A complex structure in the mRNA of Tf1 is recognized and cleaved to generate the primer of reverse transcription

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All retroviruses and LTR-containing retrotransposons are thought to require specific tRNA molecules to serve as primers of reverse transcription. An exception is the LTR-containing retrotransposon Tf1, isolated from *Schizosaccharomyces pombe*. Instead of requiring a tRNA, the reverse transcriptase of Tf1 uses the first 11 bases of the Tf1 transcript as the primer for reverse transcription. The primer is generated by a cleavage that occurs between bases 11 and 12 of the Tf1 mRNA. Sequence analysis of the 5' untranslated region of the Tf1 mRNA resulted in the identification of a region with the potential to form an RNA structure of 89 bases that included the primer binding site and the first 11 bases of the Tf1 mRNA. Systematic mutagenesis of this region revealed 34 single-point mutants in the structure that resulted in reduced transposition activity. The defects in transposition correlated with reduced level of Tf1 reverse transcripts as determined by DNA blot analysis. Evidence that the RNA structure did form *in vivo* included the result that strains with second site mutations that restored complementarity resulted in increased levels of reverse transcripts and Tf1 transposition. The majority of the mutants defective for reverse transcription were unable to cleave the Tf1 mRNA between bases 11 and 12. These data indicate that formation of an extensive RNA structure was required for the cleavage reaction that generated the primer for Tf1 reverse transcription.

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Retroviruses and long terminal repeat (LTR)-containing retrotransposons proliferate in hosts that comprise the full spectrum of eukaryotic evolution. Although the LTR-containing retroelements propagate in organisms as diverse as yeasts and mammals, the mechanisms of reverse transcription are well conserved (Gabriel and Boeke 1993; Leis et al. 1993). To initiate replication, retroviruses and LTR-containing retrotransposons require specific tRNA molecules to serve as primers. The 3'-terminal 10–18 bases of the tRNA anneal to the transcript of the element at a region adjacent to the 5' LTR called the primer binding site (PBS). The element-encoded reverse transcriptase uses the 3' OH provided by the tRNA to initiate DNA synthesis of the first product of reverse transcription, the minus-strand strong-stop DNA (–ssDNA) (Varmus and Swanstrom 1984; Kikuchi et al. 1986; Chapman et al. 1992). All LTR-containing retroelements are thought to require tRNA for priming because the elements that have been sequenced have a PBS that is complementary to known tRNA species. An interesting exception is Tf1, an LTR-containing retro-

transposon isolated from *Schizosaccharomyces pombe* (Levin et al. 1990).

Tf1 is a highly active transposon that encodes Gag, protease (PR), reverse transcriptase (RT), and integrase (IN) proteins within a single open reading frame (ORF). The PBS sequence of Tf1 has no complementarity to any known tRNA species, suggesting that no tRNA molecules are able to serve as the primer for Tf1 reverse transcription. Instead, our laboratory has proposed that Tf1 undergoes a unique mechanism of self-primed reverse transcription. We noted that perfect complementarity exists between the first 11 nucleotides of the Tf1 mRNA and its PBS. On the basis of this observation and the results of a mutagenesis study, it was proposed that the first 11 bases of the Tf1 transcript fold back and anneal to the PBS to serve as primer (Levin 1995). Data from this previous report showed that *in vivo* transposition activity and the accumulation of –ssDNA in Tf1 particles was greatly reduced as the result of mutations in the PBS or the first 11 nucleotides of the Tf1 mRNA (Levin 1995). Compensatory mutants that restored complementarity between these sequences rescued both the transposition activity and the ability to synthesize the –ssDNA. In addition, recent data from our laboratory demonstrate that the Tf1 mRNA is cleaved between

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bases 11 and 12 in a reaction that requires the RNase H domain of the Tf1 RT (Levin 1996). These results suggested that the first 11 bases of the Tf1 transcript anneal to the PBS and serve as the primer for reverse transcription.

Because the replication of retroviruses begins with the annealing of a cellular tRNA to the PBS, interactions between the primer tRNA and the viral mRNA were studied extensively to understand how retroviruses initiate reverse transcription. In addition to the annealing of the 3' end of a tRNA to the PBS, two other regions of the tRNA have been found to interact with retroviral transcripts (Aiyar et al. 1992, 1994). Formation of a binary complex through extended interactions between the tRNA primer and the PBS of the retrotransposon Ty1 have also been found to be an essential step in initiating reverse transcription (Wilhelm et al. 1994; Keeney et al. 1995; Friant et al. 1996). In light of the extensive interactions between viral RNA and tRNA that are required for the initiation of retroviral reverse transcription, we conducted a detailed analysis of the 5'-untranslated region (5' UTR) in the Tf1 mRNA. We identified a sequence in this region that had the potential to form an RNA structure of 89 bases that included the primer binding site and the first 11 bases of the Tf1 mRNA (Fig. 1A). This complex structure includes a hairpin and three duplexes formed by long-range nucleotide interactions. We systematically mutated the Tf1 sequences in the 5' UTR to test the contribution of this sequence to Tf1 transpo-

sition and to determine whether the putative RNA structure can form in vivo. The results of this study indicated that the complex RNA structure encompassing the PBS was required for the cleavage reaction that generated the primer of Tf1 reverse transcription.

Results

Sequence analysis of the 5' UTR of Tf1 and Tf2 mRNA

Sequence analysis of the 5' UTR in the Tf1 mRNA revealed that 89 nucleotides had the potential to fold into a structure that included the PBS. The proposed RNA structure is presented in Figure 1A. This structure includes a hairpin and three duplexes formed by long-range nucleotide interactions. In addition to the PBS duplex reported previously (Levin 1995), we identified sequence that could potentially form a 5-bp duplex upstream and an 11-bp duplex downstream of the PBS duplex. The 5-bp duplex and the PBS duplex are separated by a bulge of 4 nucleotides in the top strand. Adjacent to the 11-bp duplex, a hairpin with the potential to form a 12-bp stem and a 6-nucleotide loop was also found. Base position 1 in Figure 1A is the first nucleotide of Tf1 mRNA as expressed from the Tf1 promoter.

Sequence with the potential to form a similar RNA structure was also found in Tf2, a retrotransposon closely related to Tf1 that was also isolated from *S.*

Figure 1. The proposed RNA structure in the 5' UTR of the Tf1 mRNA. (A) The sequence of the three duplexes and the hairpin. The 5' end of the mRNA is indicated, and the relationship to the 3' end of the mRNA is shown. The vertical arrow indicates the position of the cleavage that creates the primer. The names of the duplexes are indicated. Every tenth nucleotide position is indicated relative to the first nucleotide of the Tf1 mRNA as expressed from the Tf1 promoter. The length of the loops between the duplexes are indicated. (B) Sequences in the RNA structure are well conserved between Tf1 and Tf2. The number above each duplex termini indicates the position of the nucleotide in the Tf1 mRNA. The differences in sequences between Tf1 and Tf2 are marked with asterisks (*).

pombe (Levin et al. 1990). Although Tf1 and Tf2 have significantly divergent *gag* sequences, nucleotides included in the RNA structure were found to be well conserved (see Fig. 1B). The putative RNA structure of Tf2 differs slightly from that of Tf1 in the hairpin. The two additional differences between Tf1 and Tf2 include a position in the bulge where Tf2 has a U instead of an A and a position in the bottom strand of the 11-bp duplex where Tf2 has an A instead of a G. The latter change allows Tf2 to form an A:U base pair instead of the G:C/U base pair in the Tf1 counterpart.

Screening a library of random mutations in the 5' UTR of Tf1 for changes that affect Tf1 transposition

More than one-fourth of the nucleotides (89 of 353) in the Tf1 5' UTR are involved in the formation of this complex structure that included the PBS of Tf1. On the basis of this observation and the results of a previous study from our laboratory supporting a model of self-primed reverse transcription, we speculated that this putative RNA structure may participate in the initiation of Tf1 reverse transcription.

To evaluate the role of the putative RNA structure in Tf1 transposition, we conducted a mutagenesis study of the 5' UTR. We generated a library of Tf1 plasmids that contained single-point mutations in a region of Tf1 that included the putative structure. This plasmid library was then screened for mutations that caused transposition defects. The method used to measure transposition was based on the resistance to G418 that cells acquire as the result of the transposition of a *neo*-marked copy of Tf1 from the *ura3*-containing plasmid into the genome. Figure 2 shows the assay plasmid with its copy of Tf1 that can be induced because the Tf1-*neo* element was fused to the *nmt1* promoter that is activated when cells are grown in the absence of thiamine. After induction, patches of cells were transferred to a plate with medium that contained 5'-fluoro-orotic acid (5-FOA), a drug that selects against the presence of the *ura3* gene and therefore against the presence of the plasmid. These patches were then replica printed to a YES 5-FOA-G418 plate to identify cells that contained copies of Tf1-*neo* that had transposed into the genome.

Figure 2 illustrates how the library was generated. We first created a *XhoI*-*SpeI* cassette in the Tf1 expression plasmid to facilitate the cloning of the mutagenized fragment. This *XhoI*-*SpeI* cassette version of the Tf1 plasmid (pHL891-19) produced wild-type levels of Tf1 transposition when transformed into *S. pombe* (data not shown). We therefore used this plasmid for the wild-type Tf1 element in the following study. The region containing the putative structure in the 5' UTR is expanded in Figure 2 to indicate which regions of complementarity were subjected to the mutagenesis. We produced mutations throughout the sequence in oligonucleotides HL100 and HL101 by creating degenerate pools of these oligonucleotides that were used to amplify this fragment by PCR. The addition of all four nucleotides during the oligo synthesis at a concentration 250-fold less than the normal

Figure 2. The structure of the Tf1-containing plasmid and the mutagenesis of the 5' UTR. The sequence of Tf1 includes two direct LTRs and a single ORF that encodes capsid protein Gag, protease (PR), reverse transcriptase (RT), and integrase (IN). The LTRs are depicted by open wedges on either side of the ORF (indicated by a rectangle that is labeled with the name of the proteins encoded). The U3, R, and U5 regions of the LTRs are indicated. The box labeled *nmt1* represents the *nmt1* promoter that replaces the U3 region in the 5' LTR; the box labeled *neo* is the inserted *neo* gene, and the box labeled *URA3* is the *URA3* gene of *S. cerevisiae*. The restriction sites in the plasmid used in the experiments are indicated. The area with the potential to form an RNA structure in the 5' UTR is expanded, and the regions of complementarity are indicated by the boxes with the same pattern of shading. Each pattern is labeled according to which duplex and which strand is represented. The lengths of the two degenerate oligonucleotides, HL100 and HL101, are represented by the horizontal arrows with the arrowheads indicating the 3' end of the oligonucleotides.

level ensured that the majority of the plasmids contained no more than a single-point mutation. The two degenerate oligonucleotides included most of the bases in the putative structure except nucleotides 103–113 and 163–167. Assuming that all four nucleotides are incorporated with equal probability, we predicted that 70.6% of the plasmids would have wild-type sequence, 24.6% of the plasmids would have single-point mutations, and 4.8% of the plasmids would contain multiple-point mutations. The degenerate PCR fragment was ligated into the *XhoI* and *SpeI* sites of pHL891-19, and the resulting 11,350 bacterial transformants obtained from electroporation were amplified and pooled for library plasmid isolation.

We transformed the mutant plasmid library into *S. pombe* and used the transposition assay to screen for transformants that showed reduced transposition activity in vivo. The results of the mutant screening are summarized in Table 1. Among 5000 transformants screened, 384 mutants (7.68%) were found to have reduced transposition activity as determined by the patch assay. One fourth of the defective transformants were randomly chosen to be extracted for plasmid isolation, and the nucleotides between the *XhoI* and *SpeI* sites were sequenced. Fifty-eight plasmids were found to con-

Table 1. Genetic screen for strains defective in transposition

<i>S. pombe</i> transformants screened	5000
Transformants defective in Tfl transposition	384
Plasmids rescued from defective transformants	96
Plasmids sequenced to identify the mutation	96
Plasmids with different single point mutations	37
Transposition phenotypes reconfirmed	35
Transformants with normal expression of Tfl proteins	34

tain base changes that represented 37 unique mutations. The sequence between the *Mlu*I [see Fig. 2] and the *Spe*I sites from all 37 plasmids was subcloned into a wild-type copy of the same vector to confirm that the Tfl sequence outside of the *Xho*I–*Spe*I cassette was wild type. We then tested the expression of Tfl proteins by immunoblot analysis of Gag and IN proteins. Thirty-four mutant plasmids were found to express normal levels of Tfl proteins.

The single-point mutations that affect Tfl transposition clustered within the putative RNA structure

Figure 3 shows the sequence subjected to mutagenesis and the distribution of the single-point mutants in the RNA structure. Represented in uppercase letters in the RNA structure are sequences subjected to mutagenesis, and letters bordering the structure are the mutations. All base changes were found to cluster within the complementary regions of the proposed RNA structure. We identified 6 mutations in the PBS and 10 mutations in the bottom strand of the PBS duplex. We also found four mutations in the bottom strand of the 5-bp duplex. The most noticeable result is the presence of the 14 mutations in the top strand of the 11-bp duplex. Not a single mutation was found in the non-base-pairing regions

(nucleotides 12, 18–30, and 170–171) that were subjected to mutagenesis. The only mutant found in the hairpin created a start codon by converting AAG to AUG. This base change resulted in generating an additional ORF upstream of Gag such that the translation of Tfl proteins was inhibited.

Targeted mutagenesis of the putative RNA structure

We explored specific aspects of the RNA structure by generating targeted mutations at eight distinct positions. The positions and the transposition effect of these site-directed mutations are indicated in Figure 3 by nucleotides marked with an asterisk. Judging from the patch assay results, the transposition defects due to mutations U11A and G14C appeared severe and were comparable to those produced by mutations in the same regions, A10G and C15A. To characterize duplex sequence not subjected to mutagenesis, three site-directed mutations were generated in the bottom strand of the 11-bp duplex and one was created in the top strand of the 5-bp duplex. All four mutants resulted in significantly reduced Tfl transposition. We also created two site-directed point mutations, C170G and A171C, in the bulge region between the PBS and the 5-bp duplexes. Both changes drastically reduced Tfl transposition activity. Because these two mutations were in the last two nucleotides of the U5 region, the base changes were likely to result in lower transposition because of the change in the IN binding site on the reverse transcripts.

The quantitative measurement of transposition defects

The magnitude of the transposition defects caused by each mutation was measured by subjecting each strain to a quantitative transposition assay. In a method similar to the transposition patch assay described above, cells on

Figure 3. The distribution of single-point mutations in the putative RNA structure. The uppercase letters in the RNA structure are sequences that were subjected to random mutagenesis, and the letters bordering the structure represent the mutations that caused transposition defects. Eight site-directed mutations are denoted by asterisks (*). The mutation A216T, (•) was found to inhibit the production of Tfl proteins. The magnitude of the transposition defects are indicated by the size of the lettering.

agar plates were grown without thiamine to induce transposition and were then resuspended in liquid media. By spreading cells on 5-FOA containing plates at a density that produced ~5000 colonies, we could detect the fraction of induced cells that acquired genomic copies of *Tf1-neo* by replica printing the 5-FOA-resistant colonies to 5-FOA-G418 plates. Table 2 is the list of the actual and relative transposition frequencies of all single-point mutants with transposition defects. The actual transposition frequency from each strain was obtained by averaging the measurements from at least three independent patches of cells. The relative transposition frequency was normalized to that of the wild-type strain and listed as %WT. The 100-fold drop in the frequency of G418-resistant colonies because of the frameshift in IN indicated that the level of recombination events that were independent of transposition was low. The results of the quantitative transposition assays are also indicated in Figure 3 by the sizes and symbols of the nucleotides. Mutations in the PBS and 5-bp duplexes were more likely to cause severe defects as only 3 of the 25 changes in these duplexes had transposition frequencies $\geq 10\%$ of wild type. In comparison, 11 of the 17 mutations in the 11-bp duplex resulted in frequencies $\geq 10\%$ of wild type. The potential of forming G:U base pairs existed in the mutants A6G, A10G, A13G, C15U, and C181U. Two of these mutants, A10G and C15U, resulted in transposition frequencies two- to threefold higher than other base change at the same position. These results suggested that at least the A10G and C15U mutations, and possibly the others, allowed the formation of duplexes that contained G:U base pairs.

The detection of base-pairing interactions within the 5' UTR of the Tf1 mRNA

The hypothesis that the complementary strands of the duplexes anneal in vivo was tested by measuring the transposition of *Tf1* elements that contained pairs of mutations predicted to restore base-pairing and therefore rescue transposition. Previously published results from this type of analysis produced strong evidence that nucleotides within the PBS base-pair with the first 11 bases of the *Tf1* mRNA (Levin 1995). We conducted the same type of study on the 5- and 11-bp duplexes. As seen in Figure 4A, the transposition defects of all three top strand mutants (U184G, U187G, and C191G) and all three bottom strand mutants (A112C, G109C, and G105C) in the 11-bp duplex were rescued at least partially by strains with compensating mutations that restored complementarity. The mutations A112C and U184G resulted in transposition frequencies of 36.8% and 13.5% of wild type, respectively. The combination of these two changes rescued the level of transposition to the remarkable level of 166% of wild type, indicating that the transposition did depend on the level of stability generated by hydrogen bonding between these two bases. The mutation U187G, which converted a potential G:U pair to G:G mismatch, caused only a minor defect in *Tf1* transposition and transposed at 70.1% of

wild-type frequency. The mutation G109C, on the opposite strand of U187G in the putative structure, resulted in a transposition frequency of 31.8% of wild type. The combination of these two mutations, U187G/G109C, partially rescued the transposition defects caused by the G109C mutant and exhibited a transposition frequency of 75.1% of wild type. The mutation C191G resulted in the most severe transposition defect in the 11-bp duplex and had a transposition frequency of only 0.67% of wild type. The mutation on the opposite strand, G105C, resulted in 10% of wild-type transposition frequency. The combined double point mutant, G105C/C191G, restored *Tf1* transposition to 44.7% of the wild-type frequency. This represented at least a 4-fold rescue above the activity of the single mutation G105C and a 66-fold rescue above the activity of the C191G mutation. These results demonstrated that nucleotide interactions exist between the two strands of the 11-bp duplex.

The increase in *Tf1* transposition caused by combining two detrimental mutations that reestablish complementarity was also observed with mutations in the 5-bp duplex (Fig. 4B). The two single-point mutations on the opposite strands of the 5-bp duplex, C15A (bottom strand) and G165U (top strand), both greatly reduced the *Tf1* transposition activity and resulted in transposition frequencies of 0.82% and 0.86% of wild type, respectively. The combined mutations, C15A/G165U, resulted in 13.92% of the wild-type transposition frequency that represented a 16-fold rescue of *Tf1* transposition activity. The absence of total rescue could be attributable to the possibility that both the formation of this complex structure and the sequences within the structure are important for *Tf1* transposition.

Sequence alterations in two upstream short ORFs and the putative hairpin exhibit no reduction in Tf1 transposition activity

An interesting feature that exists in the *Tf1* mRNA is the presence of two short ORFs located at least 260 nucleotides upstream of the initiation codon of the major *Tf1* ORF. A similar feature was found in the mRNA of Rous sarcoma virus (RSV), which contains three ORFs in the mRNA leader upstream of the AUG initiator of the *gag* gene. These three ORFs were found to contribute to viral protein translation, mRNA packaging, and viral infectivity (Donze and Spahr 1992; Moustakas et al. 1993a,b). The initiation codons of the two short ORFs in *Tf1* were removed (mutations G12A and G25C), and no effect on transposition was observed (data not shown). Thus, the transposition defects caused by other mutations found in the vicinity of the short ORFs were not attributable to effects on translation of these two short peptides.

The role of the putative hairpin (nucleotides 196–225) in the transposition mechanism remained unclear because the solitary mutation in the hairpin, A216T, created an ORF that resulted in decreased translation of the *Gag* and *Pol* proteins (data not shown). To analyze further the function of the putative hairpin, more severe

Table 2. List of transposition frequencies of all single-point mutants

Mutation	Actual transposition frequency (%) ^a		Relative transposition frequency (% WT)	Region
U4G	0.243 ± 0.065	(4)	6.40	bottom strand of PBS duplex
C5A	0.007 ± 0.012	(4)	0.22	
A6G	0.279 ± 0.154	(3)	6.54	
G7C	0.059 ± 0.038	(4)	1.37	
G7U	0.156 ± 0.175	(4)	3.64	
U8G	0.114 ± 0.087	(4)	3.54	
U8A	0.403 ± 0.099	(4)	12.55	
A10U	0.058 ± 0.052	(4)	1.82	
A10G	0.156 ± 0.142	(4)	4.87	
U11A	0.103 ± 0.036	(3)	2.49	
U11Δ	1.076 ± 0.306	(3)	26.22	
A13G	0.138 ± 0.047	(4)	3.23	bottom strand of 5-bp duplex
G14C	0.049 ± 0.046	(3)	1.18	
C15A	0.035 ± 0.024	(4)	0.82	
C15U	0.078 ± 0.054	(4)	2.42	
U16C	0.846 ± 0.280	(4)	26.34	
G105C	0.340 ± 0.130	(3)	10.00	bottom strand of 11-bp duplex
G109C	0.944 ± 0.085	(3)	31.80	
A112C	1.120 ± 0.165	(3)	36.80	
G165U	0.024 ± 0.034	(3)	0.86	top strand of 5-bp duplex
C170G	0.024 ± 0.033	(3)	0.86	bulge
A171C	0.102 ± 0.054	(3)	3.68	
U177A	0.044 ± 0.029	(4)	1.36	top strand of PBS duplex
U177G	0.080 ± 0.060	(4)	1.87	
U177C	0.188 ± 0.046	(4)	4.40	
G178A	0.054 ± 0.034	(4)	1.28	
G178U	0.095 ± 0.078	(4)	2.96	
C181U	0.054 ± 0.076	(3)	1.32	
U184A	0.118 ± 0.082	(4)	2.75	top strand of 11-bp duplex
U184G	0.310 ± 0.059	(7)	10.09	
U185A	0.475 ± 0.063	(4)	11.14	
U185G	0.471 ± 0.072	(4)	11.05	
U185C	0.740 ± 0.254	(4)	23.02	
G186C	1.071 ± 0.270	(4)	25.11	
G186U	1.175 ± 0.191	(3)	28.62	
U187G	2.465 ± 0.229	(7)	78.91	
G188U	0.173 ± 0.066	(4)	5.38	
A189C	0.197 ± 0.039	(4)	4.63	
U190G	0.225 ± 0.093	(4)	7.00	
U190A	0.636 ± 0.249	(4)	14.91	
C191G	0.026 ± 0.023	(7)	0.67	
C191A	0.164 ± 0.037	(4)	5.09	
IN-fs	0.030 ± 0.021	(12)	0.95	
PR-fs	<0.001 ± 0.001	(6)	<0.02	
WT	3.471 ± 0.672	(21)		

^aTransposition frequencies were obtained by averaging at least three measurements from each strain and expressed as the average ± S.D. The number of measurements are indicated in the parentheses.

mutations were made that included changing the stretch of purines (nucleotides 209–213) to pyrimidines, changing the stretch of pyrimidines (nucleotides 202–207) to

purines, and deleting the hairpin (nucleotides 196–225) altogether. None of these changes had any effect on Tf1 transposition.

Figure 4. Transposition assay results showing that defects caused by single-point mutations in the 11- and 5-bp duplexes were restored by the addition of mutations that reestablished complementarity. (A) Results of transposition assays of strains with mutations in the 11-bp duplex. Pairs of independent yeast transformants that contained mutant versions of Tf1 were induced for transposition and printed onto an 5-FOA plate, which was then printed onto the 5-FOA-G418 plate shown. The level of growth indicated the frequency of transposition. The sequence of the base changes is indicated, and the results of quantitative transposition assays (as percent of wild type) are shown in parentheses. (B) Results of transposition assays of strains with mutations in the 5-bp duplex. The mutations in each strain are indicated with the corresponding positions on the boxes at the right. Numbers in the parentheses indicate the results of quantitative transposition assays represented in transposition frequency relative to wild type level.

Mutations that disrupt the RNA structure affect the synthesis of Tf1 reverse transcripts

Because the RNA structure contained the PBS, we speculated that the bulk of the mutations that reduced transposition frequencies specifically reduced the priming of reverse transcription. To test this hypothesis, we used DNA blot analysis to measure the levels of the reverse transcripts (cDNA) synthesized by strains that contained these mutations (Fig. 5). Levels of the double-stranded reverse transcripts of Tf1 can be determined by performing DNA blot analysis on total *S. pombe* DNA extracts digested with *Bst*XI (Atwood et al. 1996). A 2.1-kb cDNA band derived from the reverse transcript of Tf1-*neo* can be detected using a radiolabeled *neo* fragment as probe. In the same DNA blot, the 9.5-kb plasmid band can also be visualized. The majority of the mutant strains produced either very little or no detectable Tf1 reverse transcript, whereas the levels of the plasmid bands detected were comparable to that of wild type. All mutants in the

PBS produced no detectable 2.1-kb signal. In addition, no signal was detected in the extracts made from the strains with mutations in the bottom strand of the PBS duplex, except for the strains that contained mutations U8A and U11Δ. These two mutations resulted in transposition frequencies of 12.5% and 26.22% of wild type, respec-

Figure 5. DNA blots showing reduced levels of cDNA produced by versions of Tf1 with mutations in the 5' UTR. Total DNA was extracted from induced cells grown to stationary phase and digested with *Bst*XI. The filter was probed with a 1-kb *neo* fragment. (A) A blot of DNA from strains with mutations located in the PBS. The label at the top of each lane indicates the mutation of the strain from which the DNA was extracted. The wild-type strain was the positive control and the PR-fs, the strain with a frameshift in PR, expressed no RT and was the negative control. The 9.5-kb signal was the plasmid copy of Tf1, and the 2.1-kb signal was a cDNA fragment of Tf1. The 5.5-kb signal was a single LTR-containing cDNA fragment whose structure was not confirmed. (B) A blot of DNA from strains with mutations located in the first 11 bases of the Tf1 mRNA. All descriptions in this blot are the same as in A. (C) A blot of DNA from strains with mutations located in the top strand of the 11-bp duplex and the bottom strand of the 5-bp duplex. All descriptions in this blot are the same as in A.

tively, which reflected much higher levels than were caused by the other mutations in the same region.

The levels of the 2.1-kb signal produced by all strains with mutations in the top strand of the 11-bp duplex also correlated with the transposition frequencies detected. The majority of the mutants in this region expressed reduced but visible 2.1-kb bands with the exception of the two most severe mutants, U184A and C191G, which produced very faint signal. These two mutations also generated AUG initiation codons and resulted in 3- to 7-fold lower transposition frequencies than those of other changes at the same positions. Although these mutations had no effect on the expression of Tf1 proteins, it was possible that translational factors bound to these AUGs and prevented these nucleotides from interacting with the nucleotides in the bottom strand of the 11-bp duplex. Data from mutants on the bottom strand of 5-bp duplex showed similar results. Four of five mutants in this region produced little or no Tf1 reverse transcript. However, an easily visible 2.1-kb band was detected in the strain that contained the U16C mutation, which resulted in a transposition frequency of 26.34% of wild type. These results indicated that the reduced transposition frequencies caused by the single-point mutations correlated well with and may be attributable to the reduced levels of Tf1 reverse transcripts.

We described above the rescue of defective Tf1 elements that resulted from combining two mutations that restored the complementarity of the duplexes. We tested the possibility that the restoration of the RNA structure that resulted in increased Tf1 transposition caused increased reverse transcription. The DNA blot in Figure 6 indicated that copies of Tf1 with pairs of mutations that re-established complementarity in all three duplexes generated an increase in the amount of reverse tran-

scripts. The intensities of the 2.1-kb band produced by the three strains that contained compensating pairs of mutations in the 11-bp duplex were much stronger than those produced by the strains with single-point mutations. Two previously reported sets of compensating mutants (Levin 1995) in the PBS duplex also produced significantly higher levels of Tf1 reverse transcripts than the individual point mutants (Fig. 6). This result is consistent with the previously reported transposition frequencies of 80% of wild type produced by the strains with the compensating pairs of mutations in the PBS duplex. The strain that contained a pair of compensating mutations in the 5-bp duplex produced significantly more cDNA than the strains that contained the individual point mutations. In summary, we detected higher levels of reverse transcripts produced by versions of Tf1 that contained compensating pairs of mutations than by copies of Tf1 that contained the corresponding single-point mutations.

The levels of reverse transcripts synthesized in all mutant strains were quantified by comparing the intensities of the 2.1-kb cDNA bands to those of the 9.5-kb plasmid bands using PhosphorImaging analysis. To examine the relationship between the magnitude of the transposition defects caused by the mutations and the levels of reverse transcripts that resulted, we plotted transposition frequencies against relative cDNA levels detected for each mutant strain. We found that the transposition frequencies of the mutant strains were directly related to the amount of cDNA by a second-order relationship (Fig. 7). This correlation suggested that the majority of the mutants were defective in transposition because of a defect in the production of Tf1 reverse transcripts.

We showed above that two site-directed mutants in the bulge region between the PBS and the 5-bp duplexes,

Figure 6. A DNA blot showing increased levels of cDNA produced by strains with mutations that restored complementarity within the RNA structure. The preparation and the detection of the DNA in this blot were the same as described in Fig. 5. The wild-type and the PR-fs strains served as the positive and the negative controls for reverse transcription, respectively. The 9.5-kb signal was the plasmid copy of Tf1, and the 2.1-kb signal was a cDNA fragment of Tf1. The lanes on the blot are labeled in groups of three representing two strains with single-point mutations opposite each other in the duplexes and one strain with the combined double mutations that restored complementarity. The strains in the 11- and 5-bp duplexes were strains constructed for this study. The strains in the PBS duplex were strains constructed previously (Levin 1995). The strains with the mutations located in the bulge, C170G and A171C, are also indicated.

Figure 7. A plot of transposition frequency vs. the amount of cDNA produced by each mutant strain. Each strain was subjected to a quantitative transposition assay, and the actual transposition frequency was obtained by averaging the measurements from at least three independent assays. The transposition frequency was normalized to that of the wild-type strain (represented as %WT). The amount of cDNA produced by each strain was measured quantitatively by comparing the intensity of the 2.1-kb band to the 9.5-kb plasmid band in the DNA blot using PhosphorImaging analysis. The amount of cDNA produced was also normalized to that produced by the wild-type strain. Data obtained from strains with single-point mutations, double-point mutations, and mutations in the ORFs were included in this plot. The relationship between the transposition frequency and the amount of cDNA produced could be approximated by a second-order equation listed at the top of the plot in which x represents the amount of cDNA and y represents the transposition frequency. The error bars indicate the standard deviation of the relative transposition frequency.

C170G and A171C, were defective in Tf1 transposition. We speculated that the transposition defects could be attributable in part to the changes in nucleotides that IN may bind during integration. If this is the case, these two mutations should not affect the reverse transcription of Tf1. Quantitative analysis of the 2.1-kb signal produced by these two strains, C170G and A171C, revealed that these mutations caused a 2.8- and a 3.3-fold defect in reverse transcription, respectively. These data indicated that sequence in the bulge also contribute to the reverse transcription mechanism even in the case where the duplexes are preserved.

Detection of -ssDNA

In a previous report (Levin 1995), strains that contained versions of Tf1 with single-point mutations in the PBS duplex accumulated significantly less -ssDNA than the

wild-type strain. The addition of compensating mutations that reestablished hydrogen bonding in the duplex resulted in wild-type levels of -ssDNA. Two species of -ssDNAs were detected in wild-type strains when a minus-strand-specific oligonucleotide was used as a probe (Levin 1995). The difference in electrophoretic mobility between the two species of -ssDNAs produced by wild-type Tf1 was found to be attributable to the absence of ~11 nucleotides of sequence at the 3' end of the shorter species. Together with our current understanding that the first 11 bases of the mRNA are removed (Levin 1996), we proposed that the shorter strong-stop species was the result of the reverse transcription of Tf1 mRNA that was cleaved between bases 11 and 12. To establish whether the mutations in this study affected the levels of -ssDNA, we tested a representative sample of strains with single-point mutations in different regions of the RNA structure for any defect in the synthesis of -ssDNA. No -ssDNA could be seen in samples extracted from strains that contained mutations A10G, U11Δ, and U11A in the bottom strand of the PBS duplex and the mutation C191G in the top strand of the 11-bp duplex (Fig. 8). The strain that contained Tf1 with the mutation U16C was the only mutant that produced clearly visible amounts of -ssDNA, albeit more of the shorter species was detected than the full-length DNA. In each of these cases, the levels of -ssDNA produced correlated well with the levels of 2.1-kb cDNA signal observed and the frequencies of transposition detected.

The effect of mutations in the RNA structure on the levels of primer cleavage

Thus far, nine separate point mutations in the 5' end of Tf1 mRNA [four from previous work (Levin 1995) and five from this study] resulted in low levels of -ssDNA. This provides strong support for the model that formation of this structure is required for priming of reverse transcription. Recent results from our laboratory (Levin

Figure 8. A DNA blot showing the effect of mutations in the RNA structure on the levels of -ssDNA. Nucleic acid extracted from virus-like particles was analyzed by DNA blot hybridization analysis. The minus-strand strong-stop species of DNA were detected using JB341, a radiolabeled oligonucleotide probe that was specific for the minus strand. The sequencing ladders were generated using kinased HL78 as a primer, the oligonucleotide that hybridized to the PBS (Levin 1995). Two species of -ssDNAs, the full-length and the DNA that was 11 bases shorter, were produced by the wild-type strain.

1996) showed that the primer for synthesizing –ssDNA is generated by a cleavage of the Tf1 mRNA between bases 11 and 12. A technique that was developed to measure the levels of cleaved Tf1 mRNA was reported (Levin 1996). The assay included primer extension reactions to detect the levels of Tf1 mRNA that was cleaved at base 11. As a way to improve the sensitivity of the assay, a mutation was incorporated in the polymerase active site of Tf1 RT to prevent DNA polymerization and the subsequent degradation of the cleaved Tf1 mRNA that is catalyzed by RNase H. Aspartic acid 564 (Because the PR cleavage sites that define the amino and carboxyl termini of Tf1 RT have not been precisely mapped, the number refers to the position within the primary translation product) in Tf1 reverse transcriptase is the second D in the highly conserved RT motif YMDD and when it is changed to asparagine the polymerase activity of RT is eliminated. The result of the D564N mutation is the increased accumulation of Tf1 mRNA cleaved between bases 11 and 12. Here we used this technique to determine whether any of the mutations in the RNA structure result in reduced levels of cleaved primer. Small amounts of cleaved Tf1 mRNAs were detected in samples extracted from either log-phase or stationary-phase cells of wild-type strains as seen in Figure 9. We observed no increase in cleaved Tf1 mRNA from stationary-phase cells because the cleaved Tf1 mRNA intermediates were reverse transcribed and then degraded by RNase H. As expected, we detected a substantial increase in the level of cleaved Tf1 mRNA in a strain with the mutation D564N. The D564N mutation was then cloned into the versions of Tf1 with mutations in the 5' UTR to test the effect of base changes in the RNA structure on primer cleavage. Primer extension reactions were conducted on DNA samples extracted from stationary-phase cells of strains with these double mutations. Results of these primer extension reactions are shown in Figure 9.

Most strains with mutations in the RNA structure were either defective or inefficient in cleaving off the first 11 nucleotides of Tf1 mRNA. The intensity of primer extension products were also quantified using

PhosphorImaging analysis. The fraction of Tf1 RNA cleaved was calculated by dividing the intensity of the shorter primer extension product by the combined intensity of both full-length and shorter primer extension products. These values were represented in parentheses in Figure 9 as percent primer cleavage. Because we detected –ssDNA produced by the strain that contained the mutation U16C and this strain transposed at 26% of wild-type frequency, we were not surprised to observe that it also produced cleaved Tf1 mRNA. Strains that contained either of two mutations that generated a GU pair, A10G and A13G, showed close to half of wild-type primer cleavage, indicating the formation of a weaker interaction allowed some primer cleavage. We were intrigued to find that the strain with the mutation U11A produced nearly wild-type levels of the cleaved mRNA even though this strain generated no detectable –ssDNA or 2.1-kb reverse transcription product (Fig. 8). A likely explanation for this result is that the mismatch between A11 and A172 did not affect the formation of the overall RNA structure or the primer cleavage but it prevented the extension of the primer.

Discussion

Our examination of the sequence in the 5' UTR of Tf1 revealed four regions with the potential to form RNA base pairs within an 89-nucleotide structure composed of three duplexes and a hairpin. We found the sequences included in these putative interactions were well conserved between Tf1 and Tf2, and in both cases they included the PBS. Results from both random and site-directed mutagenesis showed that the mutations that caused defective transposition clustered in sequences that had the potential to form duplex interactions.

Evidence that the regions of complementarity in the RNA did fold into duplex structures derived from the observation that the transposition defects caused by individual mutations could be rescued if point mutations were combined such that the complementarity of the duplex was restored. The base-pairing in the PBS duplex was demonstrated in a paper published previously (Levin

Figure 9. Primer extension analysis of total RNA showing the effect of mutations on the levels of the cleaved Tf1 mRNA. The RNA analyzed came from strains that contained wild-type or mutant versions of Tf1 RT. The flanking sequencing ladders were generated using kinased JB890, the same oligonucleotide that was used in the primer extension reactions. (Arrow *a*) the primer extension products templated by cleaved Tf1 mRNA; (arrow *b*) the primer extension products templated by full-length Tf1 mRNA. The top band (indicated by arrow *c*) is a nonspecific product that is also present in strains without Tf1 (data not shown). (WT log) RNA samples isolated from log-phase cells; (WT stat) RNA samples isolated from stationary-phase cells. All other samples were isolated from stationary-phase cells of strains with the D564N mutation only or in combination with mutations in the 5' UTR. Numbers in parentheses indicate the percentage of the total Tf1 signal in the lane that was templated by the cleaved mRNA.

1995). In the results described above, we found that the transposition defects caused by single-point mutations at three positions of the 11-bp duplex and one position of the 5-bp duplex could be rescued by additional mutations that restored complementarity. These results strongly suggested that the formation of the three duplexes play an important role in the Tf1 transposition pathway. It is interesting to note that alternative RNA structures other than the one we proposed could occur if two separate molecules of Tf1 RNA participate in the formation of the RNA duplexes.

The result that we could not detect any effect on Tf1 transposition of mutations in the hairpin is puzzling. It is possible that in our system the replacement of the original Tf1 promoter with the *nmt1* promoter may have bypassed a requirement for the hairpin related to transcription. Alternatively, growth conditions in the laboratory may not adequately mimic specific conditions in the wild that could affect the function of the hairpin. When a Tf1 transposition assay was conducted at room temperature instead of at 32°C, the strains that contained the hairpin deletion transposed at a slightly lower rate than the wild type (data not shown). This result suggested that the levels of Tf1 transposition could be affected by the hairpin when cells grow in the wild.

The mutations that caused lower levels of transposition also produced reduced amounts of cDNA without affecting Tf1 protein expression. Mutations that re-established the complementarity of the altered duplexes also caused an increase in the production of cDNA. These results indicated that the complex RNA structure was required for the completion of reverse transcription.

Quantitative transposition assays revealed that the magnitude of the transposition defects caused by the mutations correlated well with the amounts of Tf1 cDNA produced by these strains. When transposition activity was plotted against cDNA levels, the position of the points was best approximated by a function of the square of the cDNA concentration. The shape of this curve, as seen in Figure 7, indicated that a threshold level of cDNA was required for efficient transposition. The accumulation of a basal amount of reverse transcripts might be required to saturate nonproductive interactions with cellular factors. An alternative explanation for the shape of this curve is that the rate limiting step could be cooperatively regulated.

The low levels of –ssDNA produced by representative strains that contained point mutations in various regions of the 5' UTR supported our model that formation of the RNA structure was required for the priming of reverse transcription. The results of primer extension assays designed to measure the amount of the cleaved Tf1 mRNA show that the majority of the mutations blocked the cleavage and indicated that the complex RNA structure was required for this step in the synthesis of –ssDNA. The result that the U11A mutation caused reduced levels of –ssDNA without a significant reduction in the level of cleaved mRNA provided strong evidence that the first 11 bases of the mRNA did serve as primer. This conclusion is based on the prediction that the U11A base

change would cause a mismatch at the 3' end of the primer that would inhibit synthesis of –ssDNA.

One puzzling result is that the strain with the mutation U187G showed near wild-type levels of transposition and reverse transcription though it produced no primer cleavage under our assay conditions. Because our recent genetic data suggested that the Tf1 RNase H domain is required to cleave the mRNA (Levin 1996), one possibility is that the mutation in the polymerase active site of RT used in our cleavage assay rendered the enzyme unable to recognize and cleave the modified RNA structure that resulted from U187G mutation, whereas the wild-type RT was able to cleave the modified mRNA and complete reverse transcription. This suggested that the polymerase active site of RT may recognize and bind to the 11-bp duplex before the cleavage can occur. To initiate DNA synthesis, the RT must either reorientate the position of its active site relative to the cleavage site or allow an additional RT molecule to start polymerization.

Base-pairing between the nucleotides in the PBS and the 5-bp duplexes resulted in the formation of a bulge between the two duplexes. We speculated that the formation of this bulge structure opposite the cut site is required for cleavage because it resulted in a strained structure that destabilized the phosphodiester bond between bases 11 and 12. Alternatively, the bulge might be positioned opposite the cleavage site to provide the active site of a nuclease with access to the phosphodiester bond that is cleaved. In either case, the identity of the nucleotide at positions 11 and 12 must not be critical, as the U11A and G12A mutations resulted in near wild-type levels of cleavage. Biochemical and X-ray crystallographic studies of HIV RT (Kohlstaedt et al. 1992) showed that the distance between the polymerase active site and the RNase H active site of RT is ~18–20 bp. If Tf1 RT possesses a similar structure, the binding of the polymerase active site of Tf1 RT to the 11-bp duplex would locate the active site of RNase H near the bulge and allow for the primer cleavage to occur.

Although we have presented evidence that indicates the sequence in the 5' UTR of the Tf1 message formed a complex RNA structure that is specifically recognized and cleaved by a nuclease to generate the primer for reverse transcription, we cannot exclude the possibility that some of our point mutations may result in inefficient packaging of the Tf1 mRNA into virus-like particles (VLPs). If this is true, lower levels of Tf1 mRNA would be exposed to the enzymes in the VLPs such as RT that may be required for the cleavage reaction. This would suggest that some regions of the complex RNA structure could be required for RNA packaging. To address this issue, we have made several efforts to develop a packaging assay to measure the levels of Tf1 mRNA in VLPs. Measuring packaged mRNA has been particularly difficult in the case of all retrotransposons because their VLPs are not secreted into culture medium. Thus far, we have been unable to purify Tf1 particles away from the bulk of the cellular transcripts and therefore cannot measure any defects in packaging of Tf1 mRNA.

Extended contacts of the primer tRNA with the sequences flanking the PBS of RSV and Ty1 have been identified through in vivo and in vitro studies. The formation of individual duplexes contributes to the efficient annealing and packaging of the tRNA primers. Mutations resulting in the disruption of these interactions caused reduced propagation of RSV and Ty1 (Wilhelm et al. 1994; Keeney et al. 1995; Friant et al. 1996). In addition, the results of in vitro reverse transcription indicated that mutations in the Ty1 structure resulted in low levels of annealed tRNA (Wilhelm et al. 1994). Three short regions of Ty1 RNA downstream of the PBS, named boxes 0, 1, and 2.1, interact with the T and D stems and loops of tRNA^{Mct} (Fig. 10), respectively. In the case of the RSV, the structures that form as the result of base-pairing of the mRNA include the U5-IR stem and the U5-leader stem (Fig. 10). The structures that form between the mRNA and the tRNA^{T_{rp}} include the PBS duplex and a 7-base sequence upstream of the PBS that interacts with T ψ C arm of tRNA^{T_{rp}} (Fig. 10). The formation of a large RNA structure and a 4- or 5-base bulge near the PBS bears similarity to the structure of Tf1 (Fig. 1).

The structural and functional similarities of these extended RNA complexes suggest that RTs of retroelements recognize and bind to a specific RNA conforma-

tion to initiate reverse transcription. Interestingly, the mechanism of Tf1 cleavage and self-priming bares resemblance to the mechanism of plus-strand priming from the polypurine tracts of retroviruses and LTR-containing retrotransposons. Both mechanisms require RNase H to generate the primer for reverse transcription. It is possible that the recognition of the polypurine tract by RNase H and the production of the Tf1 primer require similar features yet to be discovered.

Materials and methods

Media

The *S. pombe* minimal liquid and plate media were composed of Edinburgh minimal medium (EMM) (Moreno et al. 1991) and were supplemented with amino acids, vitamin B1, 5-FOA and Geneticin (G418) as described previously (Levin 1996).

Transposition assay

S. pombe strains with Tf1 plasmids were first grown as patches on EMM in the absence of Vitamin B1 to induce the *mnt1* promoter that was fused to the *neo*-marked Tf1. After 4 days of 32°C incubation, the plates were replica printed to EMM medium containing 5-FOA to select against cells containing the Tf1-*neo* plasmid (Boeke et al. 1987). These plates were then replica printed to YES medium (Moreno et al. 1991) containing 5-FOA-G418 and incubated at 32°C for 2 days to detect cells with copies of Tf1-*neo* that had transposed into the genome (Levin and Boeke 1992; Levin et al. 1993).

The actual transposition frequency of each mutant strain was determined with a quantitative transposition assay (Levin 1995) and was normalized to the frequencies of wild-type strains assayed at the same time as control.

Strains and plasmid constructions

The yeast strains used in this paper are listed in Table 3. All plasmids were transformed into YHL912, a strain of *S. pombe* with the genotype *h⁻, ura4-294, and leu1-32*. All plasmids were constructed by directional cloning of PCR-amplified fragments into a Tf1-containing plasmid. All PCR reactions described were performed with the native *Pfu* enzyme (Stratagene). The specific oligonucleotides used in the construction of each mutation are shown in Table 4. Restriction sites were created or abolished by fusion PCR (Levin 1995).

The mutation sites in each plasmid generated by PCR were sequenced with dideoxynucleotide termination reactions (U.S. Biochemical Sequenase v. 2.0 DNA sequencing kit) to verify the nature of the base changes. In addition, each PCR-generated construct was made in duplicate using an independent PCR reaction to control against the possibility of nontemplated PCR-generated mutations. Each duplicated plasmid gave results in transposition and primer extension assays equivalent to that of its partner.

Construction of the XhoI-SpeI cassette in the Tf1-containing plasmid

Plasmids pHL891-19 and pHL900-1 are wild-type and PR-frameshift versions of Tf1-containing plasmids, respectively, that both lacked the XhoI site in the *neo* marker and contained a unique SpeI site that was generated to introduce mutations in the 5' UTR of Tf1. Plasmid pHL891-19 was the wild-type Tf1

Figure 10. The binary RNA complexes of RSV and Ty1 formed with primer tRNA. (Top) The structure of Ty1 as reported by Friant et al. (1996). Interactions among PBS and regions termed box 0, box 1, and box 2.1 of Ty1 RNA with acceptor stem, T stem, T loop, and D loop of tRNA^{Mct} are indicated. (Bottom) The structure of RSV as reported by Aiyar et al. (1994). Formation of U5-IR and U5-leader stems by RSV mRNA are indicated. Interactions between the acceptor stem and T ψ C arm of the tRNA^{T_{rp}} with the PBS and a sequence in the U5 region of RSV are shown.

Table 3. *Yeast strains*

Yeast strain ^a	Plasmid description			Yeast strain ^a	Plasmid description		
	number	Tf1 allele	region		number	Tf1 allele	region
YHL4093	pHL891-19	WT	—	YHL5134	pHL1142-1	U4G	PBS
YHL4179	pHL900-1	PR-fs	PR	YHL5128	pHL1139-1	C5A	PBS
YHL1306	pHL431-25	IN-fs	IN	YHL5158	pHL1154-1	A6G	PBS
YHL5038	pHL1120-1	hairpin-deletion	hairpin	YHL5150	pHL1150-1	G7C	PBS
YHL5037	pHL1119-2	purines to pyrimidines	hairpin	YHL5160	pHL1155-1	G7U	PBS
YHL5040	pHL1121-2	pyrimidines to purines	hairpin	YHL5110	pHL1130-1	U8G	PBS
YHL4273	pHL901-4	G12A	1st ORF	YHL5116	pHL1133-1	U8A	PBS
YHL4285	pHL902-4	G25C	2nd ORF	YHL5130	pHL1140-1	A10U	PBS
YHL4297	pHL903-4	G12A/G25C	both ORFs	YHL5104	pHL1127-1	A10G	PBS
YHL2706 ^b	pHL789-1	C176G	PBS	YHL5136	pHL1143-1	U11A	PBS
YHL2722 ^b	pHL791-1	G178C	PBS	YHL5019	pHL1099-2	U11A	PBS
YHL2738 ^b	pHL793-1	C5G	PBS	YHL5154	pHL1152-1	A13G	5-bp
YHL2754 ^b	pHL795-1	G7C	PBS	YHL5020	pHL1101-2	G14C	5-bp
YHL2770 ^b	pHL789-1	G7C/C176G	PBS	YHL5144	pHL1147-1	C15A	5-bp
YHL2786 ^b	pHL799-1	C5G/G178C	PBS	YHL5112	pHL1131-1	C15U	5-bp
YHL5029	pHL1106-1	A112C	11-bp	YHL5124	pHL1137-1	U16C	5-bp
YHL5025	pHL1104-1	G109C	11-bp	YHL5680	pHL1245-1	C170G	bulge
YHL5021	pHL1102-1	G105C	11-bp	YHL5676	pHL1244-1	A171C	bulge
YHL5031	pHL1107-4	A112C/U184G	11-bp	YHL5224	pHL1195-1	U177A	PBS
YHL5027	pHL1105-1	G109C/U187G	11-bp	YHL5156	pHL1153-1	U177G	PBS
YHL5023	pHL1103-2	G105C/C191G	11-bp	YHL5164	pHL1157-1	U177C	PBS
YHL5672	pHL1243-1	G165U	5-bp	YHL5146	pHL1148-1	G178A	PBS
YHL5684	pHL1246-1	C15A/G165U	5-bp	YHL5132	pHL1141-1	G178U	PBS
YHL5041	pHL1122-1	A216U	hairpin	YHL5126	pHL1138-1	C181U	PBS
YHL5328	pHL1209-1	D564N	RT	YHL5102	pHL1126-1	U184A	11-bp
YHL5337	pHL1213-2	U8G-D564N	PBS	YHL5114	pHL1132-1	U184G	11-bp
YHL5333	pHL1211-2	A10G-D564N	PBS	YHL5162	pHL1156-1	U185A	11-bp
YHL5252	pHL1200-2	U11A-D564N	PBS	YHL5152	pHL1151-1	U185G	11-bp
YHL5344	pHL1217-1	U11-Δ564N	PBS	YHL5106	pHL1128-1	U185C	11-bp
YHL5347	pHL1218-2	A13G-D564N	5-bp	YHL5142	pHL1146-1	G186C	11-bp
YHL5231	pHL1198-1	G14C-D564N	5-bp	YHL5228	pHL1197-1	G186U	11-bp
YHL5253	pHL1201-1	C15A-D564N	5-bp	YHL5108	pHL1129-1	U187G	11-bp
YHL5339	pHL1214-2	C15U-D564N	5-bp	YHL5122	pHL1136-1	G188U	11-bp
YHL5341	pHL1215-2	U16C-D564N	5-bp	YHL5148	pHL1149-1	A189C	11-bp
YHL5233	pHL1199-1	U177G-D564N	PBS	YHL5118	pHL1134-1	U190G	11-bp
YHL5331	pHL1210-2	U184A-D564N	11-bp	YHL5140	pHL1145-1	U190A	11-bp
YHL5334	pHL1212-1	U187G-D564N	11-bp	YHL5166	pHL1158-1	C191G	11-bp
YHL5255	pHL1202-1	C191G-D564N	11-bp	YHL5120	pHL1135-1	C191A	11-bp

^aAll plasmids were transformed into YHL912 (*h⁻, ura4-294, leu1-32*).^bStrains constructed by Levin (1996).

plasmid used in the experiments described in this report and plasmid pHL900-1 was used in each experiment as a negative control for transposition and reverse transcription. Plasmids pHL891-19 and pHL900-1 were constructed by the insertion of a *SpeI*-containing PCR-generated *XhoI*-*AvrII* fragment of 1.0 kb into pHL888-3 and pHL889-1, respectively. Plasmids pHL888-3 and pHL889-1 were constructed by inserting a PCR amplified *neo* fragment, which contained flanking *Bam*HI sites and a silent mutation that destroyed a *XhoI* site, into the *Bgl*III site of pHL411-62 and pHL412-63 (Levin et al. 1993), respectively. The *neo* fragment lacking the *XhoI* site was derived from pHL765-3. Plasmid pHL411-62 contained the *nmt1* promoter fused to Tf1 at the start of the transposon transcript. The construct pHL412-63 also was identical to pHL411-62 except for a frameshift mutation inserted at the *SacI* site found in the beginning of PR. Plasmid pHL414-2 (Levin et al. 1993) resulted from the insertion of a *neo* gene into the *Bgl*III site of pHL411-62. Plasmid pHL431-

25 was identical to pHL414-2 except that a reading frameshift in the beginning of IN was created. pHL431-25 was constructed by ligating the 5.2-kb *XhoI* fragment, which contained Tf1, of pHL338-9 (Levin 1995) into the 8.5 kb *XhoI* fragment, which constituted the vector, of pHL414-2.

The construction and amplification of a plasmid library that contained mutations in Tf1

The plasmid library containing mutations in the 5' UTR of Tf1 was constructed by cloning a PCR-amplified fragment of 230 bp into the unique *XhoI* and *SpeI* sites of pHL891-19. Two oligonucleotides, HL100 and HL101, were made degenerate by the addition of all 4 nucleotides during the oligonucleotide synthesis at a concentration 250-fold less than the normal level for the wild-type nucleotide. Using these two oligonucleotides, the 230-bp *XhoI*-*SpeI* fragment was PCR amplified and ligated into

Table 4. Oligonucleotides

Oligo	Sequence (5' to 3')	Purpose
HL38	GGAAGAGGAATCCTGGC	Top strand primer hybridizes to nmt sequence 5' of XhoI site, used in PCR
HL39	CCAAATGCTTCCAGTCTTTG	Bottom strand primer hybridizes to downstream of AvrII site, used in PCR
HL80	CGGAAACGCTCTGTTCGAGGCCGC	Top strand primer used to generate a silent mutations in neo gene to abolish Xho I site
HL81	ATCGCGCCCTCGA[CA]CAAGACGTTTC	Bottom strand primer used to generate silent mutations in neo gene to abolish Xho I site
HL83	GGAATTATTG[ACTAGT]ATTTTCTCTC	Top strand primer used to generate a SpeI site immediately 3' of the hairpin.
HL84	CCAGAGAGAAAAT[ACTAGT]CAATAATTCC	Bottom strand primer used to generate a SpeI site immediately 3' of the hairpin.
HL93	CATATCCTCGAGTTTCAGTTAT[AGCTATATAAATG	Primer used to generate mutant G12A, a point mutation at AU[G] of first short ORF
HL94	CATATCCTCGAGTTTCAGTTATGAGCTATATAAA[CGATAGGTAAC	Primer used to generate mutant G25C, a point mutation at AU[G] of second short ORF
HL95	CATATCCTCGAGTTTCAGTTAT[AGCTATATAAA[CGATAGGTAAC	Primer used to generate mutant G12A/G25C, mutations at both AU[G] of first short ORF and AU[G] of second short ORF
HL100	CATATCCTCGAGTTTCAGTTATGAGCTATATAAATGATAG	Top strand degenerative primer used to generate mutant plasmid library with a restriction
HL101	GAAAATAC[AGTCAATAATTCCTTTTTCGAAAAGAAATTTTGTAGATCACA	Bottom strand degenerative primer used to generate mutant plasmid library with a restriction Spe I site
	AGAGTTCAAGTTATTG	Primer used to generate U11A mutation
HL132	CATATCCTCGAGTTTCAGTTAT[AGCTATATA	Primer used to generate G14C mutation
HL133	CATATCCTCGAGTTTCAGTTATG[AGCTATATAAAT	Top strand primer used to generate G105C mutation in fusion PCR
HL136	TGATTTGTAATAATA[CA]ATCGCAAGGAAAC	Bottom strand primer used to generate G105C mutation in fusion PCR
HL137	GTTTCTCCTGCGAT[GT]ATTATATACAAATACA	Top strand primer used to generate G109C mutation in fusion PCR
HL138	GTAATAATAATAGATC[CA]AAGGAAAACTCACCG	Bottom strand primer used to generate G109C mutation in fusion PCR
HL139	CGGTGAGTTTTCCTTG[GT]GATCTATTATTAC	Top strand primer used to generate A112C mutation in fusion PCR
HL140	ATATAATAGATCGCA[CG]GAAAACTCACCGCAG	Bottom strand primer used to generate A112C mutation in fusion PCR
HL141	CTGCGGTGAGTTTTC[GT]GCGATCTATTATAT	Primer used to generate purines to pyrimidines mutations in the hairpin
HL146	GAAAATAC[AGTCAATAATTCCTTTTTCGAG]GAAAAGAAATTATT	Primer used to generate a Tf1 plasmid with deleted hairpin
HL147	GAAAATAC[AGTCTGTAGATCA]CAAGAGTTC	Primer used to generate purines to pyrimidines mutations in the hairpin
HL148	GAAAATAC[AGTCAATAATTCCTTTTTCGAG]GAAAAGAAATTATT	Primer used to generate purines to pyrimidines mutations in the hairpin
HL172	GAAAATAC[AGTCAATAATTCCTTTTTCGAG]GAAAAGAAATTATT	Primer used to generate G165U mutation
	AGAGTTCAAGTTATTG	
HL173	GAAAATAC[AGTCAATAATTCCTTTTTCGAG]GAAAAGAAATTATT	Primer used to generate A171C mutation
	AGAGTTCAAGTTATTG	
HL174	GAAAATAC[AGTCAATAATTCCTTTTTCGAG]GAAAAGAAATTATT	Primer used to generate C170G mutation
	AGAGTTCAAGTTATTG	

Brackets indicate the positions of mutations.

pHL891-19. The ligated products were electroporated into ElectroMax DH10B cells (Life Tech. Inc.).

Recovery of plasmids from S. pombe strains with defective Tf1 transposition

The mutant plasmids were transformed into YHL912. Five thousand transformed colonies were subjected to transposition patch assays. The cells identified as defective for transposition were harvested from 10-ml cultures grown to an OD_{600} of 2.0 in EMM minus uracil dropout media that contained 10 μ M thiamine. The cells were resuspended in 1.5 ml of 2 mg/ml Zymolyase 20T (ICN) in a buffer consisting of 50 mM Na_2HPO_4 , 50 mM citric acid, and 1.2 M sorbitol at pH 5.6 and incubated at 37°C for 1 hr. The enzyme-treated cells were pelleted and resuspended in 300 μ l of TE. Thirty-five microliters of 10% SDS was added and the mixture was incubated at 65°C for 5 min. After addition of 100 μ l of 5 M KOAc and incubation on ice for 30 min, 400 μ l of supernatant was collected from a 10-min microcentrifuge spin at full speed. The nucleic acids in the supernatant were bound to 30 μ l of Prep-a-gene matrix (Bio-Rad) in 1.2 ml of binding buffer and eluted with 50 μ l of TE after washing with 2 ml of washing buffer. The *XhoI*–*SpeI* fragment from each mutant strain identified was sequenced to identify base changes. The 5' UTR segment of Tf1 from plasmids isolated by the mutant screen was subcloned into pHL891-19 as a 3794-bp *MluI*–*SpeI* fragment to ensure that no mutations created during the initial yeast transformation existed in Tf1 outside of the region between *XhoI* and *SpeI*.

Construction of plasmids with site-directed mutations

The plasmids containing site-directed mutations, including the single- and multiple-point mutations and deletions, were constructed by cloning into pHL891-19 the *XhoI*–*SpeI* fragment amplified with oligonucleotides that included the specific mutations. The plasmids containing compensating double-point mutations in the 5' UTR of Tf1, including plasmids pHL1103-1, 1105-1, 1107-1, and 1246-1, were created by using DNA from the plasmids with single-point mutation as template in either regular or fusion PCR reactions that used the corresponding oligonucleotides to generate the additional mutation. The plasmids with double mutations that contained D564N in RT were constructed by subcloning the 8.25-kb *AvrII*–*BamHI* fragment from the mutant plasmids into the 5.45-kb *AvrII*–*BamHI* fragment of pHL944-1 (Levin 1996).

Particle preparation and extraction of nucleic acid for –ssDNA detection

The preparations of large-scale yeast extracts and the subsequent analysis on sucrose gradients was based on previously published protocols (Garfinkel et al. 1985; Eichinger and Boeke 1990). Cells were inoculated at an OD_{600} of 0.05 in 500 ml of EMM minus uracil drop out media and harvested at an OD_{600} of 1.0. (Levin et al. 1993). Nucleic acid was extracted from sucrose fractions containing virus-like particles. –ssDNA was detected according to a previously published protocol (Levin 1995).

Isolation of RNA and DNA from whole-cell extracts

Ten milliliters of stationary-phase cells at OD_{600} and 10 or 50 ml of log-phase cells at OD_{600} of 2.0 were harvested for nucleic acids isolation. These preparations, as described previously (Levin 1996), were used to generate materials for either DNA

blot analysis (Atwood et al. 1996) or RNA-templated primer extension.

Primer extension

One-tenth of the isolated RNA (~10 μ g) was resuspended in 15 μ l of 1 $\times$ AMV reverse transcription buffer (Boehringer Mannheim) that contained 1 pmole of 5' end- ^{32}P -labeled oligonucleotide JB890. Extension reactions with AMV RT (Boehringer Mannheim) were as described previously (Levin 1996).

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References

- Aiyar, A., D. Cobrinik, Z. Ge, H.J. Kung, and J. Leis. 1992. Interaction between retroviral U5 RNA and the T psi C loop of the tRNA(Trp) primer is required for efficient initiation of reverse transcription. *J. Virol.* **66**: 2464–2472.
- Aiyar, A., Z. Ge, and J. Leis. 1994. A specific orientation of RNA secondary structures is required for initiation of reverse transcription. *J. Virol.* **68**: 611–618.
- Atwood, A., J. Lin, and H. Levin. 1996. The retrotransposon Tf1 assembles virus-like particles with excess Gag relative to integrase because of a regulated degradation process. *Mol. Cell. Biol.* **16**: 338–346.
- Boeke, J.D., J. Trueheart, G. Natsoulis, and G.R. Fink. 1987. 5-Fluoro-orotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* **154**: 164–175.
- Chapman, K.B., A.S. Byström, and J.D. Boeke. 1992. Initiator methionine tRNA is essential for Ty1 transposition in yeast. *Proc. Natl. Acad. Sci.* **89**: 3236–3240.
- Donze, O. and P.F. Spahr. 1992. Role of the open reading frames of Rous sarcoma virus leader RNA in translation and genome packaging. *EMBO J.* **11**: 3747–3757.
- Eichinger, D.J. and J.D. Boeke. 1990. A specific terminal structure is required for Ty1 transposition. *Genes & Dev.* **4**: 324–330.
- Friant, S., T. Heyman, M.L. Wilhelm, and F.X. Wilhelm. 1996. Extended interactions between the primer tRNAi(Met) and genomic RNA of the yeast Ty1 retrotransposon. *Nucleic Acids Res.* **24**: 441–449.
- Gabriel, A. and J.D. Boeke. 1993. Retrotransposon reverse transcription. In *Reverse transcriptase* (ed. S. Goff and A. Skalka), pp. 275–328. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Garfinkel, D.J., J.D. Boeke, and G.R. Fink. 1985. Ty element transposition: Reverse transcriptase and virus-like particles. *Cell* **42**: 507–517.
- Keeney, J.B., K.B. Chapman, V. Lauermaun, D.F. Voytas, S.U. Astrom, U. von Pawel-Rammingen, A. Byström, and J.D. Boeke. 1995. Multiple molecular determinants for retrotransposition in a primer tRNA. *Mol. Cell. Biol.* **15**: 217–226.
- Kikuchi, Y., Y. Ando, and T. Shiba. 1986. Unusual priming mechanism of RNA-directed DNA synthesis in copia retrovirus-like particles of *Drosophila*. *Nature* **323**: 824–826.
- Kohlstaedt, L., J. Wang, J. Friedman, P. Rice, and T. Steitz. 1992.

- Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**: 1783–1790.
- Leis, J., A. Aiyar, and D. Cobrinik. 1993. Regulation of initiation of reverse transcription of retroviruses. In *Reverse transcriptase* (ed. A. Skalka and S. Goff), pp. 33–47. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Levin, H.L. 1995. A Novel mechanism of self-primed reverse transcription defines a new family of retroelements. *Mol. Cell. Biol.* **15**: 3310–3317.
- . 1996. An unusual mechanism of self-primed reverse transcription requires the RNase H domain of reverse transcriptase to cleave an RNA duplex. *Mol. Cell. Biol.* **16**: 5645–5654.
- Levin, H.L. and J.D. Boeke. 1992. Demonstration of retrotransposition of the Tf1 element in fission yeast. *EMBO J.* **11**: 1145–1153.
- Levin, H.L., D.C. Weaver, and J.D. Boeke. 1990. Two related families of retrotransposons from *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **10**: 6791–6798.
- Levin, H.L., D.C. Weaver, and J.D. Boeke. 1993. Novel gene expression mechanism in a fission yeast retroelement: Tf1 proteins are derived from a single primary translation product [published erratum appears in *EMBO J.* **13**: 1494 (1994)]. *EMBO J.* **12**: 4885–4895.
- Moreno, S., A. Klar, and P. Nurse. 1991. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194**: 795–823.
- Moustakas, A., T.S. Sonstegard, and P.B. Hackett. 1993a. Alterations of the three short open reading frames in the Rous sarcoma virus leader RNA modulate viral replication and gene expression. *J. Virol.* **67**: 4337–4349.
- . 1993b. Effects of the open reading frames in the Rous sarcoma virus leader RNA on translation. *J. Virol.* **67**: 4350–4357.
- Varmus, H.E. and R. Swanstrom. 1984. Replication of retroviruses. In *RNA tumor viruses* (ed. R. Weiss, N. Teich, H. Varmus, and J. Coffin), pp. 369–512. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Wilhelm, M., F.X. Wilhelm, G. Keith, B. Agoutin, and T. Heyman. 1994. Yeast Ty1 retrotransposon: The minus-strand primer binding site and a cis-acting domain of the Ty1 RNA are both important for packaging of primer tRNA inside virus-like particles. *Nucleic Acids Res.* **22**: 4560–4565.

A complex structure in the mRNA of Tf1 is recognized and cleaved to generate the primer of reverse transcription.

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