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A sequence immediately upstream of the plus-strand primer is essential for plus-strand DNA synthesis of the *Saccharomyces cerevisiae* Ty1 retrotransposon

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ABSTRACT

Priming of plus-strand DNA is a critical step in reverse transcription of retroviruses and retrotransposons. All retroelements use an RNase H-resistant oligoribonucleotide spanning a purine-rich sequence (the polypurine tract or PPT) to prime plus-strand DNA synthesis. Plus-strand DNA synthesis of the yeast *Saccharomyces cerevisiae* Ty1-H3 retrotransposon is initiated at two sites, PPT1 and PPT2, located at the upstream boundary of the 3′-long terminal repeat and near the middle of the *pol* gene in the integrase coding region. The two plus-strand primers have the same purine-rich sequence GGGTGGTA. This sequence is not sufficient by itself to generate a plus-strand origin since two identical sequences located upstream of PPT2 in the integrase coding region are not used efficiently as primers for plus-strand DNA synthesis. Thus, other factors must be involved in the formation of a specific plus-strand DNA primer. We show here that mutations upstream of the PPT in a highly conserved T-rich region severely alters plus-strand DNA priming of Ty1. Our results demonstrate the importance of sequences or structural elements upstream of the PPT for initiation of plus-strand DNA synthesis.

INTRODUCTION

Long terminal repeat (LTR)-containing retrotransposons are ubiquitous components of eukaryotic genomes. They transpose via the reverse transcription of an RNA intermediate and resemble eukaryotic retroviruses structurally and functionally (1–4). The yeast *Saccharomyces cerevisiae* contains five families of LTR retrotransposons, Ty1–Ty5 (3). Thirty-three members of the most abundant family Ty1 and 13 members of the closely related family Ty2 have been identified in the sequenced yeast genome (5). The Ty1 elements have a length of 5.9 kb consisting of two 334 bp LTRs that flank two open reading frames, TyA and TyB, homologous to the *gag* and *pol* genes of retroviruses. During replication, the Ty1 RNA genome is converted into double-stranded DNA by the process of reverse transcription. The two strands of the Ty1 DNA are synthesized by the retrotransposon reverse transcriptase which is able to utilize both DNA and RNA as templates. Like other DNA polymerases, Ty1 reverse transcriptase has an absolute requirement for primers to initiate DNA synthesis. A specific host tRNA, the initiator methionine tRNA, is used as a primer for minus-strand DNA synthesis of Ty1 (6–8). The plus-strand DNA primer is a purine-rich fragment of RNA, the polypurine tract (PPT), which persists after the RNase activity of reverse transcriptase has degraded most of the genomic RNA from the RNA–DNA duplex formed during minus-strand synthesis. Plus-strand DNA synthesis proceeds from this primer, using the minus-strand DNA as a template. We have previously demonstrated (9,10) that plus-strand DNA synthesis of the retrotransposon Ty1-H3 (11) is initiated at two sites located at the upstream boundary of the 3′-LTR and near the middle of the *pol* gene in the integrase coding region. The two plus-strand primers PPT1 and PPT2 have the same purine-rich sequence GGGTGGTA. However, this sequence is not sufficient by itself to generate a plus-strand origin since two identical sequences located upstream of PPT2 in the integrase coding region are not used efficiently as primers for plus-strand DNA synthesis. Thus, other factors must be involved in the formation of a specific plus-strand DNA primer. It has been suggested that the overall conformation, the secondary structure of the PPT domain or *cis*-acting sequences outside the PPT could be implicated in the formation of a functional plus-strand primer (12–17). A T-rich sequence immediately upstream of the PPT is highly conserved among diverse retroviruses and LTR retrotransposons, including Ty1 (18–20). The results of Illyinskii and Desrosiers (18) and Robson and Telesnitsky (19) suggest that mutation of this T-rich region in SIV and MoMLV causes a block in reverse transcription at a stage between minus-strand strong-stop transfer and plus-strand initiation. Noad *et al*. (20) have also shown that a pyrimidine-rich sequence 5′ to the PPT has an important role in PPT recognition in the plant pararetrovirus CaMV.

To examine the role of the T-rich sequence 5′ to the PPT in the yeast Ty1 retrotransposon we have analyzed the effect of mutations in this region on plus-strand DNA synthesis and transposition. Our results demonstrate the importance of

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sequences or structural elements upstream of the PPT for initiation of plus-strand DNA synthesis.

MATERIALS AND METHODS

Plasmids and constructs of mutants

Plasmid pJEF1105, kindly provided by J. D. Boeke, is a high copy number (2 μm) plasmid marked with *URA3* containing a Ty1-*neo* element fused to the GAL1 promoter. Site-directed mutagenesis was performed as described by Kunkel (31). A 3597 bp *Bam*HI–*Kpn*I fragment from pJEF1105 was subcloned into phagemid pSL1190 and mutagenized. Following mutagenesis, the 3597 bp *Bam*HI–*Kpn*I fragment was cloned back into pJEF1105.

Transposition assays

The transposition assay was performed as described by Chapman *et al.* (6). Yeast strain AGY9 (MAT**a** *leu2Δ1 ura3-52 trp1Δ63 his4-539 lys2-801 spt3-202*), kindly provided by J. D. Boeke, was used to prevent transcription of endogenous Ty1 elements. Transcription of GAL1 promoted elements is unaffected in the *spt3-202* background (32). Yeast strain AGY9 harboring the wild-type or mutant Ty1 element was patched onto SC-Ura plates containing 2% glucose. After 2 days of growth at 30°C, the patches were replica plated to SC-Ura plates containing 2% galactose. Following 3 days of growth at 22°C the cells were replica plated to non-selective medium YPD to allow for plasmid loss. Following 1 or 2 days of growth at 30°C the patches were replica-plated to YPD medium containing 1 mg/ml 5-FOA and incubated for 1 day at 30°C to select for cells that have lost the plasmid containing the *URA3* gene. Cell patches were scraped, plated as single cells onto YPD plus 5-FOA medium and incubated for 2 days at 30°C. Cells were finally replica plated to YPD containing 150 μg/ml G418 to identify colonies that had undergone transposition of the Ty1-*neo* element. Transposition is expressed as the number of Neo^rUra^- yeast colonies/total Ura^- colonies.

Analysis of Ty1 virus like particles (VLP) DNA

Ty1 VLP purification, extraction of DNA from VLPs, electrophoresis on agarose gels, blotting and hybridization with 5′-labeled oligomer were as described (8,9). Non-denaturing agarose gels (1%) in 50 mM Tris–borate, pH 8.3, 1 mM EDTA were used to analyze single-stranded DNA fragments. The DNA samples were denatured at 90°C in 90% formamide, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA before loading onto the gel. In these conditions the positions of the molecular weight markers indicate that single-stranded DNA fragments migrate as a function of molecular weight in the non-denaturing gel. A probe of antisense polarity complementary to positions 5697–5716 of the U3 region of Ty1-H3 (11) (5′-CCTTGCGTTTCAGCTTCCAC-3′) was hybridized to the Southern blot. After hybridization the blots were exposed to autoradiography films. The autoradiograms were quantified using a Bio-Rad Gel Doc 1000.

Reiterative primer extension

PPT1 or PPT4 use was analyzed by reiterative primer extension. Antisense primers complementary to positions 5697–5716 of Ty1-H3 downstream of PPT1 (5′-CCTTGCGTTTCAGCTTCCAC-3′) and complementary to positions 3438–3455 of Ty1-H3 downstream of PPT4 (5′-TACGAGTCTGAGTGTCTG-3′) were 5′ ^{32}P end-labeled with polynucleotide kinase and [α-^{32}P]ATP (NEN). The reaction mixture of 8 μl contained 75 mM Tris–HCl, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20, 2 mM $MgCl_2$, 10 mM dNTP, 1 μl 5′ ^{32}P end-labeled primer and 2 U *Taq* polymerase (Goldstar Eurogentec). For each reaction the same amount of DNA extracted from VLPs was used as the template. Primer extension products were generated by 30 cycles of denaturation (30 s at 95°C), annealing (30 s at 42°C) and extension (60 s at 70°C). At the end of the reaction 10% formamide was added to the reaction mixture. Products were denatured by heating at 80°C for 2 min prior to loading on an 8% polyacrylamide–8 M urea gel. Dried gels were exposed to autoradiography films.

RESULTS

A T-rich box 5′ of the functional PPT1 and PPT2 sequences

In most Ty1 and Ty2 elements two exact copies of the PPT sequence (GGGTGGTA) are found at the 5′-boundary of the 3′-LTR (PPT1) and near the center of the genome (PPT2) (21). These two sequences probably correspond to the two functional PPTs identified in the genome of the Ty1-H3 element (9–10). A 15 base long untranslated region (UTR) exists between the end of the Ty1 reverse transcriptase coding sequence and PPT1. Alignment of the 15 bp UTR sequences of all Ty1 and Ty2 elements reveals a striking conservation of a short T-rich region (T-box) immediately upstream of PPT1 (Fig. 1). Such a T-rich region can also be seen upstream of PPT2 of the Ty1 and Ty2 elements, upstream of PPT1 of the yeast retrotransposon Ty4 element (22), in the vast majority of retroviruses (18–20) and 5′ of the central PPT located within the integrase gene of many lentiviruses (23).

In most Ty1 and Ty2 elements two GGGTGGTA sequences, which we have called PPT3 and PPT4 (9), are found 5′ of the central PPT. A T-rich box is not found 5′ of these PPT sequences. According to previous Southern blot analyses of DNA extracted from Ty1 VLPs (9), these sequences do not prime for plus-strand DNA synthesis.

To assess the importance of the T-box located immediately upstream of the functional PPT1 of Ty1-H3, several mutants with T residues substituted by other nucleotides were constructed and analyzed for plus-strand priming efficiencies and transposition capacities.

Mutations in the T-box decrease plus-strand DNA synthesis and transposition

The sequences of the T-box mutants analyzed in this study are summarized in Table 1. In mutant 1, positions –9, –10, –11, –16, and –17 were mutated to disrupt the T-rich box (position –1 is defined as the nucleotide 5′ of the primer cleavage site, position +1 is the first nucleotide of plus-strand DNA). In mutants 1.1 and 1.2 the T residues distal (–16 and –17) or proximal (–9, –10 and –11) to the PPT sequence were mutated, respectively. In mutant 2, the sequences upstream and downstream of PPT were changed so that 8 nt 3′ and 10 nt 5′ of the PPT were identical to the sequence of the non-functional PPT4 of Ty1.

The effect of mutations on plus-strand priming and transposition was analyzed as described previously (Materials and

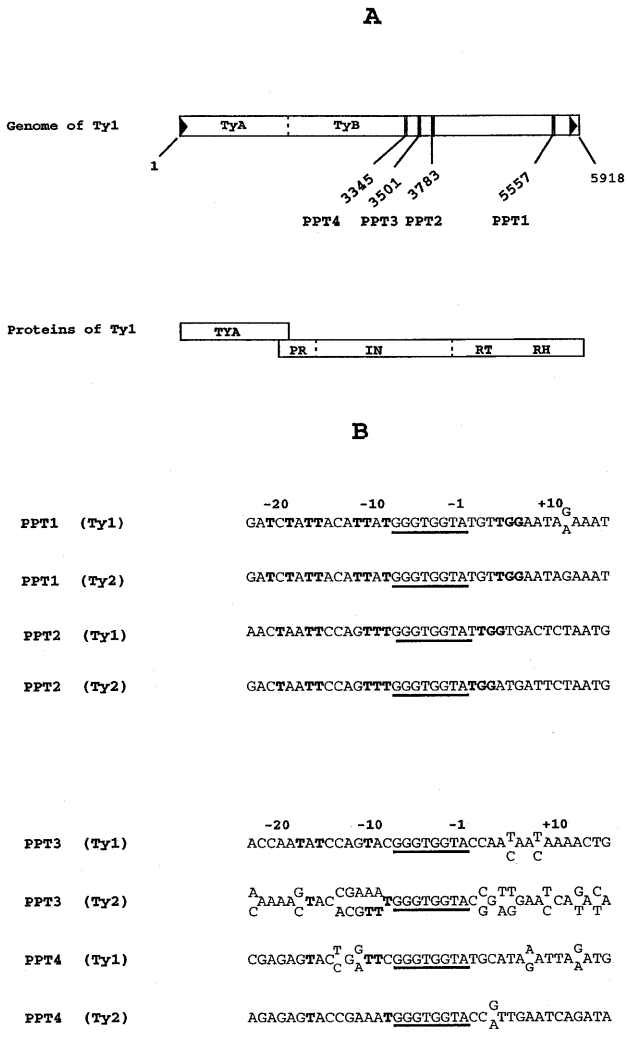

Figure 1. (**A**) Positions of the functional (PPT1 and PPT2) and non-functional (PPT3 and PPT4) polypurine tracts on the DNA genome of Ty1. The triangles are the LTR. The coding sequences TyA and TyB are analogous to the retroviral *gag* and *pol* genes. The four domains of the TYB protein have been identified: protease (PR), integrase (IN), reverse transcriptase (RT) and RNase H (RH). (**B**) Sequence comparison of the PPT regions of the Ty1 and Ty2 elements found in the yeast genome. PPT sequences are underlined. Thymidines upstream of the PPT and the TGG sequence downstream of PPT1 and PPT2 are shown in bold. The PPT1 and PPT2 regions are highly conserved in Ty1 and Ty2, with the exception of residue +12, which is G or A in PPT1 of Ty1. The PPT3 and PPT4 regions of Ty1 and Ty2 are not as well conserved. The variations in the PPT3 region of Ty2 are mainly due to one element in chromosome III.

Methods). In the wild-type element the plus-strand strong-stop DNA of 0.345 kb primed at PPT1 and a 3.0 kb fragment primed at PPT2 were revealed by Southern blot analysis of the DNA intermediates extracted from purified VLPs using a probe of antisense polarity (Fig. 2). A minor unidentified 1.0 kb fragment was also revealed by the antisense probe.

Mutations of the T-box 5′ of PPT1 had a significant effect on plus-strand strong-stop DNA priming as shown by the reduced level of the 0.345 kb band in the Southern blot (Fig. 2A). The priming activity of PPT1 in the T-box mutant elements was quantified by comparing the relative amount of the 3.0 and

Table 1. Mutations of the PPT1 region of Ty1

	-20 -10 -1 +8
WT	GATCTATTACATTATGGGTGGTATGTTGGAA
Mut1	GATCTA**AA**ACAT**CCC**GGGTGGTATGTTGGAA
Mut1.1	GATCTA**AA**ACATTATGGGTGGTATGTTGGAA
Mut1.2	GATCTATTACAT**CCC**GGGTGGTATGTTGGAA
Mut2	GATCT**GTACCGGTTC**GGGTGGTATG**CATA**AA
Mut3	GATCTATTACATTATGGGTGGTATG**CAT**GAA

The wild-type sequence is on the first line. The PPT sequences are underlined. For mutations, differences from the wild-type sequence are indicated in bold.

0.345 kb bands. Substitution of two T residues in the T-box (mutants 1.1 and 1.2) allowed a level of priming of ~20% that of the wild-type element. Substitution of four T residues (mutant 1) reduced the frequency of priming to <10% of the level of the wild-type element. An even more significant reduction in priming was seen in mutant 2. These experiments show that maximal priming activity requires all T residues in the T-box but that substitution of only two T residues is sufficient to impair synthesis of plus-strand strong-stop DNA.

Transposition frequency of T-box mutant elements was quantified by detecting the G418 resistance phenotype of plasmid-free yeast cells which had undergone transposition (Materials and Methods). The levels of wild-type and T-box mutant Ty1-*neo* element transposition are listed in Table 2. The transposition frequency was strongly reduced by substitution of the T residues distal (mutant 1.1) or proximal to the PPT1 sequence (mutant 1.2). Substitution of four T residues decreased transposition to almost the background level. These results are in line with the observation that plus-strand priming is more significantly reduced in mutant 1 than in mutant 1.1 or 1.2.

Mutations of the TGG trinucleotide 3′ of PPT1 does not affect plus-strand priming but abolishes transposition

The 3′-flanking sequence of PPT1 of all Ty1 and Ty2 elements always begins with the dinucleotide TG, which determines the U3 5′-terminus of the upstream LTR of pre-integrative DNA. This terminal TG dinucleotide is absolutely required for transpositional integration of Ty1 *in vivo* (24). Sequence comparison of the region downstream of the PPT sequences of all Ty1 and Ty2 elements reveals that a TGG trinucleotide is always found 3′ of PPT1 and PPT2 but that it is not found 3′ of PPT3 and PPT4 (Fig. 1). To test if the TGG trinucleotide is involved in plus-strand DNA synthesis and transposition, the sequence TGTTGGAA immediately 3′ of PPT1 of Ty1-H3 was changed to TGCATGAA (mutant 3, Table 1). We observed no effect of this mutation on plus-strand priming (Fig. 2B). This observation is consistent with the *in vitro* results of Rattray and Champoux (25–27) showing that sequences dowstream of the PPT have no detectable effect on priming specificity. In contrast, transposition of the mutant element was decreased to the background level (Table 2). Therefore, integration of the Ty1 DNA into the host genome must be affected at a stage after plus-strand priming. As mentioned above, the sequence of plus-strand

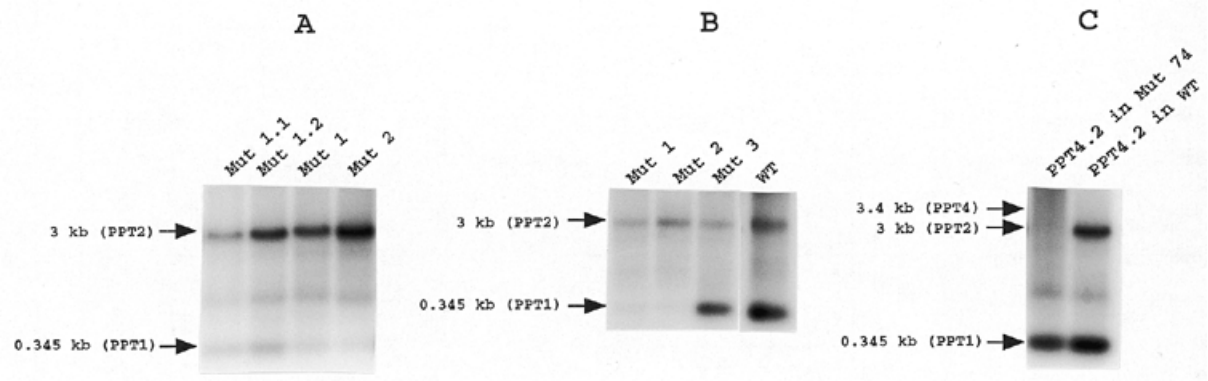

Figure 2. Analysis of plus-strand DNA in mutant Ty1 VLPs. Plus-strand DNA extracted from VLPs was separated by electrophoresis on 1% agarose gels, blotted and detected using a radiolabeled antisense probe. Plus-strand strong-stop DNA initiated at PPT1 has a length of 0.345 kb. Plus-strand DNA initiated at PPT2 has a length of 3.0 kb. A minor unidentified 1.0 kb fragment is also seen on the blot. (**A**) Plus-strand DNA in T-box mutant VLPs: the sequences of mutants 1, 1.1, 1.2 and 2 are shown in Table 1. (**B**) Plus-strand DNA in mutant 3 with a substitution in the TGG trinucleotide 3′ of PPT1 (Table 1). Plus-strand DNA in mutants 1 and 2 and in a wild-type element is also shown. (**C**) Plus-strand DNA in PPT4 mutants: the PPT4.2 mutation shown in Table 3 was introduced into a wild-type element (PPT4.2 in WT) or a PPT2 mutant element (PPT4.2 in Mut 74). No 3.4 kb fragment corresponding to plus-strand initiated at PPT4 can be detected on the Southern blot. An arrow indicates where the 3.4 kb band should run.

Table 2. Transposition of wild-type and PPT1 mutant elements

		Neo^{r}Ura$^-$colonies/Ura$^-$colonies		Transposition frequency
		Exp.1	Exp. 2	
WT	PPT1	137/445	364/1200	30,4%
Mut 1	PPT1	3/764	13/990	0,91%
Mut 1.1	PPT1	29/1000	72/1118	4,76%
Mut 1.2	PPT1	15/695	79/1618	4,06%
Mut 2	PPT1	0/536	2/1248	0,11%
Mut 3	PPT1	4/764	11/726	1,00%

Two experiments were carried out with independent transformants.

strong-stop DNA determines the sequence of the upstream LTR, which contains *cis*-acting sequences required for integration of the pre-integrative DNA into the host cell DNA. Early studies with Ty1 and retroviruses showed that the region in U3 required for integration lies within 12 bp of the 5′-terminus (28,29). The mutation described here identifies a determinant of Ty1 integration within 5 bp of the 5′-terminus of the reverse transcript. Mutation of this determinant likely creates an unusable *att* site which does not allow recognition of the upstream LTR of the pre-integrative DNA by the Ty1 integrase.

Initiation of plus-strand synthesis cannot be restored by creating a T-box 5′ of PPT4

The importance of the T-rich flanking sequence in the priming function of the PPT sequence could explain the failure of PPT3 and PPT4 to prime plus-strand DNA synthesis. We wanted to determine whether priming activity could be restored at one of these ectopic genome sites by introducing mutations creating a T-rich region 5′ of PPT4. Two mutants (PPT4.1 and PPT4.2) including –9 to –18 and –9 to –38 substitutions were constructed so that the sequence upstream of PPT4 was similar to the sequence upstream of PPT2 (Table 3). Since PPT4 is only 430 bases upstream of PPT2 (Fig. 1) we introduced the PPT4 mutations into a mutant element (mutant 74 in ref. 9) which does not allow priming of plus-strand synthesis from PPT2, in order to avoid competition between the two adjacent primers. The PPT4 mutations were also introduced into a wild-type element. The functionality of the mutated PPT4 was then tested by Southern blotting. As shown in Figure 2C, no 3.4 kb fragment corresponding to plus-strand initiated at PPT4 could be detected on the Southern blot. When a more sensitive assay was used to analyze plus-strand DNA synthesis, a low level of

Table 3. Mutations in the PPT4 region of Ty1

	-50 -40 -30 -20 -10 -1 +10
WT PPT4	GATCTAGCACCCCCCAAATTTCCAATATCGAGAGTACCGGTTCGGGTGGTATGCATAAATT
Mut PPT4.1	GATCTAGCACCCCCCAAATTTCCAATATCGAGA**ATT**CC**A**GTTT**T**GGGTGGTAT**TGG**TAAATT
Mut PPT4.2	GATCTAGCACCCC**GATAAA**TTC**TCGTCAAACTAATT**CC**A**GTTT**T**GGGTGGTAT**TGG**TAAATT
WT PPT2	AAGAACTCCCACCGATAAATTCTCGTCAAACTAATTCCAGTTTGGGTGGTATTGGTGACTC

Two mutants (PPT4.1 and PPT4.2) including –9 to –18 and –9 to –38 substitutions were constructed so that the sequence upstream of PPT4 was similar to the sequence upstream of PPT2. The wild-type PPT4 region is on the first line. The wild-type PPT2 region is on the bottom line. The PPT sequences are underlined. For the two mutants, differences from the wild-type PPT4 region are indicated in bold.

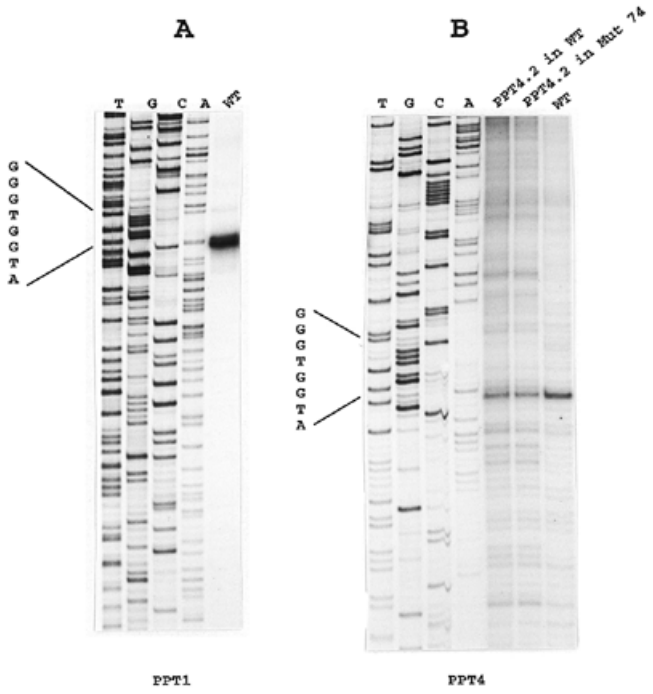

Figure 3. Primer extension analysis of PPT use. Assay conditions and primers are as described in Materials and Methods. (**A**) Products primed at PPT1 in wild-type elements (lane WT). The location of the PPT1 sequence is indicated alongside the plus-strand sequence lanes (TGCA). Plus-strand sequence was determined with the primer used in the primer extension assay. (**B**) Products primed at PPT4 in the control wild-type element (lane WT) and in two PPT4 mutant elements: PPT4.2 in WT (the PPT4.2 mutation shown in Table 3 was introduced into a wild-type element) and PPT4.2 in Mut 74 (the PPT4.2 mutation was introduced into a PPT2 mutant element, mutant 74 in ref. 9, which does not allow priming of plus-strand synthesis from PPT2). The location of the PPT4 sequence is indicated alongside the plus-strand sequence lanes (TGCA). Plus-strand sequence was determined with the primer used in the primer extension assay.

priming from PPT4 in the wild-type element was detected. In this assay, use of PPT is represented by a band on a polyacrylamide gel corresponding to the primer extension product of an end-radiolabeled antisense primer. A weak band corresponding to plus-strand initiated at PPT4 in the wild-type element is detected on the gel shown in Figure 3 (compare the intensity of the bands initiated at PPT4 in Fig. 3B, lanes PPT4.2 in WT, PPT4.2 in Mut 74 and WT, to the band obtained with a primer allowing detection of priming at PPT1 in Fig. 3A, lane WT). The mutation creating a T-rich region 5′ of PPT4 had no effect on the level of priming at PPT4: the intensities of the bands initiated at the mutant and wild-type PPT4 are similar (lanes PPT4.2 in WT, PPT4.2 in Mut 74 and WT in Fig. 3B). This result suggests that other sequences around the PPT affect plus-strand priming efficiency. In other systems it has been shown that plus-strand priming can be initiated *in vivo* by insertion of the PPT at an ectopic genomic site (19,20,23,30). We do not know if the low level of priming at PPT4 in Ty1 is due to the overall conformation of the PPT4 region which inhibits priming or whether other factors are important for priming of plus-strand DNA synthesis in Ty1.

The primer extension assay used to detect plus-strand priming of the PPT also allows mapping of the site of plus-strand DNA initiation. It is worth noting that although priming efficiency of PPT4 is drastically reduced, the priming fidelity is maintained at this site, i.e. the 5′-end of the plus-strand begins with the nucleotide immediately 3′ of the A residue of the GGGTGGTA sequence (Fig. 3B).

DISCUSSION

Priming of plus-strand DNA synthesis is a critical step in reverse transcription of retroviruses and retrotransposons. The precision of plus-strand initiation from the primer located immediately upstream of U3 is particularly important because it defines the 5′-border of the upstream LTR which plays a role in integration of the retroelement pre-integrative DNA into the host cell genome. All retroelements use an RNase H-resistant oligoribonucleotide spanning a purine-rich sequence (the polypurine tract or PPT) to prime plus-strand synthesis. The sequence and length of the PPT vary from one retroelement to another. Several reports have demonstrated the importance of the PPT sequence on plus-strand priming. In model reactions Rattray and Champoux (25–27) have shown that single base changes in the PPT of MoMLV and HIV-1 destroy the precision of priming. Robson and Telesnitsky (19) have demonstrated *in vivo* that the conserved nucleotides within the PPT of MoMLV contributed to optimal plus-strand priming. A few years ago we reported that mutations changing two pyrimidines to purines within PPT1 or PPT2 of the yeast *S.cerevisiae* Ty1 element abolished priming from these sites *in vivo* (9).

The results discussed in this report demonstrate that sequences outside the PPT contribute to plus-strand priming. Mutations upstream of the PPT in a highly conserved T-rich

region severely alter plus-strand priming and transposition of Ty1. A similar role for a T-rich region upstream of the PPT of SIV and MoMLV was observed recently by Illyinskii and Desrosiers (18) and Robson and Telesnitsky (19). Similarly, Noad *et al*. (20) have shown that a short pyrimidine tract 5′ to the PPT of the plant pararetrovirus CaMV plays an important role in PPT recognition *in vivo*. Illyinskii and Desrosiers (18) concluded that the effects of the T-box are determined principally by the three bases immediately upstream of the PPT. In Ty1, the effects of the T-box extend further upstream since mutations of two T residues 8 and 9 nt upstream of PPT have the same effect on efficiency of plus-strand DNA priming as mutating the T residues immediately upstream of PPT.

A sensitive reiterative primer extension assay has allowed us to show that the PPT4 sequence, which is not flanked by a T-rich box, is able to prime plus-strand DNA synthesis at a low level. Interestingly, the priming fidelity at this site is not affected (Fig. 3B). This suggests that the structure of the polypurine tract might be sufficient to induce specific cleavage at the 3′-end of the PPT by RNase H. Recently Fedoroff *et al*. (15) have shown that the structure of an RNA/DNA PPT-derived hybrid, as determined by 2-dimensional NMR, contains a prominent bend in the double helix. The site of the bend, combined with a widening of the minor groove width towards the 5′-end of the RNA strand in the duplex, may be a factor determining the position of RT on the substrate and ultimately the site of cleavage by RNase H at the 3′-terminus of the PPT.

After the 3′-terminus of the PPT has been cleaved, synthesis of minus-strand DNA continues and the RNase H active site cleaves the PPT at the 5′-end. During cleavage of the primer, the polymerase domain of RT which extends in the 5′ direction contacts the T-rich region upstream of the PPT. These contacts might be important for positioning the enzyme on the substrate. It is possible that mutations in the T-rich region could affect recognition of the PPT flanking region by the polymerase active site of RT. As a consequence, the RNase H active site would not be correctly positioned to make a specific cut at the 5′-end of the PPT. *In vivo* experiments in other systems have shown that the PPT has a minimal and maximal length for optimum efficiency (12,20). We therefore suggest that mutations in the T-box would not allow the generation of a primer for plus-strand synthesis with the optimal length for efficient plus-strand DNA synthesis. This could explain the low levels of priming and transposition of the T-box mutant Ty1 elements observed in this work.

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