Plus-Strand Strong-Stop DNA Synthesis in Retrotransposon Ty1

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Reverse transcription in the yeast retrotransposon Ty1 follows the general “rules” of retroviral replication overall. However, some details of the retroviral and Ty1 reverse transcription processes are different. We have identified and determined the structure of plus-strand strong-stop DNA and examined the effect of polypurine tract deletion mutations on its synthesis. Furthermore, we have defined the stop signal for plus-strand strong-stop DNA synthesis as an unusual 2'-O-ribosylated nucleotide in the primer tRNA. Full-length plus-strand strong-stop DNA, following strand transfer, would have a terminal 2-base mismatch with minus-strand DNA. These findings indicate that the mechanism of plus-strand strong-stop DNA transfer in Ty1 differs from that of the retroviral transfer and suggest that full-length plus-strand strong-stop DNA is not a direct intermediate in Ty1 retrotransposition.

The transpositional movement of retrotransposon Ty1 has many similarities to the replication and integration processes of retroviruses. However, there are several aspects of the structure and life cycle of Ty1 that are distinct from those of retroviruses. It is instructive to consider the sequences immediately adjacent to the long terminal repeats (LTRs), which differ significantly from those found in retroviruses (Fig. 1). Specifically, the primer-binding site (PBS) at which minus-strand reverse transcription is primed (10) is only 10 nucleotides long, whereas it is 18 nucleotides long in all retroviruses. Similarly, the putative polypurine tract (PPT) in Ty1 is only 8 nucleotides long, and only 6 of these 8 residues are purines, whereas the typical retroviral PPT consists of 11 to 14 straight purines (26). These facts raise questions about whether the PBS and PPT sequences function in the same way in Ty1 as in retroviruses.

In retroviruses, the plus-strand primer, a PPT, is generated by reverse transcriptase (RT)-associated RNase H activity. This enzyme degrades viral genomic RNA, which is hybridized to newly synthesized minus-strand DNA. A PPT, positioned just upstream of the 3' LTR, resists hydrolysis by RNase H. Furthermore, the PPT is specifically cleaved at its 3' end by RNase H, creating an oligoribonucleotide sequence which serves as a plus-strand primer. This PPT-derived oligonucleotide is selectively extended by RT. Since the overall organization of purine bases within retroviral PPTs is conserved among viruses infecting different hosts, it has been suggested that the structure of PPT might be more important than simply the base composition (26). It is difficult to estimate the exact length of the so-called PPT in yeast retrotransposon Ty1 because it does not consist of purines alone. Previous analyses (4, 22, 25) suggest that it might be only approximately 8 nucleotides long, but this sequence contains only 6 purines (Fig. 1).

In the LTR retroelements, the PBS is defined as a region with perfect complementarity to the 3' end of the primer tRNA molecule. It is generally accepted that the synthesis of plus-strand strong-stop DNA on the minus-strand template

proceeds through a portion of the attached primer tRNA molecule. The modified nucleotide nearest the 3' end of the tRNA causes the RT to stop (13, 30). However, in most cases, the modified nucleotide in question is a 1-methyladenosine at position 58 (A-58), a modification conserved among all eukaryotic tRNA molecules (29). This modified nucleotide prevents incorporation of thymidine across from A-58 by RT, presumably by sterically preventing base pair formation. In all retroviruses and almost all retrotransposons, the length of the PBS corresponds exactly to the number of tRNA nucleotides between the modified nucleotide nearest the 3' end of the tRNA and its actual 3' end (21). In Ty1, the PBS is 2 bases shorter than expected from the known primer tRNA modification pattern (11, 12). Interestingly, an even more pronounced difference can be found in another *Saccharomyces cerevisiae* retrotransposon, Ty3, with a PBS 4 bases shorter than expected (14, 16). Both of these elements use tRNA^{Met} (initiator tRNA) as the minus-strand primer. Initiator tRNAs uniquely possess an unusual modification, a 2'-O-ribosylphosphate on adenosine at position 64 (A-64) (12). This modification plays an important role as a discriminator of initiator and elongator tRNAs in translation (1, 17). A-64 is the modified nucleotide nearest to the 3' end of this primer tRNA^{Met} molecule.

The plus-strand DNA is synthesized on a minus-strand template by extending the PPT-derived oligoribonucleotide primer toward the 5' end of the minus strand and the attached tRNA moiety. Because the 2'-O-ribosylphosphate on A-64 is an unusually bulky modification, Ty1-encoded RT might stop 2 bases before reaching this modified nucleotide A-64 because of steric hindrance. Thus, this unusual modification might explain the discrepancy in Ty PBS length and the tRNA modification pattern, perhaps with Ty3 RT being more sensitive to steric interference than Ty1 RT.

In this study, we examined the effect on transposition of two deletion mutations encompassing the Ty1 PPT. We also identified plus-strand strong-stop DNA and demonstrated that the PPT is a functional region in Ty1, essential for plus-strand priming and transposition. We examined a previous hypothesis that the lariat debranching enzyme might process primer/DNA junctions (9); we found no evidence for this theory. Finally, we defined the precise site of plus-strand strong-stop termination as the first modified nucleotide encountered within the primer tRNA molecule. This result suggests that full-length plus-strand strong-stop DNA is not an essential intermediate in the

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TABLE 2. Transposition frequency of *pptΔ* mutants

Yeast strain ^a	Relevant genotype	Raw data ^b	Transposition frequency (%) ^c	PPT ^d
VL18	<i>SPT3</i>	92/514	17.4	WT
YKN103	<i>SPT3</i>	18/661	2.6	<i>pptΔ1</i>
VL56	<i>SPT3</i>	9/854	1.0	<i>pptΔ2</i>
VL3	<i>spt3</i>	50/407	12.3	WT
YKN104	<i>spt3</i>	0/820	<0.1	<i>pptΔ1</i>
YKN105	<i>spt3</i>	0/608	<0.2	<i>pptΔ2</i>

^a VL3, YKN104, and YKN105 are transformants of YH50, i.e., *spt3*; VL18, YKN103, and VL56 are transformants of YH8, i.e., *SPT3*; YH8 and YH50 are isogenic.

^b Number of Trp⁺ Ura⁻ colonies/total number of Ura⁻ colonies. Numbers shown are the sum of colonies from three independent measurements.

^c Percent transposition frequency is calculated as the number of Ura⁻/Trp⁺ colonies divided by the total number of Ura⁻ colonies (×100).

^d Type of PPT mutation. WT, wild type.

We examined transposition of the mutant and wild-type elements in *spt3* strains, in which chromosomal Ty1 element transcription is greatly reduced (31). This circumvents the complication discussed above. Transposition in the *spt3* strain can still be assayed with *GAL*-Ty1 plasmids, because the *GAL1* promoter is quite active in these strains (6). In the *spt3* strain background, transposition of the mutant elements was undetectable, although wild-type Ty1-*TRP1* transposed at a high frequency (Table 2). Thus, there is an essential site for transposition defined by the *pptΔ1* and *pptΔ2* mutants.

Identification and characterization of plus-strand strong-stop DNA. To determine the nature of the transposition defect in the *pptΔ1* mutant, we examined the nucleic acid species present in wild-type and *pptΔ1* mutant VLPs from *spt3* strains. We prepared VLPs from the wild type and the mutant and obtained similar yields of RT activity and *A*₂₅₄ material in the VLP peak, indicating that there was no general defect in VLP formation or enzymatic activity. We examined VLP nucleic acids for plus-strand strong-stop DNA in two ways. First, we 5'-end labeled (with polynucleotide kinase) total VLP nucleic acids and examined them by polyacrylamide gel electrophoresis (Fig. 2). An approximately 350-nucleotide (nt) DNase-sensitive and RNase-resistant species was observed in the wild-type preparations but was absent in *pptΔ1* mutant preparations. The approximately 350-nt species is DNase sensitive and RNase resistant, as expected for a full-length plus-strand strong-stop DNA.

To confirm that the approximately 350-nt species is Ty1 plus-strand strong-stop DNA, we carried out a Southern blot hybridization analysis with a riboprobe complementary to plus-strand Ty1 LTR DNA. Unlabeled total VLP nucleic acid preparations were run on a sequencing gel together with a variety of LTR and other DNA size standards, blotted to a nylon membrane, and hybridized to a plus-strand-specific probe (Fig. 3). We found that a band of approximately 350 nt hybridized to the probe and that this band was absent from the *pptΔ1* mutant. We conclude that the 350-nt species is plus-strand strong-stop DNA and that the *pptΔ1* mutant is defective in its synthesis.

Plus-strand strong-stop DNAs from wild-type and *dbp1* mutant VLPs are identical and lack detectable RNA primers. The nature of the primer for Ty1 plus-strand strong-stop DNA is unknown. Priming may occur either (i) via a conventional 3'-5' bond to a small oligoribonucleotide as predicted by standard models of retroviral plus-strand priming or (ii) via a 2'-5' bond as in the case of the bacterial multicopy single-stranded DNAs

FIG. 2. End-labeling of total VLP nucleic acid. Total nucleic acids were extracted from VLP preparations from several yeast strains bearing either a wild-type PPT or the *pptΔ1* mutation and 5'-end labeled. The labeled preparations were then subjected to digestion by RNase (R), DNase (D), or purified yeast-debranching enzyme (B) or no treatment (-); electrophoresis on a 5% polyacrylamide-urea gel; and autoradiography. The top "band" is the well. The second band is probably the 2.1-kb plus-strand product primed at the internal (PPT2) priming site described by Pochart et al. (25). Strains used were as follows: YKN130, *DBP1*⁺, wild-type PPT, lanes 1; YKN133, *DBP1*⁺, *pptΔ1*, lanes 2; YKN128, *dbp1*, wild-type PPT, lanes 3; YKN134, *dbp1*, *pptΔ1*, lanes 4. ENZ, enzyme; MW, molecular weight.

(msDNAs) (15, 19). When yeast debranching enzyme (yDBP1) was discovered as a host factor for Ty1 transposition, it was suggested that 2'-5' priming might take place in Ty1 (9). If this were the case, we would expect to find the primer still attached to the strong-stop DNAs made in a *dbp1* mutant of *S. cerevisiae*, which lacks the 2'-5' phosphodiesterase activity of yDBP1 and has an approximately ninefold defect in Ty1 transposition. The *dbp1* mutant makes normal levels of Ty1 VLPs, with no obvious biochemical defects detected thus far (22a).

To determine whether plus-strand strong-stop DNA has any

FIG. 3. Confirmation that the approximately 350-nt species is plus-strand strong-stop DNA. VLP nucleic acid preparations were treated with various enzymes as for Fig. 2 and run on a 6% polyacrylamide-urea sequencing gel with molecular weight standards. The preparations were subjected to digestion by RNase (R), purified yeast-debranching enzyme (B), or CIP (C) or were left untreated (-). The molecular size (MW) of the plus-strand strong-stop DNA was estimated to be approximately 350 nt by comparison with the Ty1 LTR molecular weight standards. Strains used were as follows: YKN130, *DBP1*⁺, wild-type PPT (lanes 1); YKN133, *DBP1*⁺, *pptΔ1* (lanes 2); YKN128, *dbp1*, wild-type PPT (lanes 3); and YKN134, *dbp1*, *pptΔ1* (lanes 4). The aberrant migration of the samples in lanes 1 was an artifact of this particular gel resulting from differences in the buffer compositions of the samples. Aliquots of these same samples were reprecipitated and run on a second gel, and all comigrated (lower panel, lanes as above). Molecular size standards (LTR sequences of various lengths) are in nucleotides.

primer RNA attached, we pretreated some of the samples in the experiments in Fig. 2 and 3 with either RNase A or purified yDBR1 (23) prior to electrophoresis. These pretreatments had no effect on the mobility of the plus-strand strong-stop DNA, even when it was analyzed on a sequencing gel (Fig. 3).

The lack of effect of enzyme pretreatments also applied when VLPs were prepared from a *dbp1 spt3* host strain. Similar experiments demonstrated that minus-strand strong-stop DNA (with its full-length tRNA primer attached), synthesized in vitro (10), was resistant to the action of yDBR1 (data not shown). These experiments indicate that yDBR1 does not play a role in removing primers from Ty1 strong-stop DNAs.

We have identified and characterized the Ty1 DNA molecule that corresponds to retroviral plus-strand strong-stop DNA. Synthesis of plus-strand strong-stop DNA requires an intact PPT sequence; deletion of this region of the genome results in a complete loss of detectable plus-strand strong-stop DNA and transposition in *spt3* strains. We have not been able to detect any RNA primer at the 5' end of this molecule by treatment with RNases or RNA lariat debranching enzyme. The most likely explanation for this is that the primer is an oligoribonucleotide generated by RNase H and derived from the PPT, which is cleaved from the plus-strand strong-stop DNA (again, presumably by RNase H) before its synthesis is completed. Previous work has shown that there is a second site of plus-strand priming near the middle of the Ty1 element (22, 25); there is a perfect match between the 9-bp sequence that immediately precedes the 3' LTR and a sequence called PPT2, which is believed to prime this additional species. We have not investigated the nature of the PPT2 sequence in intact Ty1 elements, but we note that it can be deleted without any obvious effect from mini-Ty1 elements (32); thus, we believe that this sequence is not essential for transposition. T. Heyman and colleagues (14a) have obtained independent data supporting the conclusion that PPT2 is nonessential.

The yeast *DBR1* gene encodes the lariat debranching enzyme. Surprisingly, *dbp1* mutants are partially defective in Ty1 transposition (9). We have not detected any effect of purified yDBR1 protein on either minus-strand or plus-strand strong-stop DNAs in vitro. Furthermore, these species have exactly the same gel electrophoretic properties whether they are derived from wild-type or *dbp1* mutant strains. Hence, we believe that the 2' priming observed in bacterial msDNAs does not occur during Ty1 reverse transcription. A recent study showed that 2'-priming is not carried out by retroviral RT either (28). Thus, yDBR1 must play another role in the Ty1 life cycle.

Termination of plus-strand strong-stop DNA at the unique ribosylated adenosine residue within the primer tRNA^{Met}. The plus-strand stop signal for the Ty1 RT, presumed to be a modified nucleotide within the tRNA^{Met}, has not been previously investigated. In the tRNA^{Met}, the first modified nucleotide to be encountered by Ty1 RT is a 2'-O-ribosyl phosphate on A-64, 13 nucleotides from the tRNA 3' end. It is not very easy to predict the behavior of RT when it polymerizes on a template bearing this modification. The 2'-O-ribosyl phosphate lies on the outer surface of the tRNA acceptor stem helix (3). Therefore, it seemed possible that Ty1 RT could bypass this modification and stop at the next modified nucleotide, namely, the conserved A-58. On the other hand, this 2'-O-ribosyl phosphate attached to the ribose is a bulky molecule that might stop the RT polymerization reaction prior to position A-64.

To determine directly and exactly how far into the tRNA molecule plus-strand synthesis proceeds, we developed a new type of primer extension analysis performed inside VLPs by using Ty1 RT and endogenous nucleic acid template (Fig. 4A).

FIG. 4. Termination of plus-strand strong-stop DNA. Ty1-VLPs were used for "intra-VLP" primer extension experiments. (A) The production of these particles upon galactose induction was driven by a Ty1 element lacking its 3' LTR (pJEF938). In these particles, the minus-strand strong-stop DNA accumulates since it cannot be transferred to the 3' end of the Ty1 RNA owing to a lack of RNA complementary sequences within the 3' LTR. The primer extension reaction was performed with Ty1 RT within the VLPs. The primer was ³²P-labeled JB66, an oligonucleotide complementary to the R region of the minus-strand strong stop. The stop signal for RT is expected to be the first modified nucleotide within the primer tRNA^{Met}. (B) Following the primer extension reaction, Ty1-VLP DNA was phenol-chloroform extracted, ethanol precipitated, and run on a 6% sequencing gel. A sequencing reaction performed with a Ty1 DNA template and kinased JB66 was used as the DNA *M_r* standard. The primer extension reaction mixture was mixed with the sequencing reaction mixture to precisely determine product lengths (lanes designated GATC). Unmixed sequencing reactions were run on the same gel (not shown). Dots mark the first base of the PBS sequence. Lane Ty1 RT shows extension products generated by Ty1-RT within the VLPs. Lane MuLV RT shows extension products generated by Moloney murine leukemia virus RT on Ty1-VLP DNA.

A *GAL1*-Ty1 plasmid, pJEF938 (32), which lacks the 3' LTR, was used to direct the synthesis of Ty1-VLPs. In these VLPs, the only DNA product made is the minus-strand strong-stop DNA, which accumulates since it cannot be translocated as a result of the lack of a 3' R complementary sequence. This strong-stop DNA apparently becomes single stranded following RNA template degradation by RNase H.

The expected "stop signal" for the RT is the first modified nucleotide within the primer tRNA. After incubation and deproteinization, the reaction products were run on a sequenc-

ing gel. The exact length of the DNA products was determined by mixing the primer extension reaction mixture with a sequencing reaction mixture containing a Ty1 DNA template and the same kinased primer (Fig. 4B). To investigate whether the bands observed were specific to Ty1 RT, we isolated nucleic acids from Ty1-VLPs and ran an *in vitro* primer extension reaction with Moloney murine leukemia virus RT. In this Moloney murine leukemia virus RT reaction, we obtained a similar banding pattern. When the VLPs were pretreated with RNase A, removing the tRNA template attached to the minus-strand DNA, prior to primer extension with Ty1 RT, all the tRNA templated bands disappeared (data not shown). In separate experiments, we demonstrated that elimination of the backbone modification in the tRNA (by using a *rit1* mutant strain [2]) allows the RT to read through and stop at the next modified base, proving that it is the modification and not a tRNA structure that blocks synthesis (20a). We conclude that 12 bases of the tRNA molecule is copied into the plus-strand strong-stop DNA and that the 13th (modified) base, nearest to the 3' end, at position A-64 is a stop signal for both Ty1 and Moloney murine leukemia virus RTs. This modified nucleotide itself is not copied into DNA.

It has been demonstrated that retroviral RTs reverse transcribe the portion of primer tRNA, presumably extending to the first modified nucleotide encountered by RT (13). In the case of all tRNAs serving as a primer for retroviruses, this first modification is a 1-methyl group on A-58. This modification is found at position 58 in all eukaryotic tRNA molecules. Since this methyl is attached to the 1 carbon of the adenine base, this modification should prevent base pairing with an incoming thymine base.

Using our new intravirion primer extension method, we have unambiguously determined that the majority of Ty1 RT-derived plus-strand synthesized inside the VLPs harbors 12 tRNA-templated bases at its 3' end. A second conclusion from this experiment relates to the state of Ty1 RNA following completion of minus-strand strong-stop DNA synthesis. Since the primer used in this reaction was annealed without prior denaturation of the template, this suggests that minus-strand strong-stop DNA is in a single-stranded form; i.e., the RNA template was already removed, presumably by RNase H.

Putative terminal mismatch between plus-strand strong-stop DNA and minus-strand DNA. DNA synthesis in Ty1-VLPs is initiated by Ty1 RT with the 3' end of tRNA^{Met} as a primer. This tRNA is annealed to the complementary region of the Ty1 RNA called the PBS. The minus-strand strong-stop DNA is completed when RT reaches the 5' end of the template RNA molecule. The 5' end of the template RNA is degraded (21a), presumably by the Ty1 RNase H activity. Single-stranded minus-strand DNA is then transferred to the 3' end of the Ty1 RNA template via the R region and is extended along the Ty1 RNA template. Plus-strand DNA synthesis is presumably primed by a PPT oligoribonucleotide generated by a specific RNase H cleavage, proceeding along the minus-strand template into the primer tRNA molecule. By the conventional model for retroviral reverse transcription, this full-length plus-strand strong-stop DNA should be transferred and annealed to the opposite end of the element via the PBS complementary sequence in the minus-strand DNA molecule.

In the case of Ty1, the PBS region (strictly defined as the sequence with perfect complementarity to the primer tRNA 3' end) is 10 bases long. This is 2 bases shorter than expected from the known primer tRNA^{Met} modification pattern (i.e., there is a modification on the 13th nucleotide from the tRNA 3' end, at residue A-64). If Ty1 RT stops DNA synthesis upon reaching the first modified nucleotide in the tRNA, it would

FIG. 5. Terminal mismatch between plus-strand strong-stop DNA and minus-strand DNA. DNA synthesis in Ty1-VLPs starts by elongation of primer tRNA^{Met} with Ty1 RNA template. Template RNA is hydrolyzed by RNase H after RNA-DNA hybrid formation (21a). Resulting single-stranded strong-stop DNA transfer to the opposite end of the RNA template would allow continued minus-strand synthesis. Plus-strand synthesis is presumably primed by a PPT-derived oligoribonucleotide and extended along the minus-strand template toward the primer tRNA^{Met}. The stop signal for Ty1 RT is the first modified base, i.e., A-64. The resulting full-length plus-strand strong-stop has 12 tRNA templated bases at its 3' end. Following plus-strand transfer, a terminal mismatch of two bases would be formed.

extend 12 bases into the tRNA template. Consequently, after transfer of full-length plus-strand strong-stop DNA (harboring 12 tRNA-templated bases at its 3' end), an unusual structure would result (Fig. 5). Rather than the fully base-paired DNA strand transfer product that forms in retroviruses, a terminal mismatch of two unpaired nucleotides would result. Such a mismatch presumably would not be efficiently extended or removed by Ty1 RT, which, like other RTs, is believed to lack a proofreading exonuclease activity. Thus, this full-length plus-strand strong-stop DNA either must be processed to generate a shorter homoduplex or, more likely, is not a direct intermediate in Ty1 retrotransposition.

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