Plus strand strong-stop DNA transfer in yeast Ty retrotransposons

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The yeast Ty1 LTR retrotransposon replicates by reverse transcription and integration; the process shows many similarities to the retroviral life cycle. However, we show that plus strand strong-stop DNA transfer in yeast Ty1 elements differs from the analogous retroviral process. By analysis of the native structure of the Ty1 primer binding site and by a series of manipulations of this region and assessment of the effects on retrotransposition, we show that primer binding site inheritance is not from the tRNA primer, which is inconsistent with classical retroviral models. This unusual inheritance pattern holds even when the Ty1 primer binding site is lengthened in order to be more retrovirus-like. Finally, the distantly related Ty3 element has an inheritance pattern like Ty1, indicating evolutionary conservation of the alternative pathway used by Ty1. Based on these results we arrive at a plus strand primer recycling model that explains Ty1 plus strand strong-stop DNA transfer and inheritance patterns in the primer binding site.

Keywords: evolution/primer binding site/retrotransposon/reverse transcriptase/tRNA/yeast

Introduction

Reverse transcription of retroviruses and LTR retrotransposons is an evolutionarily conserved process (Telesnitsky and Goff, 1993). However, studies on replication of the yeast Ty1 retroelement have revealed an important difference from the generally accepted retroviral replication model; primer binding site inheritance is from element sequences and not from tRNA sequences (Lauermann and Boeke, 1994). Interestingly, sequence comparisons suggest that these deviations are preserved among yeast retroelements, even among very distantly related retrotransposons like Ty1 and Ty3.

The genome of Ty retrotransposons, like that of other LTR retroelements, consists of a positive single-stranded RNA molecule that at both ends is shorter than the chromosomal DNA donor copy (Figure 1A). This RNA is flanked by short terminal repeats (R). Regeneration of the full-length retroelement DNA flanked by long terminal repeats (LTRs) is a complex process (Figure 1) carried out by the element-encoded reverse transcriptase (RT) and its associated RNase H activity (RH).

Conversion of retroelement RNA into double-stranded DNA begins by elongating the primer molecule, a specific cellular tRNA, annealed to the genomic RNA at the complementary primer binding site (PBS; Figure 1B). The PBS is positioned at the 5’-LTR border. This precise point of reverse transcription initiation defines the final right end of the transposon DNA which will eventually be integrated into a new location in the yeast genome. The first replication step consists of extension of the tRNA primer into the U5 and R regions, generating minus strand strong-stop DNA. Genomic RNA hybridized with newly synthesized DNA is then degraded by RH (Figure 1B). To allow further elongation, the minus strand DNA must be translocated to the genomic DNA 3’-end, where it can anneal to the complementary R sequence (Blain and Goff, 1995; Figure 1C). This transfer can occur as an intramolecular jump within the same template RNA molecule or intermolecularly using the second, co-packaged genomic RNA (Panganiban and Fiore, 1988; Hu and Temin, 1990). After this first strand transfer the minus strand is extended using the retroelement RNA template, reaching the sequences at the RNA 5’-end (Figure 1D). The primer for plus strand synthesis is created by specific RH cleavage at the sequence called the polypurine tract (PPT), found just upstream of the 3’-LTR boundary (Figure 1E). This cleavage precisely defines the left end of the element DNA. In retroviruses the PPT-derived primer is removed by RH, after it is extended along the minus strand DNA template, creating the plus strand strong-stop DNA. Plus strand strong-stop DNA in retroviruses is terminated when the RT reaches the first modified base from the 3’-end of the primer tRNA, a 1-methyladenosine at position 58 in the primer tRNA. In Ty1, plus strand single-stranded DNA has been identified but the exact nature of the primer and the mechanism by which it is removed are unknown; Ty1 plus strand DNA in virus-like particles (VLPs) lacks any detectable primer. Plus strand synthesis is terminated at the first modified nucleotide encountered in the tRNA, which is a 2’-phosphoribosyl adenosine 12 nucleotides (nt) from the end (Lauermann et al., 1995). Depending on the nature of the preceding minus strand strong-stop DNA transfer, the extent of complementarity between plus strand strong-stop DNA and extended minus strand DNA differs. If the minus strand strong-stop DNA transfer was intramolecular, the extended minus strand DNA could include the R and U5 sequences. If it was intramolecular, complementarity would be restricted to the RNA left behind after the RH cleavage that facilitated minus strand strong-stop transfer. This corresponds to the PBS. In the case of Ty1 and HIV-1, DNA plus strand synthesis is also primed at an internal site, PPT2 (Charneau et al., 1992; Heyman et al.,...
Fig. 1. A general model for LTR retroelement reverse transcription. (a) LTR retroelement DNA structure: solid lines, LTRs consisting of U3, R and U5 regions. The PBS is complementary to the 3'-end of primer tRNA. The PPT is the region giving rise to the plus strand primer. Two genomic RNA transcripts (wavy lines) are present in viral core particles prior to initiation of reverse transcription. (b) The primer tRNA (bicycle) is annealed to the PBS, where it initiates minus strand strong-stop DNA synthesis. Minus strand strong-stop DNA is completed when the RT reaches the 5'-end of the RNA template; the RNA in complex with DNA is degraded (dashed line) by the RT-associated RH. (c) Minus strand strong-stop DNA transfer is facilitated by the repeated sequence R found on the ends of retroelement RNA. (d) Minus strand DNA is then extended along the element RNA template. During the transfer either RNA molecule can serve as the recipient of minus strand strong-stop DNA. Parentheses surround the R/U5 region, which may or may not be present depending on whether the RNA template was intact or partially degraded as a consequence of RH activity as in (B). (e) The primer for the plus strand strong-stop synthesis derives from the PPT. (f) The plus strand strong-stop is transferred to the 5'-end of minus strand DNA, possibly by displacement synthesis from an upstream plus strand priming event. (g) and (h) Plus strand and minus strand DNA synthesis are completed. Although these are shown as separate steps they probably occur simultaneously. (i) The full-length retroelement DNA is integrated into the genome.

This resultant product is an interrupted plus strand. After plus strand transfer (Figure 1F) synthesis of both strands can be completed (Figure 1G and H). The full-length DNA, with precisely defined ends, is then ready to be integrated into the host genome.

In retroviruses during plus strand strong-stop synthesis the genetic information flows from the primer tRNA into the plus strand of the newly made virus DNA (Berwin and Barklis, 1993). We have shown, in contrast, that the tRNA sequences are not inherited by progeny Ty1 copies (Lauermann and Boeke, 1994), even though a portion of the tRNA can be efficiently reverse transcribed during full-length plus strand strong-stop DNA formation (Heyman et al., 1995; Lauermann et al., 1995). There were several possible explanations for this apparent discrepancy. In this study we examine the transfer of plus strand strong-stop DNAs in Ty1 and provide models that can explain the observed inheritance patterns. By analyzing the distantly related Ty3 element, as well as the structure of the endogenous Ty1 and Ty2 transposon copies sequenced as part of the yeast genome project, we conclude that this unusual reverse transcription strategy is evolutionarily conserved among the *Saccharomyces cerevisiae* Ty retroelements.

### Results

**Does genetic information flow from primer tRNA to progeny Ty1 elements?**

We examined the nucleotide sequence of the PBS region of endogenous Ty1 and Ty2 elements sequenced as part of the *Saccharomyces* genome project. Using this approach we analyzed two categories of transposons: (i) inactive Ty elements that may have accumulated debilitating mutations and deletions with time (Boeke et al., 1988a) and which might have been embedded in the yeast genome for a very long period of time; (ii) active Ty elements which probably transposed much more recently (Curcio and Garfinkel, 1994).

The known Ty1 and Ty2 elements differ from retroviruses in the relationship between PBS length and length of the portion of the primer tRNA 3'-end that can be reverse transcribed during plus strand synthesis. The Ty1 and Ty2 elements have 10 nt PBSs although 12 nt of tRNA 3'-end can be reverse transcribed, whereas in retroviruses these two lengths are identical. This length is ultimately determined by the distance between the 3'-terminus and the nearest modified nucleotide in the primer tRNA. The PBSs of all but one of the 31 Ty1 and 12 Ty2 elements in the complete *Saccharomyces* genome sequence (*Saccharomyces* genome database, http://genome-www.stanford.edu/saccharomyces) have precisely 10 bases of perfect complementarity to the primer tRNA. A single Ty2 element on chromosome IV has a 12 nt complementarity.

However, we have shown previously that Ty1 RT can efficiently copy the 12 nt comprising the primer tRNA 3'-end; the first modified nucleotide from the 3'-terminus apparently stops the RT (Lauermann et al., 1995). If the plus strand of the PBS copy is tRNA-templated then many Ty elements would be expected to have all 12 bases copied from the tRNA primer. Consequently, after the numerous rounds of Ty retrotransposition that must have occurred over evolutionary time, the 12 nt PBS version should have replaced the 10 nt PBS in active Ty elements, since the elements which acquire a PBS matching 12 bases of tRNA would have no efficient mechanism to revert to the original 10 nt PBS version. It has been reported that a large fraction of the endogenous Ty1 elements are capable of transposition (Curcio and Garfinkel, 1994). Therefore, if the reverse transcription process utilized primer tRNA as a source of genetic information at all, there should be evidence for this in the yeast genome. The sequence data indicate that there is strong selection for maintaining a 10 nt PBS in Ty1 and Ty2. Similarly, we found sequence evidence supporting a similar reverse transcription strategy for retrotransposon Ty3. This element, like Ty1, uses the initiator tRNA$_{3, Met}$ as its primer, but has a PBS only 8 nt long; both sequenced Ty3 genomic copies have this 8 nt PBS sequence.

Using this molecular archaeology approach we can
Ty plus strand transfer

**Fig. 2.** Extension of Ty1 PBS length to 12 nt. Extending the Ty1 PBS to a length of 12 nt would mimic the retroviral structural paradigm; the length of the PBS would correspond exactly to the distance between the 3′-end of the tRNA and the first modified nucleotide in the tRNA. (A) If plus strand strong-stop DNA synthesis extends to reach the first modified nucleotide encountered on the tRNA template before transfer, a terminal mismatch of two bases would be created between the full-length plus strand strong-stop DNA and minus strand Ty1 DNA. (B) In construct pVIT81 one amino acid in Ty1 Gag is altered while the putative mismatch is still maintained after the second strand transfer. In pVIT85 the same Gag amino acid residue is changed but the putative mismatch is corrected. (C) The nucleotide sequences of the Ty1 PBS regions and the amino acid sequences of the overlapping Ty1 Gag proteins of wild-type Ty1 and the two mutant Ty1 elements. The PBS sequence matching the tRNA primer is underlined; the nucleotide and amino acid changes are highlighted.

Fig. 3. An extended Ty1 PBS improves Ty1–TRP1 transposition frequency at higher temperatures. With increasing temperatures during transposition induction the Ty1–TRP1 element with an extended PBS transposes at higher frequencies than the wild-type. To reflect this, the relative transposition frequency is expressed as a percentage of the mutant (transposition frequency of wild-type Ty1–TRP1 divided by the value for the pVIT85 extended PBS mutant at each temperature tested). The raw data on transposition frequencies are tabulated below the graph; there were no transposition events observed in either case at 36°C.

This first PBS mutation served as a negative control for effects on Gag protein sequence. The second construct, pVIT85, harbors changes in both bases 11 and 12 of the PBS (the same single Gag amino acid is changed), but fully correcting the putative terminal mismatch that would result from full-length plus strand transfer in a wild-type Ty1 element (see Figure 2). The transposition frequency of the control (11 nt) mutant pVIT81 did not change significantly, indicating that this particular amino acid residue is not critical for Gag function. The transposition frequency of the 12 nt PBS mutant, pVIT85, did not change significantly either, indicating that the 10 nt PBS in wild-type Ty1 does not limit transposition frequency. We conclude that in Ty1 plus strand synthesis no mismatch is created following strand transfer which is capable of limiting transposition efficiency.

**Primer–PBS annealing is a temperature-sensitive step in retrotransposition**

We have previously shown that a mutant Ty1 element with a G–U mismatch in the middle of the PBS–primer tRNA duplex gives rise to a linear reduction in transposition frequency as a function of increasing temperature of induction relative to wild-type Ty1 (Lauermann and Boeke, 1994). In contrast, when we extended the PBS sequence complementarity to primer tRNA from 10 to 12 nt, in pVIT85, transposition of the extended PBS Ty1 became less sensitive to increased induction temperatures than the wild-type (Figure 3). The relative transposition frequency increase correlated almost linearly with increasing temperature, suggesting that PBS–tRNA primer formation is rate limiting for transposition in the wild-type at higher temperatures.

These data suggest that formation of the initial primer tRNA–genomic RNA complex is a temperature-limited step in Ty1 transposition and that stabilization of this structure, by extension of the region of complementarity, results in elevated transposition frequencies at higher temperatures. It is conceivable that there is some selective disadvantage to transposition at higher temperatures for

**Examine the evolution of the reverse transcription process in yeast.** These analyses indicate that the tRNA primer almost never forms a template for the plus strand PBS region of Ty1, Ty2 and Ty3 elements. This peculiar inheritance pattern is apparently conserved among distantly related Ty transposons and has persisted in yeast cells for a long period of time during their evolution.

**Construction and analysis of Ty1 mutants with elongated PBS sequences**

To determine the possible constraints on the naturally occurring 10 nt PBS on Ty1 replication we constructed two versions of Ty1 with elongated PBSSs. In the process of specifically changing the PBS sequence we could not avoid changing the amino acid sequence of the Gag protein. Such a mutated Gag protein might affect the structure of the VLPS and therefore also affect Ty1 transposition efficiency. In the first construct, pVIT81, only base 11 of the PBS was changed to match the tRNA sequence, changing the amino acid sequence but still retaining the feature of a putative mismatch between the plus and minus strands following second strand transfer.

<table>
<thead>
<tr>
<th>Strain</th>
<th>PBS type</th>
<th>Transposition frequency (raw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YH03</td>
<td>Normal</td>
<td>0.36 0.23 0.035 -0.0003</td>
</tr>
<tr>
<td>VL54/55</td>
<td>Extended</td>
<td>0.37 0.34 0.06 0.03</td>
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S. cerevisiae retrotransposons and that this selection results in shorter PBSs. Recent data from Friant et al. (1996, 1997) suggest that the Ty1 primer–PBS structure extends beyond the PBS and includes additional regions of base pairing which could provide further stability to the priming complex. Lengthening the PBS apparently further increases the thermal stability of this complex.

**Does lengthening the Ty1 PBS make its replication more retroviral-like?**

The Ty element PBSs differ from retroviral PBSs in that the former would be terminally mismatched following full-length plus strand strong-stop DNA transfer whereas the latter would not. Might this explain the unusual inheritance pattern of the Ty1 PBS, which unlike the retroviral one is not inherited from the tRNA primer? To investigate further the plus strand sequence of the PBS region we separated the plus and minus strands from VLP DNA using thermocycle-based primer extension (Lauermann and Boeke, 1994). In this method we first isolate VLP DNA and run multiple cycles of primer extension to amplify either the plus or minus strand DNA segments containing the PBS. The plus strand DNA copies terminate at the end of the Ty1 double-stranded DNA; the DNA used to prepare the minus strand copies is linearized with a restriction enzyme prior to primer extension. Thus both primer extensions give discrete sized products which are then excised from a preparative gel and used as a substrate for conventional PCR amplification. This method allowed us to examine directly the sequence of each strand independently. After separation the PBS regions of the plus and minus strands were separately amplified by PCR and directly sequenced by thermocycler sequencing. The minus strand sequence was, as expected by either hypothesis, the mutant one. The plus strand sequence was pure mutant sequence with no evidence of the wild-type C base at position 7 of the PBS. This experiment independently introduced at position 7 should revert back to the wild-type in the plus strand sequence.

After Ty1 DNA is integrated into the genome any mismatches that might have been created as a result of strand transfer, including the entire PBS region, would be rendered homoduplex either by cellular DNA repair mechanisms or simply by replicating the target chromosome; in the latter case the two daughter cells would inherit homoduplexes differing at the previously mismatched position. In order to study directly the result of the Ty1 reverse transcription process and minimize the likelihood of alterations of Ty1 DNA by host repair enzymes, we independently analyzed the PBS sequences of minus and plus strands isolated from Ty1 VLP DNA, i.e. before integration of the Ty1 element. These particles accumulate in the yeast cytoplasm (Garfinkel et al., 1985; Mellor et al., 1985) while DNA repair enzymes function in the nucleus. The membrane of the yeast nucleus, unlike membranes of other eukaryotic nuclei, does not disassemble during mitosis, so VLP DNA is not expected to be subject to the action of repair enzymes.

For the initial PBS analyses we purified VLPs from cell lysates on a sucrose gradient. The host cells used were spt3 mutants in which expression of all the endogenous Ty1 elements is reduced ~20-fold. The Ty1 VLP DNA was phenol/chloroform extracted and amplified in a subsequent PCR reaction using primers flanking the PBS region. The amplified DNA was cut with restriction enzyme HaeIII or BstBI. The resulting products were separated using agarose gel electrophoresis (Figure 4). If the plus strand DNA was tRNA templated it would acquire the wild-type PBS sequence, i.e. the HaeIII site in a fraction of PCR products would be cut by this enzyme. No HaeIII-sensitive DNA could be detected, indicating that tRNA does not form the template for the VLP DNA derived from this mutant Ty1. As expected, wild-type control DNA was cut to completion by HaeIII, ruling out the possibility that the DNA samples contained enzyme inhibitors (Figure 4, pX3, lane H). The control BstBI restriction site contained in all constructs was fully cleaved (Figure 4, pVIT221 and 222, lanes B), showing that these sequences are inherited, as expected, by both strands.

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**Fig. 4.** The Ty1 primer tRNA sequence is not inherited *in vitro*. (A) VLP DNA was subjected to PCR using primers (arrows) which amplify a region containing the PBS (shaded box). Cleavage with HaeIII (H) indicates presence of a wild-type PBS and lack of cleavage of a mutant PBS. Cleavage with BstBI indicates presence of the marker mutation present in test plasmids pVIT221 and pVIT222. This silent marker mutation is introduced just to the right of the PBS region. (B) Restriction digestion of the PCR-amplified PBS region isolated from Ty1 VLPs. As expected, in the wild-type sequence BstBI does not cut the DNA whereas HaeIII does. In the mutant constructs the introduced (silent marker) BstBI site is cut while the HaeIII site destroyed by the PBS mutation is not cut. If only the tRNA sequence was used as template in plus strand synthesis then a large fraction of the PCR products would have regenerated the HaeIII site and would be cut. pX3 is the wild-type Ty1 sequence and pVIT221 and pVIT222 are the PBS mutants. U, uncut; B, BstBI cut; H, HaeIII cut; C, control; 1, 123 bp ladder; λ, λ HindIII marker. The slightly incomplete BstBI digest in this experiment resulted from partial digestion and not from the presence of endogenous Ty1 elements, since we did not observe this in other experiments.
confirms, with higher resolution, the previous result that primer tRNA does not form the template for the plus strand of Ty1 VLP DNA.

The tRNA sequence is not inherited by progeny Ty1 elements in vivo regardless of PBS length

The pVIT222 GAL1–Ty1–TRP1 element was used to investigate the fate of the mutation introduced into the middle of the extended PBS after Ty1 reverse transcription and integration into the yeast genome. This approach is designed to reveal the role of primer tRNA as a source of genetic information for the Ty1 transposon in vivo. Ty1 transcription and consequent transposition was induced by galactose in the spt3 strain VL191 for a short period of time to obtain yeast cells with a minimal number of transposition events per cell. The number of Ty1–TRP1 transposition events per randomly chosen Trp+ colony was determined by DNA blot analyses using a TRP1 gene probe. A collection of 12 clones with a single Ty1–TRP1 integration event was analyzed for the presence of the HaeII site in the PBS by DNA blot analyses using the Ty1 442 bp PvuII–SspI DNA fragment, a GAG sequence, as probe. The genomic DNA was digested with SspI, a restriction enzyme with sites flanking the PBS region, releasing a 696 bp fragment. Digestion with a combination of SspI and HaeII shortens the 696 bp fragment to 629 bp. All of the endogenous Ty1 elements possess this 629 bp fragment. Unlike the wild-type, all but one mutant progeny Ty1–TRP1 element analyzed retained the 696 bp fragment after double digestion with SspI and HaeII, indicating that the HaeII site had not been restored (data not shown). The only exception found (which had a 629 bp fragment) could represent a restoration of the PBS templated by the tRNA sequence or, alternatively, could have resulted from a recombination event between the 5′-end of a pre-existing, chromosomally embedded endogenous Ty1 element and the 3′-end of the newly synthesized Ty1–TRP1 DNA mediated by a Rad52p-dependent pathway. Such homologous recombination of marked Ty1 elements with chromosomal copies has been shown to occur at relatively high frequencies (Sharon et al., 1994).

Cloning and PBS sequence analysis of newly transposed Ty1 elements

To rule out contamination of the Ty1–TRP1 element PBS sequence by recombination with endogenous Ty1 elements we cloned and analyzed the sequences of progeny Ty1 elements derived from donor elements with a 12 nt PBS.

To facilitate cloning of progeny Ty1 elements we used yeast–Escherichia coli shuttle plasmid pAB100 as the target for Ty1 integration (Boeke et al., 1985). The donor plasmid was pVIT223, a GAL1–Ty1–neo plasmid. pAB100 harbors a mutant his3 gene, named his3Δ4, which has a deletion that removes its native promoter region and replaces it with phage λ sequences (Struhl, 1985), rendering the his3Δ4 gene transcriptionally inactive. When pAB100 plasmid derivatives are recovered from His+ revertant prototrophs they often contain Ty1 elements which activate the previously silent his3Δ4 gene (Scherer et al., 1982; Boeke et al., 1985). When a Ty1 element integrates upstream of his3Δ4 its enhancer restores transcription of the his3Δ4 gene and His+ prototrophy.

We recovered 18 plasmids bearing progeny Ty1–neo elements from such yeast His+ prototrophs derived from cells containing the two plasmids. These were selected as follows. DNA preparations were made from prototrophic yeast and transformed into E.coli after the original donor Ty1 plasmid had been cured using 5-FOA selection (Boeke et al., 1984). Kanamycin-resistant (derived from Ty1–neo)/ampicillin-resistant E.coli contained the plasmids of interest. Every plasmid recovered was from an independent His+ reversion event, representing a single colony of yeast cells from a separate patch. Plasmids were screened by digestion with BglII or HindIII and agarose gel electrophoresis. The PBS sequences of 18 pAB100 plasmids which had acquired a new Ty1–neo integration element were analyzed. All 18 plasmids analyzed in this screen retained the mutant PBS sequence, confirming our previous result that the primer tRNA does not form a template for the Ty1 progeny elements even when the PBS is extended to the full length of 12 nt to match the primer tRNA sequence up to the first modified base. The probability of obtaining this result by random chance assuming (i) the actual product is a heteroduplex rather than a mutant/ mutant homoduplex and (ii) that there is no bias in correction of mismatches (this latter assumption is well supported in yeast for an extensive number of different mismatches; Bishop et al., 1989) is (0.5)18 or 3.8×10−6. We conclude that the product is in fact a homoduplex and thus the PBS region of the Ty1–neo DNA is entirely templated by the PBS and not by primer tRNA, even when the PBS is extended to 12 nt in length.

PBS inheritance in the distantly related retrotransposon Ty3

To investigate PBS inheritance in the Ty3 element we exploited two yeast genetic strategies developed earlier. The first step uses a yeast strain in which all four genomic copies of the initiator methionine tRNA gene (IMT, the gene encoding tRNA3Met, which is also the primer for the Ty3 element) have been deleted. In this strain either wild-type or any mutant IMT gene of choice can be provided on a plasmid (Chapman et al., 1992; Keeney et al., 1995). The second consists of a Ty3-specific integration assay (Kinsey and Sandmeyer, 1995). In our experiment the only IMT gene present in the cell, pLMT112 (Keeney et al., 1995), differs in a single base (corresponding to the fifth base of the PBS) from the PBS complementary sequence (Figure 5). If the mutant tRNA primer templates the plus strand of the PBS region the resulting Ty3 progeny should have a mutant PBS in ~50% of the newly transposed elements (Figure 5), again assuming that the resulting mismatch in the newly integrated DNA would be corrected without bias.

The Ty3 element integrates its new DNA copy into the genome at a very specific position, at the transcription start site of tRNA genes (Chalker and Sandmeyer, 1990). An assay for Ty3 transposition and simultaneous cloning of progeny Ty3 elements has been described earlier (Kinsey and Sandmeyer, 1995). In this two-plasmid system transposition of Ty3 on the donor plasmid is controlled by the GAL1 enhancer, as in the case of Ty1. The target plasmid harbors two tRNA genes, an ochre suppressor tRNA (SUP2bo) and a tRNAVal. These genes are positioned on the plasmid head-to-head (i.e. divergently transcribed) with a polypyrimidine spacer preceding the suppressor
tRNA gene. RNA polymerase III, which transcribes all tRNA genes, does not initiate transcription efficiently at the pyrimidine-rich region and therefore the suppressor tRNA is not expressed. When a Ty3 element integrates upstream of the tRNA_Met, the pyrimidine tract is disrupted and transcription of SUP2bo is activated. The host yeast strain also bears the suppressible stop codon suppression and transcription of SUP2bo from that of the element utilizing wild-type tRNAiMet, as the mutant tRNA pIMT112 was not significantly different from 15 independent transposition events (i.e. independent patches). We have demonstrated in our study that extension of the primer tRNA sequence is activated. The host yeast cells become prototrophic for lysine.

Thus Ty3 DNA also contains homoduplex DNA inherited from the PBS region.

**Discussion**

Yeast Ty transposons are members of the LTR retroelement family. Their reverse transcription process closely resembles that of retroviruses and it has been proposed that this process is strictly conserved in these elements throughout evolution (Telesnitsky and Goff, 1993). The study of GAL−Ty elements benefits from several distinct features of their life cycle, specifically the ability to induce their transposition, non-infectivity resulting in only a single round of replication after induction and straightforward methods for selective cloning of progeny elements.

Ty1 retrotransposition is temperature sensitive; transposition frequencies are highest at ~22°C and much lower at higher temperatures, although overexpression from a GAL−Ty1 element can suppress this temperature sensitivity considerably (Paquin and Williamson, 1984; Boeke et al., 1986). In this study we observed that extension of the Ty1 PBS region from a 10 to 12 base complementarity to the tRNA primer caused an increase in transposition frequency at higher temperatures. This stabilization complements our earlier observations, namely that when the PBS/tRNA complementarity is disturbed by a single mismatch the mutant Ty1 becomes more temperature sensitive than the wild-type transposon (Lauermann and Boeke, 1994). These experiments suggest that annealing and stabilization of primer tRNA to the PBS to allow initiation of DNA synthesis is one of the reverse transcription steps limited by higher temperatures.

In this study we investigated the fate of the initial primer tRNA sequences. We have demonstrated in our previous experiments using Ty1 elements with a normal PBS length that genetic information does not flow from the tRNA to Ty1 element progeny (Lauermann and Boeke, 1994), despite the fact that the RT can copy the tRNA sequences during plus strand synthesis in vivo (Heyman et al., 1995; Wilhelm et al., 1997) and in vitro (Lauermann et al., 1995). It has been determined that the 3′-terminal 12 bases of primer tRNA are very efficiently reverse transcribed and that the first modified nucleotide encountered by the RT, a 2′-O-phosphoribosyl adenosine, acts as
the stop signal (Lauermann et al., 1995). In contrast, the PBSs found in all cloned Ty1 elements are only 10 nt long. Consequently, if only tRNA-templated sequences give rise to the plus strand PBS sequence region, then the PBS is correspondingly expected to be 12 bases long. We analyzed all the endogenous Ty elements in the yeast genome, the complete sequence of which recently became available. Only one of the 45 endogenous Ty1, Ty2 or Ty3 sequences had the 12 nt version of the PBS. This provides evidence from a long-term evolutionary ‘experiment’ that indicates that primer tRNA does not form the template for the Ty PBS plus strand sequence during transposition under natural conditions.

If a terminal mismatch was created between minus strand DNA and the nascent transferred plus strand DNA following full-length plus strand strong-stop DNA transfer (see Figure 2), this might give rise to a strong selection for the progeny elements to use prematurely transferred plus strand strong-stops, which would lack such a mismatch and therefore could be readily extended (Lauermann and Boeke, 1994). Consequently, if the very abundant full-length plus strand strong-stop DNAs harboring the extended 12 nt PBSs are dead-end products of reverse transcription which actively inhibit further synthesis, converting them to a base paired form (which could be readily extended by RT) should augment the flow of genetic information from primer tRNA to the Ty1 genome and greatly increase transposition efficiency. We constructed a Ty1 element with an extended 12 nt PBS. This construct did not transpose at elevated frequencies, suggesting that the production of fully base paired strong-stop DNA does not limit the efficiency of Ty1 reverse transcription. Rather, it suggests that plus strand strong-stop DNA that is extended into the tRNA sequence is not a direct intermediate in Ty1 replication.

We further explored the fate of the primer tRNA sequences in a Ty1 transposon with the extended PBS by examining PBS sequence inheritance in this context. We introduced a marker mutation into the middle of the 12 nt PBS and followed its fate during a single transposition cycle. Primer tRNA did not provide genetic information to the Ty1 PBS region in Ty1 VLP DNA nor to the Ty1–TRP1 element progeny inserted into yeast chromosomes nor to Ty1–neo elements transposed into a target plasmid. These results are similar to those obtained previously with a Ty1 PBS of normal (10 nt) length. We conclude that even when there is terminal complementarity in plus strand strong-stop DNA, its sequence is not inherited by the progeny Ty1 cDNA.

We have considered two models that could explain the observed inheritance patterns. The first of these is the premature strand transfer model, first proposed by Temin (1993). Ty1 RT probably facilitates an efficient strand transfer reaction and therefore a majority of the newly synthesized plus strand strong-stop DNAs are transferred before they reach the end of the template. Earlier work shows that Ty1 RT readily template switches on homologous templates, consistent with this model (Xu and Boeke, 1987). Such a strategy might prevent the creation of terminal mismatches after strand transfer in cases in which the RT does reach the template end and, like many other polymerases, adds an untemplated base, a widely observed and quite efficient process (Gabriel et al., 1996, and references therein; E.Mules, O.Uzun and A.Gabriel, personal communication). Independent experiments on Ty1 replication in which we inserted extra DNA sequences into the 3’-LTR region suggest that plus strand transfer may be a carefully concerted and synchronized process. When such insertions are larger than ~500 bp, large reductions in Ty1 transposition frequencies were detected. Formation of multimers of these constructs was observed, perhaps because the timing of plus strand transfer was disturbed (Lauermann et al., 1997). However, we have been unable to detect biochemically any prominent plus strand species that are shorter than full length. It is also difficult to reconcile this model with the large amounts of full-length plus strand strong-stop DNA present in Ty1 VLPs. Finally, results with Moloney retrovirus suggest that the vast majority of minus strand strong-stop transfers occur precisely at the template end (Kulpa et al., 1997).

One of the most abundant products in Ty1 reverse transcription is in fact the plus strand strong-stop DNA complete with tRNA complementary sequences at its 3’-end (Heyman et al., 1995; Lauermann et al., 1995); a molecule which seems from our experiments to be a ‘dead-end’ product rather than a direct intermediate of full-length Ty1 DNA formation. What then might be the natural function of full-length plus strand strong-stop DNA in Ty1 replication? One very likely role is in tRNA primer removal from the 5’-end of minus strand DNA (Smith et al., 1997). The plus strand DNA forms a hybrid with the primer tRNA which then creates a substrate that can be recognized and cut by the Ty1 RH. Such cleavage then forms a minus strand DNA template that would specify a correct plus strand terminus for Ty1. Furthermore, a second round of plus strand strong-stop DNA synthesis on such a template would produce a molecule lacking the PBS region that could form the left end of the transposon in a manner consistent with the observed inheritance pattern.

These observations, together with recent experiments from the Heyman/Wilhelm and Gabriel groups, lead us to the model we favor: the plus strand primer recycling model (Figure 7). This model proposes that the initial molecule of plus strand strong-stop DNA synthesized is in fact full length and functions in primer tRNA removal as outlined in the previous paragraph. As the 3’-end of this molecule would remain base paired to primer tRNA, this may block its ability to participate in plus strand transfer.

Analysis of the 5’-end of Ty1 plus strand strong-stop DNA indicates that there is no primer covalently attached to it (Lauermann et al., 1995). This suggests that the plus strand primer is removed (presumably by RH) prior to completion of the strong-stop DNA (Figure 7F). This is in contrast to Ty1 minus strand strong-stop DNA, which initially remains covalently attached to primer tRNA (Figure 7C–H). There is very strong evidence from retroviral systems that the viral RNase H activity can remove the primer precisely (Omer and Faras, 1982; Finston and Champoux, 1984; Rattray and Champoux, 1987, 1989; Huber and Richardson, 1990). Although the absence of primer sequences on plus strand strong-stop DNA makes it difficult to know the exact nature of the primer, by analogy with retroviruses it is likely to be an oligoribonucleotide derived from the PPT region (Figure 7F). We propose that immediately following cleavage of the plus
Fig. 7. A plus strand primer recycling model for Ty1 reverse transcription. (a) Ty1 DNA structure with LTR U3, R and U5 regions, PBS and PPT1 as described in the legend to Figure 1. In Ty1 the PPT2 site in the middle of the genome is not necessary for Ty1 replication but represents a second site of plus strand priming (Heyman et al., 1995). (b) Two genomic RNA transcripts (green wavy lines) are most likely packaged in the VLPs (Boeke et al., 1986). (c) The primer tRNA (green bicycle) is annealed to the PBS and initiates minus strand strong-stop DNA (blue line) synthesis. Minus strand strong-stop DNA is completed when the RT reaches the 5'end of the RNA template; the RNA in complex with DNA is inferred to be degraded by RT-associated RH. (d) Minus strand DNA transfer is facilitated by the repeated sequence R found at the ends of Ty1 RNA. (e) The minus strand is then extended along the RNA template. During the transfer either Ty1 RNA molecule can in principle serve as the recipient. As in Figure 1, the parentheses indicate sequences that may or may not be included in the extended minus strand DNA, depending on whether the full-length or partially degraded RNA template, respectively, is used. In all subsequent steps the second Ty1 RNA strand is omitted for clarity. (f) The primer for initial plus strand strong-stop DNA (red) synthesis derives from PPT1; DNA synthesis generates a substrate for RH which can cleave off the primer, allowing it to be recycled for a second round of plus strand priming. Plus strand synthesis also initiates at a second site, PPT2 (blue line). (g) After cleavage a second round of plus strand strong-stop DNA synthesis can be initiated (blue line). (h and i) First plus strand strong-stop DNA synthesis is completed, generating an RH substrate that allows cleavage of the primer tRNA from the minus strand DNA. The second plus strand strong-stop DNA begins to displace the first. (j) The first plus strand strong-stop DNA is a dead-end intermediate and is displaced; it is known to be an abundant component of VLP DNAs (Pochart et al., 1993) (this primer template complex may be extended to produce a double-strandedLTR sequence). The second plus strand strong-stop DNA is displaced by plus strand DNA primed upstream at PPT2 (or by a third round of priming at PPT1). (k) The second plus strand strong-stop DNA is transferred via complementarity to R/U5 or to the residual 3-10 nt U5 RNA sequence that remains upstream of the PBS following RH trimming (E. Mules, O. Uzun and A. Gabriel, personal communication). (l) Ty1 reverse transcription is completed by extending the 3'ends of plus and minus strand DNAs. Note that there is a discontinuity in the plus strand of the final product at PPT2 (Heyman et al., 1995).

strand primer, a second round of plus strand strong-stop DNA synthesis is primed (Figure 7G and H). This, and any subsequent rounds of synthesis, would produce a plus strand strong-stop DNA molecule lacking tRNA complementarity (Figure 7J and K) which, if it was a direct reverse transcription intermediate incorporated into the final product, would be completely consistent with our observed inheritance data. Furthermore, we observed molecules terminating exactly at the end of the Ty1 sequence in our analysis of in vitro Ty1 reverse transcription products (Lauermann et al., 1995), and Wilhelm et al. (1997), who cloned and sequenced Ty1 plus strand 3'-termini, found that 75% of the plus strand molecules terminated precisely at the Ty1 3'-end, as predicted by this model. This model also makes sense in considering the mechanism of Ty1 integrase, which, unlike its retroviral counterparts, is apparently incapable of removing terminal dinucleotides from Ty1 DNA substrates to define the transposon terminus (Moore et al., 1995). In Ty1, RNase H may have acquired this function.

An intriguing question is whether the reverse transcription strategy described above for Ty1 is shared with other yeast transposons. We examined the Ty3 element (which belongs to the gypsy group of transposons), which is evolutionarily very distant from Ty1 (a member of the copia family) (Xiong and Eickbush, 1990). Like Ty1, the Ty3 element exploits the initiator methionine tRNA as the primer for minus strand synthesis. In the Ty3 experiment we used a mutant tRNA molecule, provided by the only IMT gene present in the cells, and analyzed whether the wild-type Ty3 PBS would convert to the mutant sequence corresponding to the mutant primer tRNA. All 15 independent cloned elements had the wild-type PBS sequence. This result, taken together with the fact that the yeast genome contains only Ty3 transposons harboring a PBS of 8 nt (instead of the predicted 12), indicates that the Ty3 element, just like Ty1, fails to inherit tRNA-template sequences.

The overall reverse transcription process is strikingly similar between retroviruses and retrotransposons, making it surprising to find some features of Ty replication which seem opposite to the retroviral ones. These include aspects such as PBS inheritance (Lauermann and Boeke, 1994) and lack of IN-mediated cleavage of the element termini, releasing a dinucleotide (Moore et al., 1995; Wilhelm et al., 1997). All studied retroviruses with mutations in the PBS revert rather quickly, after several passages, to the wild-type sequence. This sequence is restored by reverse transcription of the primer tRNA sequence (Whitcomb et al., 1990; Wakefield et al., 1995, 1996). In contrast, the Ty elements do not inherit the tRNA sequences. When the Ty1 element PBS is artificially lengthened to mimic the retroviral PBS, so that its sequence matches the tRNA primer up to first modified base, it transposes at levels comparable with wild-type Ty1, suggesting that there is no absolute requirement for having a shorter Ty1 PBS. We suggest that there may be selection for repression of Ty1 transposition at higher temperatures and, consequently, selection for a shorter PBS which helps to down-regulate such transposition.

Materials and methods

Yeast strains, media and plasmids

Yeast strains were maintained according to standard techniques and media were prepared as described previously (Rose et al., 1990).
To grow on medium lacking lysine. Upon specific Ty3 integration a mutation. Therefore, the cells containing the target plasmid are unable as indicated by its inability to suppress the genomic context and as a consequence generated. Polymerase III does not initiate synthesis in the polypyrimidine extended 11 nt PBS, was created by PCR using oligonucleotides JB262 5′-ATGAGAATTGGG-3′. The Ty3 transposition assay has been described elsewhere (Kinsey and Sandmeyer, 1995). In summary, a special target plasmid containing two the promoter. Following galactose induction, Ty3 transposition is monitored by the frequency of Lys^+ prototrophs. Strains to be tested were patched on SC-Ura-His glucose plates at 30°C and after 1 day replica plated onto SC-Ura-His galactose plates, incubated for 5 days at 22°C to induce transposition and then scraped and plated onto SC-Lys and YPD plates. Ty3 transposition frequency is expressed as number of Lys^+ colonies divided by total colonies.

### Separate analysis of Ty1 plus and minus strands by cycle primer extension

The separation of plus and minus strand Ty1 DNA is based on multiple rounds of primer extension, which uses radiolabeled strand-specific primers and Taq DNA polymerase; the products are separated on a polyacrylamide gel, excised, PCR amplified and then directly sequenced (Lauermann and Boeke, 1994). The primers used for the extension reaction were JB177 (5′-TATCGACGAATTCTGTTGGAATAGAAATGCA-3′) and JB499 (5′-GCGTTTAGGGAAGCCCTTCTC-3′).

### Cloning Ty1–neo progeny elements

Yeast strain JB83 (Boeke et al., 1985) was transformed with pVIT223 and pAB100, yielding VL362. Patches of this strain were grown on SC-Ura-Trp medium containing 2% galactose. His^+ prototrophs were isolated from independent patches and DNA was prepared from the pooled His^+ prototrophs of each patch. The DNA was transformed into E.coli and Ap^+ Km^+ colonies were selected. A single such colony was isolated from each patch, ensuring independence. The Ty1 PBS region was PCR amplified and directly sequenced from each isolate.

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