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Ty3 requires yeast La homologous protein for wild-type frequencies of transposition

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Summary

The *Saccharomyces cerevisiae* retrovirus-like element Ty3 inserts specifically into the initiation sites of genes transcribed by RNA polymerase III (pol III). A strain with a disruption of *LHP1*, which encodes the homologue of autoantigen La protein, was recovered in a screen for mutants defective in Ty3 transposition. Transposition into a target composed of divergent tRNA genes was decreased eightfold. In *lhp1* mutants, Ty3 polyproteins were produced at wild-type levels, assembled into virus-like particles (VLPs) and processed efficiently. The amount of cDNA associated with these particles was about half the amount in a wild-type control at early times, but approached the wild-type level after 48h of induction. Ty3 integration was examined at two genomic tRNA gene families and two plasmid-borne tRNA promoters. Integration was significantly decreased at one of the tRNA gene families, but was only slightly decreased at the second tRNA gene family. These findings suggest that Lhp1p contributes to Ty3 cDNA synthesis, but might also act at a target-specific step, such as integration.

Introduction

The current study was undertaken in order to identify host factors that participate in the life cycles of yeast retrotransposons. Ty3 is one of five retrotransposons found in *Saccharomyces cerevisiae* (reviewed by Boeke and Stoye, 1997; Sandmeyer *et al.*, 2001). Ty3 is a 5.4kbp DNA sequence composed of an internal domain flanked by long-terminal repeats (LTRs) of 340bp (Clark *et al.*, 1988). The element encodes Gag3p and Gag3–Pol3p polyproteins that are processed into mature proteins by the Ty3 protease (PR) (Hansen *et al.*, 1992; Kirchner and Sandmeyer, 1993). Gag3p is processed into major structural proteins, capsid (CA) and nucleocapsid (NC). *POL3* is expressed as a Gag3p–Pol3p fusion dependent upon a

+1 frameshift at the end of *GAG3* (Kirchner *et al.*, 1993). This polyprotein is processed into structural as well as catalytic proteins, PR, reverse transcriptase (RT) and integrase (IN) (Hansen *et al.*, 1992). Ty3 RT is responsible for reverse transcription of RNA into double-stranded DNA in a process facilitated by NC (Orlinsky and Sandmeyer, 1994; Gabus *et al.*, 1998), and IN mediates integration of DNA into the host genome (Kirchner and Sandmeyer, 1996). Ty3 integrates specifically at the transcription initiation sites of genes transcribed by RNA polymerase (pol) III (Chalker and Sandmeyer, 1990; 1992), and pol III transcription factors have been implicated in targeting by *in vitro* and *in vivo* studies (Chalker and Sandmeyer, 1993; Kirchner *et al.*, 1995; Yieh *et al.*, 2000; Aye, 2001).

Because of limited coding capacity, retroelements rely on host cells for functions including RNA processing, nuclear import and export and translation (Coffin, 1996; Sandmeyer and Menees, 1996; Boeke and Stoye, 1997). In addition, several RNA species play quite specific roles in the retroelement life cycle, including the tRNA primer for reverse transcription and tRNA species required for frameshifting and, potentially, small RNAs involved in splicing. In the case of Ty3, tRNA^{iMet} was shown to function as the primer for reverse transcription (Keeney *et al.*, 1995). Other tRNA species control the frequency of Ty3 +1 frameshifting required for Gag3–Pol3p translation (Farabaugh *et al.*, 1993; Sundararajan *et al.*, 1999).

We identified a mutation in *LHP1* in a screen for genes that affect transposition of Ty3. *LHP1* encodes the yeast La homologous protein (Hendrick *et al.*, 1981; Yoo and Wolin, 1994), one of a number of proteins involved in the synthesis and processing of small structural RNAs. Mammalian La was first identified as a component of an autoantigen, Ro RNP, and was shown to have RNA-binding activity (Hendrick *et al.*, 1981; Stefano, 1984). It was initially implicated in termination of pol III transcription (Gottlieb and Steitz, 1989). Subsequently, the mammalian protein was reported to facilitate pol III transcript release and reinitiation (Maraia *et al.*, 1994; Maraia, 1996), although it is not absolutely required *in vitro* for pol III reinitiation (Kassavetis *et al.*, 1990; 1999; Weser *et al.*, 2000). Lhp1p is not essential. The role of Lhp1p as an RNA chaperone in yeast was uncovered beginning with the demonstration that a mutation in *LHP1* is synthetically lethal with a mutation in the anticodon stem that destabi-

lizes the structure of an essential tRNA$^{Ser}_{CGA}$ encoded by a single gene (Yoo and Wolin, 1997). Since that time, Lhp1 has been implicated in a variety of RNA chaperone activities (reviewed by Wolin and Cedervall, 2002). Lhp1p was shown to stabilize pretRNAs for the endonucleolytic cleavage leading to 3′ end maturation (Yoo and Wolin, 1997) and to co-operate with Gcd14p in the maturation of some tRNA precursors (Calvo *et al.*, 1999). In addition to binding pretRNAs, Lhp1p binds to processing intermediates of other small structural RNAs: certain U snRNP RNAs, including the pol III-transcribed U6 (Pannone *et al.*, 1998; 2001), and pol II-transcribed U1, U2, U4 and U5 RNAs (Xue *et al.*, 2000); snoRNP complex RNAs (Kufel *et al.*, 2000); and Ro RNP complex RNAs (Pruijn *et al.*, 1990). In addition to its role in binding structural RNAs, a variety of other RNA-related functions have been ascribed to La. Mammalian La binds in the vicinity of the translation initiation codon of some RNAs (McBratney and Sarnow, 1996). It has been documented to promote internal translation initiation of polio virus (Meerovitch *et al.*, 1993; Svitkin *et al.*, 1994a) and hepatitis C virus (Ali *et al.*, 2000) RNAs. In addition, it reverses interference by the TAR element with translation of human immunodeficiency virus (HIV)-1 RNA (Svitkin *et al.*, 1994b). La interacts with the 5′-terminal oligopyrimidine sequence found in a collection of growth-related genes, the so-called TOP genes (Crosio *et al.*, 2000), and appears to enhance their translation. La also stabilizes hepatitis B virus against RNase degradation (Heise *et al.*, 2001).

In this study, we found that disruption of *LHP1* decreased Ty3 transposition into a plasmid-borne, divergent tRNA gene target and differentially affected transposition at two chromosomal tRNA gene families. In addition, Ty3 cDNA accumulated at a slower rate in the mutant strain than in the wild-type strain. Based on these observations, we propose that Lhp1 may have two functions in Ty3 transposition: one in cDNA production and one at the target site.

Results

A genetic screen was performed in order to identify host factors that show genetic interactions with Ty3. Briefly, a library of yeast DNA fragments previously mutagenized with a mini-Tn*3* element marked with bacterial *lacZ* and yeast *LEU2* was transformed into yeast strain YPH500 (Table 1), and transformants were selected (Burns *et al.*, 1994; Aye, 2001). The high frequency of homologous recombination in yeast ensured that most of the transformants represented cells in which a disrupted copy of a gene has replaced the wild-type locus. A large number of isolates were screened for Ty3 transposition phenotype. We report here the characterization of one mutant. A less detailed description of Ty3 phenotypes of the larger set of mutants will be reported in a separate publication (M. Aye and S. B. Sandmeyer, manuscript in preparation).

A genetic assay in which Ty3 position-specific transposition between two divergently transcribed tRNA genes activates a suppressor tRNA gene (*sup2bo*) (Kinsey and Sandmeyer, 1995) was used in a trial screen of 2000 Leu$^+$ transformants for Ty3 transposition mutants. Leu$^+$ mutant strains and wild-type strain yMA1235, a *LEU2* version of the parental strain YPH500, containing a galactose-inducible Ty3 element on low-copy plasmid pTM843 (Table 2) and a tDNA target, on plasmid pPK689, were picked and arrayed in patches onto SC[glu] lacking histidine, tryptophan and leucine. Cultures were replicated to SC[gal] lacking histidine, tryptophan and leucine in order to induce Ty3 expression and select for cells retaining the plasmids. In pPK689, tDNA^{Val}, which functions as the Ty3 target, is positioned so that it interferes with the expression of a neighbouring, divergently transcribed ochre suppressor tDNA^{Tyr}, *sup2bo*. S*up2bo* expression is further attenuated by a tract of pyrimidines on the non-transcribed strand in the region of transcription initiation. Ty3 position-specific integration at the tDNA^{Val} target alleviates interference between the divergent genes and changes the sequence composition upstream of *sup2bo*, thereby activating suppressor expression. YPH500 contains a nonsense allele, *ade2o*, which is suppressed by expression of *sup2bo*. Patches of cells induced to undergo transposition were replicated to minimal medium supplemented with uracil and lysine. On this medium, cells that have undergone transposition and activated suppressor expression are observed as papillae (data not shown).

Table 1. Yeast strains used in this study.

Strain	Description	Reference
YPH500	*Matα ade2-101$_o$, lys2-801$_{am}$, his3Δ200, trp1-Δ63, leu2-Δ1, ura3-52*	Sikorski and Hieter (1989)
yMA1235	*LEU2* derivative of YPH500	This work
yMA1237	*lhp1*::mTn*3* disruption in YPH500 (22–19)	This work
yMA1322	*Matα, ade2$_o$, lys2$_o$, his3, trp1, leu2, ura3* (derived from YPH500)	Aye *et al.* (2001)
yMA1342	*LEU2* derivative of yMA1322	Aye *et al.* (2001)
yMA1345	*lhp1*::mTn*3* in yMA1322 (reconstructed mutant)	This work
ySBS1353	*Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0* (BY4741)	Research Genetics
ySBS1380	*lhp1Δ* derivative of BY4741	Research Genetics

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Table 2. Plasmids used in this study.

| Plasmid	Description	Reference
pDLC315	2 micron, *HIS3*, *sup2b* C56G	Chalker and Sandmeyer (1992)
pDLC356	2 micron, *HIS3*, *sup2b* tDNA	Chalker and Sandmeyer (1992)
pDLC374	2 micron, *HIS3*, *sup2*-based target	Chalker and Sandmeyer (1992)
pPK685	*sup2o* tDNA with 5′ oligopyrimidine tract	Kinsey (1994)
pPK687	tDNA^{Val}	Kinsey (1994)
pPK689	CEN4, *sup2bo*-tDNA^{Val} target, *HIS3*	Kinsey and Sandmeyer (1995)
pTM842 (pTM42)	pDLC374 with a Ty3 insertion	Menees and Sandmeyer (1994)
pTM843 (pTM45)	CEN, *TRP1*, *GAL1*$_{UAS}$-Ty3	Menees and Sandmeyer (1994)
pMA1707	*LHP1* cloned into pCRII	This work
pMA1708	2 micron, *URA3*, *GAL1*$_{UAS}$-*LHP1*	This work
pMA1890	CEN, *TRP1*, *GAL1*$_{UAS}$-Ty3 (IN catalytic mutant)	This work
pSBS2125	2 micron, *URA3*, *GAL1*$_{UAS}$-Ty3-m*his3*AI	Sadeghi *et al.* (2001)
pSBS2126	2 micron, *URA3*, *GAL1*$_{UAS}$-Ty3-m*his3*AI (IN catalytic mutant)	Sadeghi *et al.* (2001)
pYES2.0	2 micron, *URA3*, *GAL1*$_{UAS}$	Invitrogen
pCRII	Cloning vector for PCR products	Invitrogen
pTZ1	*SUP4* G62C tDNA	Braun *et al.* (1989)
YEp351	2 micron, *LEU2*	Hill (1986)

Truncation of Lhp1p reduces Ty3 transposition

Mutant 22-19 exhibited significantly less transposition than wild-type cells in the patch assay (data not shown). In order to identify the gene disrupted by insertional mutagenesis, the strain was transformed with YIp5 plasmid linearized by digestion with *Pvu*I. Homologous recombination between beta-lactamase gene sequences in this vector and in the mTn*3* insertion resulted in the introduction of an *Escherichia coli* origin of replication, allowing the region of DNA containing the insertion to be recovered by transformation. The sequence of genomic DNA at the site of the disruption was determined as described in *Experimental procedures*. This sequence was used in a BLASTN search of the *Saccharomyces* Genome Database (http://genome-http://www.stanford.edu/Saccharomyces). The mTn*3* insertion in mutant 22-19 was found to be in the middle of *LHP1* (Fig. 1A), the gene encoding an orthologue of human La autoantigen (Yoo and Wolin, 1994). This mutant was designated YMA1237. Sequence analysis showed that mTn*3* was inserted 447bp downstream of the *LHP1* initiation codon. Thus, Lhp1p produced in the mutant strain would lack the carboxyl-terminal half of the protein, including part of the RNA recognition motif and the putative nuclear localization signal (Yoo and Wolin, 1994; Rosenblum *et al.*, 1998) (Fig. 1A). Southern blot analysis using probes against *LHP1* and *lacZ* indicated that this was the sole insertion of mTn*3* in the mutant strain (Fig. 1B; data not shown). A quantitative version of the suppressor activation assay showed that the transposition frequency in YMA1237 was ≈12% of that in wild-type cells (Fig. 1C and D).

Because the transformation process itself is mutagenic, two methods were used to test whether the *LHP1* locus

disruption corresponded to the mutation causing the defect in Ty3 transposition. First, the linearized, rescued plasmid was used to disrupt the *LHP1* locus in YPH500 and YMA1322, and transposition assays were performed. Each reconstructed *lhp1* mutant had patterns in Southern blot analysis indistinguishable from the predicted pattern and the pattern of the original mutant (Fig. 1B) and had a defect in transposition comparable to the original mutant (Fig. 1C and D; data not shown). Secondly, wild-type *LHP1* was tested for its ability to reverse the Ty3 transposition phenotype. Overexpression of *LHP1* under a galactose-inducible promoter on a high-copy plasmid, pMA1708, elevated the frequency of transposition of the *lhp1* mutant relative to that of wild-type cells carrying vector alone. Wild-type cells overexpressing *LHP1* also had higher frequencies of Ty3 transposition than controls (Fig. 1C and D). To investigate whether differences in transposition frequency correlated with different levels of Lhp1p in these strains, immunoblot analysis was performed on whole-cell extracts (WCE) with Lhp1 antisera (a gift from C. Yoo, Yale University). Lhp1p was readily detectable in the wild type, but not in the mutant strain with vector plasmid (Fig. 1E). In addition, Lhp1p expression was considerably higher in wild-type and mutant strains in which *LHP1* carried on pMA1708 was induced. Thus, expression of Lhp1p correlated well with Ty3 transposition frequency.

Because the divergent tRNA gene target is an artificial target that is selective for position specificity and orientation (Kinsey and Sandmeyer, 1995; Aye *et al.*, 2001), it was also of interest to monitor Ty3 transposition into chromosomal tRNA gene targets. Cells carrying pTM843 were induced for Ty3 expression for 6h, a time at which transposition is still increasing in the wild-type background (data not shown). Chromosomal insertions were monitored in a

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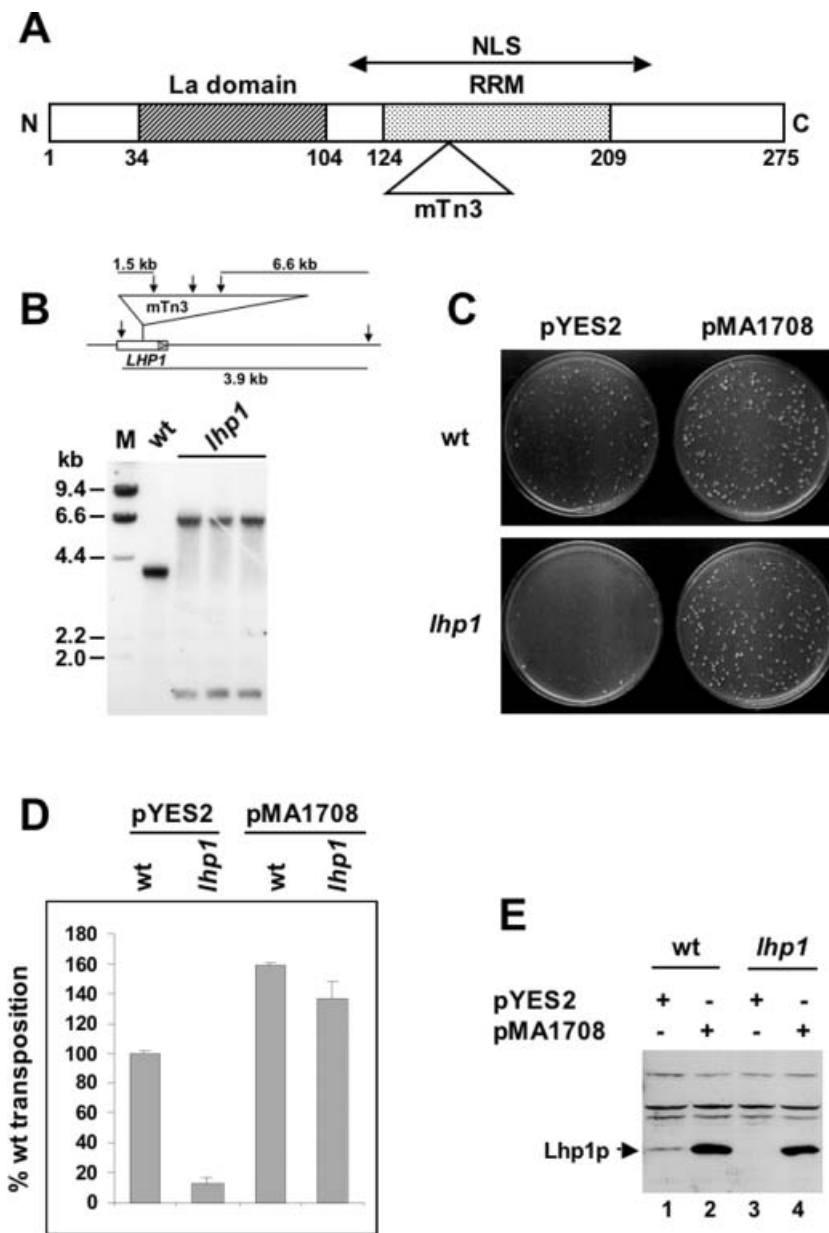
Fig. 1. Lhp1p promotes Ty3 transposition.
A. Schematic representation of Lhp1p protein domains. The coding region for Lhp1p is represented by the open box, the La-like domain by the hatched box, and the RNA recognition motif (RRM) by the stippled box. The region containing the putative nuclear localization signal (NLS) is indicated by a double-headed arrow. The location of the mTn*3* insertion is indicated by an open triangle. The numbers below the boxes represent amino acid residues as described previously (Yoo and Wolin, 1994; Rosenblum *et al.*, 1998). The La domain was determined based on similarity between the yeast and human proteins.
B. *LHP1* disruption in the mutant strain. Genomic DNA from the wild type (wt) or the mutant YMA1237 and the two reconstructed strains (*lhp1*) were cleaved with *Eco*RI, and Southern blot analysis was performed with a probe specific for *LHP1*. Lambda DNA cleaved with *Hin*dIII was used as the size marker (M).
C. Overexpression of *LHP1* elevates Ty3 transposition in mutant and wild-type cells. Cells were plated at a density of 10^6 cells per plate and replica plated to final selective medium as described in *Experimental procedures*. Growth on medium selecting for cells with Ty3 transposed into the target plasmid for wild-type (wt) and mutant (*lhp1*) strains transformed with either the control vector (pYES2.0) or expression plasmid (pMA1708). Plating was in triplicate, and examples are shown.
D. Quantification of transposition. The average number of transposition events from triplicate plates of the type shown in (C) was determined, and the average and standard deviations are shown. The level of transposition is expressed as a percentage of the frequency in the wild-type strain transformed with vector pYES2.0.
E. Expression of Lhp1p in wild-type and mutant strains. Immunoblot analysis was performed with anti-Lhp1p antibodies on − extracts from yeast strains tested for transposition. *LHP1* plasmid and vector are as described above.

polymerase chain reaction (PCR) assay in which one primer annealed to the Ty3 IN sequence upstream of the 3′ LTR, and a second primer annealed to a conserved sequence common to 10 tDNA^{Gln} or 14 tDNA^{Val} genes (Fig. 2A; Table 3). These tDNA families were chosen for the integration assay based on the observation that they had relatively few upstream Ty3 LTRs and no Ty3 insertions in the strain for which genomic sequence was determined. They performed better than two other such families in trial assays (data not shown). The amplified Ty3–tDNA junction fragment was visualized using a radioactive, Ty3 LTR-specific probe (Fig. 2B). The amount of product produced in reactions templated on DNA from cells expressing a

Ty3 IN catalytic site mutant on plasmid pMA1890 was used to estimate background in the assay. The background observed presumably arises primarily from annealing of nascent DNAs primed from endogenous tRNA gene family members with upstream Ty3 LTRs and nascent DNAs primed from the Ty3 cDNA (Fig. 2B, lanes 2 and 6). Integration of Ty3 upstream of chromosomal copies of tDNA^{Gln} was modestly reduced (Fig. 2B, compare lanes 3 and 4). In this assay, the control tDNA^{Gln} showed significant amounts of background product (Fig. 2B, lane 2). Background was less in the case of tDNA^{Val}, and insertions associated with tDNA^{Val} sequences were significantly reduced in the mutant strain (Fig. 2B, compare lanes 7 and
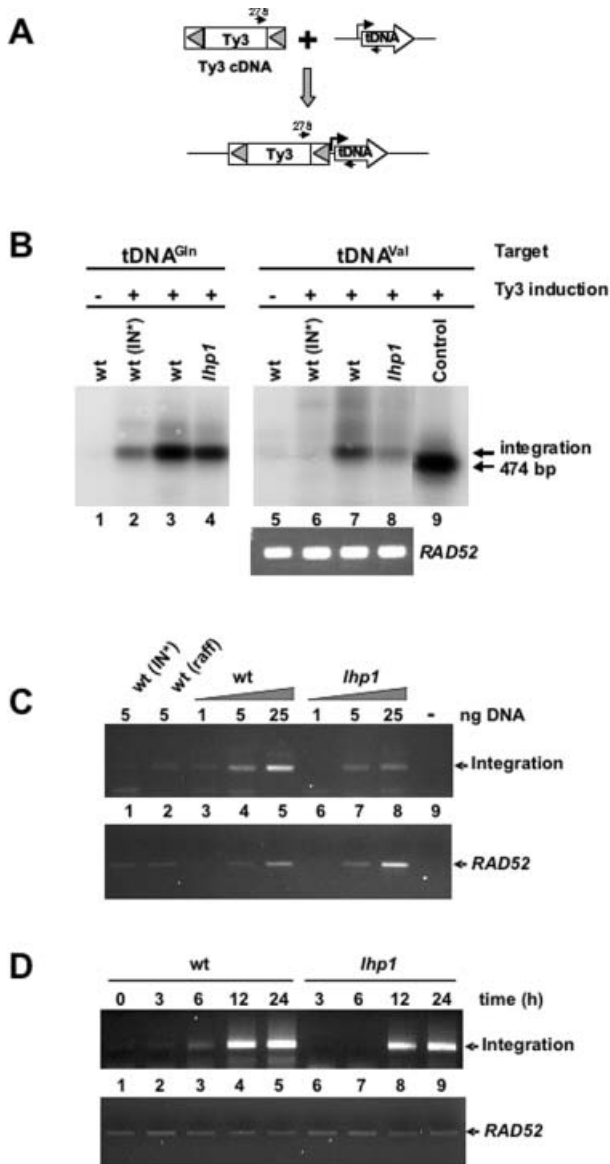
Fig. 2. PCR analysis of Ty3 genomic insertions in wild-type and *lhp1* mutant strains.
A. Strategy for PCR detection of Ty3 integration into a target. Primers are specific to Ty3 (278) and to the tDNA sequences. After insertion of Ty3, a diagnostic junction fragment can be amplified in PCR from the two primers.
B. Ty3 transposition into chromosomal targets. Wild-type and *lhp1* mutant strains transformed with Ty3 plasmid pTM843 were grown in a synthetic medium containing galactose for induction of Ty3 expression. Total DNA was extracted from cultures, and 25 ng of each was used as the template for PCR with primers specific for Ty3 and glutamine (tDNA^Gln) (lanes 1–4) or valine (tDNA^Val) tRNA gene family (lanes 5–8) genes. PCR products were subjected to Southern blot analysis with a probe specific for the Ty3 LTR (top). A Ty3 element with mutations in catalytic residues of IN (IN*) expressed from plasmid pMA1890 was used as a negative control (lanes 2 and 6). Positive control PCR (control; lane 9) was performed with pTM842, pDLC374 with a Ty3 insertion adjacent to the tDNA target (Menees and Sandmeyer, 1994). The *RAD52* locus was examined as the control PCR for the input DNA (bottom).
C. Different amounts of genomic DNA from cultures induced for Ty3 expression (6 h) were used to establish the linear range of the PCR detecting Ty3 integration. DNA (1–25 ng) from wild type (lanes 3–5) and from *lhp1* (lanes 6–8) was used as the template for PCR. For the negative control, 5 ng of each DNA was used from a culture expressing the Ty3 IN mutant (IN*; lane 1) and from an uninduced culture (raff). PCR for integrations (40 cycles, top); PCR templated by *RAD52* (20 cycles, bottom).
D. DNA samples (5 ng of each) from cultures collected after 3, 6 12 and 24 h of induction were subjected to the same analysis as in (C). Times of induction for the wild-type (lanes 2–5) and *lhp1* (lanes 6–9) samples are indicated. The *RAD52* locus was examined as the control PCR for the input DNA (bottom).

lanes 4 and 7). PCR analysis of DNA from wild-type and mutant cultures collected after different times of induction showed that integration into tDNA^Val genes occurred more frequently in the wild type than in the *lhp1* strain (Fig. 2D, compare lanes 2–5 and lanes 6–9) over a period ranging from 6 h to 24 h. The difference was most dramatic after 6 h of induction (Fig. 2D, compare lanes 3 and 7). The *LHP1* open reading frame (ORF), downstream of the mTn*3* insertion site, overlaps YDL050C, potentially complicating the interpretation of experiments with *lhp1* deletion strains.

8). Input DNA from different cultures used to template amplification of the *RAD52* locus resulted in comparable amounts of product from each sample (Fig. 2B, bottom). In order to quantify the difference observed in the case of the tDNA^Val gene family, amounts of genomic DNA from 1 to 25 ng were used to template the reaction, and the results were evaluated using QUANTITY ONE software (Bio-Rad). Analysis showed that, within this range, the amount of Ty3-tRNA^Val gene or *RAD52* control product was roughly proportional to input DNA for wild type and *lhp1* mutant (Fig. 2C). Within this range, using 5 ng of genomic DNA collected from cells induced for 6 h, the amount of Ty3-tDNA^Val product generated from wild-type DNA, normalized to *RAD52* product, was more than fourfold the amount generated from the *lhp1* mutant DNA (Fig. 2C, compare

Table 3. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′ to 3′)
12	gatttcgtaggttacctgataaaattacag
83	tggtagcgccgctgcgtttcgatccgag
278	cctcctgggatttgatccataaagctc
279	cttggctatgtttcttataattaatatcaatg
477	gcaaaggatccatgtctgaaaaaaccacaacaagagg
478	cgatagagctcccagaagaaacacaagaatcactcc
676	gttctgcgtgttaagcagatgccataacc
677	ccgaaagtgataaccactacactatagg
712	ttacgcgaccggtatcga
713	tatttgtttcggccaggaag
747	tatttgtttcggccaggaag
783	tggtagatatggccgcaacc
785	cacgccattttgagaatcga
786	atggagccaaagcggtgat
814	cctaagactgtttatattaggattgt
849	gtatataaagtaaaaaagatgcgcaagc

Nevertheless, in the genetic background used for the yeast knock-out collection (BY4741; Research Genetics), this assay yielded similar differences between wild type and a *lhp1* null mutant (*lhp1Δ*; data not shown). Thus, the Ty3
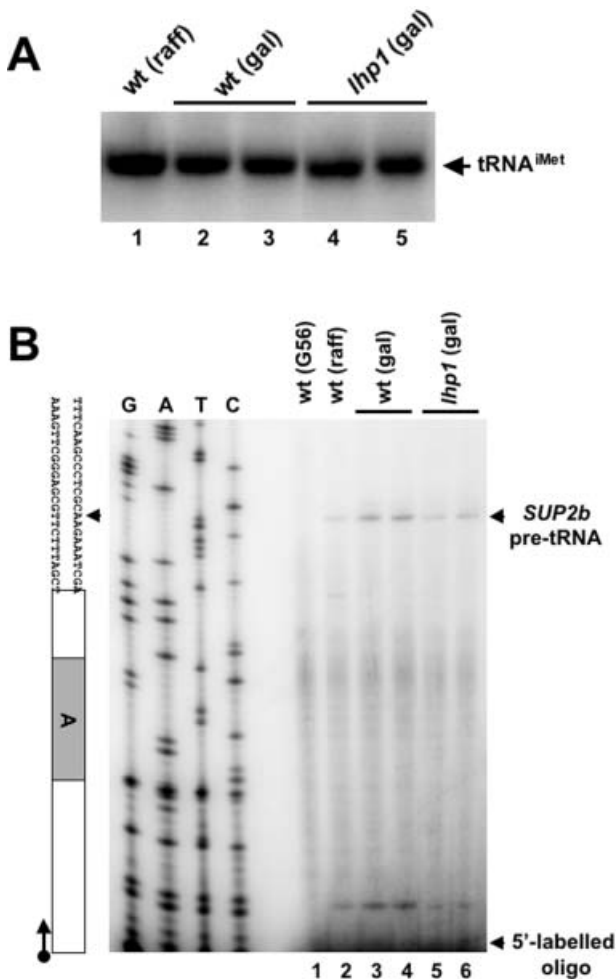
Fig. 3. Truncation of *LHP1* does not affect production of tRNA[iMet] and initiation site selection of *sup2b* pretRNA.

A. Northern blot analysis of tRNA[iMet]. Equivalent amounts of total RNA were fractionated and transferred as described in Experimental procedures. An oligonucleotide specific for mature tRNA[iMet] was used as the probe to perform Northern blot analysis of total RNA samples from the wild-type (wt) and mutant (*lhp1*) strains grown with medium containing either raffinose (raff) or galactose (gal) as the carbon source. Samples in lanes 2 and 4 were from cells induced at slightly lower densities than those in lanes 3 and 5. Samples are the same as those shown in Fig. 4.

B. Detection of pretRNA[Tyr] by primer extension. Twenty micrograms of total yeast RNA was used as the template, and ^{32}P-labelled *SUP2b* intron-specific oligonucleotide as the primer for each extension reaction. A DNA sequencing ladder was generated from a reaction templated by the *SUP2b* tDNA in pDLC356 using the same oligonucleotide. Plasmid pDLC315, containing a *SUP2* gene with a C56G mutation in the *boxB* promoter element (G56), was used as a negative control. A portion of *SUP2b* tDNA (open box) with a *boxA* element (shaded box) and upstream sequences are shown to the left of the autoradiograph. Transcription initiation site, primer extension product from pretRNA and the 5′ end-labelled, free oligonucleotide are indicated by arrows.

transposition defect observed for the synthetic target extends to at least one family of chromosomal targets.

Effect of LHP1 *truncation on cellular growth and pol III transcription*

The effect of *LHP1* truncation on growth was tested in rich and synthetic dropout medium and on medium containing glucose, galactose or raffinose as the carbon source. These experiments, as well as growth curves (data not shown), indicated that the mutant has no significant growth defect. In addition, the *lhp1* strain was not temperature sensitive as it grew as well as the wild-type strain at 37°C (data not shown). Because Lhp1p is implicated in stabilization and endonucleolytic processing of nascent pol III transcripts (Yoo and Wolin, 1997), the possibility of gross alterations in tRNA transcripts resulting in effects on Ty3 transposition was evaluated. The amount of tRNA[iMet], the Ty3 reverse transcription primer (Keeney *et al*., 1995) was examined first. Total RNA was prepared from the mutant and the wild-type cells grown to between $A_{600}=0.3$ and 0.4 and induced with the addition of galactose to a final concentration of 2% for 6h. Northern blot analysis was performed with an oligonucleotide probe specific for tRNA[iMet] (Fig. 3A). The blot was scanned, and the bands were quantified using IMAGEQUANT software. The amount of tRNA[iMet] in the *lhp1* cultures was ≈95% of that in the wild type when normalized to actin mRNA in the same preparations (Fig. 4A). Thus, it seemed unlikely that differences in tRNA[iMet] could completely account for the differences observed in transposition.

Because Ty3 inserts specifically at the transcription initiation site of genes transcribed by pol III and because mammalian La protein has been implicated in transcription reinitiation, it was possible that the *lhp1* mutant was sloppy in the transcription initiation site selection, which in turn reduced the apparent efficiency of Ty3 insertion as measured by assays demanding position specificity. To investigate transcription initiation site selection by pol III in the mutant strain, primer extension analysis was performed on the RNA samples prepared from the cells carrying a plasmid with *SUP2b* tRNA gene (pDLC356) as described in *Experimental procedures*. This tRNA gene contains an intron with a unique sequence so that pre-tRNAs are preferentially detected by primer extension. Identical sizes of the primer extension products from wild-type and mutant cells indicated that the major transcription initiation site was not altered in the mutant strain (Fig. 3B).

Effect of LHP1 *mutation on Ty3 expression, frameshifting and proteolytic processing*

Because La has been implicated in the stability of RNAs
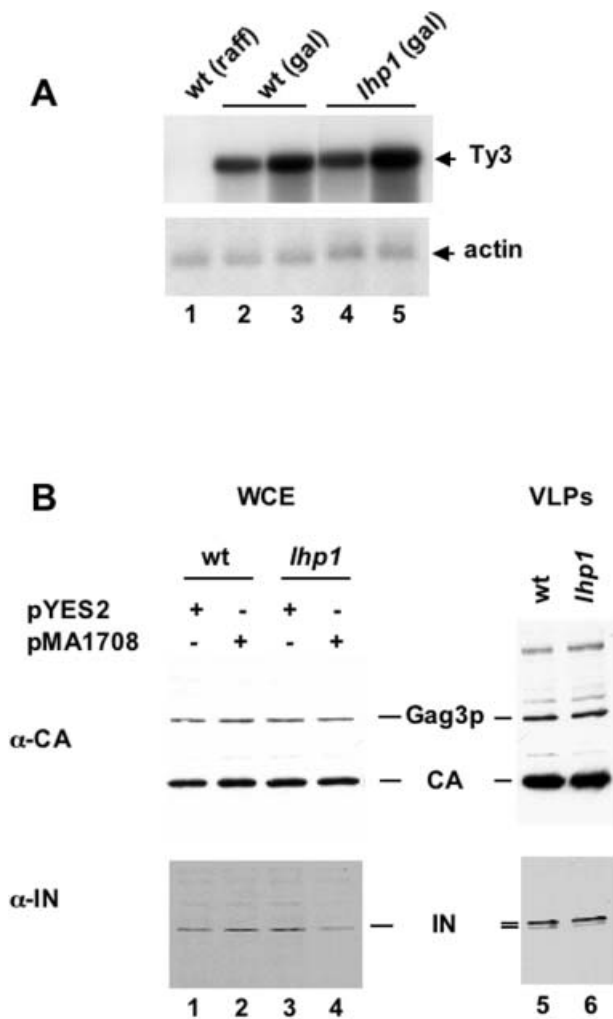
Fig. 4. Truncation of *LHP1* has no detectable effect on Ty3 expression and VLP protein production.
A. Northern blot analysis of Ty3 RNA. Total RNA samples from wild-type (wt) and mutant (*lhp1*) strains grown in medium containing raffinose (raff) or galactose (gal) were subjected to Northern blot analysis with a ^{32}P-labelled, internal *Bgl*II fragment of Ty3 (top) and a ^{32}P-labelled fragment containing the *ACT1* gene (bottom). Samples from two independent inductions, one at an A_{600} of 0.3 (lanes 2 and 4) and another at 0.4 (lanes 3 and 5), are shown.
B. Immunoblot analysis of Ty3 proteins. Wild-type (wt) and mutant (*lhp1*) strains were transformed with control plasmid (pYES2.0) or *LHP1* expression plasmid (pMA1708) in addition to pTM843 and grown in medium with galactose as the carbon source for preparation of extracts. VLPs were prepared as described in *Experimental procedures*. Immunoblots with antibodies to Ty3 CA or IN proteins were performed on 20 μg each of WCE (left) and 1 μg each of wild-type and *lhp1* Ty3 VLPs (right). Antibodies to CA recognize mature CA protein (26 kDa) and precursor Gag3p (38 kDa). Antibodies to IN recognize mature IN species of 61 and 58 kDa in VLP preparations. The slightly lower amount of IN in the *lhp1* WCE sample observed in this experiment was not typical of the majority of experiments.

and in translation efficiency, whether the amount of a specific Ty3 intermediate is decreased by the *lhp1* mutation was investigated. Ty3 RNA, protein and cDNA were measured in wild-type and mutant strains. The wild-type

and mutant strains carrying pTM843, with Ty3 under the *GAL1*$_{UAS}$, were grown in SC[raff] lacking tryptophan to a density of A_{600} of 0.3 and 0.4. Galactose was added to these cultures to a final concentration of 2% to induce Ty3 expression. After 6h of induction, cells were harvested and proteins and nucleic acids extracted. Northern blot analysis showed that the amount of Ty3 transcripts did not differ significantly between the mutant and wild-type strains (Fig. 4A, compare lanes 2 and 3 with lanes 4 and 5 respectively).
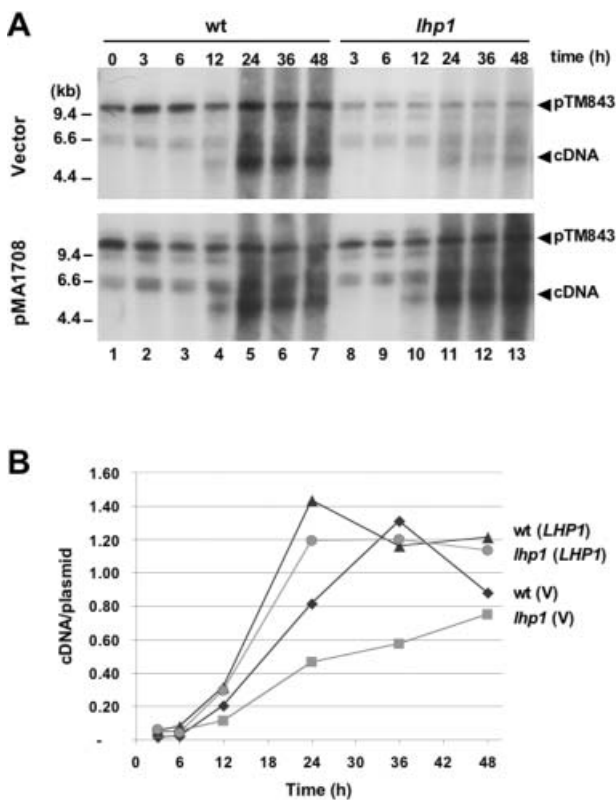
Ty3 encodes structural and catalytic proteins in *GAG3* and *POL3* ORFs respectively. Catalytic proteins are processed from a Gag3–Pol3p fusion protein precursor, the synthesis of which depends upon a +1 frameshift at the end of the *GAG3* reading frame, which occurs at a frequency of about 10% (Kirchner *et al.*, 1992; Farabaugh *et al.*, 1993). Not surprisingly, disruption of the ratio of Gag3p to Gag3–Pol3p interferes with Ty3 virus-like particle (VLP) formation (Kirchner *et al.*, 1992). In Ty3 frameshifting, low abundance of a critical, charged tRNA$^{Ser}_{GCU}$ species creates a 'hungry codon' at the frameshift site, and wobble basepairing of a near cognate peptidyl-tRNA$^{Ala}_{IGC}$ to the mRNA is proposed to allow +1 frameshifting of the ribosomes (Sundararajan *et al.*, 1999). In order to monitor Ty3 frameshifting, two *lacZ* reporter plasmids, in which the coding region of *lacZ* downstream of the Ty3 frameshift sequence was in frame with or in +1 reading frame relative to the upstream *HIS4* ORF (a generous gift from P. Farabaugh, University of Maryland), were used to measure β-galactosidase activity in wild-type and *lhp1* cells. Comparison of the activity of these two constructs indicated that Ty3 frameshifting occurred slightly more frequently in the *lhp1* mutant than in the wild-type cells (data not shown). Thus, it is possible that the loss of Lhp1 resulted in an increase in frameshifting. If the change in frameshifting was responsible for the disruption of Ty3 transposition, then the ratio of Gag3p- to Gag3–Pol3p-derived proteins or VLP formation and processing would be expected to be affected. Immunoblot analysis with antibodies against Ty3 CA and IN, encoded in *GAG3* and *POL3*, respectively, indicated that the level of Ty3 proteins in WCE and VLPs was not significantly altered by the mutation or overexpression of *LHP1* (Fig. 4B). Immunoblots of dilutions of extracts of cells expressing Ty3 confirmed that a twofold difference in CA or IN could have been detected and was not detected between extracts of wild-type and *lhp1* cells (data not shown). We conclude that, in spite of potential differences in frameshifting, production and processing of proteins was not significantly affected by the *lhp1* mutation.

Ty3 cDNA is reduced in the lhp1 *mutant*

Subsequent to particle assembly and protein maturation,

Ty3 DNA is reverse transcribed from the genomic RNA template. To investigate the effect of Lhp1p truncation on reverse transcription, expression of Ty3 was induced in the wild-type and the mutant strains, and samples were taken at different time points. Total DNA extracted from these samples was subjected to Southern blot analysis with a Ty3-specific probe (Fig. 5A). The results of this experiment were measured and quantified using a phosphorimager and IMAGEQUANT software (Molecular Dynamics). This analysis showed that Ty3 cDNA, normalized to Ty3 plasmid DNA, accumulated faster in the cells where full-length Lhp1p was present. Between 12 and 36 h of induction, the level of Ty3 cDNA in the mutant strain was about half the wild-type level, but continued to accumulate and approached the wild-type level by 48 h, when the accumu-

Table 4. Frequencies of histidine prototrophy for the wild-type (BY4741) and *lhp1Δ* strains generated by m*his3*AI-marked Ty3 elements.

Strain	Ty3 element	His⁺ frequency (×10⁻⁴)
wt	pGAL-Ty3 m*his3*AI	2.47±0.47
lhp1Δ	pGAL-Ty3 m*his3*AI	1.22±0.14
wt	pGAL-Ty3(IN*) m*his3*AI	2.45±0.24
lhp1Δ	pGAL-Ty3(IN*) m*his3*AI	1.48±0.13

IN* indicates catalytic mutant of Ty3 integrase.

Fig. 5. Lhp1 mutants produce less Ty3 cDNA.
A. Truncation of *LHP1* reduces the level of Ty3 reverse transcripts (cDNA). Wild-type (wt) and the mutant (*lhp1*) strains transformed with pTM843 and control plasmid, pYES2.0, or *LHP1* expression plasmid pMA1708 were grown in SC[raff] lacking tryptophan and uracil (0 h). After the addition of galactose, samples were taken at the indicated times. Yeast DNA was digested with *Eco*RI, and Southern blot analysis was performed with a ³²P-labelled, Ty3-specific probe, which hybridizes to full-length 5.4 kb cDNA, as well as to Ty3 donor plasmid (pTM843) and chromosomal Ty3 elements (unmarked bands).
B. The Southern blots shown in (A) were quantified in a phosphorimager and analysed with IMAGEQUANT software (Molecular Dynamics). Ty3 cDNA level, normalized to pTM843 from the same sample and expressed in arbitrary units, was plotted against the time of Ty3 induction. Cells transformed with vector or pMA1708 are indicated by (V) or (*LHP1*) respectively.

lation of Ty3 cDNA had reached a steady state or declined in the cells with normal or higher levels of Lhp1p (Fig. 5B). Moreover, the level of cDNA accumulation in different strains showed a direct correlation with the level of Lhp1p (Figs 5B and 1E), suggesting that, even in normal cells, Lhp1p may be limiting the accumulation of cDNA.

Mobilization of Ty3 was also measured using the Ty3-m*his3*AI assay (Sadeghi *et al.*, 2001) originally developed for Ty1 (Curcio and Garfinkel, 1991). Expression of Ty3 from this construct yields Ty3 RNA fused to the antisense RNA of *HIS3* interrupted by an artificial intron. Intron splicing and subsequent reverse transcription produces Ty3-*HIS3* cDNA, which can be integrated or recombined into the genome or the plasmid. Although Lhp1 is an RNA chaperone, its loss does not affect splicing (Xue *et al.*, 2000). The frequency of His⁺ prototrophy reflects recombination as well as Ty3 integration. However, in strains containing endogenous elements, the frequency of His⁺ prototrophy appears primarily to reflect recombination (Sadeghi *et al.*, 2001; unpublished results). In this context, the assay serves as a reporter for the delivery of Ty3 cDNA to the nucleus. The Ty3-m*his3*AI assay was used to measure mobilization of a wild-type element compared with an IN mutant element in the wild-type and *lhp1Δ* background (Table 4). Mobilization of the wild type and IN mutant Ty3 occurred in the wild type with a frequency of 2.47×10⁻⁴ and 2.45×10⁻⁴ per cell respectively. The wild-type Ty3 was mobilized at 1.22×10⁻⁴ per cell in the *lhp1* background. In neither the wild-type nor the *lhp1Δ* background was the result different for a catalytic site mutant of the Ty3 IN protein. No His⁺ cells were recovered in the absence of galactose induction of Ty3 expression in either strain (data not shown). Thus, using the Ty3-m*his3*AI assay, Ty3 recombination occurred with about half the frequency in the *lhp1Δ* strain as it did in the wild-type strain. The accumulation of Ty3 cDNA showed a similar decrease in the *lhp1Δ* strain background as in the YPH500 background (data not shown). Thus, Ty3 cDNA and the recombination of that cDNA showed similar reductions in the *lhp1Δ* mutant compared with wild type.

If Lhp1p affects the amount of Ty3 reverse transcripts, it might function in association with the VLP where reverse transcription occurs. Immunoblot analysis with anti-Lhp1p

antibodies was performed in order to test for the presence of Lhp1p in the VLP fraction. A small amount of Lhp1p was detected in the wild-type VLPs (Fig. 6A, right). However, it is possible that this Lhp1 is not physically associated with the VLPs.

Mutant extracts support in vitro *integration of Ty3*

The cDNA defect is less than the defect observed in the genetic assay using the divergent tRNA gene target, sug-

gesting that Ty3 could be affected at an additional step, subsequent to cDNA synthesis in the *lhp1* mutant. An *in vitro* assay was used to examine integration. The *in vitro* reaction requires, in addition to target tDNA and VLPs, TFIIIB and TFIIIC (Kirchner *et al.*, 1995). Pol III competes with VLPs for access to the target (Connolly and Sandmeyer, 1997). Immunoblot analysis was performed to determine whether Lhp1p was present in the transcription extract fractions used in these assays. Lhp1p was detected in the S100, BR500 and TFIIIB fractions (Fig. 6A,
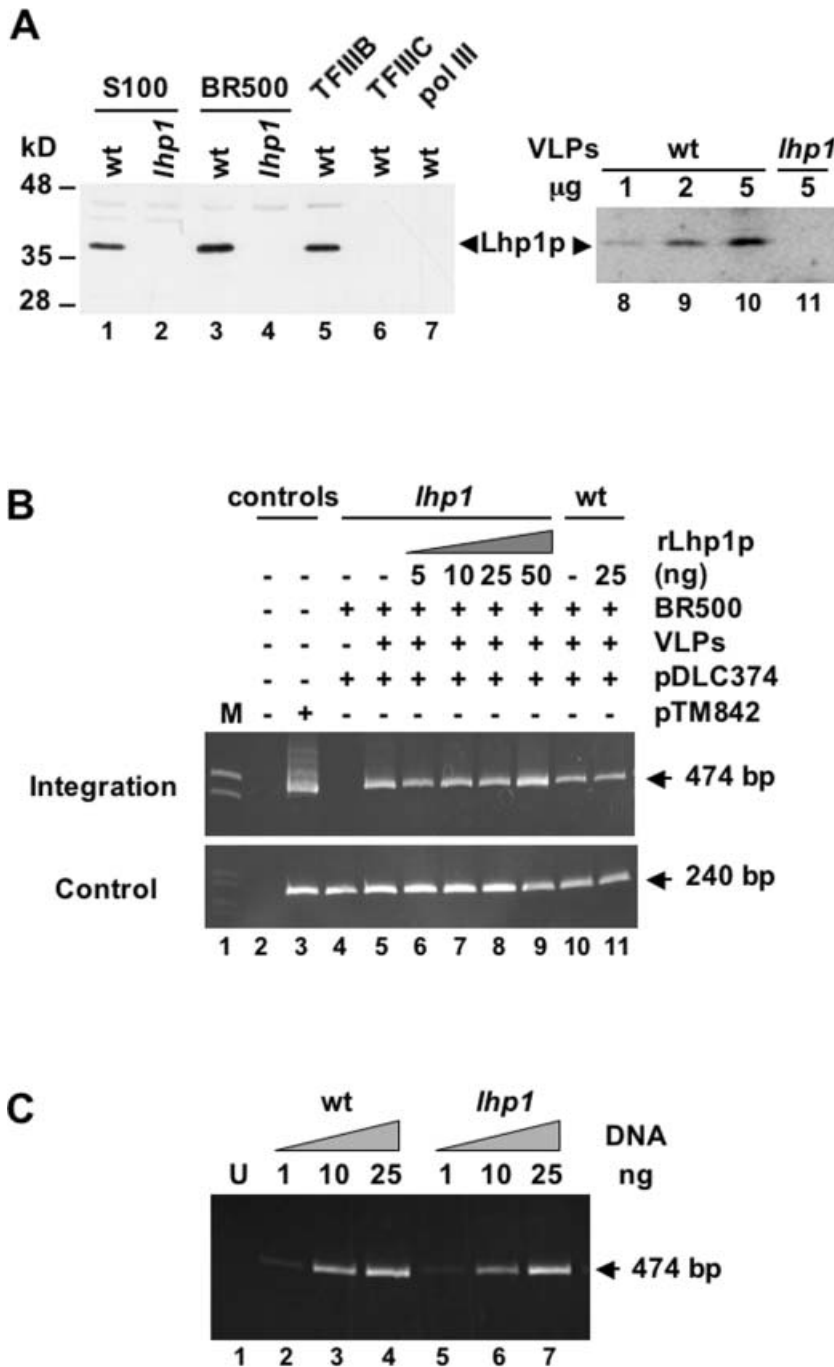
Fig. 6. *In vitro* integration assays using extracts and VLPs from wild-type and *lhp1* mutant strains.
A. Immunoblots with anti-Lhp1p antisera were performed on S100, BR500 extracts and subfractions of BR500 (TFIIIB, TFIIIC and pol III) (left) and on VLPs (right).
B. *Lhp1* extracts support *in vitro* integration of Ty3. *In vitro* integration reactions were performed with wild-type and *lhp1* BR500, recombinant Lhp1p (rLhp1p), *lhp1p* VLPs and a synthetic tRNA gene target plasmid (pDLC374). The amount of rLhp1p used is indicated. DNA extracted from each reaction was used as the template for PCR amplification. Positive and negative PCR controls were performed with pTM842 plasmid or no DNA as the template. PTM842 is pDLC374 with a Ty3 insertion. The control for loading is the product of PCR amplification of a fragment of the *HIS3* gene present on pDLC374 (bottom). DNA was fractionated by PAGE. The sizes of PCR products for integration reactions (top) and control PCR (bottom) are shown in bp. Lambda DNA cleaved with *Pst*I was used as the size marker (M).
C. *In vivo* integration of Ty3 into pDLC374 plasmid. Wild-type and *lhp1* strains transformed with pTM843 and pDLC374 were grown in synthetic medium containing galactose for the induction of Ty3 expression. Increasing amounts of total DNA template extracted from wt (lanes 2–4) and *lhp1* (lanes 5–7) reactions show increasing amounts of integration product, indicating that reactions were within a linear range.

left). Because the TFIIIB fraction is required for tDNA integration, the possibility that Lhp1p might play a role in position-specific integration of Ty3 was explored further. Transcription extracts and VLPs prepared from the wild-type and *lhp1* mutant strains were compared for the ability to mediate integration into a plasmid-borne tRNA gene *in vitro*. Integration was detected at the tRNA gene using PCR and primers in the Ty3 and tRNA gene target (Kirchner *et al*., 1995). Integration activity was similar for *lhp1* VLPs tested with either wild-type or *lhp1* extract (Fig. 6B, compare lanes 5 and 10). No difference was observed if wild-type VLPs were used in place of *lhp1* VLPs in these reactions (data not shown). The effect of incubation of BR500 or VLPs with rLhp1p was also examined. Within the concentration range of rLhp1p that altered the pretRNA processing of transcripts produced from plasmid-borne tRNA gene templates (data not shown), no significant stimulation of Ty3 integration was observed in reactions with mutant or wild-type extracts (Fig. 6B, lanes 5–8, 10 and 11). A small increase detected at a higher concentration, 50 ng of rLhp1p, in the experiment shown (Fig. 6B, lane 9) was not observed consistently. *In vivo* transposition of Ty3 into the target plasmid used for the *in vitro* assays was also examined as described previously (Menees and Sandmeyer, 1994). PCR detection of the Ty3 LTR–tDNA junction (Fig. 6C) showed that integration in the *lhp1* strain (Fig. 6C, lane 5–7) is not dramatically decreased compared with the wild-type strain (Fig. 6C, lane 2–4). Although these experiments do not exclude the possibility that Lhp1p is limiting *in vivo* for some chromosomal targets, we conclude that, *in vitro*, Lhp1p is not essential for Ty3 integration.

Discussion

In this study, we found that a disruption of the *LHP1* gene reduced Ty3 transposition into synthetic and chromosomal tRNA targets. Investigation of Ty3 life cycle intermediates showed that amounts of the Ty3 RNA and mature VLP proteins were not significantly affected by *LHP1* disruption. The disruption was associated with a twofold decrease in the amount of Ty3 cDNA during the first 24 h of induction. Greater decreases were observed for integration into a synthetic tRNA gene target plasmid and into one of two chromosomal tRNA gene targets. These results are consistent with two models: (i) that cDNA, which could be limiting for transposition overall, is used preferentially at some targets so that a reduction in cDNA differentially affects individual targets; and (ii) that Lhp1 participates at points in the life cycle subsequent to reverse transcription.

Although the *LHP1* disruption mutant isolated in our study was not a complete deletion of the *LHP1*, it is likely that the disruption resulted in loss of Lhp1p function. The NLS of Lhp1p resides within a region that encompasses the RNA recognition motif (Yoo and Wolin, 1994; Rosenblum *et al*., 1998). The mTn*3* insertion in our mutant strain truncated the coding region of *LHP1* such that the mutant protein lacked the C-terminal half, including part of the NLS region and RNA recognition motifs. The mutant Lhp1p may simply be unstable as it was not detectable using polyclonal serum raised against a recombinant protein representing residues 1–252 of Lhp1p (Yoo and Wolin, 1997). Processing of pretRNA in the *LHP1*::mTn mutant extracts was similar to processing reported for a null mutant (data not shown; Yoo and Wolin, 1997). In addition, for the properties tested, a *lhp1Δ* mutant was similar to our disruption mutant: decreased cDNA, integration into a chromosomal target (data not shown) and Ty3 mobilization in a genetic assay (Table 4). Finally, expression of wild-type *LHP1* reversed the mutant phenotype. Although *LHP1* was overexpressed under a heterologous promoter, complementation is consistent with a loss-of-function mutation.

The La protein and Lhp1p have been implicated in a broad variety of functions related to RNA-binding activity, and we speculate that this activity is also relevant in the Ty3 transposition context. As reviewed in the *Introduction*, these functions include pol III transcription termination, release and reinitiation, acting as a chaperone for a number of RNP RNAs, facilitating internal translation and protecting RNAs from degradation. Small RNAs and pol III transcription are directly critical to several stages of the Ty3 life cycle, including frameshifting, reverse transcription and integration targeting. It is therefore attractive to consider models in which the role of Lhp1p in the Ty3 life cycle is related to its role as a pol III transcript chaperone.

After particle assembly and protein processing, reverse transcription is initiated. tRNA^{iMet} is the Ty3 reverse transcription primer (Keeney *et al*., 1995). Calvo *et al*. (1999) have shown that Lhp1 is important for maturation of tRNA^{iMet} and reported decreases of about 27% in mature tRNA^{iMet} levels and of about 77% in precursor tRNA^{iMet} in an *lhp1* null mutant. Because Lhp1p is an RNA chaperone, it is possible that it functions to facilitate proper folding or binding of tRNA^{iMet} or inclusion of the tRNA^{iMet} or Ty3 RNA in the VLP. Although the decrease that we observed in Ty3 cDNA levels could be consistent with a small decrease in mature tRNA^{iMet} or in its activity as the Ty3 primer, we did not observe a comparable decrease in amounts of tRNA^{iMet} in the *lhp1* truncated strain (Fig. 3A).

RNA chaperone activity might be required in unwinding and annealing of RNA–DNA duplexes for template switching events during reverse transcription. Human La was shown to possess ATP-dependent helicase activity on RNA–DNA duplexes (Bachmann *et al*., 1990). However, yeast Lhp1p lacks the ATP-binding motif of mammalian La (Rosenblum *et al*., 1998), and no helicase activity has been reported. If Lhp1p facilitates reverse transcription in

conjunction with NC and RT, it would probably be associated with the VLP. Although there is no evidence for physical association with the VLPs, Lhp1p was detected in the VLP fraction of wild-type cells (Fig. 6A). Despite lacking the exact mechanism of Lhp1 action, it is clear that the level of functional Lhp1 is critical for the initial accumulation of Ty3 cDNA (Figs 1E and 5B) and for wild-type frequencies of transposition (Figs 1C–E and 2).

The different magnitudes of effects on cDNA accumulation and transposition that we observed suggest that the role of Lhp1 in Ty3 transposition might not be exclusive for replication, and may involve a post-replication step. However, reduction in cDNA by a small magnitude could also result in a larger magnitude of defect in transposition. In the case of Ty1, host gene mutations that affect cDNA, in some instances, are also associated with disproportionate effects on transposition. Previously, disruption of *DBR1*, the gene for the intron-debranching enzyme, was shown to result in a ninefold reduction in Ty1 transposition with no detectable effect on RNA or proteins (Chapman and Boeke, 1991). More recently, *dbr1* mutants have been reported to be deficient for Ty3 transposition (Karst *et al.*, 2000). The defect in transposition in the case of both Ty1 and Ty3 is associated with modest decreases in reverse transcripts after 24 h of induced expression (Karst *et al.*, 2000). In addition, a strict correlation between the Ty1 cDNA level and transposition was not observed among a large set of mutants for Ty1 transposition identified recently based on a genetic assay (Scholes *et al.*, 2001). Unfortunately, in those cases, the basis of the effect(s) is also not clear.

An alternative explanation for the discrepancy between the different magnitudes of effects of Lhp1 loss on Ty3 cDNA and transposition frequency is that Lhp1p has a role subsequent to cDNA production. A role at integration, for example, would be consistent with different effects of the *lhp1* mutation observed for different targets. There was an eightfold decrease in use of the synthetic target and a more than fourfold decrease at one chromosomal target in the *lhp1* mutant compared with wild type, but only modest effects at another chromosomal tRNA gene family and another synthetic plasmid target. We speculate that, if Lhp1p affects integration at the target site, the synthetic divergent target is especially sensitive to Lhp1p deficiency. The two tDNAs on this target are inefficiently transcribed (Kinsey and Sandmeyer, 1995; Aye, 2001). Because the positions of the divergent tDNAs preclude the formation of transcription initiation complexes simultaneously over both genes, they may form a target that is inherently unstable, making them particularly sensitive to conditions that destabilize the transcription complex. Although no integration defect correlated *in vitro* with either VLPs or factors prepared from the *lhp1* mutant, the *in vitro* and *in vivo* results are not directly comparable

because technical limitations of the primer combinations prevented us from specifically testing the divergent target *in vitro*. In fact, the target used *in vitro* was tested *in vivo* using a PCR assay and did not show the same magnitude of defect as the synthetic divergent tRNA gene target.

In this study, we showed that disruption of *LHP1* resulted in a substantial decrease in Ty3 transposition into a plasmid-borne tRNA target and one chromosomal tRNA gene family, but not at another. The level of Ty3 reverse transcripts was decreased by half in the mutant strain and accumulated more slowly. Although the biochemical mechanism underlying the *lhp1* effect remains to be identified, the RNA-binding function of Lhp1p seems likely to contribute to reverse transcription. The differential integration defect among target tDNAs also suggests that Lhp1p plays a post-replication role as well. Thus, Lhp1 may act at multiple points in the Ty3 life cycle.

Experimental procedures

Yeast strains and plasmids

Media and standard techniques for yeast were as previously described (Sherman *et al.*, 1986). Strains are described in Table 1. Mutants were generated in the haploid yeast strain YPH500 by integration of a yeast library disrupted with insertions of mini-Tn*3* carrying the yeast *LEU2* gene (Burns *et al.*, 1994). The isolated *LEU2* gene excised with *Bam*HI and *Nar*I restriction enzymes from YEp351 was also transformed into YPH500 to obtain the leucine prototrophic strain yMA1235, which is referred to as wild type in this study. Plasmids are described in Table 2. The mutant isolated as 22-19 (yMA1237) is a mutant with a reduced frequency of Ty3 transposition into a synthetic, plasmid-borne target. A derivative of YPH500, yMA1322, was used to reconstruct *lhp1* mutants. Yeast strains BY4741 and its *lhp1Δ* derivative (# 3748) were acquired from Research Genetics.

The *LHP1* ORF was amplified by PCR from wild-type genomic DNA, using primer oligonucleotides 477 and 478, and cloned into the pCRII vector (Invitrogen) to create pLHP1. Oligonucleotides are described in Table 3. Plasmid pMA1708 was created by cloning a 0.9 kb *Bam*HI fragment containing the *LHP1* coding region from pLHP1 into the *Bam*HI site of the high-copy, galactose-inducible, yeast expression vector pYES2.0 (Invitrogen).

Yeast mutagenesis

Disruption mutagenesis was performed as described previously. A yeast library was mutagenized by subjecting it to mini-Tn*3*::*lacZ*::*LEU2* transposition in bacteria (Burns *et al.*, 1994). DNA was prepared from library pools (kindly provided by M. Snyder, Yale University), cleaved with *Not*I and transformed using the lithium acetate procedure into YPH500, carrying Ty3 expression vector pTM843 (Menees and Sandmeyer, 1994) and Ty3 target plasmid pPK689 (Ito *et al.*, 1983). Disruption mutants were enriched by selection of Leu+ transformants on SC medium lacking leucine, tryptophan and histidine.

Ty3 transposition assays

A genetic assay for Ty3 integration (Kinsey and Sandmeyer, 1995) was adapted to screen for Ty3 transposition mutants. Leu[+] mutant transformants and wild-type strain yMA1235, carrying pTM843 and pPK689, were patched onto synthetic dropout medium with glucose as a carbon source (SC[glu] lacking histidine, tryptophan and leucine). After incubation for 24h at 30°C, each plate was replicated to SC with galactose as the carbon source (SC[gal] lacking histidine, tryptophan and leucine) for induction of Ty3 expression. After 48h of growth at 30°C, yeast cells expressing Ty3 were replicated to minimal medium with glucose as the carbon source supplemented with uracil and lysine for detection of transposition events. This medium selected for cells in which Ty3 insertion at the synthetic target activated expression of the ochre suppressor tRNA[Tyr], *sup2bo*, resulting in the suppression of *ade2o.* Cultures were incubated at 30°C for 5days, and the number of papillae within mutant and wild-type patches of cells were compared. Cells plated onto SC[glu] lacking histidine, tryptophan and leucine were incubated for 1day at 30°C (no induction) and replicated to minimal medium supplemented with glucose, uracil and lysine for the negative control. In a quantitative version of this assay (Kinsey and Sandmeyer, 1995), 1×10^6 cells were plated in triplicate on SC[glu] lacking histidine, tryptophan and leucine. Cultures were replicated onto media as described for the patch assay, and the average number of colonies per plate on final selective medium was determined. Mutants isolated in this screen were retained for analysis only if they grew on selective medium when transformed with a target plasmid containing a Ty3 insertion.

A Ty3 mobilization assay using *GAL*-Ty3-m*his3*AI elements was performed as described previously (Sadeghi *et al.*, 2001) using BY4741 and its *lhp1Δ* derivative (#3748). Briefly, at least three transformants for each strain were patched to SC[glu]-ura plates. After 2days at 30°C, patches were replicated to SC[gal]-ura and incubated at room temperature for 3days. Each patch was scraped off the plate and resuspended in 1ml of water. The cell suspension was serially diluted and plated to SC[glu]-ura and SC[glu]-his plates to titre the cells. After 3days at 30°C, His[+] and Ura[+] prototrophs were counted, and the frequency of His^{+} cells was expressed as the number of His^{+} cells divided by the number of Ura^{+} cells.

Identification of genomic loci disrupted by mTn3

Genes disrupted in each mutant were identified by recovering the disrupted locus in *E. coli.* Each candidate mutant was transformed with *Pvu*I-linearized, YIp5 plasmid to integrate an *E. coli* origin of replication into the mTn*3* construct. Genomic DNA prepared from each Ura[+] Leu[+] transformant was cleaved with *Nsi*I or *Bgl*II and treated with T4 DNA ligase to circularize DNA fragments. The ligation mixture was transformed into *E. coli* strain HB101, and transformants with *LEU2*-marked plasmids were selected. This selection was facilitated by the ability of the yeast *LEU2* gene to complement growth of HB101, which is a *leuB* mutant. Plasmids with the characteristic restriction pattern of the mTn*3* construct were sequenced with M13 (−40) primer, which anneals

to proximal *lacZ* sequence. Each sequence generated was compared with complete *S. cerevisiae* genomic DNA using the BLASTN search of the *Saccharomyces* Genome Database (http://genome-http://www.stanford.edu/Saccharomyces/) to identify the site of mTn*3* insertion. The recovered *LHP1* insertion plasmid was cleaved with *Bgl*II to linearize the plasmid and transformed into YPH500 and yMA1322 to reconstruct this disruption. Transformants were selected on SC[glu] lacking uracil and leucine; cells that lost the YIp5 *URA3* marker by recombination were selected on SC medium containing 5-fluoroorotic acid (5-FOA). Gene disruptions were confirmed by Southern blotting with probes specific for *LHP1* and *lacZ*. These isolates were retested for Ty3 transposition.

Assays for Ty3 chromosomal transposition

Ty3 transposition into chromosomal loci was measured using PCR to amplify a diagnostic fragment generated with primers annealed to Ty3 and to the tDNA. Yeast strains transformed with pTM843 were grown in SC[raff] lacking tryptophan and histidine to an A_{600} between 0.2 and 0.4. Galactose was added to a final concentration of 2% to induce Ty3 expression. After 6h at 30°C, the cells were harvested, and total DNA extracted. The concentration of yeast DNA was quantified by fluorometry in a TKO 100 (Hoefer Scientific Instruments); 1–25ng of DNA was used as the template for PCR with primer oligonucleotides 278 (Ty3) and 676 (14 valine tDNAs) or 677 (10 glutamine tDNAs). PCR conditions were essentially the same as described previously (Menees and Sandmeyer, 1994), except that the Mg^{2+} concentration was 2mM and the annealing temperature was 60°C. In order to visualize exclusively PCR products that included a Ty3 LTR, as expected for junction fragments of interest, 10μl of 75μl PCR was separated on an agarose gel, and Southern blot analysis was performed with a labelled, *Nde*I–*Xho*I fragment of Ty3 LTR. To control for input DNA, the *RAD52* locus was amplified by PCR using primers 712 and 713 under the same PCR conditions, except that 20–25 cycles of amplification were used instead of 40.

Immunoblot analysis

In order to examine the expression of Ty3 proteins, whole-cell extracts (WCE) and VLPs were prepared as described previously (Menees and Sandmeyer, 1994). Twenty micrograms of WCE or 1μg of concentrated VLP protein was fractionated by SDS-PAGE, transferred to nitrocellulose membranes (Hybond ECL; Amersham) and incubated with rabbit polyclonal antibodies against CA and IN (Menees and Sandmeyer, 1994). Secondary antibodies to rabbit IgG were detected by the ECL system (Amersham). In a separate set of experiments, twofold dilutions of whole-cell extracts from wild-type and *lhp1* cells expressing Ty3 were compared showing that assays sensitive to twofold differences did not detect differences in amounts of Ty3 proteins between the two cell types (data not shown). Immunoblot analysis of Lhp1p was performed using antisera against Lhp1p (a gift from C. Yoo, Yale University) as described previously (Yoo and Wolin, 1994).

DNA analysis

Ty3 cDNA was visualized as described previously (Menees and Sandmeyer, 1994). RNA-free total yeast DNA (1 μg) was digested with *Eco*RI to linearize the Ty3 plasmid and separated by electrophoresis on an agarose gel, transferred to a nylon membrane (Duralon UV; Stratagene) and immobilized by UV cross-linking in a Stratalinker 1800 (Stratagene). Hybridization was performed with a ^{32}P-labelled, internal *Bgl*II fragment of Ty3, which hybridizes with full-length cDNA of 5.4 kbp, as well as Ty3 donor plasmid and chromosomal Ty3 elements, but not with LTRs. To confirm that mTn*3*::*lacZ*::*LEU2* was inserted into *LHP1*, yeast DNA was digested with *Eco*RI and processed as described above. Hybridization was performed with ^{32}P-labelled, *LHP1* coding sequence amplified by PCR or a ^{32}P-labelled, *Bam*HI fragment of *lacZ*. Lambda DNA digested with *Hin*dIII was ^{32}P labelled and used as the probe to visualize the lambda size markers for Southern blots.

RNA analysis

In order to examine the effect of *lhp1* on Ty3 RNA levels, yeast cells transformed with pTM843 were grown for 6 h with 2% raffinose or 2% galactose as the carbon source, harvested and total RNA extracted (Clark *et al.*, 1988). RNA samples denatured by glyoxylation and fractionated by agarose gel electrophoresis were transferred to a GeneScreen membrane (Stratagene) and probed with the ^{32}P-labelled, internal *Bgl*II fragment of Ty3. For detection of tRNA^{iMet}, total RNA was separated in 8% polyacrylamide–8.3 M urea gel by electrophoresis, transferred to a GeneScreen Plus membrane (Stratagene) and probed with ^{32}P-labelled oligonucleotide 83, specific for mature tRNA^{iMet}.

PretRNA analysis

Primer extension analysis was performed as described previously (Kinsey and Sandmeyer, 1991) to identify the transcription initiation site of su*p2b*. Yeast cells transformed with high-copy plasmid (pDLC356) bearing the *sup2b* gene (Chalker and Sandmeyer, 1992) were grown in SC[glu] lacking histidine to an A_{600} between 0.8 and 1.0. Sup2b contains a small insertion in the intron. A primer that uniquely anneals to that insertion was used to prime reverse transcription from the intron-containing pretRNA (Chalker and Sandmeyer, 1992). Cells were harvested, and total RNA was extracted as described previously (Clark *et al.*, 1988). Twenty micrograms of total RNA was used as the template and ^{32}P-labelled *sup2b*-specific oligonucleotide 12 as the primer for each extension reaction. The DNA sequencing ladder was generated by annealing oligonucleotide 12 to denatured pDLC356 DNA and extending in the presence of dideoxynucleotides and Sequenase (US Biochemicals). Primer extension products were separated in an 8% polyacrylamide–7 M urea gel by electrophoresis and visualized by autoradiography.

BR500 transcription extract preparations and *in vitro* integration assays

Yeast transcription extracts were prepared from 12 l of stationary phase cultures as described previously (Kassavetis et al., 1989). Briefly, cell pellets were washed, resuspended and lysed with glass beads in a bead-beater chamber. Centrifugation of lysates for 1 h at 100 000 *g* yielded S100 supernatant. The S100 was fractionated by $(NH_4)_2SO_4$ precipitation. The 35–70% ammonium sulphate precipitate was dissolved and loaded on to a BioRex70 ion-exchange column. The 500 mM NaCl eluate was designated BR500. Extracts were demonstrated to be active by *in vitro* transcription of the *SUP4* tDNA^{Tyr} promoter-up mutant on plasmid pTZ1 and the isolated tDNA^{Val} and *sup2bo* carried on plasmids pPK687 and pPK685 (Kinsey and Sandmeyer, 1995). Further experiments showed that, as reported previously (Yoo and Wolin, 1997), processing activity in mutant extracts differed from that in wild-type extracts, and these differences were reversed by the addition of recombinant Lhp1p (rLhp1p; data not shown), a generous gift from C. Yoo and S. Wolin (Yale University, New Haven, CT, USA).

In vitro integration assays using BR500 extracts were performed as described previously (Kirchner *et al.*, 1995). A standard integration reaction consists of 30 μg of BR500, 2.5 μg of VLPs and 400 ng (0.075 pmol) of target plasmid pDLC374, 20 mM Hepes (pH 7.8), 80 mM NaCl, 1 mM dithiothreitol, 5 mM $MgCl_2$ and 10% glycerol in a 50 μl reaction volume. The reactions were performed at 15°C for 30 min. DNA from integration reactions was extracted with phenol–chloroform and ethanol precipitated with NH_4OAc. The pellets were resuspended in 50 μl of TE, pH 8.0, and 15 μl was used as the input for PCR. PCR primers and conditions were the same as described previously (Menees and Sandmeyer, 1994). PCR products were separated on an 8% non-denaturing polyacrylamide gel, stained with ethidium bromide and visualized by UV light.

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