Increased Length of Long Terminal Repeats Inhibits Ty1 Transposition and Leads to the Formation of Tandem Multimers

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ABSTRACT
The Ty1 retrotransposon of Saccharomyces cerevisiae is bounded by long-terminal repeats (LTRs). We have constructed a variety of Ty1 elements in which the LTR length has been increased from the normal length of 334 bp to >2 kb. Although small insertions in the LTR have minimal effects on transposition frequency, larger insertions dramatically reduce it. Nevertheless, elements with long LTRs are incorporated into the genome at a low frequency. Most of these rare insertion events represent Ty1 tandem (head to tail) multimers.

THE Ty elements of yeast are characterized by long-terminal repeats (LTRs) that flank their coding regions and contain critical cis-acting signals. These signals include transcriptional promoters and 3′ end formation signals as well as the sequences that define the tips of the element and are recognized by the transposon-encoded IN protein to mediate insertion of Ty DNA into host target DNA.

The Ty elements structurally resemble retroviral proviruses, which are also flanked by LTRs. Furthermore, numerous studies have confirmed that the transposition mechanisms of Ty1–Ty3 and Ty5 functionally resemble the replication/integration processes used by retroviruses. Ty1, the element studied here, produces a transcript whose 5′ end is specified in part by promoter sequences in the 5′ LTR (Liao et al. 1987; Hirschman et al. 1988) and whose 3′ terminus is specified by 3′ LTR sequences (Yu and Elder 1989; Hou et al. 1994). This transcript is packaged into virus-like particles (VLPs), where it is converted into a linear Ty1 DNA copy by the Ty1-encoded reverse transcriptase (RT) (Boeke et al. 1985; Garfinkel et al. 1985; Mellor et al. 1985; Adams et al. 1987; Eichinger and Boeke 1988). This DNA copy contains intact LTR sequences at both termini. The termini of the Ty1 LTRs define the Ty1 DNA as a substrate for Ty1 IN (integrate) protein (Eichinger and Boeke 1990; Devine and Boeke 1994; Moore and Garfinkel 1994; Moore et al. 1995).

The five families of Ty elements from Saccharomyces cerevisiae, designated Ty1–Ty5, vary dramatically in primary sequence, but interestingly, the length of the LTRs in these families is relatively well conserved, from 251 (Ty5) to 371 bp (Ty4) (Table 1). Retrotransposons from Schizosaccharomyces, Candida, and Pichia fall in the same range. In nonyeast elements, LTR lengths range from as short as 77 [Bombyx mori mug element (Michaille et al. 1990)] to 2136 bp (Drosophila Ulysses element (Evgen′ev et al. 1992)).

Investigations of Moloney murine leukemia virus have indicated that LTR length can be dramatically increased in this virus with only modest effects on replication efficiency (Reddy et al. 1991; Faustinella et al. 1994). We have begun investigations of the effects of altering LTR length on Ty1 retrotransposition. The “marked” LTRs generated also serve as useful substrates for evaluating the inheritance patterns of sequences in the LTRs.

We have examined the effect of lengthening the Ty1 LTR from 334 bp upward and find that transposition is dramatically inhibited when the LTR length exceeds ~500 bp. Nevertheless, these long LTR elements are mobilized into the genome at a low frequency. These rare events represent Ty1 multimers rather than normal Ty1 simple insertion events. Ty1 multimers have previously been reported as arising at a relatively low frequency in cells undergoing transposition of a normal Ty1 element (Weinstock et al. 1990; Mastrangelo et al. 1992), and at a relatively high frequency in cells in which normal integration is blocked by either mutations in IN or mutations in LTR termini (Sharon et al. 1994).

MATERIALS AND METHODS

Yeast strains, media and measurement of transposition frequencies: All yeast strains used are described in Table 1. Media used were as described by Rose et al. (1990). SC Foa and SC–Trp Foa plates contained 0.1% 5-fluoro-orotic acid (Foa) (Boeke et al. 1984). Transposition frequency measurements were made as follows. Transformants bearing the TRPI-marked GAL-Ty1 plasmid of interest were maintained as patches on SC–Ura glucose plates at 30°C (these plasmids...
contain \textit{URA} as the selection marker on the plasmid backbone). The patches were then replica-plated to SC – \textit{Ura} galactose plates and incubated for 5 days at 22\textdegree C. The patches were then sequentially replica-plated to SC – \textit{Ura} glucose (overnight at 30\textdegree C) and YPD medium (overnight at 30\textdegree C) to allow plasmid loss. The patches were then treated in one of two ways to determine percentage transposition, which is defined as \text{[number of Trp\textsuperscript{+} \textit{Ura}\textsuperscript{-} cells]}/\text{[total number of \textit{Ura}\textsuperscript{-} cells]} \times 100. Method 1: Cells were scraped from the YPD patch into water and then diluted and plated on YPD plates at \(-200–400\) colonies per plate at 30\textdegree C; the colonies were then replica-plated to SC – \textit{Ura} glucose and SC – \textit{Trp} glucose plates and the frequencies of the two types of colonies were tallied. This method was used when transposition frequencies were relatively high (> 1\%). For those constructs that gave rise to lower transposition frequencies, method 2 was used. Method 2: The cells were scraped from the YPD patches as above and appropriate dilutions were plated onto SC Foa plates (these colony counts give the population of total \textit{Ura}\textsuperscript{-} cells) and SC – \textit{Trp} Foa plates (these colony counts give the population of \textit{Trp}\textsuperscript{+} \textit{Ura}\textsuperscript{-} cells). Methods 1 and 2 gave similar results when performed in parallel with strain VL3 (data not shown). For qualitative transposition assays, the YPD patches were simply replica-plated to SC – \textit{Trp} Foa plates (Figure 2).

\textbf{Oligonucleotides:} Oligonucleotides used were as follows: JB14, 5’ CTAGTGTAGAGTGGATATGCAGTCTCAGTTGTTAG- TGGC 3’; JB746, 5’ CAGTGGGCGCGGTAATTGAGCAGCT- CACTATAG 3’; JB772, 5’ CCCGGATCCGGTTAGTTTTAGGATCAATATTGCAAT- TTAG 3’; JB868, 5’ CCCGGATCCGTCTTTGAGACACTGATTAATGCT- 3’; JB878, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB879, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB880, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB881, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB882, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB883, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB884, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’.

\textbf{Construction of GAL-Ty1 plasmids with altered LTR lengths:} All marked plasmids used were derived from pX3, which is a \textit{GAL1-Ty1-Trp1} 2\mu plasmid containing \textit{URA} as the vector/backbone marker (Xu and Boeke 1987). A small SSB polynkier, referred to as “SSB” and containing the \textit{SacI}, \textit{SmaI} and \textit{BamHI} sites, was introduced into the 3’ Ty1 LTR by site-directed mutagenesis as described by Kunkel (1985) using oligonucleotide JB14 (5’ CTAGTGTAGAGTGGATATGCAGTCTCAGTTGTTAGTGGC 3’; underlined bases represent the SSB polynkier). A 1.8-kb \textit{HindIII-Sall} fragment containing the 3’ LTR from pGTY1-H5 (pJEF724) was subcloned into similarly digested M13mp18, creating recombinant plasmid \textit{fVT25} DNA, creating mutant plasmid \textit{fVT26}. To construct \textit{GAL-Ty1} plasmids in which the \textit{BamHI} site in the SSB polynkier would be unique, the preexisting site downstream of Ty1 in the pJEF724 plasmid was first removed by cutting pJEF724 with \textit{BamHI}, filling in with Klenow fragment, and religating, generating plasmid pJEF1706. The full-length \textit{GAL1-Ty1-trp1} containing plasmid pVT14, containing LTR:: SSB, was then constructed by three-piece ligation of 8.8-kb \textit{BstEII-Sall} and 2.5-kb \textit{BstEII-HindIII} fragments from pJEF706 and the \textit{HindIII-Sall} fragment bearing LTR:: SSB from IV IT26. The \textit{GAL1-Ty1-Trp1} marked plasmid pVT14 that was the progenitor for all of the other constructs (Figure 1) was constructed by three-piece ligation of 3-kb \textit{BstEII-BamHI} and 8.8-kb \textit{BstEII-Sall} fragments from pX3 and the 0.85-kb \textit{SalI-BglII} piece from pVT41. Thus all Ty1 coding sequences came from pX3 and were not exposed to PCR amplification. The LTR sequence was confirmed by DNA sequencing.

\textbf{TABLE 1} Yeast retrotransposon LTR lengths

<table>
<thead>
<tr>
<th>Element</th>
<th>Host</th>
<th>LTR length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ty1</td>
<td>\textit{S. cerevisiae}</td>
<td>334</td>
<td>CLARE and FARABAUGH (1985)</td>
</tr>
<tr>
<td>Ty2</td>
<td>\textit{S. cerevisiae}</td>
<td>332</td>
<td>WARMINGTON et al. (1985)</td>
</tr>
<tr>
<td>Ty3</td>
<td>\textit{S. cerevisiae}</td>
<td>340</td>
<td>HANSEN et al. (1988)</td>
</tr>
<tr>
<td>Ty4</td>
<td>\textit{S. cerevisiae}</td>
<td>371</td>
<td>JANETZKY and LEHLE (1992); STUCKA et al. (1992)</td>
</tr>
<tr>
<td>Ty5</td>
<td>\textit{S. paradoxus}</td>
<td>251</td>
<td>ZOU et al. (1996)</td>
</tr>
<tr>
<td>Tcl</td>
<td>\textit{C. albicans}</td>
<td>388</td>
<td>CHEN and FONZI (1992)</td>
</tr>
<tr>
<td>Tfi</td>
<td>\textit{Sch. pombe}</td>
<td>358</td>
<td>LEVIN et al. (1990)</td>
</tr>
<tr>
<td>T12</td>
<td>\textit{Sch. pombe}</td>
<td>349</td>
<td>WEAVER et al. (1993)</td>
</tr>
<tr>
<td>Pm-T1</td>
<td>\textit{P. membranaefaciens}</td>
<td>289</td>
<td>PEARSON et al. (1995)</td>
</tr>
</tbody>
</table>

The patches were then replica-plated to SC – \textit{Ura} glucose (overnight at 30\textdegree C) and YPD medium (overnight at 30\textdegree C) to allow plasmid loss. The patches were then treated in one of two ways to determine percentage transposition, which is defined as \text{[number of Trp\textsuperscript{+} \textit{Ura}\textsuperscript{-} cells]}/\text{[total number of \textit{Ura}\textsuperscript{-} cells]} \times 100. Method 1: Cells were scraped from the YPD patch into water and then diluted and plated on YPD plates at \(-200–400\) colonies per plate at 30\textdegree C; the colonies were then replica-plated to SC – \textit{Ura} glucose and SC – \textit{Trp} glucose plates and the frequencies of the two types of colonies were tallied. This method was used when transposition frequencies were relatively high (> 1\%). For those constructs that gave rise to lower transposition frequencies, method 2 was used. Method 2: The cells were scraped from the YPD patches as above and appropriate dilutions were plated onto SC Foa plates (these colony counts give the population of total \textit{Ura}\textsuperscript{-} cells) and SC – \textit{Trp} Foa plates (these colony counts give the population of \textit{Trp}\textsuperscript{+} \textit{Ura}\textsuperscript{-} cells). Methods 1 and 2 gave similar results when performed in parallel with strain VL3 (data not shown). For qualitative transposition assays, the YPD patches were simply replica-plated to SC – \textit{Trp} Foa plates (Figure 2).

\textbf{Oligonucleotides:} Oligonucleotides used were as follows: JB14, 5’ CTAGTGTAGAGTGGATATGCAGTCTCAGTTGTTAGTTAGTGGC 3’; JB746, 5’ CAGTGGGCGCGGTAATTGAGCAGCTCACCATAG 3’; JB772, 5’ CCCGGATCCGGTTAGTTTTAGGATCAATATTGCAATTTAGTGGTTAGTTAGTGGC 3’; JB868, 5’ CCCGGATCCGTCTTTGAGACACTGATTAATGCT 3’; JB878, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB879, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB880, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB881, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB882, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB883, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’; JB884, 5’ CCCCCATCCGATATAATCTATATGAGTCTTGG 3’.
48, -72, and -96. These SSBs were constructed by ligating together two PCR products in each case. A "left" PCR product, extending from the HindIII site in pVT43 (position 5145, Figure 1) to the new SSB, was amplified using oligonucleotide JB772 to define its left boundary and one of three oligonucleotides JB879, 881 or 883 that define the SSB end. The left PCR product was combined with a "right" PCR product, extending from the new SSB to the SalI site in pVT43, and was amplified using oligonucleotide JB740 to define the right boundary and one of three oligonucleotides JB878, 880 or 882 to define the SSB end. The left end products were digested with HindIII and BamHI, the right end products were digested with SalI and BamHI, and the left and right products were ligated into pVT219 digested with HindIII and SalI. BgII-SalI fragments containing each altered LTR were then ligated into pX3 that had been digested with BamHI and SalI, generating plasmids isosctructural to pVT43. The resulting pGTyl-TRP1-LTR::SSB plasmids were dubbed pVT481, 483, and 482, respectively. The gfp fragment was subcloned into these three vectors as described for pVT218 above. The resulting pGTyl-TRP1-LTR::gfp plasmids were dubbed pVT484, 485, and 486, respectively. The 1-kb neo BamHI fragment from plasmid pGH14 (JOYCE and GRINDLEY 1984) was inserted into the BamHI site of pVT482 to generate pVT487.

Construction of mini-Tyl element with long LTRs: The pGTyl-1-LTR::gus plasmid pVT56 was digested with HpaI and BgII, and the large fragment was ligated to an EcoRV-BamHI fragment containing HIS3 previously used to construct mini-Tyl elements (from plasmid pX130) (XU and BOEKE 1990), resulting in plasmid pVT1211.

Southern blot analysis: Yeast genomic DNA was prepared as described (HOFFMAN and WINSTON 1987). The DNA was digested overnight with appropriate restriction enzymes in the supplier specified buffer at 37°C and run on a 1.2% agarose gel at 40 V overnight. The gel was treated with HCl for 15 min in 0.1X SSC, UV-crosslinked to the membrane using the random priming method (FEINBERG and VOGELSTEIN 1983) using the following input DNAs. The gus probe was a PstI restriction fragment from pRAJ260. The lacZ probe was a 698-bp SacI-BglII fragment from M13mp18.

RESULTS

A family of GAL-Tyl plasmids with altered LTRs: GAL-Tyl plasmids are a family of plasmids (pGTy plasmids) that can be used to study the structure and function of yeast transposons. In these plasmids, the controllable GAL1 promoter replaces the U3 region of the 5′ LTR, which normally provides the promoter sequences for Tyl. Furthermore, marker sequences of 1–2 kb can be conveniently inserted into a nonessential region near the 3′ end of the transposon and downstream of the POL ORF, but upstream of the 3′ LTR. These marked Tyls can be launched at high efficiency into either genomic or plasmid targets by induction of the GAL1 promoter, and the properties of the progeny elements can be studied (BOEKE et al. 1985, 1988, 1991; XU and BOEKE 1987).

We have constructed a new set of pGTyl-TRP1 plasmids that bear selective markers and other DNA fragments in the U3 region of the 3′ LTR (Figure 1, Table 2). According to previous experiments and the accepted models for reverse transcription of retroviruses as well as Tyl, the U3 regions of both progeny LTRs are expected to be inherited from the 3′ LTR.

Transposition frequencies: The transposition frequencies of the pGTyl-TRP1 elements with LTR alterations were measured in both wild-type strains and spt3 strains (Figure 2, Table 3). In wild-type strains, the analysis of Tyl transposition is complicated by the fact that genomic Tyl elements (whose LTRs are unmarked) are expressed at high levels. Previous studies have shown that the progeny Tyl elements that arise in such strains can be hybrids between the marked element and an unmarked chromosomally derived element (BOEKE et al. 1986). However, in the spt3 strains, the chromosomal elements are not transcribed (WINSTON et al. 1984), and the transposition of the marked GAL-Tyl-TRP1 element can be studied in isolation, because GALI-driven transcription is unaffected by the spt3 mutation (BOEKE et al. 1986).

We introduced a variety of pGTy plasmids with LTR-marked elements into isogenic SPT3+ and spt3 strains and observed that transposition frequency was inversely related to LTR length (Table 3). Elements with the short SSB polylinker insertion transposed at essentially wild-type efficiency, whereas elements with LTR insertions >1 kb in length had extremely low transposition frequencies. The Tyl-TRP1 element with the LTR::lacZa marker had an intermediate phenotype, namely ~10% of wild-type LTR in the SPT3+ strain background, but this element transposed at ~0.3% of the wild-type LTR in the spt3 strain background. Thus it appears that transposition of at least this lacZa-marked element can be "helped" in some currently unknown way by genomic Tyl elements.

LTR length and position of marker insertion inhibits transposition: The above experiments were carried out with elements that carried various markers inserted in the SSB polylinker inserted at position 24 of the 3′ LTR. It remained possible that an important element or coding region might be interrupted by these insertions. To address this point, we created a frameshift mutation in the SSB polylinker that had no effect on transposition frequency (data not shown). In addition, we built a set of additional SSB-containing plasmids with the SSB inserted at positions 48, 72, and 96 bp in the 3′ LTR (see MATERIALS AND METHODS). A set of transformants containing either SSB or gfp inserted at the various positions in the 3′ LTR U3 region were qualitatively assayed for transposition. All of the SSB plasmids transposed as well as the wild type, (data not shown). However, none of the LTR::gfp plasmids derived from these LTR::SSB plasmids transposed at high efficiency in spt3 strains, indicating either that the gfp marker was incompatible with transposition or that long LTRs, regardless of the position of insertion of the marker, interfered with efficient transposition (Figure 2). To rule out the former, we constructed pVT487, which contains the neo marker inserted
**TABLE 2**

**Yeast strains and plasmids**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid name</th>
<th>Plasmid description</th>
<th>Reference</th>
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<tr>
<td>VL3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pX3</td>
<td>Native LTR, TRP1 marked body</td>
<td>Xu and Boeke (1987)</td>
</tr>
<tr>
<td>VL4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pVIT14</td>
<td>LTR::lacZa</td>
<td>This study</td>
</tr>
<tr>
<td>VL11&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pAG1403</td>
<td>Native LTR; protease frameshift mutant&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>VL12&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pVIT43</td>
<td>LTR::SSB</td>
<td>This study</td>
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<tr>
<td>VL18&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pX3</td>
<td>Native LTR, TRP1 marked body</td>
<td>Xu and Boeke (1987)</td>
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<tr>
<td>VL24&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pVIT15</td>
<td>LTR::SSB-frameshift</td>
<td>This study</td>
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<tr>
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<td>VL264&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>Δ3'LTR&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>VL328&lt;sup&gt;+&lt;/sup&gt;</td>
<td>pVIT487</td>
<td>LTR::neo96&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>JB369&lt;sup&gt;+&lt;/sup&gt;</td>
<td>—</td>
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<td>This study</td>
</tr>
</tbody>
</table>

<sup>+</sup> All plasmids except pJE938 are marked in the body of the Ty1 element with TRP1 (as in pX3).

<sup>*</sup> VLH50 is parent, MAPra ura3-167 his3Δ200 leu2Δ1 trp1Δ1 spo3-202.

<sup>+</sup> YH8 is parent, MAPra ura3-167 his3Δ200 leu2Δ1 trp1Δ1.

<sup>+</sup> VL245 is parent, MAPra ura3-52 his3Δ200 leu2Δ1 his2Δ202 trp1Δ63 spo3-101.

<sup>+</sup> Genotype is MAPra ura3-52 trp1-289.

<sup>+</sup> Frameshift introduced into pX3 by filling in BspEII site; mutant kindly provided by A. Gabriel.

<sup>+</sup> pJE938 lacks a 3' LTR.

<sup>+</sup> These LTR insertions, unlike the rest in this table, are not at position 24 in the LTR, but are at position 48, 72, or 96 as indicated by the suffix. See MATERIALS AND METHODS for further details.
into the LTR; neo is known to be compatible with efficient transposition when inserted into the body of Ty1 at the BglII site at position 5561 (Xu and Boeke 1987; Boeke et al. 1988). Like pVT1486 (LTR::gus96), pVT1487 (LTR::neo96) contains a ~1-kb-long marker inserted at the same site in the LTR; however neither plasmid allows efficient transposition. Therefore, LTR length and not position of insertion in the LTR is sufficient to explain the low transposition frequency observed in these long LTR plasmids.

**Inheritance of U3-marked LTRs:** We studied the inheritance of the lacZα marker inserted into the 3’ LTR. Eighteen independent Trp+ Ura- segregants of an SPT3+ strain were analyzed by Southern blotting with a lacZ probe in such a way that 5’ LTR and 3’ LTR lacZα elements could be distinguished. Strains bearing insertions of Ty1-TRPI elements with marked 3’ LTRs only, marked 5’ LTRs only and both 5’ and 3’ LTRs marked were observed. The majority of the strains analyzed (14/18, Figure 3B) appear to have undergone a single transposition event and about half of these (7/14) have both LTRs marked, but there are clear examples of 3’ LTR only marked (lanes 6 and 16) and 5’ LTR only marked (lanes 1, 5, 10, 13 and 15). The latter species presumably arose from transposition events that involved RNAs derived from both the marked element and a chromosomal Ty1 element with unmarked LTRs, presumably through template switches occurring during reverse transcription (Boeke et al. 1986; Pangandaran and Fiore 1988). Alternatively, homologous recombination at the DNA level between newly transposed and resident unmarked Ty1 elements might have given rise to these hybrid elements.

**Analysis of rare long LTR Ty1-TRPI integration events:** The Trp+ Far+ (Ura-) colonies obtained at a very low frequency with the pGTyl-TRPI-LTR::gus constructs were analyzed genetically as well as by genomic Southern blotting (Figure 4). We noted that, surprisingly, these strains contained multiple copies of Ty1-TRPI-LTR::gus, in spite of the fact that these events occurred at a very low frequency (Table 3). Moreover, when these Trp+ colonies were back-crossed to a Trp- strain (lacking marked Ty1 elements), the Trp+ phenotype segregated 2:2 in 17/18 tetrads derived from crosses of three independent Trp+ colonies, suggesting tight linkage of the multiple gus genes. One tetrad (tetrad 11) apparently represents a conversion tetrad in which the marked Ty1 copies and the Trp+ phenotype segregated 3:1 (Figure 4). Southerns of the tetrads from these crosses confirmed that in fact all copies of gus cosegregated meiotically in all the tetrads analyzed. Interestingly, in two of the 18 tetrads analyzed (tetrads 10 and 11) a single gus band present in the other tetrads was absent from both Trp+ spores, suggesting that it had been lost mitotically before sporulation. Three such linked arrays of Ty1-TRPI-LTR::gus elements were put through three generations of back-crossing to wild type; again all tetrads segregated 2 Trp+ : 2 Trp-, and no additional changes in the Southern blot pattern were observed in several Trp+ spores checked at each generation of backcrossing (data not shown).

These observations suggested that these Ty1-TRPI-LTR::gus elements were present in a linked tandem array, such as has been reported at HML (Weinstock et al. 1990) and under other special conditions, such as when normal Ty1 integration is blocked (Sharon et al. 1994). These arrays consist, for the most part, of Ty1 elements in tandem, with single LTRs at the junctions, although truncated and inverted Ty1 elements are occasionally inserted willy-nilly into these arrays (Weinstock et al. 1990).
To test directly for the presence of multimers, we analyzed a number of independent Trp+ colonies from the above experiment by genomic Southern blotting using a restriction enzyme (Clal) that has only a single site in Ty1, and has no sites in TRP1 or gus. This blot was then probed with a gus probe. Thus any members of a tandem array containing a gus-marked LTR should be revealed as an 8.1-kb fragment, and the intensity of such a band should be indicative of the number of such elements in the array. In 14/14 strains tested, a band of 8.1 kb was observed, and in most of these, this band was of an intensity that suggested the presence of a tandem array containing a gusmarked LTR should actually depend on RAD52, but instead use an alternate pathway for mobilization.

When either integrase or LTR tips were mutant, the frequency of Ty1 integration was shown to be greatly reduced in rad52 strains (SHARON et al. 1994), so we examined whether mobilization of the LTR::gus-marked element was affected by a rad52 mutation (Table 3). We observed that while mobilization of the LTR::gus element did not absolutely depend on RAD52, it was about threefold more sensitive to the effect of a rad52 mutation than conventional Ty1-TRP1 element transposition (which was itself reduced only threefold). Thus in this experiment most of the mobilization events occurred in a RAD52-dependent manner, whereas perhaps about 1/3 of the events were RAD52-independent, and presumably used an integrase-dependent mechanism.

We examined the structure of the rare integration events that occurred in the rad52 mutant, to determine whether they showed the tandem array structure or not. We used the Clal genomic DNA blotting strategy described above, and found that six of 10 Trp+ strains examined had an 8.1-kb fragment (Figure 5B). However the intensity of this band was lower than observed in the RAD52+ strain background, and the bands whose mobilities were consistent with dimers and trimers of the 8.1-kb fragment were much less common if not absent all together. Our interpretation of these results is that a dimer of the long LTR element is common in the rad52 strains, but higher-order multimer formation is at least partially suppressed.

**Transposition defect of long LTR elements is not alleviated by deletion of internal sequences:** To test whether the inhibition of transposition of long LTR elements is a consequence of overall DNA length of the marked Ty1 element, we constructed mini-Ty derivatives of the long LTR elements, using a similar strategy to that described by Xu and BOEKE (1990). The internal Ty1 sequences extending from the Hpal site at position 815 to the BglII site at position 5561 were deleted from an LTR::gus plasmid. Previous studies showed that this deletion does not remove any essential cis-acting sequences for transposition (Xu and Boeke 1990). Rather than provide the helper functions from the same plasmid as we have done previously, we expressed the helper functions from a second 2-μ plasmid (Figure 6). This strategy made it easier to study complementation of the mini-Ty element with either a wild-type or various mutant elements, such as IN- (integrase) mutants.

The results of these studies are reported in Table

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**Table 3**

<table>
<thead>
<tr>
<th>Strain, plasmid</th>
<th>Relevant genotype</th>
<th>LTR length</th>
<th>Transposition frequency</th>
<th>N (number of colonies tested)</th>
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<tr>
<td>YH8, pX3 (native LTR)</td>
<td>SPT3</td>
<td>334</td>
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<td>556</td>
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<td>13</td>
<td>256</td>
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<td>SPT3</td>
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<td>44</td>
<td>1380</td>
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<td>SPT3</td>
<td>349</td>
<td>17</td>
<td>156</td>
</tr>
<tr>
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<td>SPT3</td>
<td>531</td>
<td>2</td>
<td>674</td>
</tr>
<tr>
<td>YH8, pVIT44 (LTR::lacZa)</td>
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<td>1047</td>
<td>6.2</td>
<td>291</td>
</tr>
<tr>
<td>YH50, pVIT44 (LTR::lacZa)</td>
<td>SPT3</td>
<td>1047</td>
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<td>9.0 x 10^4</td>
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<tr>
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<td>SPT3</td>
<td>~2140</td>
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<td>7.7 x 10^5</td>
</tr>
<tr>
<td>YH58, pVIT55 (LTR::gus)</td>
<td>SPT3 rad52</td>
<td>~2140</td>
<td>2.0 x 10^-2</td>
<td>1.2 x 10^6</td>
</tr>
<tr>
<td>YH50, pAG1403 (POLAs)</td>
<td>SPT3</td>
<td>334</td>
<td>5 x 10^-4</td>
<td>6.0 x 10^4</td>
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</tbody>
</table>

*Host strains YH8 and YH50 are described in Table 2; YH58 is an isogenic derivative of YH8 bearing a rad52::LEU2 disruption.

* Transposition frequency in % is as defined in MATERIALS AND METHODS; method 1 was used for frequencies >1%, method 2 for frequencies <1%.
The reverse transcription process is illustrated to depict the expected fate of insertions engineered into the LTR of a GAL-Tyl element. Assuming that there is only LTR-marked RNA present for reverse transcription, both wild-type and LTR-marked RNAs could lead to other expected inheritance patterns. (A) The cartoon depicts the expected fate of insertions engineered into the 3' LTR of a GAL-Ty1 element. (B) A Southern blot of yeast genomic DNA digested with SalI and probed with the lacZ alpha fragment. (A) LTR and laczZa probe. (B) LTR and lacZa probe. (A) Full length minus strand DNA is transferred to the 5' end of the RNA. (c) Nascent minus-strand strong-stop of R-U5 is transferred and annealed to the 3' end of the companion RNA molecule via the complementary R sequence (or it could be transferred intramolecularly). (d) Minus-strand DNA is extended to the extreme 5' end of its template. (e) Synthetic plus-strand DNA commences from just upstream of the 3' LTR and proceeds to the end of minus-strand template DNA. (f) The full length plus-strand strong-stop DNA is transferred to the 5' end of the minus-strand via its R, U5 and PBS regions. (g) Plus-strand DNA is completed on the minus-strand template. (h) Full-length duplex DNA with two marked LTRs is generated. (B) A Southern blot of yeast strains in which transposition of pVIT44 (pGTyl-TRPZ-LTR::lacZa) had been induced is shown. DNA from 18 plaque-free Trp' derivatives (1-18) was digested with SacI and run on an agarose gel, which was blotted and probed with the lacZa probe. Such digestion results in the release of a 2.8-kb internal fragment (arrow) containing the 5' lacZa LTR and a variable-length junction fragment with a minimum length of ~1 kb containing the 3' lacZa LTR. There were no bands between 500 bp and ~2 kb other than those shown in the figure. The strain in lane 16 must contain a TRP1-marked Ty1 element because it is Trp'. Markers are in kilobase-pairs.

**DISCUSSION**

Transposons marked with subterminal reporter genes can be useful for identifying transcriptional regulatory regions (Cooley et al. 1988; Reddy et al. 1991) and this was our initial motivation for carrying out these studies, which involved placement of a variety of genes adjacent to the U3 LTR tip. However, Ty1 has dramatically nonrandom patterns of insertion (Ji et al. 1993) with hotspots upstream of RNA polymerase III transcribed genes (Devine and Boeke 1996). This, together with the inhibitory effects of long LTRs on Ty1 end of the RNA. (c) Nascent minus-strand strong-stop of R-U5 is transferred and annealed to the 3' end of the companion RNA molecule via the complementary R sequence (or it could be transferred intramolecularly). (d) Minus-strand DNA is extended to the extreme 5' end of its template. (e) Synthetic plus-strand DNA commences from just upstream of the 3' LTR and proceeds to the end of minus-strand template DNA. (f) The full length plus-strand strong-stop DNA is transferred to the 5' end of the minus-strand via its R, U5 and PBS regions. (g) Plus-strand DNA is completed on the minus-strand template. (h) Full-length duplex DNA with two marked LTRs is generated. (B) A Southern blot of yeast strains in which transposition of pVIT44 (pGTyl-TRP1-LTR::lacZa) had been induced is shown. DNA from 18 plaque-free Trp' derivatives (1-18) was digested with SacI and run on an agarose gel, which was blotted and probed with the lacZa probe. Such digestion results in the release of a 2.8-kb internal fragment (arrow) containing the 5' lacZa LTR and a variable-length junction fragment with a minimum length of ~1 kb containing the 3' lacZa LTR. There were no bands between 500 bp and ~2 kb other than those shown in the figure. The strain in lane 16 must contain a TRP1-marked Ty1 element because it is Trp'. Markers are in kilobase-pairs.
transposition reported here, makes such an approach with Ty1 in yeast of dubious value.

The length of Ty1 LTRs appears to be critical for efficient retrotransposition. Very small insertions, such as the 15-bp SSB polylinker, had no effect on transposition efficiency (Table 3). In a previous study, we noted that a 200-bp insertion further downstream in U3 (position 187) was compatible with efficient retrotransposition (Braiterman et al. 1994). Once LTR length exceeds 1000 bp, there is a dramatic decrease in the efficiency with which Ty1-TRPI is inserted into the ge-

![Figure 4](image1.png)

**Figure 4.**—Progeny marked long LTR Ty1 elements are genetically linked. (A) Eighteen plasmid-free Trp' strains derived from VL77 were analyzed by Southern blotting using the gus probe. Digestion was with HindIII, which releases a ~6 kb internal fragment diagnostic of a 5' LTR::gus element (arrow) and 3' LTR::gus junction fragments of various lengths. Note that all of the strains (except lane 13) contain multiple Ty1-TRP1LTR::gus elements. (B) A plasmid-free Trp' strain derived from VL77 and containing multiple progeny Ty1-TRP1LTR::gus elements was crossed to a strain lacking marked Ty1 elements (JB369) and a number of tetrads were dissected. The Trp phenotype segregated 2:2 in all but one of 18 tetrads dissected. A presumed conversion tetrad, tetrad 11, segregated 3 Trp':1 Trp-. A Southern blot of six tetrads is shown. DNA was extracted from the tetrads, including the presumed conversion tetrad, and digested with HindIII. The blot was probed with the gus probe. In all of the tetrads, the gus-hybridizing bands cosegregated with the Trp' phenotype, indicating tight genetic linkage of all of the bands. The missing bands in tetrads 10 and 11 are presumed to have arisen from a mitotic loss event that preceded the meiosis, as the patterns are identical within the Trp' spores within these tetrads. Spore 12C has a light pattern identical to that of spore 12A that did not reproduce well.

![Figure 5](image2.png)

**Figure 5.**—Long LTR elements form Ty1 multimers. A Southern blot of yeast strains in which transposition of pVT155 (pGTy1-TRP1LTR::gus) had been induced is shown. (A) DNA from 15 plasmid-free Trp' derivatives was digested with CiaI and run on an agarose gel, which was blotted and probed with the gus probe. Samples 6 and 10 appear to have suffered incomplete digestion, and sample 7 is too light to interpret. CiaI has a single site in Ty1, and does not cut in TRPI or gus. Thus tandem arrays of LTR::gus-Ty1-TRP1LTR::gus are expected to release an 8.1-kb fragment (solid arrow). Indeed fragments of this size are prominent in most of the strains and present in all of them. Dimers and trimers of this fragment are expected if some of the Ty1 elements lose the CiaI site; some chromosomal Ty1 elements lack this site. Fragments consistent with such dimers and trimers (dark and light stippled arrows) are observed in many of the strains analyzed. (B) A similar experiment to that outlined above was performed in a rad52 mutant strain. DNA from 10 independent plasmid-free Trp' derivatives was digested, blotted and probed as above. The position of the 8.1-kb band is indicated by a solid arrow.
had no effect on transposition efficiency in either SPT3 or spt3 strains indicates that there is no essential cis element at those positions in the LTR. Efficient transposition in an spt3 strain ensures that a defective LTR sequence is not simply being “rescued” by wild-type Ty1 transcripts coming from chromosomal Ty1 copies (Boeke et al. 1986; Lauermann and Boeke 1994; Lauermann et al. 1995). There are no long ORFs in the 3' LTR U3 region; the longest one (which could potentially encode a peptide of only 44 amino acids) extends to bp 96 of the 3' LTR. It is extremely unlikely that this ORF encodes a protein required for transposition: (1) this ORF would be encoded downstream of GAG and POL, making it the third ORF in the Ty1 mRNA; (2) insertion of a variety of foreign DNAs into the BglII site upstream of the 3' LTR does not interfere with transposition but disrupts this ORF (Boeke et al. 1985, 1988; Garfinkel et al. 1988; Curcio and Garfinkel 1991); and (3) filling in the BamH site within the SSB polylinker (creating a frameshift in this ORF) had no effect on transposition efficiency (data not shown). Furthermore, insertions of gfp DNA at positions 24, 48, 72 and 96 in the U3 region of the 3' LTR inhibited transposition similarly, indicating that it is the length of the LTR and not the position of insertion within it that inhibits transposition.

Although we have not yet directly investigated the molecular mechanism of inhibition of transposition resulting from long LTRs, we have studied the structure of the rare Ty1-TRPI mobilization events that do occur. Rather than the individual, unlinked insertion events that are normally associated with conventional retrotranspositions of Ty1-TRPI (Boeke et al. 1985; V. Lauermann 1996, unpublished results), the long LTR variants give rise to multiple insertions that are tightly linked genetically. Southern blot analysis indicated that many of the elements in these arrays were present as head to tail Ty1 multimers. Ty1 and Ty2 multimers have previously been shown to arise in response to a selection for the disruption of the silent mating locus HML and to occur at a low frequency during normal transposition (Weinstock et al. 1990; Mastrangelo et al. 1992). In addition, Ty1 multimers are frequently observed when normal Ty1 integration is blocked. Ty1 integration (like retrovirus integration) is normally mediated by the element-encoded integrase protein, which recognizes the termini of newly synthesized Ty1 DNA and joins them to target site DNA in a reaction that is independent of the homologous recombination protein Rad52p. When mutations that block integration are introduced into either the integrase coding region or into the LTR tips, Ty1 can still be mobilized into the genome at a significant frequency, but this form of marked Ty1 mobilization is Rad52p-dependent, and gives rise only to multimers (Sharon et al. 1994). Our data show that another defect that leads to multimer formation is lengthening of the Ty1 LTR.

Although we have ruled out the presence of an essen-

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**Figure 6.** Scheme for evaluating whether reducing the overall length of Ty1 elements suppresses the transposition defect of long LTR elements. Two plasmids are introduced into yeast cells, a helper plasmid (top circle) that provides Gag and Pol proteins in *tenuis*, and a mini-Ty1 element that provides the marked Ty1-HIS3 element and all necessary cis-acting signals (lower circle). The helper may or may not include a 3' LTR; deletion of the 3' LTR results in ~10-fold higher transposition frequency (Table 4). The mini-Ty1 plasmids contain a mini-Ty1-HIS3 element with either a native or long (gus-marked) 3' LTR. The two plasmids are maintained by growing the cells on medium lacking histidine and tryptophan. Following induction of transposition on galactose, both plasmids are counterselected on Foa medium, and selection is applied for insertions into the chromosomes (heavy line with oval), which confers a His" phenotype. Results of these experiments are presented in Table 4.

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nome, and the frequency continues to drop as LTR length increases. Thus it appears that when LTR length reaches a value somewhere between 500 and 1000 bp, there is a significant inhibition of retrotransposition efficiency.

Could the inhibition observed in the current series of altered Ty1 elements result not from a length increase but from disruption of either a critical cis element or an ORF? The fact that the small SSB insertions
TABLE 4

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<tr>
<th>Mini-Tyl-HIS3 LTR type</th>
<th>Helper type (plasmid name)</th>
<th>Total Foa&lt;sup&gt;k&lt;/sup&gt;</th>
<th>Foa&lt;sup&gt;k&lt;/sup&gt; His&lt;sup&gt;i&lt;/sup&gt;</th>
<th>% Transposition&lt;sup&gt;b&lt;/sup&gt;</th>
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<td><strong>Experiment 1: Mini-Tyl with native vs. long LTRs</strong></td>
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<tr>
<td>Native LTR (pX136)&lt;sup&gt;0&lt;/sup&gt;</td>
<td>Ty1-TRPI-LTRA (pVIT 213)</td>
<td>274</td>
<td>12</td>
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<td>LTR::gus (pVIT211)</td>
<td>Ty1-TRPI-LTRA (pVIT 213)</td>
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<td>LTR::gus (pVIT211)</td>
<td>Ty1-TRPI (pX3)</td>
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<td>575</td>
<td>0.34</td>
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<td><strong>Helper plasmid</strong></td>
<td><strong>Helper Ty1-TRPI type</strong></td>
<td><strong>Total Foa&lt;sup&gt;k&lt;/sup&gt;</strong></td>
<td><strong>Foa&lt;sup&gt;k&lt;/sup&gt; His&lt;sup&gt;i&lt;/sup&gt;</strong></td>
<td><strong>% Transposition&lt;sup&gt;b&lt;/sup&gt;</strong></td>
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<td><strong>Experiment 2: Mini-Tyl-HIS3.LTR::gus (pVIT 211) with various helper elements</strong></td>
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* Both experiments were done using SPT<sup>3</sup> diploid host strain JB508 (MATα ura3-52 his3Δ200 trplΔ1 lys2) × YH8.
* Foa<sup>k</sup> His<sup>i</sup>/total Foa<sup>k</sup> × 100
* pX136 has been described (Xu and Boeke 1990). Helper plasmids pGM313-317 bear linker insertion mutations that eliminate PR or IN function (Monorian et al. 1994).

Tial cis-acting signal as the explanation for the low transposition frequency of the long LTR elements, the formation of multimers in the case of long LTR elements may result from a different type of integration defect. It may be difficult to fully replicate the terminal DNA sequences of long LTR elements because of packaging constraints introduced by the lengthy marker DNAs. This possibility has been addressed by three experiments. First, we checked whether the simultaneous presence of an internal marker (TRPI) and an LTR marker (lacZα) interfered with transposition. The TRPI marker was removed from the LTR::lacZα element and this plasmid was transformed into yeast. Following induction of transposition and plasmid curing, 20 randomly chosen colonies were assayed by Southern blotting for new transposition events (using the lacZ probe); no insertions were detected. Had transposition been restored to the level found in elements with normal length LTRs, we would have expected to see one or more new transposition events in ~70% of these colonies. Second, we attempted to increase the transposition efficiency of the Tyl-TRPI-LTR::gus element by removing the cis-dispensable GAG and POL sequences and expressing the transposon proteins in trans using a mini-Tyl system. However, the transposition competence of this element was not restored by such shortening. These results suggest that the transposition defects of long LTR elements do not result solely from constraints imposed by nucleic acid packaging. Finally, we showed that a fraction of the events depend on the cellular RAD52 gene product, suggesting that these might result from recombination between the marked Tyl element and genomic copies of Tyl or LTRs.

We propose instead that transposition inhibition and the formation of multimers might result from aberrant replication processes that can occur when LTR length exceeds a certain threshold (Figure 7). LTR length may ensure the proper timing/coordination of the complex strand transfer events required during reverse transcription. Alteration of LTR length may alter the timing of specific events in this potentially ordered pathway and this may deregulate the steps of reverse transcription and lead to loss of transposition efficiency, formation of multimers, or both.

Both ends of Tyl RNA molecules are shorter than Tyl DNA, having just 45 nucleotide terminal repeats may ensure the proper timing/coordination of the complex strand transfer events required during reverse transcription. Alteration of LTR length may alter the timing of specific events in this potentially ordered pathway and this may deregulate the steps of reverse transcription and lead to loss of transposition efficiency, formation of multimers, or both.

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![Figure 7](image_url) - Model for stimulation of formation of Tyl multimers by long LTRs. The extra-long U3 region present in these elements would result in a delay of completion of plus strand DNA synthesis relative to a wild-type Tyl element. This would allow extra time for the formation of an aberrant replication intermediate to form (step f); formation of this intermediate would normally be blocked by completion of plus strand synthesis and plus strand strong-stop DNA transfer. Extension of the minus-strand (f) would lead to formation of a double-length Tyl element with a single LTR at the junction. See Discussion for further details. RNA, wavy lines; DNA, straight lines; 3'OH, dot; primer tRNA, bicycle. LTRs (►) are subdivided into U3, R and U5 (dashed lines)
The full-length Tyl cDNA product with complete LTRs is generated during reverse transcription as the result of two strand transfers. LTRs are divided into three domains, R, corresponding to the repetitive sequence found on the RNA ends; U3, derived from the unique 3' region of the RNA; and U5, derived from the unique 5' region of the RNA. Retroviruses [and probably LTR-retrotransposons (Boeke et al. 1986)] package two genomic RNA molecules, at least one of which serves as a template for reverse transcriptase (Figure 7a). Reverse transcription is primed by the 3' OH of a host tRNA bound to a primer binding site just downstream of the 5' LTR; the initial product of reverse transcription, minus strong-stop DNA, is completed when the RT reaches the 5' end of the RNA. During this process the RNA portion of the RNA-DNA hybrid is removed by the reverse transcriptase-associated RNase H activity (Figure 7b). Nascent minus-strand strong-stop of R-U5 is transferred and annealed to the 3' end of the companion RNA molecule via the complementary R sequence (Figure 7c). Minus-strand DNA is extended to the extreme 5' end of its template, U5-R (Figure 7, d and e). Meanwhile, synthesis of plus-strand DNA commences from a plus-strand priming site just upstream of the 3' LTR and would normally proceed to the end of minus-strand template DNA; this process would take much longer in elements with lengthened LTRs (Figure 7c). Normally, the completed plus-strand strong stop DNA would be transferred to the completed minus strand DNA, but if no completed plus-strand DNA were available, the 3' end of minus-strand DNA would be available to base pair with the 3' end of the second RNA molecule (Figure 7f); extension of this primer/template would lead to the formation of a dimeric minus-strand (Figure 7g). In principle, this could give rise to a full-length dimeric double-stranded Tyl element with a single shared LTR at the junction.

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