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The yeast retrotransposon Ty5 uses the anticodon stem-loop of the initiator methionine tRNA as a primer for reverse transcription

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ABSTRACT

Retrotransposons and retroviruses replicate by reverse transcription of an mRNA intermediate. Most retroelements initiate reverse transcription from a host-encoded tRNA primer. DNA synthesis typically extends from the 3′-OH of the acceptor stem, which is complementary to sequences on the retroelement mRNA (the primer binding site, PBS). However, for some retrotransposons, including the yeast Ty5 elements, sequences in the anticodon stem-loop of the initiator methionine tRNA (IMT) are complementary to the PBS. We took advantage of the genetic tractability of the yeast system to investigate the mechanism of Ty5 priming. We found that transposition frequencies decreased at least 800-fold for mutations in the Ty5 PBS that disrupt complementarity with the IMT. Similarly, transposition was reduced at least 200-fold for IMT mutations in the anticodon stem-loop. Base pairing between the Ty5 PBS and IMT is essential for transposition, as compensatory changes that restored base pairing between the two mutant RNAs restored transposition significantly. An analysis of 12 *imt* mutants with base changes outside of the region of complementarity failed to identify other tRNA residues important for transposition. In addition, assays carried out with heterologous IMTs from *Schizosaccharomyces pombe* and *Arabidopsis thaliana* indicated that residues outside of the anticodon stem-loop have at most a fivefold effect on transposition. Our genetic system should make it possible to further define the components required for priming and to understand the mechanism by which Ty5's novel primer is generated.

Keywords: cDNA synthesis; *copia*; priming; retrovirus; retrotransposition

INTRODUCTION

Retrotransposons are a class of genetic elements that replicate through an mRNA intermediate. They are structurally and functionally analogous to retroviruses and therefore provide an important model for understanding retroviral replication (Brown & Varmus, 1989; Boeke & Sandmeyer, 1991). During reverse transcription, both retrotransposons and retroviruses typically use a host-encoded tRNA as a primer for first strand cDNA synthesis. Immediately downstream of the 5′ long terminal repeat (LTR) is a region called the minus-strand primer binding site (PBS), which base pairs with sequences at the 3′ end of the primer tRNA. Reverse transcriptase initiates cDNA synthesis from the 3′ OH of the tRNA and extends the cDNA to the 5′ end of the element mRNA. Reverse transcription continues through a series of two strand transfers, ultimately resulting in a double-stranded linear cDNA. This cDNA is integrated into the genome by the element-encoded integrase.

Whereas most retroviruses and retrotransposons, including HIV-1 and the *Saccharomyces cerevisiae* Ty1 elements, use the 3′ end of a tRNA as a primer for reverse transcription (Chapman et al., 1992; Leis et al., 1993), other novel priming mechanisms have been described. For example, Hepatitis B virus uses a protein primer, and DNA synthesis initiates from an OH provided by a tyrosine residue (Wang & Seeger, 1992; Tavis & Ganem, 1993). The Tf1 retrotransposon of *Schizosaccharomyces pombe* uses a self-priming mechanism (Levin, 1995, 1996). The 5′ end of the template mRNA folds back and anneals to the PBS; the template mRNA is then cleaved to release an RNA fragment that serves as a primer. For the *Drosophila melanogaster copia* element, the PBS pairs to the anticodon stem-loop of the *D. melanogaster* initiator methionine tRNA.

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The tRNA is cleaved by some unknown mechanism and a half tRNA molecule is used to initiate DNA synthesis (Kikuchi et al., 1986).

Saccharomyces cerevisiae and its Ty retrotransposons have become an important model system for understanding mechanisms by which tRNAs prime reverse transcription. In *S. cerevisiae*, both the retrotransposons and their primer tRNA can be genetically manipulated. Efficient transposition assays have been developed for Ty1 and Ty3, and both elements have PBSs complementary to the 3′ end of an initiator methionine tRNA (IMT; Boeke et al., 1985; Hansen et al., 1988; Chapman et al., 1992; Keeney et al., 1995). A yeast strain has been developed in which all four copies of the *IMT* genes are disrupted, and cells survive by carrying a functional *IMT* gene on a plasmid (Bystrom & Fink, 1989). This makes it possible to test *imt* mutants for their effect on transposition. Using this system, Ty1 and Ty3 have been shown to use the 3′ acceptor stem of the IMT as a primer for reverse transcription (Chapman et al., 1992; Keeney et al., 1995). Additional IMT residues important in priming have also been identified in the D and TΨC arms (Keeney et al., 1995; Friant et al., 1998; Gabus et al., 1998). These residues base pair with other regions of the retroelement mRNA and thereby help to stabilize primer/template interactions (Friant et al., 1998; Gabus et al., 1998).

The putative PBS of the yeast Ty5 retrotransposon is complementary to the anticodon stem-loop of the *S. cerevisiae* IMT (Fig. 1; Voytas & Boeke, 1992). Strikingly, the region of complementarity is identical to that observed between the *copia* element mRNA and the *D. melanogaster* IMT (Kikuchi et al., 1986). Other retrotransposons from a variety of organisms, including 1731 from *D. melanogaster*, *Osser* from *Volvox carteri*, and Tp1 and Tp2 from the slime mold *Physarum polycephalum* have PBSs that are complementary to the same region of the IMT anticodon stem-loop (Fourcade-Peronnet et al., 1988; McCurrach et al., 1990; Rothnie et al., 1991; Lindauer et al., 1993). This suggests that the mechanism of half-tRNA priming is highly conserved among these fungal, protist, and animal retrotransposons. In this study we have exploited the *S. cerevisiae* system to better understand how half-tRNAs are used by Ty5 to prime reverse transcription.

RESULTS

The sequence of the putative Ty5 PBS is complementary to 14 bases within the anticodon stem-loop of the *S. cerevisiae* IMT (Fig. 1). To test the significance of this complementarity in Ty5 transposition, we adopted an assay previously used to evaluate the role of the IMT in priming Ty1 reverse transcription (Keeney et al., 1995). In this assay, Ty5 and the *IMT* gene are carried

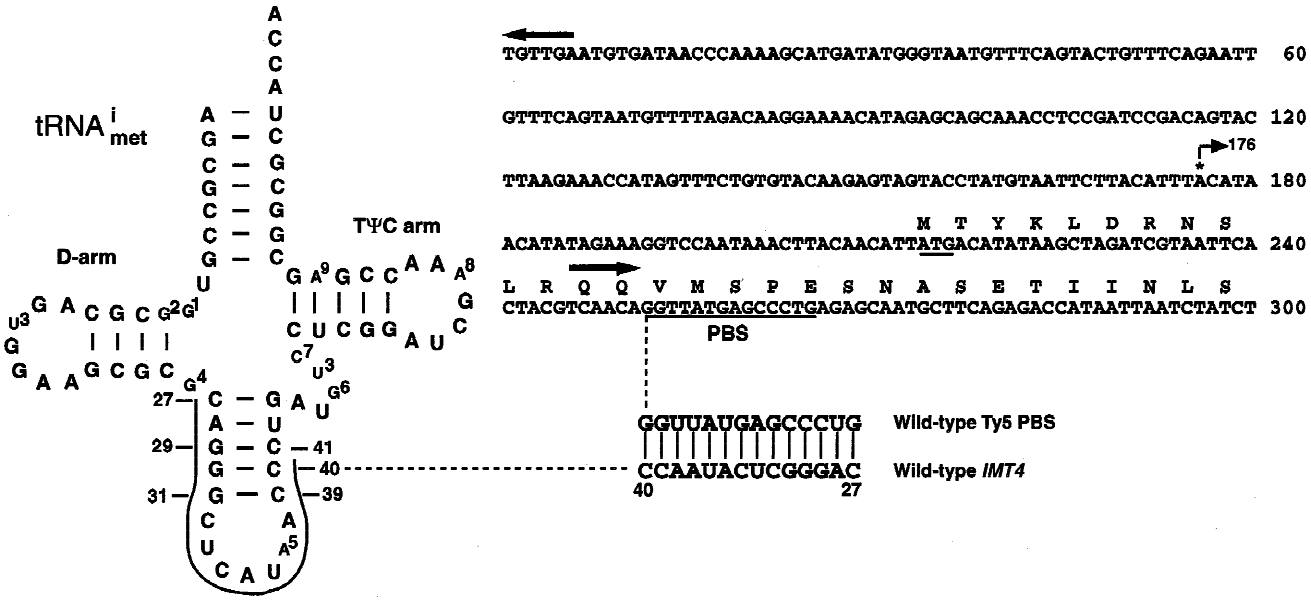

FIGURE 1. Sequences of the Ty5 PBS and the initiator methionine tRNA. On the left is shown the sequence and secondary structure of the *Saccharomyces cerevisiae* IMT. The underlined IMT sequences can base pair with the Ty5 PBS. The numbering of the IMT residues is based on the system for the elongator methionine tRNA (von Pawel-Rammingen et al., 1992). The numbers adjacent to individual bases denote positions of modification. On the right is shown the sequence of the first 300 bases of the Ty5 retrotransposon (GenBank accession number: U19264). The arrows indicate the inverted repeats at the ends of the Ty5 LTR. The asterisk at base 176 denotes the Ty5 transcription start site. The derived amino acid sequences are shown above the nucleotide sequence. The region of complementarity with the IMT (the PBS) is underlined, and base pairing is illustrated below the sequence.

on plasmids to facilitate the testing of a variety of Ty5 and *imt* mutants for their effect on transposition. The plasmid-borne Ty5 element can be transcriptionally induced by growth on galactose, and it also carries a *HIS3* marker gene (*his3*AI) that is nonfunctional because of the presence of an artificial intron. The *HIS3* marker becomes functional after intron loss through Ty5 transcription, intron splicing, and reverse transcription. Integration of Ty5 cDNA into the genome confers a His^+ phenotype (Zou et al., 1996).

The assay system uses a yeast strain with disruptions in all four copies of the initiator methionine tRNA genes (Bystrom & Fink, 1989; Fig. 2A). Translation is supported by a wild-type *IMT* gene on a *URA3*-based plasmid. Mutant *imt* genes (on *LEU2*-based plasmids) are introduced into this strain by plasmid shuffling: the strain is transformed with a plasmid carrying a mutant *imt* gene, and the plasmid with the wild-type *IMT* is lost by growing the cells on medium containing 5-fluoroorotic acid (5-FOA), which selects against the *URA3* marker (Boeke et al., 1987). This plasmid shuffling strategy requires, however, that the mutant *imt* can support translation. The effect of given *imt* mutants on Ty5 transposition is measured by introducing the Ty5-containing plasmid and carrying out our standard transposition assay. To ensure that only transposition and not cDNA recombination is evaluated, we disrupted the *RAD52* gene, which is responsible for high frequency homologous recombination of Ty5 cDNA (Ke & Voytas, 1999).

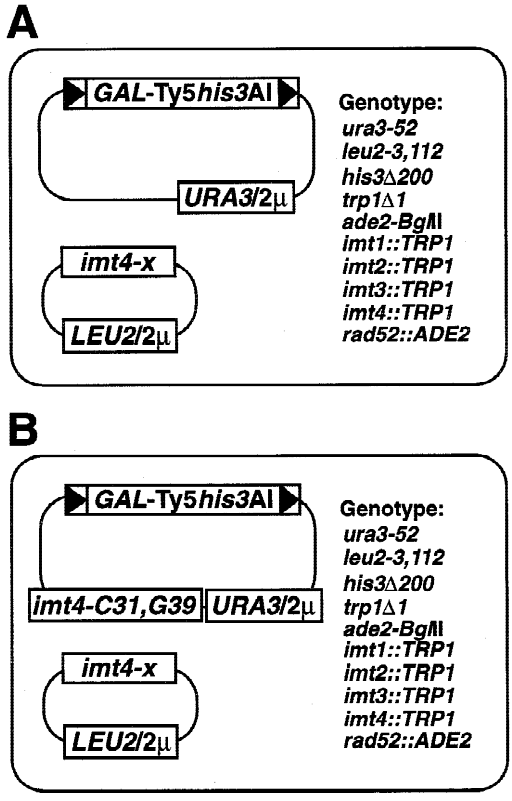

FIGURE 2. Assay systems used to determine the effect of IMT mutations on Ty5 transposition. **A**: The assay system used for *imt* mutants that support translation. The strain shown has all four of its *IMT* genes disrupted by *TRP1* and carries a mutant *imt4-x* on a *LEU2*-based plasmid. The mutant *imt* supports translation and is tested for its effect on transposition. Ty5 is carried on a *URA3*-based plasmid. **B**: The assay system used for *imt* mutants that cannot support translation. The Ty5 element and *imt4-C31,G39* (which supports translation but not transposition) are carried on a *URA3*-based plasmid. A second *imt-x* that cannot support translation is introduced to test its effect on transposition.

Mutations in the putative Ty5 PBS abolish transposition

To test whether the putative PBS is important for transposition, two PBS mutations were generated by site-directed mutagenesis that disrupt complementarity with the IMT (*pbs-1*, five bases altered; *pbs-2*, four bases altered) (Fig. 3). Since the PBS lies within the Ty5 coding region, bases were changed that did not affect the derived amino acid sequence in these mutants. Quantitative transposition assays were carried out in a strain with a wild-type, plasmid-borne *IMT* gene. For both PBS mutants, transposition frequencies were at least ~800-fold lower compared to a wild-type Ty5 element. The most severe defect was observed for *pbs-1*, in which the 5′-most base of the putative PBS was no longer complementary to the IMT. These experiments indicate that the putative Ty5 PBS is an important *cis* element for Ty5 transposition.

Transposition is abolished by mutations in the IMT anticodon stem-loop that disrupt complementarity with the Ty5 PBS

Because the Ty5 PBS is complementary to 14 bases in the IMT anticodon stem-loop (positions 27–40), we next assayed transposition in strains with mutant *imt* genes that reduce this complementarity. Our initial experiments focused on *imt* mutants with base changes near

Ty5 PBS mutants	Transposition frequencies ($X10^{-4}$)	Fold decrease relative to wild type Ty5
GGUUAUGAGCCCUG WT PBS CCAAUACUCGGGAC WT *IMT4* 40 27	2.70 ± 0.98	1
AGUAAUGUCACCUG *pbs-1* CCAAUACUCGGGAC WT *IMT4* 40 27	0.01 ± 0.00	3150
GGUAAUGUCACCUG *pbs-2* CCAAUACUCGGGAC WT *IMT4* 40 27	0.03 ± 0.02	794

FIGURE 3. The effect of Ty5 PBS mutations on transposition. Base pairings between the IMT and the wild-type PBS, *pbs-1*, and *pbs-2* are shown. Transposition frequencies are calculated as described in Materials and Methods.

the 3′ end of the putative Ty5 primer, including two mutants that had previously been characterized (*imt4-U31,U39* and *imt4-A29,U41,U31,U39*) (von Pawel-Rammingen et al., 1992; Keeney et al., 1995; Fig. 4). Positions 29, 31, and 39 are predicted to base pair with the Ty5 PBS, and because position 39 is the penultimate 3′-base of the putative primer, we predicted that disruption of base pairing at this position would impair DNA synthesis. Because the above two *imt* mutants have a C-to-T transition at position 39 and could potentially form a G-U pair with the Ty5 PBS (Fig. 4), two additional *imt* mutants (*imt4-U31,A39* and *imt4-C31,G39*) were made that disrupted this G-U pair. All four mutant tRNA genes supported translation and were expressed at levels similar to the wild-type *IMT* genes (von Pawel-Rammingen et al., 1992; data not shown).

Ty5-containing plasmids were introduced into the four strains with the mutant *imt* genes, and transposition frequencies were determined (Fig. 4). For *imt4-U31,U39*, transposition dropped threefold relative to wild-type. Further destabilizing primer/PBS complementarity with a mutation at position 29 (*imt4-A29,U41,U31,U39*) caused transposition to drop 19-fold. Because both *imt4-U31,U39* and *imt4-A29,U41,U31,U39* can form a G-U base pair at position 39 that may stabilize the primer/template complex, the role of G-U pairing was directly tested by changing U39 to either A39 (*imt4-U31,A39*) or G39 (*imt4-C31,G39*). In strains carrying these tRNA genes, transposition dropped more than 200-fold and 500-fold, respectively. All four of the mutant tRNA genes were expressed at levels comparable to wild-type (von Pawel-Rammingen et al., 1992; data not shown), and so the decrease in transposition was not due to a decrease in tRNA availability. These data indicate that the IMT anticodon stem-loop is important for transposition, and transposition is particularly sensitive to mutations that disrupt base pairing at the region near the 3′ end of the putative primer.

imt4 mutants	Transposition frequencies ($X10^{-5}$)	Fold decrease relative to wild type *IMT4*
GGUUAUGAGCCCUG WT PBS CCAAUACUCGGGAC WT *IMT4* 40 27	11.30 ± 4.11	1
GGUUAUGAGCCCUG WT PBS CUAAUACUCUGGAC *imt4-U31,U39* 40 27	3.76 ± 0.60	3
GGUUAUGAGCCCUG WT PBS CUAAUACUCUGAAC *imt4-A29,U31,U39* 40 27	0.61 ± 0.78	19
GGUUAUGAGCCCUG WT PBS CAAAUACUCUGGAC *imt4-U31,A39* 40 27	0.05 ± 0.01	226
GGUUAUGAGCCCUG WT PBS CGAAUACUCCGGAC *imt4-C31,G39* 40 27	0.02 ± 0.01	565

FIGURE 4. The effect of IMT mutations on Ty5 transposition. Base pairings between the Ty5 PBS and the wild-type and mutant IMTs are shown. Also shown are the overall and the relative transposition frequencies.

Restoring complementarity between the PBS and the IMT restores Ty5 transposition

We next tested whether the transposition defect caused by a mutant *imt* could be suppressed by restoring base pairing between the IMT and the putative PBS. We focused on mutant *imt4-C31,G39*, because it had the most severe effect on transposition. Two PBS mutants were made that were complementary to *imt4-C31,G39* at either position 39 (*pbs-4*) or at positions 39 and 31 (*pbs-3*) (Fig. 5A). Because the PBS lies within the Ty5 coding region, these changes alter the derived amino acid sequences: *pbs-4* has a Val-to-Leu substitution at amino acid 14, and *pbs-3* has a Val-to-Leu change at position 14 and a Ser-to-Arg change at position 16. Plasmids carrying Ty5 with either wild-type or mutant PBSs were tested in strains with either a wild-type *IMT* or *imt4-C31,G39*. Consistent with our previous observations, mutations in either the PBS or the IMT largely abolished transposition (Fig. 5B). However, in the strains carrying *imt4-C31,G39*, transposition was restored to almost wild-type levels for *pbs-4* and to some extent for *pbs-3*. The difference in the extent of restoration was probably due to the type and number of changes in the Ty5 amino acid sequence, which may affect Gag protein function. The single Val-Leu substitution in *pbs-4* is conservative and less likely to compromise protein func-

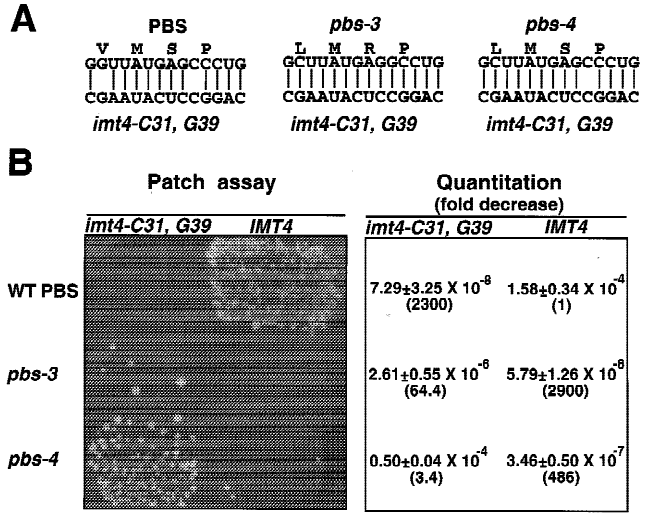

FIGURE 5. Complementarity between the IMT and the Ty5 is essential for Ty5 transposition. **A**: Base pairings between Ty5 PBSs (wild-type, *pbs-3*, and *pbs-4*) and the IMTs (wild-type and *imt4-C31,G39*) are shown. **B**: Transposition assay results for the strains with different combinations of the Ty5 elements and *IMT* genes. The numbers in parentheses indicate fold decrease compared to the strain with a wild-type Ty5 and *IMT4*.

tion compared to the two changes in *pbs-3*. Alternatively, the observed differences in transposition could be due to changes in interactions with other proteins such as reverse transcriptase, which may be sensitive to the nucleotide sequence of the paired primer/template. Nonetheless, the extensive restoration of transposition conferred by *pbs-4* indicates that base pairing between the tRNA and the Ty5 PBS is essential for transposition.

The effect of mutations in the anticodon stem-loop on Ty5 transposition

To identify tRNA residues important for Ty5 transposition, we tested other *imt* genes with mutations throughout the anticodon stem-loop for their affect on transposition. Six of these mutants could support translation (Table 1): *imt4-C33* and *imt4-U38* had no significant effect on transposition, and *imt4-U41*, *imt4-A29,U41*, *imt4-C29,G41*, and *imt4-U29,A41* had less than a fourfold effect on transposition, despite the fact that position 41 is the putative cleavage site. Because many *imt* genes with anticodon stem-loop mutations had translation defects, a modified assay was developed (Fig. 2B). This assay used two plasmid-borne *imt* genes: *imt4-C31,G39* supports translation but cannot support transposition, and this *imt* gene was cloned into a Ty5-containing plasmid; a second *imt* mutant that cannot support translation was introduced to test its effect on transposition. The mutant *imt* genes that failed to support translation were previously found to support Ty1 transposition (Keeney et al., 1995). We also found that their expression levels approximate that of the wild-type *IMT4*, indicating that they are available to serve as primers (data not shown). Using our modified assay, mutant *imt* genes with translation defects were found, in general, to have a more severe effect on transposition when the altered base was close to the 3′ end of the tRNA primer (Table 1).

Mutations in regions other than the anticodon stem-loop have no effect on Ty5 transposition

To initiate reverse transcription, the primer tRNA needs to be packaged into virus or virus-like particles, loaded onto the template mRNA, and, in the case of Ty5, it may be processed by cleavage. We next looked at mutations in other regions of the IMT to determine whether steps other than primer annealing may be important for Ty5 transposition (Table 2). Most of the mutants analyzed were made previously to study features that distinguish the IMT from the elongator methionine tRNA (EMT) and to identify residues important for Ty1 transposition (von Pawel-Rammingen et al., 1992; Keeney et al., 1995). All mutants support translation with the exception of *imt4-U54 C60*.

TABLE 1. The effect of *imt* anticodon stem-loop mutations on Ty5 transposition.

Plasmids	Translation	Transposition	IMT
pKC35	+	1.00	*IMT4*
pNK540	+	0.53	*imt4-C33*
pKC74	+	0.64	*imt4-U38*
pNK493	+	1.32	*imt4-U41*
pNK496	+	0.64	*imt4-A29,U41*
pNK547	+	0.27	*imt4-C29,G41*
pNK548	+	0.41	*imt4-U29,A41*
pKC77	−	0.15[a]	*imt4-G32*
pKC81	−	0.08[a]	*imt4-G34*
pKC80	−	0.32[a]	*imt4-C35*
pKC75	−	0.05[a]	*imt4-C36*
pKC78	−	0.03[a]	*imt4-U37*
pKC79	−	0.02[a]	*imt-ΔA38*

[a]Transposition frequency was calculated by the two-*IMT* assay shown in Figure 2B. All mutant *imt* genes tested are stable and support Ty1 transposition (Keeney et al., 1995).

Mutations in the acceptor stem had little effect on Ty5 transposition. *imt4-9*, which has nine mutations in the acceptor stem and reduces Ty1 and Ty3 transposition frequency more than 100-fold (Chapman et al., 1992; Keeney et al., 1995), supports Ty5 transposition to approximately wild-type levels (Table 2). Two mutations in the D arm were also tested: *imt4-+A17* has an A inserted at position 17 that enlarges the D loop by 1 nt; *imt4-U12,A23* has a G–C-to-U–A base pair change in the D stem, which is found in the *EMT* gene. Neither of the D stem-loop mutations affected transposition significantly. Several residues in the TΨC arm have been implicated in distinguishing the IMT from the EMT, and some are critical for priming Ty1 reverse transcription (von Pawel-Rammingen et al., 1992; Astrom & Bystrom, 1994; Keeney et al., 1995). For example, A54 and A60 are conserved among all cytoplasmic IMTs. A64 (of the

TABLE 2. The effect of *IMT* mutations in regions other than the anticodon stem-loop on Ty5 transposition.

Plasmids	Translation	Transposition	IMT
pKC35	+	1.00	*IMT4*
pKC10	+	0.74	*imt4-9*
pNK494	+	0.77	*imt4-U12, A23*
pIMT116	+	0.66	*imt4-+A17*
pIMT123	+	1.65	*imt4-U64,A50*
pVIT83	+	0.65	*imt4-C64,G50*
pNK495	+	0.90	*imt4-A62,U52*
pIMT118	+	1.44	*imt4-C54*
pIMT118a	+	1.41	*imt4-G54*
pIMT120	−	0.62[a]	*imt4-C60,U54*
pIMT121	+	1.02	*imt4-U60,C54*
pIMT119	+	0.48	*imt4-C60*
pIMT119a	+	1.15	*imt4-G60*

[a]Transposition frequency was calculated by the two-*IMT* assay shown in Figure 2B.

U50/A64 pair) is ribosylated at the 2′ position, a modification unique to IMTs from plants and fungi. This modification has been shown to be critical in preventing the IMT from being used in elongation both in vivo and in vitro (Kiesewetter et al., 1990; Astrom & Bystrom, 1994). We tested mutations at these and other positions in the TΨC arm, including mutations at position 52 and 62, which we generated by changing the G-C base pair to a U-A pair (*imt4-U52,A62*). The transposition frequencies for all mutants tested ranged from 0.48 to 1.65 (compared to 1.0 for wild-type). It appears, therefore, that of the mutations we tested, those in the anticodon stem-loop have the most significant effect on Ty5 transposition.

The effect of heterologous *IMT* genes on Ty5 transposition

Because *Arabidopsis thaliana* and *S. pombe IMT* genes can support translation in *S. cerevisiae*, we assayed their effects on Ty5 transposition (Fig. 6; Keeney et al., 1995). Seven- and tenfold lower transposition frequencies were observed for these heterologous tRNAs, respectively (Table 3), despite the fact that these tRNAs are expressed at near wild-type levels (Keeney et al., 1995; data not shown). To identify the regions responsible for the decreases, we changed their anticodon stem-loops or their acceptor stems to match the sequence of the *S. cerevisiae* IMT. The acceptor stem changes did not significantly affect transposition (Table 3). The anticodon stem-loop changes, however, restored transposition to some extent (from tenfold to fivefold lower for the *S. pombe IMT*; from seven- to twofold lower for the *A. thaliana IMT*). This again indicates that the anticodon stem-loop sequences are the most important determinants in Ty5 priming, and base changes elsewhere among the heterologous IMTs have at most a fivefold effect on transposition.

DISCUSSION

During retroelement replication, a tRNA is typically used to prime reverse transcription. Priming involves multiple steps: the tRNA is first packaged into virus or virus-like particles, then loaded onto the messenger RNA, and finally reverse transcriptase initiates cDNA synthesis (Voytas & Boeke, 1993). Each step may involve multiple element or host-encoded proteins. *Saccharomyces cerevisiae* provides an attractive system to dissect retroelement priming mechanisms. Transposition assays have been developed for the yeast Ty1, Ty3, and Ty5 retrotransposons (Boeke et al., 1985; Hansen et al., 1988; Zou et al., 1996). In addition, the gene that encodes the IMT can be genetically manipulated to identify residues important in priming (Bystrom & Fink, 1989; Keeney et al., 1995). We have taken advantage of this system to investigate the mechanism by which the Ty5 element initiates cDNA synthesis.

The Ty5 PBS is complementary to 14 bases within the anticodon stem-loop of the initiator methionine tRNA (positions 27–40) (Voytas & Boeke, 1992). Our initial experiments focused on testing the effect of mutations in either the Ty5 PBS or the IMT anticodon stem-loop that disrupt the complementarity between these two RNAs. We observed at least an 800-fold decrease in transposition frequencies for strains carrying a PBS with four mismatched bases. A range of transposition defects was observed for *imt* mutants that disrupt the G-C base pair at position 39, the penultimate 3′ base in the primer. When position 39 was changed to either a G-A pair (*imt4-U31,A39*) or a G-G pair (*imt4-C31,G39*), transposition decreased at least 200-fold. However, for *imt4-U31,U39* and *imt4-A29,U41,U31,U39*, which can form a G-U base pair with the Ty5 PBS at position 39, transposition frequency was only three- and 19-fold lower, respectively (in the latter case, the base pairing was further destabilized by a C-A mismatch). A G-U pair between the Ty1 PBS and IMT was previously shown to have little effect on Ty1 transposition (Keeney et al., 1995).

Base pairing between the Ty5 PBS and the IMT anticodon sequences is essential for Ty5 transposition. Two Ty5 PBS mutants were made (*pbs-3* and *pbs-4*) that allow for base pairing with *imt4-C31,G39* at either position 39 or at both positions 31 and 39. Individual mutations in either the IMT or PBS had dramatic effects on Ty5 transposition, causing at least a 486-fold decrease. Restoring the base pairing, however, by combining the *imt* and *pbs* mutants resulted in transposition frequencies of only 3.36- and 64.4-fold lower for *pbs-4* and

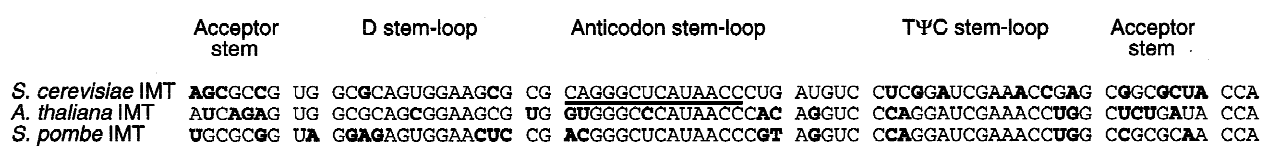

FIGURE 6. The alignment of the *S. cerevisiae*, the *A. thaliana*, and the *S. pombe* initiator methionine tRNA sequences. The underlined sequences indicate the region that base pairs with the Ty5 PBS. The bold sequences in the *S. cerevisiae* IMT indicate the residues that were changed and tested in this study (Table 2). The bold sequences in the other IMTs indicate the residues that differ from the *S. cerevisiae* IMT; bold sequences within the anticodon stem-loop were changed to match the *S. cerevisiae* sequence and tested for their effect on transposition (Table 3).

TABLE 3. The effect of heterologous *IMT* genes on Ty5 transposition.

Plasmids	Translation	Transposition	IMT
pKC35	+	1.00	*IMT4*
pNK514	+	0.10	*S. pombe IMT*
pNK515	+	0.18	*S. pombe IMT* with *S. cerevisiae* anticodon stem-loop
pJK258	+	0.10	*S. pombe IMT* with *S. cerevisiae* acceptor stem
pNK518	+	0.14	*A. thaliana IMT*
pNK519	+	0.60	*A. thaliana IMT* with *S. cerevisiae* anticodon stem-loop
pJK244	+	0.10	*A. thaliana IMT* with *S. cerevisiae* acceptor stem

pbs-3, respectively. Because the Ty5 PBS lies within the coding region of Ty5, the residual transposition defect observed for the *pbs-3, imt4-C31,G39* combination is likely due to the changes in the amino acid sequences caused by the PBS mutation. Whereas *pbs-4* has a relatively conserved Val-to-Leu change, *pbs-3* has both this change and a Ser-to-Arg change. A second possibility is that the defect in the position 39 mutations results from failure to efficiently cleave the tRNA to produce the primer. Alternatively, the sequence of the primer/template pair may be important by facilitating the interaction with factors involved in replication such as reverse transcriptase. At this time we cannot discriminate among these possibilities.

We have demonstrated that base pairing between the IMT anticodon stem-loop and the Ty5 PBS is required for transposition, yet several questions regarding the mechanism of priming remain to be answered. For example, we cannot distinguish whether priming is initiated from a 2′ OH at position 40 or from a 3′ OH at position 40 after a cleavage between position 40 and 41. We also do not know whether the cleavage event occurs specifically between position 40 and 41 or whether the tRNA is digested from the 3′ end by an exonuclease. Distinguishing among these possibilities is the goal of ongoing investigations.

Several closely related elements from diverse organisms appear to use the same region of the initiator tRNA as primer, suggesting that half-tRNA priming is a conserved mechanism for initiating reverse transcription (Kikuchi et al., 1986; Fourcade-Peronnet et al., 1988; McCurrach et al., 1990; Rothnie et al., 1991; Lindauer et al., 1993). Among these elements, *copia* of *D. melanogaster* has been studied most extensively. *Copia* uses the identical region of the anticodon stem-loop as a primer, and sequence analysis of the initial product of reverse transcription (strong stop DNA) has indicated that a tRNA fragment is the *bona fide copia* primer (Kikuchi et al., 1986). There is in vitro evidence that the catalytic RNA of RNase P can cut IMTs between positions 39 and 40, as well as at other positions, suggesting that overprocessing by RNase P produces the *copia* primer (Kikuchi et al., 1990; Kikuchi & Sasaki, 1992). Priming of the *S. pombe* Tf1 element also involves cleavage of its primer, and this cleavage requires Tf1 RNase H (Levin, 1996). An RNase H-dependent scission event could generate the Ty5 primer.

For those IMT mutations that do not support translation, a modified assay was developed to test their effect on transposition. For this assay, *imt4-C31,G39*, which supports translation but not transposition, was cloned into a Ty5-containing plasmid. A second tRNA gene that cannot support translation was introduced on a high-copy plasmid and tested for its effect on transposition. Using both our original and this modified assay, we tested mutations in residues throughout the IMT, none of which significantly altered tRNA expression or stability. The anticodon stem-loop mutations in *imt4-U41*, *imt4-A29,U41*, *imt4-C29,G41*, and *imt4-U29,A41* had at most a fourfold effect on transposition. It is somewhat surprising that mutations at position 41 had only a slight effect when changed to all three other nucleotides, even though this position is at the putative cleavage site. Other anticodon mutations generally had a greater effect on transposition when the mutations were close to the 3′ end of the primer. For example, *imt4-ΔA38* and *imt4-U37* affected transposition frequencies dramatically (0.02), whereas *imt4-G32* and *imt4-C35* had more modest effects (0.15 and 0.32, respectively). An exception to this trend is *imt4-U38*, which had a minimal effect on transposition (0.64), even though it is near the 3′ end of the primer. *imt4-U38* may form a U-U base pair between the PBS and tRNA; U-U pairs have been observed in other RNAs (Gutell et al., 1993; Cech et al., 1994; Gutell, 1994). However, *imt4-U37* (0.02) should also be able to form a U-U pair, suggesting that if U-U pairing occurs, it may be context dependent. Alternatively, because A37 is the only modified base in the anticodon stem-loop (Basavappa & Sigler, 1991), the U37 mutation may affect transposition indirectly (i.e., not through base pairing). Taken together, our results suggest that the activity that cleaves the tRNA is not highly sequence specific.

In contrast, none of the 12 mutants with mutations outside the IMT anticodon region affected transposition more than twofold. The altered bases include nine residues in the acceptor stem (positions 1, 2, 3, 6, 67, 70, 71, 72, and 73), six in the TΨC arm (positions 50, 52, 54, 60, 62, and 64) and three in the D-arm (12, 17, and 23) (Fig. 6). This suggests that only the anticodon stem-

loop region is essential for transposition. The lack of importance for bases outside of the region of PBS complementarity is also supported by our experiments with heterologous tRNAs. The *S. pombe* and *A. thaliana* IMT each differ from the *S. cerevisiae* IMT at 20 positions and therefore they are useful as probes to determine globally which regions or residues of the IMT are important for priming (Fig. 6). Relative transposition frequencies for these IMTs, however, were 0.10 for the *S. pombe* and 0.14 for the *A. thaliana* IMT. Among the bases that differ are positions within the anticodon stem-loop (two for *S. pombe* and three for *A. thaliana*). When these anticodon sequences were changed to match the *S. cerevisiae* IMT, the hybrid IMTs supported transposition at relative frequencies of 0.18 for the *S. pombe* hybrid IMT and 0.60 for the *A. thaliana* hybrid IMT. Therefore, when the anticodon sequences can base pair with the Ty5 PBS, other differences in the heterologous IMT have at most a fivefold effect on Ty5 transposition.

Although IMT anticodon stem-loop sequences are necessary for priming, they are not sufficient. For example, we have found that a hybrid elongator methionine tRNA that carries the IMT anticodon stem-loop cannot support Ty5 transposition (data not shown). What are the other structural features of the IMT that are important for transposition? Ty1 and Ty3 mRNAs pair with regions other than the 3′ acceptor stem sequences, and this interaction helps stabilize the primer/template complex (Keeney et al., 1995; Friant et al., 1998; Gabus et al., 1998). Multiple interactions between element mRNAs and their primers occur among other retroelements, including Tf1 and Rous sarcoma virus (Aiyar et al., 1992; Lin & Levin, 1997). Ty5 mRNA is also complementary to other regions within the IMT: nine bases within the D-arm and eight bases within the TΨC stem are complementary to sequences within the Ty5 coding region (GAG) and U3, respectively (data not shown). Some of the mutant tRNAs tested disrupt this complementarity at a single base without consequences on transposition. It may be that more extensive disruption of pairing is required before a phenotype can be observed. Interactions between the Ty5 mRNA with other regions of the IMT are currently being tested more rigorously. We hope that a comprehensive understanding of tRNA bases required for priming, coupled with biochemical assays for each step in the priming reaction, will ultimately enable us to obtain a better understanding of half-tRNA priming mechanisms.

MATERIALS AND METHODS

DNA plasmids

Many of the *imt4* mutants were previously used to identify residues important for translation and for Ty1 transposition (von Pawel-Rammingen et al., 1992; Keeney et al., 1995). These include pKC35 (*IMT4*), pIMT116 (*imt4-+A17*), pKC74 (*imt4-U38*), pIMT114 (*imt4-U31,U39*), pIMT115 (*imt4-A29,U41,U31,U39*), pKC75 (*imt4-C36*), pKC76 (*imt4-A33*), pKC77 (*imt4-G32*), pKC81 (*imt4-G34*), pKC79 (*imt4-ΔA38*), pKC78 (*imt4-U37*), pKC80 (*imt4-C35*), pIMT118 (*imt4-C54*), pIMT119a (*imt4-G60*), pIMT118a (*imt4-G54*), pIMT119 (*imt4-C60*), pIMT120 (*imt4-C60,U54*), pIMT121 (*imt4-U60,C54*), pIMT123 (*imt4-U64,A50*), pVIT83 (*imt4-C64,G50*), pJK258 (*S. pombe* IMT with *S. cerevisiae* IMT acceptor stem), and pJK244 (*A. thaliana* IMT with *S. cerevisiae* IMT acceptor stem). Other *imt4* mutants in this study were made by a two-step PCR-based mutagenesis method with wild-type *IMT4* as a template (Chen & Przybyla, 1994): pNK494 contains *imt4-U12,A23* (made with DVO490, 5′-CGCCGTGGCTCAGTGG AAGAGCGCAGGGC-3′); pNK344 contains *imt4-U31,A39* (made with DVO291, 5′-GGACATCAGGTTTATGAGACCT GCGCGCT-3′); pNK346 contains *imt4-C31,G39* (made with DVO280, 5′-ACATCAGGCTTATGAGGCCTGCGCG-3′); pNK493 contains *imt4-U41* (made with DVO489, 5′-CTCAT AACCTTGATGTCC-3′); pNK496 contains *imt4-U41,A29* (made with DVO488, 5′-GAAGCGCGCAAGGCTCATAACC TTGATGTCC-3′); pNK547 contains *imt4-C29,G41* (made with DVO725, 5′-GGAAGCGCGCACGGCTCATAACCGTGATGT CCTCG-3′); pNK548 contains *imt4-U29,A41* (made with DVO726, 5′-GGAAGCGCGCATGGCTCATAACCATGATGT CCTCG-3′); pNK540 contains *imt4-C33* (made with DVO708, 5′-GCGCAGGGCCCATAACCCTGATG-3′); and pNK495 contains *imt4-U52,A62* (made with DVO491, 5′-GATGTCCTCT GATCGAAACAGAGCGGCGC-3′).

The plasmids containing heterologous *IMT* genes were constructed as follows. pDV111 carries the *S. pombe IMT* gene on a 1.3-kb *Hin*dIII fragment in YEp351. The 237-bp *IMT*-containing *Dra*I fragment of pDV111 was cloned into the *Eco*RV site of pBluescript (Stratagene) to generate pNK507. The *S. pombe IMT* was then cloned into YEp351 using *Bam*HI and *Hin*dIII to generate pNK514. For pNK515, the anticodon sequence of the *S. pombe IMT* was changed to the corresponding sequences of *S. cerevisiae IMT* by a PCR-based site-directed mutagenesis strategy using primer DVO569 (5′-GGAACTCCGCAGGGCTCATAACCCTGAGGTCCCAG-3′) (Chen & Przybyla, 1994). pSZ1 contains the *A. thaliana IMT* in YEp351. A 600-bp *Hin*dIII-*Sma*I from pSZ1 was cloned into YEp351 to generate pNK518. pNK519 was generated by changing the *S. pombe* anticodon sequences to match the corresponding *S. cerevisiae IMT* sequences using DVO568 (5′-GGAAGCGTGCAGGGCTCATAACCCTGAGGTCCCAG-3′).

Ty5 PBS mutants were also made by PCR-based site-directed mutagenesis (Chen & Przybyla, 1994). pLG1 contains the wild-type *GAL*-Ty5*his3*AI in pRS426; it has *GAL1-10* upstream activation sequences (UASs) fused in front of Ty5 5′ long terminal repeat (LTR) and a *his3*AI marker inserted between the end of the Ty5 open reading frame and the 3′ LTR (Zou et al., 1996). pLG2 contains *pbs-1*, a Ty5 element with five mutations in the PBS that was constructed using primer DVO285 (5′-ACTACGTCAACAAGTAATGTCACCTG AGAGCAAT-3′). pLG3 contains *pbs-2*, a Ty5 element with four mutations in the PBS that was constructed using primer DVO286 (5′-ACGTCAACAGGTAATGTCACCTGAGAGCAAT-3′). pLG2 and pLG3 were constructed using pLG1 as a template. pNK488 contains *pbs-3*, which restores base pairing with *imt4-C31,G39* at position 39, and was constructed using

primer DVO436 (5′-ACGTCAACAGCTTATGAGCCCTG-3′). pNK469 contains *pbs-4*, which restores the base pairing with *imt4-C31,G39* at both positions 31 and 39, and was constructed using primer DVO435 (5′-ACGTCAACAGCTTATG AGGCCTGAGAGCAATG-3′). pNK488 and pNK469 were constructed using as a template pNK254, which carries the *GAL*-Ty5*his3*AI on pRS416 (Ke & Voytas, 1997).

Plasmids used in the two-*IMT* assay were constructed as follows. *imt4-C31,G39* was cloned into the Ty5-containing plasmid pNK254 by first cloning a *Bam*HI and *Hin*dIII fragment from pNK346 into the corresponding sites in pBluescript; this generated pNL2. PCR-based mutagenesis was carried out (using primer DVO474, 5′-TCCCCGCGGGACG GTATCGATAAGCTT-3′) to create *Sac*II restriction sites flanking *imt4-C31,G39* (Chen & Przybyla, 1994). The resultant *Sac*II fragment was cloned into pNK254 to generate pNK502.

Transposition assays

All strains used in this study are isogenic derivatives of JKc543 (*MATα ura3-52 trp1Δ1 leu2-3,112 his3Δ200 ade2-BglII imt1-imt4::TRP1/Y*Ep351-*IMT2*). To facilitate plasmid shuffling, pKC1, which carries *IMT3* on a *URA3*-based plasmid, was introduced into JKc543. The preexisting, plasmid-borne *IMT2* gene (YEp351-*IMT2*; Keeney et al., 1995) was lost by selecting colonies that grew on synthetic complete media lacking uracil (SC-U) but not on SC-L media; this generated YNK611. YNK611 was transformed with *Bam*HI-digested pNK437, which contains *rad52::ADE2*. It was constructed by cloning the *ADE2*-containing *Bam*HI fragment from pJK204 into the *Bgl*II site of pSM20. This replaced the *LEU2* marker that interrupts *RAD52* gene in pSM20 with *ADE2*. Ade$^+$ colonies were picked and confirmed to be *rad52* by Southern blot analysis; this strain was designated YNK616 and was used for all subsequent transposition assays.

To determine the importance of PBS sequences in Ty5 transposition, the *IMT4*-containing plasmid, pKC35, was introduced into YNK616. The preexisting *IMT3* gene carried on pKC1 was lost by selecting on SC-L, 5-fluoroorotic acid media (SC-L/5-FOA) (Boeke et al., 1987). The Ty5-containing plasmids pLG1 (wild-type Ty5), pLG2 (*pbs-1*), and pLG3 (*pbs-2*) were introduced, and three Ura$^+$ colonies resulting from each transformation were picked and used for transposition assays. The cells were grown as patches on SC-U/glucose plates for 2 days before being replica-plated onto SC-U/galactose media to induce Ty5 transcription. After 3 days of induction at room temperature, the cells were replica-plated to SC-H media to select for transposition events. For quantitative assays, the cells from the SC-U/galactose plates were scraped and resuspended in ddH_2O. Serial dilutions of the resuspended cells were made and plated on either SC-U plates to determine the total cell number or on SC-H plates to determine the number of transposition events.

To test transposition in strains with IMT mutations that support translation, *imt* mutants on *LEU2*-based YEp351 were transformed into strain YNK616. The *IMT3* gene carried on pKC1 was then lost by selecting cells on SC-L/5-FOA media. Ty5 elements carried on the *URA3*-based plasmid pRS416 (pNK254, wild-type Ty5; pNK488, *pbs-3*; pNK469, *pbs-4*) were then introduced into the strains with either the wild-type or mutant *imt* genes. Three Ura$^+$ colonies were picked and used in transposition assays as described above.

For *imt* mutants that cannot support translation, the two-*IMT* assay was used. pKC35 (*IMT4*) was shuffled into YNK616. Plasmid (pNK502), which contains *imt4-C31,G39* and *GAL*-Ty5*his3*AI, was then introduced, and pKC35 (YEp351-*IMT4*) was lost by plasmid shuffling prior to introducing plasmids with mutant *imt* gene. Transposition assays were then conducted and transposition frequencies calculated as described above.

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