 and to one of the substrate oligonucleotides, HL194 through HL198. After incubation with T4 DNA ligase for 1 or 3 h, the resulting products were subjected to primer extension with JB890 in order to characterize the ligation products. The primer extension products are shown labeled with the number of the substrate oligonucleotide present during ligation. The band labeled A was the shorter product that comigrated with the 12th base of the native Tf1 transcript. The band labeled B was the full-length product that comigrated with the first base of the *nmt1*-expressed Tf1 mRNA, and the band labeled C was templated by the product of the ligation reaction. A ladder generated with kinase-treated JB890 was included to measure the size of the extension products. (C) Size measurement of the ligation product. This contains a sequence ladder generated with kinase-treated JB890 run next to the extension made from the ligation reaction that contained HL197. The band labeled B is the full-length extension product, and the band labeled C is 6 bases larger than B and resulted from the ligation product.

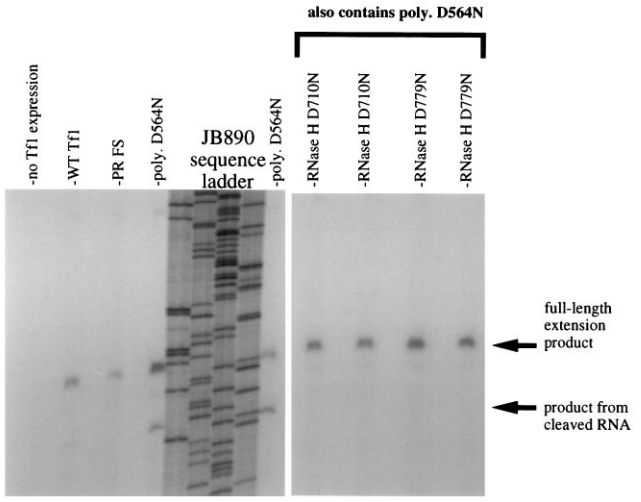


FIG. 5. Analysis of RNase H mutations for defects in the cleavage reaction. Total RNA was extracted from a strain that did not express Tf1, a strain that expressed a version of Tf1 containing a frameshift in PR, and from strains that contained versions of Tf1 with point mutations in either the RT polymerase domain or the RT RNase H domain. The pairs of strains with the same point mutations in RT were generated from independent PCRs. The four samples on the right were derived from strains that contained two point mutations, the D564N polymerase mutation and an RNase H point mutation. WT, wild type.

activity in mutant and wild-type virus-like particles (VLPs) by in vitro RNase H assays (data not shown). However, the purity of the VLP fractions is not sufficient to detect the activity of Tf1 RNase H over the high levels of background produced by the cellular RNases H. This is not surprising because the cellular enzymes are known to have specific activities 100- to 1,000-fold higher than retroviral RNases H.

DISCUSSION

The complementarity between the PBS and the first 11 bases of the Tf1 transcript suggested the self-priming mechanism of reverse transcription that is outlined in Fig. 1A. One simple prediction that resulted from the model was that priming actually requires a 3′ OH that is generated by a nuclease that clips off the first 11 bases from the transcript. Although initial attempts to detect the products of cleavage were unsuccessful, primer extension analysis of RNA produced by Tf1 elements with mutations in the conserved YXDD motif of RT resulted in products that were the size expected if cleavage had occurred between the 11th and 12th bases. Confirmation of this cleavage was produced with ligation reactions that extended the length of shortened RNAs by predicted amounts. The ligation reactions also demonstrated the specificity of the cleavage. The result that HL197 converted almost all of the shorter extension product into a uniform-sized ligation product demonstrated that the vast majority of the Tf1 mRNA was cleaved between the 11th and 12th bases. The existence of this reaction is consistent with the model that cleavage is required to generate a primer for reverse transcription that uses the newly acquired 3′ OH to attach the nascent DNA chain. Although the simplest interpretation of our results is that priming occurs from the 3′ OH of the 11-base RNA, we cannot rule out the possibility that the short RNA uses a 2′ OH to prime DNA synthesis. The accumulation of the cleaved RNA in cells that carried Tf1 with a mutation in the DNA polymerase domain of RT and not in cells with wild-type RT indicated that cleavage, reverse transcription, and RNase H-mediated degradation are coupled.

Previously reported evidence that favored the self-priming mechanism shown in Fig. 1A over a tRNA priming mechanism included the observation that no tRNA candidate existed for Tf1 priming. Instead, complementarity was found between the PBS and the first 11 bases of the Tf1 transcript (23). In addition, mutational evidence that the RNA duplex was essential for transposition and the accumulation of minus-strand reverse transcript was presented (23). Taken together with the result that the Tf1 transcript is cleaved between the 11th and 12th bases, strong evidence now exists for the proposed model of self-primed reverse transcription.

Once Tf1 mRNA cleavage was established, the identity of the nuclease that cut the Tf1 RNA became a significant question. The splinted-ligation assay established that the RNA cleavage activity resulted in one large RNA product that had a 5′ phosphate and one short species that likely possessed a 3′ OH. Because RNases H also produce cleaved RNA with 5′ phosphates, Tf1 RNase H was considered a candidate for the activity that cleaved Tf1 mRNA. By making mutations in two aspartic acid residues of RNase H thought to be essential and combining them with a mutation in the YXDD motif of RT, evidence that suggested that RNase H activity was required for the cleavage process was obtained. Although a strong correlation was observed between the strains with the RNase H mutations and the lack of primer cleavage, biochemical characterization will be required to obtain direct evidence that Tf1 encodes RNase H activity and that the mutations we made destroy this activity. In addition, purified components will be required to establish whether RNase H is the nuclease that cuts the Tf1 RNA between the 11th and 12th bases.

The possibility that RNase H is responsible for cleaving the Tf1 transcript at the end of an RNA duplex is intriguing because the in vivo activities of all RNase H enzymes are thought to be specific for RNA substrate that is annealed to DNA. Recent reports have indicated that in in vitro reactions, retroviral RNases H can degrade double-stranded RNA if Mn^{2+} is present (2, 13). The in vivo relevance of this activity is obscure, and therefore it has been termed RNase H* by analogy with the convention for restriction enzymes with relaxed specificity (13). Detailed examinations of the in vitro activities of HIV-1 RNase H revealed that double-stranded RNA can be degraded even without the addition of Mn^{2+} if the enzyme is artificially arrested during polymerization (11). This activity was shown to be 30-fold slower than the cleavage of RNA in RNA:DNA duplexes. The implication from these experiments is that RNases H may have the ability to cleave double-stranded RNA, but only if its active site is tightly bound to the substrate to allow sufficient time for the slow cleavage of duplex RNA. The cleavage at the Tf1 duplex RNA by Tf1 RNase H may require the enzyme to be specifically tethered to the cleavage site to allow a slow cleavage reaction to occur. If Tf1 RNase H does cut at the end of double-stranded RNA during priming, it would be a novel case of a physiological role for RNase H activity that recognizes RNA duplexes. An alternative explanation for the ability of Tf1 RNase H to cleave a specific nucleotide at the end of an RNA structure is that the RNA itself may possess specific structural features that mimic RNA:DNA duplexes.

A novel case of an LTR-containing retrotransposon that does not prime reverse transcription from the 3′ end of a tRNA is the copia element of *Drosophila melanogaster*. Instead, copia RT initiates minus-strand synthesis from an internal site within the initiator $tRNA^{Met}$ (16). A model suggesting that the $tRNA^{Met}$ is cleaved at the priming site by an RNase P

activity to provide a 3′ OH has been put forth (17, 18). The data reported above regarding the cleavage of the Tf1 transcript suggest the alternative hypothesis that the tRNA primer for copia reverse transcription might be cleaved by the copia-encoded RNase H.

The use of a self-priming process to initiate cDNA synthesis of Tf1 represents an unusual modification to the complex mechanisms of reverse transcription that are highly conserved among all LTR-containing retrotransposons and retroviruses yet characterized. As described in an earlier publication, CfT-I and Tf2 are two other retrotransposons that lack tRNA candidates for priming and have the potential to form the self-priming loop structure, indicating that this unusual mechanism of priming defines a family of retrotransposons. The presence in these elements of LTRs, an RT, and a polypurine track indicates that the steps of reverse transcription subsequent to minus-strand strong-stop priming are typical of the broad class of retroelements that contain LTRs. The self-priming mechanism may represent an evolutionary adaptation to the loss of a specific tRNA primer by a common progenitor of the self-priming family of transposons. Alternatively, there may be a specific feature of Tf1 reverse transcription that obstructs the use of tRNA primers, such as the localization to an unusual compartment.

Although LTR-containing viruses and transposons prime reverse transcription from tRNA molecules, non-LTR retroelements exhibit alternative mechanisms of priming. The hepatitis B virus is similar to retroviruses and can prime minus-strand DNA synthesis with the hydroxyl group of a tyrosine residue near the N terminus of the reverse transcriptase (37, 45). The Mauriceville plasmid, a retroelement that replicates as a closed circular DNA in the mitochondria of some *Neurospora* strains, can initiate DNA synthesis without a primer (38). The Mauriceville RT may have evolved from an RNA-dependent RNA polymerase that did not require specific primers. Recent work has suggested that a broad class of retroelements lacking LTRs prime their reverse transcription from nicks made in the target site DNA. Elements in this category include the yeast mitochondria DNA group II intron aI2 and the non-LTR retrotransposon R2Bm from *Bombyx mori* (27, 43, 44).

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