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The Yeast Retrotransposons Ty1 and Ty3 Require the RNA Lariat Debranching Enzyme, Dbr1p, for Efficient Accumulation of Reverse Transcripts

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A mutant screen has been initiated to identify host genes important for the replication of retrotransposons in *Saccharomyces cerevisiae.* Two mutants were identified that undergo Ty1 and Ty3 transposition at <10% of the wild-type frequency. Both these mutants have deficiencies in the accumulation of full-length Ty1 and Ty3 cDNAs, although Ty proteins (including reverse transcriptase) accumulate at wild-type levels. The *DBR1* gene, encoding the yeast debranching enzyme, complements both mutants. This suggests that Dbr1p is important for either reverse transcription or the stability of Ty cDNA, roles that have not been previously reported for this protein. The deficiency in accumulation of Ty cDNAs in *dbr1* mutants is apparent when engineered Ty elements are expressed for short time periods (6–10 h) but is not apparent following long expression periods (>24 h). © 2000 Academic Press

Long terminal repeat (LTR)-containing retrotransposons such as the Ty elements found in *Saccharomyces cerevisiae* are structurally and functionally related to retroviruses and, like retroviruses, depend on host cell factors for successful propagation. Specific cellular proteins are known to play positive roles at multiple steps in the Ty replication cycle. Ty elements in the yeast genome are transcribed along with cellular genes. A set of *SPT* genes and *SNF2, SNF5,* and *SNF6* are all required for normal transcription of certain Ty elements (1–3). Translation of the resulting retroelement RNA produces a set of proteins that assemble in the cytoplasm to form virus-like particles, or VLPs, in which the process of reverse transcription occurs. It is known that Ty elements use specific host tRNAs as primers for minus-strand DNA synthesis during reverse transcription (4). The Ty cDNA product of reverse transcription can then be integrated into the host chromosome. Recently, the Ku protein, a DNA end-binding protein involved in cellular DNA double-strand (ds) break repair, has been shown to play a role in Ty1 retrotransposition, possibly during the integration of cDNA (5). Identification of additional host factors required in the Ty replication cycle would allow a better understanding of the complex interplay between host and retroelement. Accordingly, a genetic screen for mutants causing decreased Ty transposition was initiated. The effects of mutations in the yeast *DBR1* gene, isolated in this screen, on both Ty1 and Ty3 life cycles are presented.

MATERIALS AND METHODS

Yeast strains, media, and general procedures. The *S. cerevisiae* strain TMY30 (*MAT***a** *ura3-52 trp1-Δ63 his3-Δ200 ade2-101^{OC} leu2-Δ1*) was used for the mutant screen. TMY73 (*MATα ura3-52 lys2-801^{am} ade2-101^{oc} trp1-Δ63 his3-Δ200 leu2-Δ1*) was mated with mutant strains to determine segregation patterns of mutant phenotypes. Yeast selective medium, rich medium, and sporulation medium were all prepared as described by Kaiser *et al.* (6). Liquid cultures used in induction experiments were grown in YNB/CAA medium (7). Yeast transformations were done using methods developed in the Gietz laboratory (8, 9).

A standard method for assaying β-galactosidase activity in yeast was used (6). Two reporter constructs were used in these assays. pS424 (a gift from S. Honigberg) contains a *lacZ* gene fused to a *GAL1* promoter. The other construct, pTM17, contains the Ty3 *GAG3* ORF inserted between the *GAL1* promoter and the *lacZ* gene.

Transposition assays. A Ty1 transposition assay utilizing a galactose-inducible Ty1 element marked with *his3*AI has been previously described (10). The *URA3*-marked plasmid bearing this Ty1 element, pGTy1-H3m*HIS3*AI, was a gift from J. Curcio and is referred to as Ty1*his3*AI in this paper. Plasmid DG784, a derivative of pGTy1-H3m*HIS3*AI marked with *TRP1* instead of *URA3,* was also used (a gift from D. Garfinkel). Plasmid TM146, carrying a galactose-

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inducible Ty3 element marked with *his3*AI (Sadeghi *et al.*, in preparation) was used to assay Ty3 transposition.

Transposition assays were done as follows. Three patches of each transformant were replica-plated from glucose medium (SD-ura or SD-trp) to galactose medium. Following a 24-h incubation at 22°C to induce Ty1 expression, or at 28°C for Ty3, the patches were replica-plated to medium lacking histidine and scored after 3 days for Ty1 and 5 days for Ty3. To determine transposition frequencies, cells were scraped off the galactose plates, suspended in water, and dilutions were plated on both SD-ura and SD-ura-his. Transposition frequencies were then calculated as His^+ colonies/total cells. An assay for determining transposition rates (number of transposition events per cell per generation) has been previously described (11, 12).

An alternative Ty1 transposition assay utilizing a galactose-inducible Ty1 element marked with *TRP1* (on pX3, a gift from J. Boeke), and has been previously described (13).

Ty protein and cDNA analysis. Immunoblots of yeast whole cell extracts were done using standard procedures (7, 14). The ECL system (Amersham) was used in all qualitative Western blots. The AttoPhos system (JBL Scientific) was used for quantitative immunoblots. Fluorescence was detected using a STORM imager (Molecular Dynamics).

Southern blot hybridizations were done using standard methods (14). For Ty3 cDNA detection, a 1.6-kb fragment of the Ty3 sequence encoding the RT protein was used as a probe. For Ty1 cDNA detection, an 0.9-kb fragment of the *his3*AI gene was used as a probe to avoid detection of unmarked genomic copies of Ty1.

Complementation of transposition defects. An *S. cerevisiae* genomic library, containing 4–8 kb randomly sheared genomic DNA fragments in the vector λYES-R (which is marked with *URA3*), was obtained from ATCC (American Type Culture Collection, catalog No. 77256) (15, 16). Sequencing of library plasmids was done at the UMKC Molecular Biology Core Facility using the primer, 5′-CAACAAAAAATTGTTAATATACCTC-3′, which corresponds to a region of the *GAL1* promoter that is directly upstream of the yeast genomic fragments (16).

RESULTS

Isolation of Two Mutants That Cause Decreased Ty Transposition

To identify specific cellular genes involved in the Ty replication cycle, a genetic screen for mutations causing a decrease in Ty1 transposition was performed. Mutations were induced by UV irradiation of the yeast strain TMY30 containing the Ty1*his3*AI reporter construct (10). In this reporter system, the Ty1 element is under the control of the yeast *GAL1* promoter and marked with a *his3* allele interrupted by an artificial intron (AI). Due to opposing transcriptional orientations, the AI can only be spliced out of the *his3* gene when transcription proceeds from the *GAL1* promoter. Reverse transcription then produces a Ty1 element fused to a functional *HIS3* gene and, upon transposition, cells are converted from a His− to a His+ phenotype. Accordingly, mutants with a decreased level of His+ papillation after growth in the presence of galactose are candidates for positive genes involved in Ty transposition.

In the initial stages of the mutant screen, transposition assays were performed on ~4000 colonies arising from UV irradiated cells (~60% killing). Colonies giving rise to decreased numbers of His+ papillae were retested. Candidates showing reduced growth on galactose were not considered further. The remaining candidates were retested with fresh reporter plasmids to eliminate those with plasmid mutations. Two mutants, initially called mut198 and mut208, showed 50-fold and 25-fold decreases in the frequency of Ty1 transposition compared to wild-type levels, respectively. The frequency of Ty3 transposition was also found to be significantly reduced in both mutants. To factor out transposition variation due to growth rate, Ty1 and Ty3 transposition rates (the number of transposition events per cell per generation) were determined for both wild-type and mutant populations. As seen in Table 1, the rates of Ty1 and Ty3 transposition in both mutants using the *his3*AI assays are dramatically reduced compared to wild-type rates.

TABLE 1

Transposition Rates in mut198 and mut208

	*his3*AI assay[a] (%)		pX3 assay[b] (%)
	Ty1	Ty3	Ty1
Wild-type	100	100	100
mut198	7.8	<4.2	<0.28
mut208	<0.58	2.7	2.7

Note. Rates of mut198 and mut208 are given as percentages of wild-type transposition.

[a] Transposition rates = His^+ events/cell generation.

[b] Transposition rates = Trp^+ events/cell generation.

An alternative Ty1 transposition assay was performed to ensure that the mutant phenotypes were not specific to the *his3*AI-marked elements. This Ty1-pX3 assay utilizes a Ty1 element marked with a *TRP1* gene and transposition is measured as the rate of Trp^+ prototroph formation after plasmid loss (13). The results of this experiment, also seen in Table 1, agree with the results from the *his3*AI assays.

mut198 and mut208 Produce Wild-type Levels of Ty Proteins, but Decreased Levels of Ty cDNA

To characterize the blocks to Ty1 and Ty3 transposition in mutants 198 and 208, Ty life cycle intermediates were examined. To examine Ty3 intermediates, pairs of cultures for mutants and wild-type were grown and one culture of each set was induced to express Ty3*his3*AI for 6–8 h by the addition of galactose. Cells were harvested, total cellular protein was extracted from one half of all cultures, and immunoblots were performed using anti-Ty3 CA antiserum. There are comparable amounts of CA and its precursors in all three strains when Ty3 is expressed (Fig. 1A).

Total yeast DNA was extracted from the other half of the cultures described above, and Southern hybridiza-

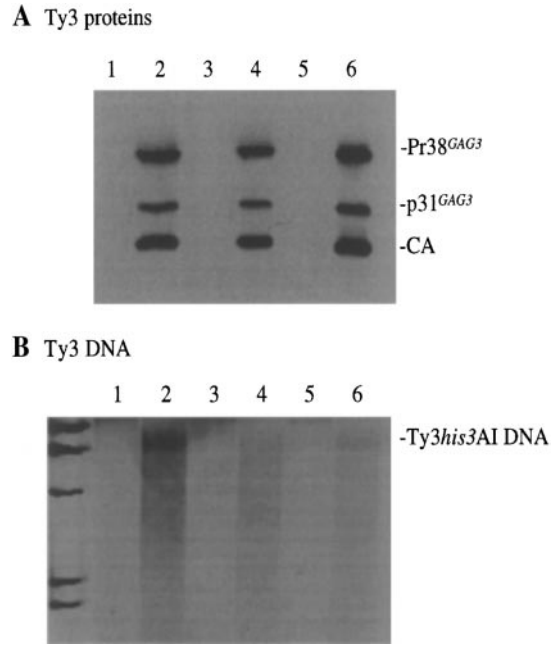

FIG. 1. Ty3 protein and cDNA levels in mut198 and mut208. Total cellular protein and DNA were isolated from pairs of TMY30 (lanes 1 and 2), mut198 (lanes 3 and 4), and mut208 (lanes 5 and 6) cultures transformed with pTM146. One culture of each pair was induced to express Ty3 by incubation with 2% galactose for 6.5 h at 28°C (lanes 2, 4, and 6) while the other culture of each pair was incubated with 2% raffinose for those 6.5 hr as negative controls (lanes 1, 3, and 5). (A) Immunoblot analysis of protein samples (5 μg per lane) using anti-Ty3 CA antibody. (B) Southern blot analysis of DNA (1 μg per lane) using a Ty3-specific probe. The molecular weight marker lane is λ DNA digested with *Hin*dIII.

tion was performed with a Ty3-specific probe. Although a significant amount of Ty3 cDNA is produced in wild-type cultures expressing Ty3, very little or no Ty3 cDNA is produced in the corresponding mutant cultures (Fig. 1B).

Ty1 protein and cDNA levels were also examined in wild-type and mutant strains by performing experiments similar to those described for Ty3 analysis. The results of an immunoblot, probing with anti-Ty1 RT antiserum, are shown in Fig. 2A. As for Ty3, equivalent amounts of the Ty1 protein are produced in mutant and wild-type cultures expressing Ty1.

A Southern blot using a *his3*-specific probe shows that the mutants also produce less Ty1*his3*AI cDNA (Fig. 2B). Thus, mut198 and mut208 affect two different retroelements in a similar manner, blocking the Ty replication cycle at the stage of reverse transcription or at the level of cDNA stability.

Complementation of mut198 and mut208 by DBR1

Each mutant was crossed with a wild-type strain (TMY73) and segregation patterns for the mutants' phenotype were analyzed after sporulation of the diploids. For mut208, the Ty1 transposition phenotype segregated 2:2 for all 6 of the dissected tetrads, indicative of a single gene mutation in this strain. For mut198, the segregation of the transposition defect was obscured by segregation of a second character affecting growth. The data suggested that one mutation affects Ty transposition while a second mutation is responsible for decreased growth rates.

To isolate the corresponding wild type genes, mut198 and mut208, both containing the Ty1*his3*AI plasmid, were transformed with a yeast genomic DNA library. Ty1 transposition was measured in transformant colonies by assaying His+ formation following galactose induction. Two positive clones out of 6300 total transformants of mut208, and one positive clone out of 5000 total transformants of mut198, were isolated. For these clones, loss of library plasmids resulted in loss of the wild-type transposition phenotype. The library plasmids from the three positive clones were isolated by transformation of *E. coli* with total yeast DNA. Retransformation of the original mutants with the appropriate individual library plasmids resulted in complementation of the transposition defect once again (Fig. 3).

The three complementing library plasmids were sequenced. All three plasmids contain segments of chromosome XI sequences containing *DBR1*, encoding the yeast debranching enzyme. In fact, *DBR1* is the only complete gene in the region defined by the overlapping portions of each plasmid (Fig. 4). Accordingly, each of the three library plasmids complemented mutant phenotypes in both mut198 and mut208. Mutation of the

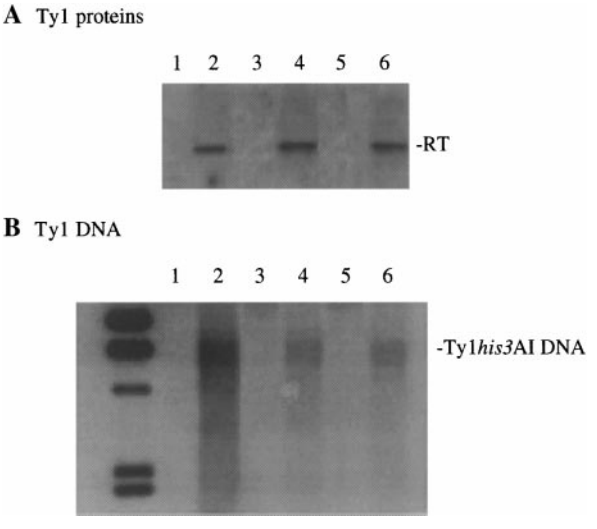

FIG. 2. Ty1 protein and cDNA levels in mut198 and mut208. The same experiment as described in the legend to Fig. 1 was performed with wild-type (lanes 1 and 2), mut198 (lanes 3 and 4), and mut208 (lanes 5 and 6) transformed with the Ty1*his3*AI plasmid instead of pTM146. Incubations were done for 8 h at 22°C in this case. (A) Immunoblot analysis (1 μg per lane) using anti-Ty1 RT antibody. (B) Southern blot analysis (2 μg per lane) using a *his3*-specific probe.

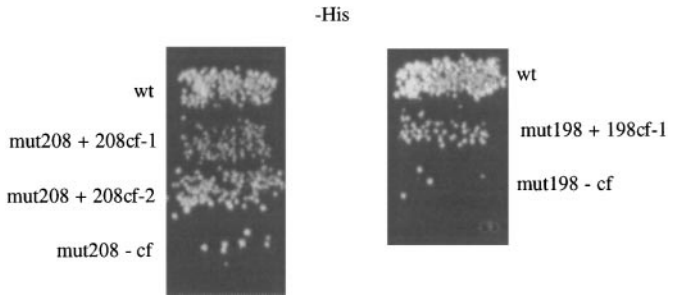

FIG. 3. Specific library fragments complement the Ty1 transposition defect in mut198 and mut208. Mut208 and mut198 were transformed with the *TRP1*-marked Ty1*his3*AI plasmid, pDG784 and then transformed with the library plasmids containing their respective complementing fragments (mut208 + 208cf-1 and 208cf-2; mut198 + 198cf-1), or with a *URA3*-marked CEN-based vector lacking an insert as negative controls (mut208 − cf and mut198 − cf). TMY30, the parental strain of the mutants, was transformed with pDG784 and the negative control construct as well (wt). Transformants patched on SD-ura-trp medium were replica-plated to SG-ura-trp and incubated at 22°C for 24 h. This plate was then replica-plated to SD-ura-trp-his and incubated at 28°C for 3 days.

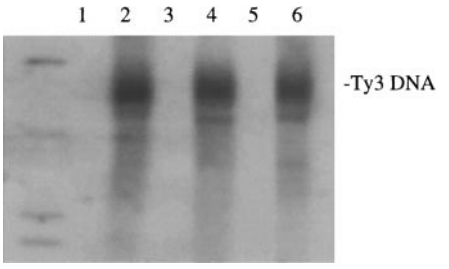

FIG. 5. Ty3 cDNA synthesis in *dbr1-198* and *dbr1-208* during long inductions. The same experiment as described in the legend of Fig. 1 was performed, with the sole exception being a 24 h vs a 6–1/2 h incubation with galactose. Again, lanes 1 and 2 are wild-type samples, lanes 3 and 4 are *dbr1-198* samples, and lanes 5 and 6 are *dbr1-208* samples. The first sample for each strain was isolated from uninduced cultures and the second from induced cultures.

DBR1 gene is responsible for the transposition defects seen in both mutants, now referred to as *dbr1-198* and *dbr1-208*.

dbr1 Mutations Result in Slower Accumulation of Ty cDNA

Previous work has reported that Ty1 VLPs have no obvious biochemical defects in *dbr1* strains (17). However, our results show a strong decrease in the levels of Ty1 and Ty3 cDNA due to *dbr1* mutations. One explanation for the disparity between previous and present data is that the deficiency in Ty cDNA synthesis due to *dbr1* mutations may be masked if Ty is expressed for a long time period. Our experiments were performed with induction periods of 6–10 h while previous experiments on *dbr1* mutants used induction periods of 24 h or greater. Therefore, levels of Ty1 and Ty3 cDNA produced in wild-type vs *dbr1-198* and *dbr1-208* cultures induced for 24 h were analyzed. The results for Ty3 are shown in Fig. 5, where it can be seen that the amounts of Ty3 cDNA in mutant and wild-type samples are much more comparable than for short inductions (see Fig. 1B). The same results were obtained for Ty1 cDNA (data not shown). Thus, Ty cDNA accumulates in mutant cells but at a much slower rate than in wild-type cells.

Perhaps a small but significant decrease in galactose-induced transcription could have significant effects on Ty replication even though cellular growth is unaffected. It has been noted that the small twofold increase in overall Ty1 RNA in the cell due to galactose induction of a Ty1 reporter construct results in a much more dramatic (20- to 100-fold) increase in other Ty1 components (18, 19). So a more moderate increase in Ty RNA than full galactose induction would produce disproportionately lower levels of Ty proteins and

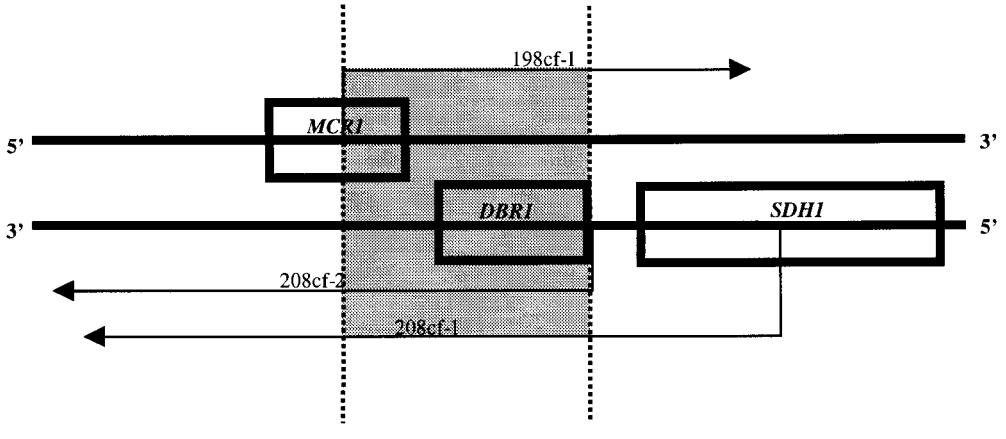

FIG. 4. *DBR1* complements the transposition defects seen in mut198 and mut208. The two complementing fragments for mut208 (208cf-1 and 208cf-2) and the one complementing fragment for mut198 (198cf-2) were sequenced. Their locations on chromosome XI are shown here. The overlapping region of all three fragments is shaded.

cDNA than those observed during full galactose induction. Therefore, galactose induction of two reporter constructs in which the *lacZ* gene is transcribed from the *GAL1* promoter, pS424 and pTM17, were tested in the *dbr1* mutants and wild-type cells. The β-galactosidase activity, and thus the amount of galactose induction, produced from both constructs upon addition of galactose was found to be equal in the wild-type and both mutant strains (data not shown). Consistent with these results, quantitative immunoblots demonstrate that Ty1 and Ty3 protein levels in *dbr1-198* and *dbr1-208* are equivalent to levels in wild-type cells (data not shown).

DISCUSSION

We have identified two mutations generated by UV-irradiation which diminish both Ty1 and Ty3 transposition. These mutations are both complemented by *DBR1,* encoding the yeast debranching enzyme. It has been previously reported that *dbr1* mutants exhibit decreased Ty1 transposition (20). In the present study, we report that Ty3 transposition is also significantly decreased in *dbr1* strains. However, a specific role for *DBR1* in the Ty life cycle has not been determined. We have shown that in *dbr1* mutant strains Ty protein levels are unaffected whereas Ty cDNA levels are significantly reduced. It was also shown that full-length Ty cDNA can accumulate in *dbr1* strains but the rate of accumulation is significantly slower than in wild-type cells. Comparison of Ty1 and Ty3 intermediates shows that mutation of *dbr1* affects both elements similarly.

Dbr1p cleaves 2′–5′-phosphodiester linkages which occur at intron RNA lariat branchpoints (20, 21). Debranching (cleavage of the 2′–5′ linkage) appears to be a rate-limiting step in the intron RNA degradation pathway because excised intron lariats accumulate in *dbr1* mutants (20). Analysis of the substrate specificity of Dbr1p has shown that multiple branched substrates, including prokaryotic multicopy single-stranded DNAs (msDNAs), a *S. cerevisiae* mitochondrial group II intron, and synthetic branched RNAs, can be cleaved by Dbr1p as long as there is a 2′–5′-phosphodiester linkage at the branchpoint (21).

We show in this work that Dbr1p is required for either Ty reverse transcription or cDNA stability. Dbr1p is presumed to be a nuclear protein due to its role in intron lariat cleavage. However, Ty1 and Ty3 reverse transcription occurs in the cytoplasm. Therefore, some Dbr1p may be localized to Ty VLPs through an interaction with a VLP component. One possible role for Dbr1p in Ty1 replication that has been previously explored is that priming of Ty1 reverse transcription may occur via a 2′-OH and Dbr1p is required to remove this primer from the cDNA product (17). Lauermann *et al.* analyzed the length of plus-strand strong-stop DNAs produced *in vivo* in wild-type and *dbr1* strains with and without treatment with purified Dbr1p. The sizes of plus-strand DNAs detected in both strains were unaffected by Dbr1p treatment. In fact, the plus-strand DNAs detected have no RNA primer still attached since RNAse treatment also had no effect on their sizes. It was also reported that the size of the Ty1 minus-strand strong-stop DNA synthesized *in vitro* with its tRNA primer attached is unaffected by treatment with purified Dbr1p (17). However, synthesis of minus-strand strong-stop DNA *in vitro* may not proceed as it does *in vivo.* It is possible that priming could occur through either of two mechanisms, a predominant one involving a 2′-OH and another less common one utilizing a 3′-OH. *In vitro* assays could favor the use of the 3′-OH in which case Dbr1p would have no effect on the DNA produced. Comparison of minus-strand strong-stop DNAs made *in vivo* in wild-type and *dbr1* strains, with and without Dbr1p treatment, may help determine if Dbr1p plays a role in Ty minus-strand priming.

An alternate possibility is that, in the context of a Ty VLP, Dbr1p may perform a function different from its cellular role. For example, Dbr1p may bind and stabilize Ty cDNA or transport Ty cDNA to the nucleus.

Dbr1p may play a less direct role in Ty transposition. The ribonucleotides in intron lariats eventually get recycled. In the *dbr1* mutant there may be lower levels of deoxyribonucleotides (the substrates for DNA synthesis) outside of S phase because their precursors are tied up in intron lariats. This might limit the synthesis of Ty cDNA to S phase. The levels of cellular nucleotide pools have been shown to affect reverse transcription for human immunodeficiency virus type 1 (HIV-1) (22) as well as Moloney murine leukemia virus (MoMLV) (23). Another possibility is that accumulation of introns produces as yet unidentified effects in the cell that in turn affect Ty replication.

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