A PROTEOMIC APPROACH FOR STUDYING LTR RETROTRANSPOSON HOST FACTORS IN *SACCHAROMYCES CEREVISIAE*

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DOCTOR OF PHILOSOPHY

by
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A PROTEOMIC APPROACH FOR STUDYING LTR RETROTRANSPOSON HOST FACTORS IN *SACCHAROMYCES CEREVISIAE*

Le Ding, Candidate for the Doctor of Philosophy Degree

University of Missouri-Kansas City, 2011

ABSTRACT

The Ty retrotransposons of *S. cerevisiae* are mobile elements that resemble retroviruses, both in their genetic structures and replication cycles. Ty retrotransposition begins in the nucleus with the transcription of Ty RNA. Following translation of Ty proteins, virus-like particles (VLPs) assemble in the cytoplasm. VLPs contain Ty RNA and enzymes surrounded by a capsid shell. Specific cellular tRNAs are also packaged within VLPs and serve as primers for reverse transcription of Ty element RNA into dsDNA. Ty dsDNA enters the nucleus and becomes inserted into the cellular genome, either by integrase-mediated integration or by homologous recombination, completing a cycle of retrotransposition. A wide range of cellular host factors involved in the Ty replication cycles has been identified by genetic screens; however little is known about the mechanism by which most of these affect Ty element replication. To complement these genetic approaches we have identified the host proteins associated with Ty1 VLPs by performing affinity purification of VLPs followed by mass spectrometric analyses. Almost 100 host proteins are associated with Ty1 VLPs. This list only minimally

overlaps with the lists generated by previous genetic screens for host factors and includes many essential host proteins. As with the previous genetic screens, the challenge is to determine which VLP-associated host proteins play a sensible biological role in retrotransposition. Proteins that have also appeared in other screens are obvious candidates for further study. In addition, we hypothesize that there is an increased probability of identifying host proteins that play a distinguishable role in retrotransposition among the subgroup that is specifically packaged into VLPs.

In chapter 1, I describe my discovery that two RNA binding proteins, Puf6p and Khd1p, play roles in Ty1 RNA localization and I produce evidence that supports the idea of a "specialized ribosome".

In chapter 2, I describe my analysis of a population of uncapped Ty1 RNA in VLPs. I also describe experiments on the role of the RNA lariat debranching enzyme Dbr1p in Ty1 retrotransposition, including my work establishing the association of Dbr1p with Ty1 VLPs.

APPROVAL PAGE

The faculty listed below, appointed by the Dean of the School of Graduate Studies, have examined a dissertation titled, "A Proteomic Approach for Studying LTR Retrotransposon Host Factors in *Saccharomyces Cerevisae*" presented by Le Ding, candidate for the Doctor of Philosophy degree, and certify that in their opinion it is worthy of acceptance.

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ABBREVIATIONS

2μ	2-micron origin of replication
5-FOA	5-fluoroorotic acid
A260	Absorbance at 260 nanometers
BMV	Brome mosaic virus
Bp	Base Pair
cDNA	Complementary DNA
CEN	Centromere
DNA	Deoxyribonucleic acid
Ds	Double strand
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene-diamine-tetra-acetate
FISH	Fluorescence in situ hybridization
HIV	Human immunodeficiency virus
hnRNP K	heterogeneous nuclear ribonucleoprotein K
IN	Integrase
kD	KiloDalton
LTR	long-terminal repeat

Mb	Mega base
-sssDNA	minus strand strong-stop DNA
ml	milliliter
mRNA	Messenger Ribonucleic Acid
Nt	nucleotide
OD600	Optical density at 600 nanometers
ORF	Open Reading Frame
P-body	processing body
PBS	primer-binding site
PCBP	poly(C)-binding protein
PIC	pre-integration complex
PPT	polypurine tract
PR	Protease
Puf	Pumilio-FBF
Pum-HD	Pumilio homology domain
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNase H	ribonuclease H
RNP	Ribonucleoprotein

RPs	ribosomal proteins
RT	Reverse transcriptase
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulfate
TAP	Tandem-affinity purification
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEV	tobacco etch virus
tRNA	transfer RNA
UTR	untranslated region
VLP	virus-like particle
YPD	yeast–peptone–dextrose

ACKNOWLEDGEMENTS

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I thank my lovely wife Lingjuan Zeng, my daughter Emma and parents Ping Xu, and Hanwu Ding for their support in these years.

GENERAL INTRODUCTION

Transposable elements, or mobile elements, are genetic fragments that can move from one location in a host genome to another. There are two classes of transposable elements: DNA transposons and retrotransposons. Retrotransposons mobilize themselves through RNA intermediates, which are reverse-transcribed into double-stranded (ds) cDNA by the retroelement-encoded reverse transcriptase (RT) protein and inserted into the genome by the encoded integrase (IN) protein. Retrotransposons can be further classified into long-terminal repeat (LTR) retrotransposons (e.g., Ty elements) and non-LTR retrotransposons (e.g., LINE1) based on whether they have long terminal repeat sequences at their ends.

A large number of organisms such as bacteria, fungi, plants, animals, and humans contain retroelements, including LTR retrotransposons in eukaryotes. A specific set of LTR retrotransposons found in the baker's yeast *Saccharomyces cerevisiae* are called the Ty elements. Five members of this family, Ty1-5, have been identified (Boeke and Sandmeyer, 1991; Clare and Farabaugh, 1985; Hansen and Sandmeyer, 1990; Janetzky and Lehle, 1992 Stucka et al., 1989; Voytas and Boeke, 1992,1993; Warmington et al., 1985).

Ty1 is the most abundant and best characterized LTR retroelement in *S. cerevisiae* with 32 full size elements (Lesage and Todeschini, 2005). It shares many structural

similarities with retroviruses, such as HIV (Figure 1), with the exception that Ty1 does not encode an *ENV* gene. The *ENV* gene of retroviruses encodes the envelope protein essential for their extracellular (infectious) phase (Figure 2).

The 5.9 Kbp Ty1 retroelement contains two open reading frames (ORFs), referred to *TYA* and *TYB*, flanked by two ~340 bp long terminal repeat (LTR) sequences at both 5' and 3' ends. The *TYA* ORF, which is equivalent to the retroviral *gag* ORF, encodes a 49 kDa Capsid precursor protein that is cleaved to a 45 kDa mature Capsid protein. The *TYB* ORF, which is equivalent to the retroviral *POL* ORF, encodes 20 kDa protease (PR), 71 kDa integrase (IN), and 63 kDa reverse transcriptase/RNase H (RT/RH) proteins (Boeke et al., 1985; Garfinkel et al., 1985; Mellor et al., 1985). The LTRs consist of U5 (Unique to the 5' end of the RNA), R (Repeated at both ends in RNA), and U3 (Unique to the 3' end of the RNA) regions (Figure 1). LTRs contain transcription initiation and terminiation sequences as well as the sites for strand transfer events that occur during the reverse transcription step.

The intracellular stages of retroelement replication are similar for Ty1 and retroviruses such as HIV. The typical Ty1 replication cycle can be roughly divided into 4 steps: transcription, assembly, reverse transcription and integration (Figure 2).

Ty1 genomic DNAs are first transcribed into mRNA by the host RNA polymerase II transcription machinery. The mRNA is exported into the cytoplasm, where it is translated into two types of proteins, a Gag protein and a Gag-Pol polyprotein.

The assembly step consists of the formation of virus-like particles (VLPs) which, in addition to Gag and Gal-Pol protein, contain initiator $tRNA^{Met}$ ($tRNA_i^{Met}$), two identical copies of Ty1 RNA and multiple yeast host proteins. VLPs are obligate transposition intermediates and are capable of carrying out reverse transcription and integration *in vitro* (Meller et al., 1985; Eichinger and Boeke, 1985; Garfinkel et al., 1985). Subsequent proteolytic cleavage of Gag and Gag-Pol polyproteins by the Ty1 encoded PR enzyme leads to mature VLPs in which Ty1 RNA is reverse transcribed into a linear, double-stranded cDNA by the Ty1 encoded RT using a host initiator $tRNA^{Met}$ ($tRNA_i^{Met}$) as the primer. Ty1 cDNA associates with Ty1 encoded integrase to form a pre-integration complex (PIC) which is imported into the nucleus and mediates cDNA integration into the yeast genome, usually upstream of tRNA genes or other RNA polymerase III-transcribed genes (Kim et al., 1998; Hani and Feldmann, 1998).

Ty1 elements are abundant in *S. cerevisiae* genomes. A survey of the complete genome sequence of *S. cerevisiae* (S288C strain) identified a total of 331 Ty-related insertions, which together account for 3.1% of the 12-Mb genome (Kim et al., 1998). Most of these insertions (85%) consist of a single LTR, which result from recombination between the two LTRs of a full-length element (Lesage and Todeschini, 2005). Endogenous Ty1 elements are actively transcribed. Ty1 transcripts account for 0.1 to 8% of poly(A) $^+$ RNA in different haploid yeast strains (Curcio et al., 1990). The Ty1 transcript initiates from the beginning of the 5' R region and terminates at the end of the

3’ R region, resulting in a terminally redundant RNA (Elder et al., 1983). Ty1 RNA is capped (Mules et al., 1998) and polyadenylated (Elder et al., 1983) by the yeast cellular machinery. Furthermore, Ty1 RNA is especially long-lived with a stable half-life of at least 3 hours (Nonet et al., 1987).

The conversion of genomic Ty1 RNA into ds cDNA occurs inside of VLPs (Figure 3) and is a complicated process. The Ty1 cDNA molecule is longer than its RNA template, with the cDNA having full length LTR sequences at both ends. Ty1 reverse transcription requires two identical R regions at the 5’ and 3’ ends of the Ty1 RNA template and includes two strand transfer events. Ty1 RT exhibits RNA-dependent and DNA-dependent DNA polymerase activities as well as ribonuclease H (RNase H) activity (Tanese and Goff, 1988).

Reverse transcription is initiated from a host-encoded initiator $tRNA^{Met}$ that anneals to the minus strand complementary primer-binding site (PBS) near the 5’LTR (Barat et al., 1989; Chapman et al., 1992). Elongation from this primer continues into the U5 and R regions of the RNA template to form minus strand strong-stop DNA (-sssDNA). Along with elongation, RNase H activity of the RT enzyme degrades the 5’ end of RNA. The first strand transfer event is the movement of the –sssDNA from 5’ end to 3’ end of the same or a second Ty1 RNA, which allows for further elongation of the minus strand DNA. Again, concomitant with this elongation, RNase H activity of the RT enzyme degrades the RNA template except for two short polypurine tract (PPT) sequences, which

are resistant to the cleavage by RNase H, and remain annealed to the elongating minus strand DNA. One PPT is located just upstream of U3 (PPT1) and another one is at a more central position 3' to the coding sequence for integrase (PPT2). Both of them serve as primers for Plus strand synthesis (Friant et al., 1996; Lauermann et al., 1995), but only PPT1 is essential for reverse transcription of Ty1 (Heyman et al., 1995; Xu and Boeke et al., 1990). Elongation of PPT1 to the 5' end of the minus strand cDNA produces a plus strand strong stop DNA (+sss DNA). Later, +sss DNA undergoes the second strand transfer, moving from the 5' end to the 3' end of the minus strand, forming the 5' boundary of Ty1 cDNA. The transferred plus strand is further extended using the minus strand as a template. For most retroviruses, PPT1 RNA is thought to be removed by retroviral RNase H. However, in the case of Ty1, this PPT1 primer is thought to prime multiple rounds of +sss DNA synthesis until eventually being replaced by synthesis of a second longer +sss DNA primed at PPT2 (Lauerman and Boeke, 1997).

After completion of the reverse transcription step, the resulting ds cDNA together with IN is then transported into the host nucleus and integrated into the host genome. IN integrates blunt-end substrates that contain the proper 3' ends (Eichinger and Boeke, 1990). It joins the processed Ty1 cDNA to host target DNA by making a staggered cleavage in the target DNA and ligating the 3' ends of the Ty1 cDNA to the 5' phosphate ends of the target DNA (Brown et al., 1989; Fujuwara and Mizuuchi, 1988; Katzman et al., 1989). Although integration of retroviral cDNA is random, without site preference, Ty1

cDNAs show target site specificity for the upstream region of tRNA genes (Ji et al., 1993). Once integrated into the host genome, Ty1 retroelments can be transcribed along with cellular genes, completing the intracellular phase of retroelement of replication.

Ty1 RNA is essential for Ty1 retrotranspostion. It codes for the Gag and Pol proteins which are required for the assembly and reverse transcription steps. Ty1 RNA itself also serves as the template for reverse transcription. In Ty1 RNA, several *cis* elements important for retrotransposition have been identified. Such *cis* elements include the LTRs, PBS and PPT which allow for strand transfer of minus/plus strong stop DNA, priming of reverse transcription, and priming of plus stand elongation, respectively.

In retroviruses a specific cellular tRNA is annealed to an 18 nucleotide (nt) region called the primer binding site (PBS) near the 5' end of genomic RNA. In Ty1, the primer for reverse transcription, $tRNA_i^{Met}$, binds to a PBS that is much shorter (10nt) than the 18 nt retroviral PBS. A search for additional contacts between $tRNA_i^{Met}$ and Ty1 RNA that could account for the stability and specificity of the RT priming process found that $tRNA_i^{Met}$ anneals to boxes 0, 1 and 2.1 in the 5' coding region of Ty1 RNA in addition to the canonical PBS (Chapman et al., 1992; Friant et al., 1998).

Furthermore, an *in vitro* Ty1 reverse transcription system revealed that the 3' region of Ty1 RNA (termed CYC3) also greatly enhances the initiation efficiency, a role that is independent from its role in minus-strand DNA transfer. This functional interaction requires base pairing between a 5' Gag coding sequence of 14 nt (CYC5) and a

complementary sequence located in the 3′ UTR (CYC3) and is essential *in vivo* for efficient Ty1 transposition. The importance of the 5'–3' RNA interaction (CYC3::CYC5) is strengthened by conservation or covariation in all full-length Ty1 and Ty2 elements present in the yeast genome. This observation has led to the proposal that the 5'-3' interaction results in Ty1 genomic RNA circularization, which could be a common feature of reverse transcription in all LTR-retrotransposons (Cristofari et al., 2002).

While genomic Ty1 elements are highly transcribed, they transpose at a very low frequency, merely 10^{-6} per element per cell division (Curcio and Garfinkel, 1991). However, expression of Ty1 fused to the yeast *GAL1* promoter overcomes transpositional dormancy and results in inducible high level of transposition, nearly a 100-fold increase in the presence of galactose even though the level of Ty1 mRNA increases only 5- to 15-fold (Boeke et al., 1985; Boeke et al., 1988). In these *GAL1* driven plasmids, the *GALl* promoter replaces the U3 region of the 5' LTR of the Ty1 element. The result is that the major Ty1 promoter is removed but the native Ty1 transcription start site, at the beginning of the R region of the 5' LTR, is still present and utilized. Accordingly, the Ty1 transcripts produced from elements with the native Ty1 promoter and the *GAL1* promoter are identical such that protein synthesis and reverse transcription are unaffected.

The use of this promoter also allows Ty1 transcription to be easily controlled because the expression of this type of Ty element is regulated by the carbon source. Expression is induced by galactose but not by raffinose and is strongly repressed by

glucose. The p*GAL1-Ty1* element on a low-copy number *CEN* plasmid (Griffith et al., 2003) has been useful for transposition assays.

Due to the similarites between Ty1 and retroviruses and the ease with which yeast can be manipulated, yeast retrotransposons are suitable models for the study of mammalian retroviruses. The strategy I employed in this work is to utilize the Ty1 elements as a model for the identification and characterization of host factors and then to study the roles of these host factors in retroelement replication.

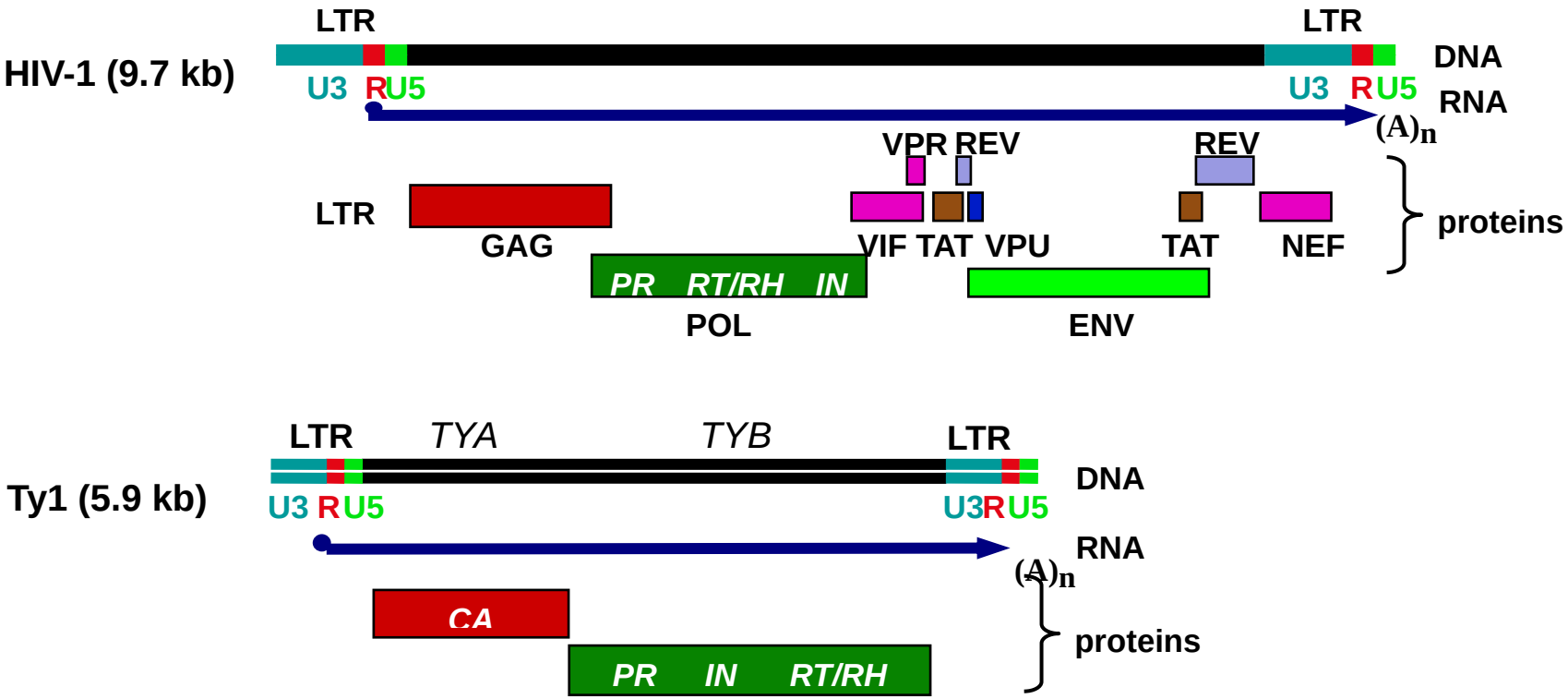

HIV-1 (9.7 kb)
LTR
LTR
U3 RU5
U3 RU5
DNA
RNA
(A)n
VPR REV
REV
LTR
GAG
VIF TAT VPU
TAT
NEF
proteins
PR RT/RH IN
POL
ENV
Ty1 (5.9 kb)
LTR
TYA
TYB
LTR
U3 RU5
U3RU5
DNA
RNA
(A)n
CA
PR IN RT/RH
proteins

Figure 1. Genetic structures of the Ty1 retrotransposon and the HIV-I retrovirus

A diagram of the genetic structure of chromosomal Tyl and HIV elements and their RNA transcripts. For Ty1, long terminal repeats (5' LTR and 3' LTR) flank a central coding region containing two partially overlapping open reading frames, *TYA*, encoding the structural Capsid (CA) protein, and *TYB*, encoding the enzymatic proteins protease (PR), integrase (IN), and reverse transcriptase/RNaseH (RT/RH). The LTRs consist of three nonoverlapping regions, U3 (skyblue box), R (red box), and U5 (lime box). Transcription of the Tyl element produces a terminally redundant mRNA that extends from the R region in the 5' LTR to the R region in the 3' LTR

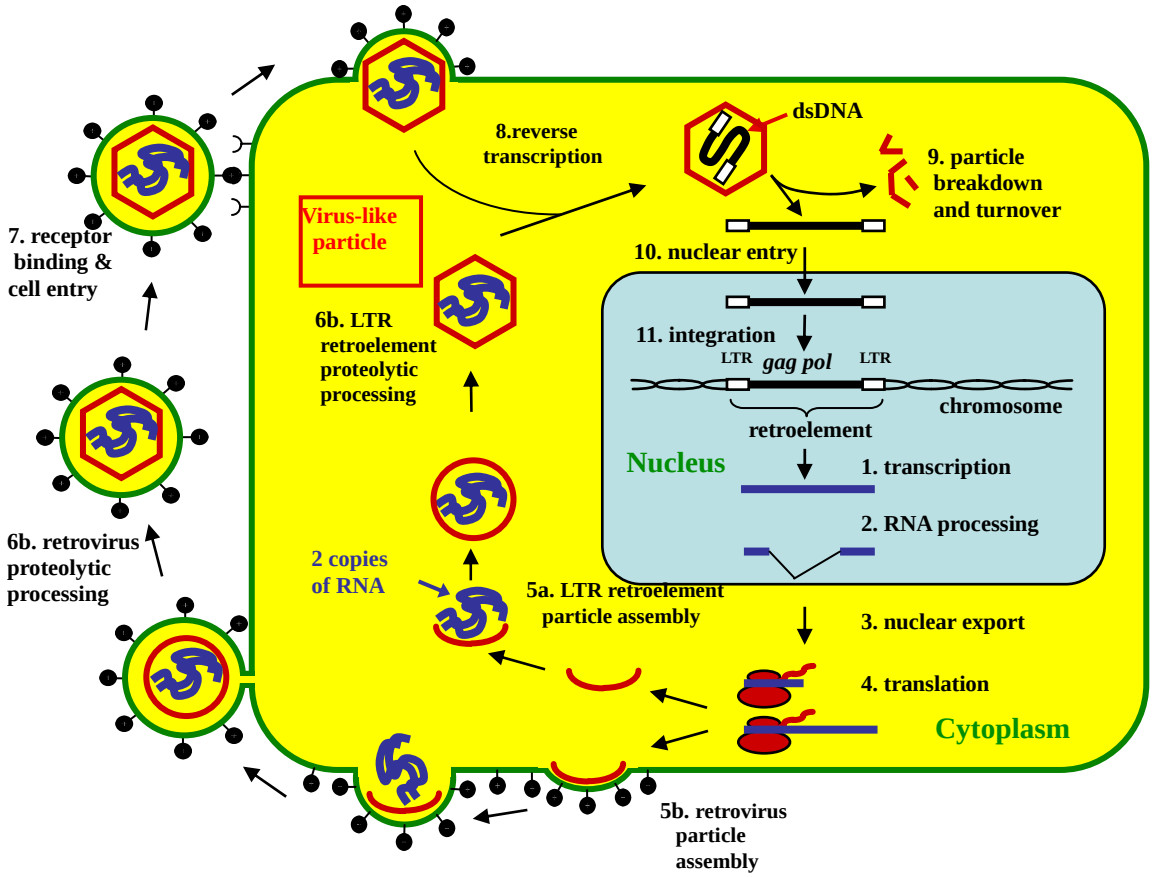


Figure 2. Tyl / HIV replication cycles. Individual steps are described in the text.

1-3) Ty1 element transcription and mRNA export from the nucleus; 4) translation of the mRNA to produce Gag and Gag-Pol polyproteins; 5-6) formation of a virus-like particle (VLP), RNA packaging, and proteolytic processing of Gag and Gag-Pol polyproteins; 8) synthesis of complementary DNA by reverse transcriptase in the VLP; and 9-11) import of the cDNA-integrase complex into the nucleus and integration into the chromosome.

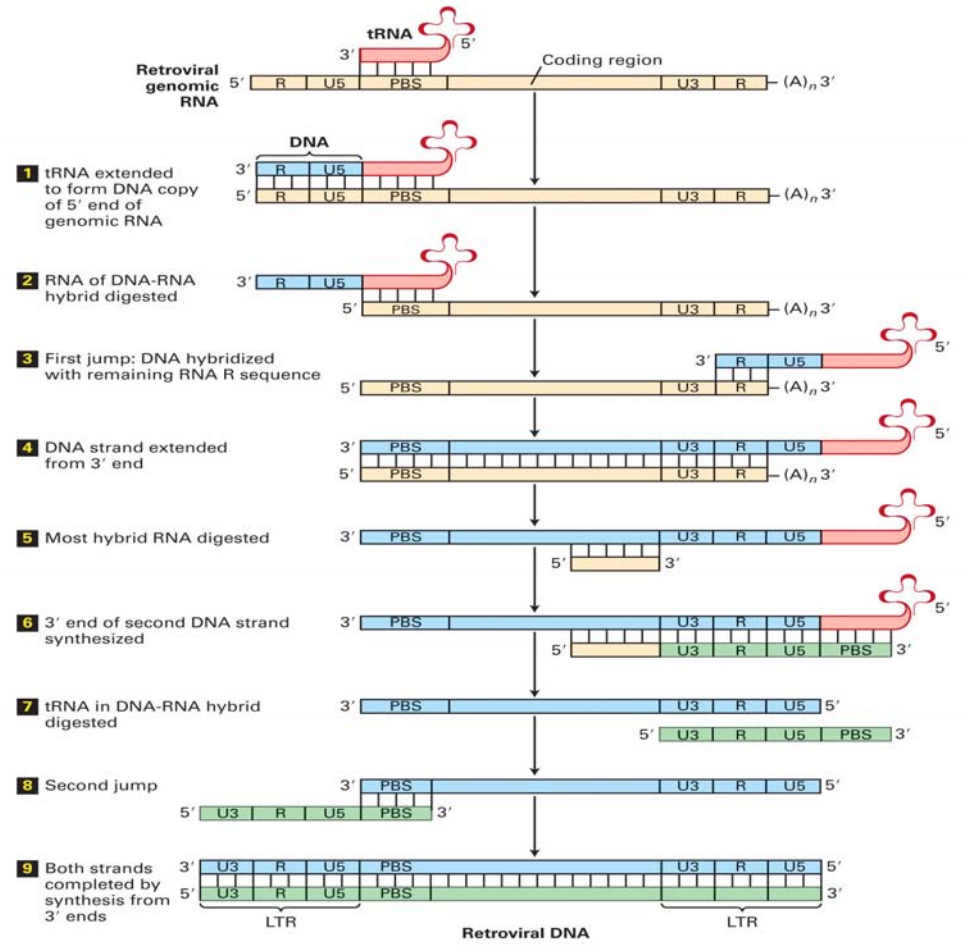


Figure 3. Model for reverse transcription of Ty1 genomic RNA into DNA.

Ty1 genomic RNA is packaged in the VLPs with a Ty1 specific cellular $tRNA_i^{Met}$ hybridized to a complementary sequence near its 5' end called the primer-binding site (PBS). The Ty1 RNA has a short direct-repeat terminal sequence (R) at each end. The overall reaction is carried out by Ty1 RT, which catalyzes polymerization of deoxyribonucleotides. RNaseH activity of RT digests the RNA strand in a DNA-RNA hybrid. The entire process yields a double-stranded DNA molecule that is longer than the template RNA and has a full length LTR at both ends. The different regions are not shown to scale. The PBS and R regions are actually much shorter than the U5 and U3 regions, and the central coding region is very much longer than the other regions.

(Lodish, H. *et a*l., 2004, *Molecular Cell Biology*, 5th edition, New York, NY, W.H. Freeman and Company)

CHAPTER 1

ASSOCIATION OF Puf6p AND Khd1p WITH VLPs OF THE YEAST RETROTRANSPOSON TY1

Introduction

Ty1 retrotransposition cycle occurs in 4 steps: transcription, assembly, reverse transcription and integration. The cycle spans several intracellular compartments from nucleus to cytoplasm, then back to nucleus for integration. As obligate parasites, retrotransposons and their host cells coevolved mechanisms that mutually enable their own survival. On the one hand, due to limited gene information contained in their genomes and a complex mode of replication, it is reasonable to envison that retrotransposons rely on a multitude of host factors. A set of *SPT* genes and *SNF2, SNF5* and *SNF6* are all required for normal transcription of Ty1 (Winston et al., 1984; Happel et al., 1991). It is also well known that Ty1 uses host initiator $tRNA_i^{Met}$ as the primer for minus strand cDNA synthesis during the reverse transcription step. *PMR1* encodes a calcium/manganese exporter which pumps Mn^{2+} out of the cell to keep Ty1 reverse transcriptase active (Bolton et al., 2002). DNA end-binding protein Ku, which is involved in many processes that ensure genome integrity such as chromatin assembly, double strand break repair via the end-joining pathway, telomere maintenance and transcriptional gene silencing appears to regulate the Ty1 integration process by ensuring Ty1 integrase substrate specificity (Kiechle et al., 2000).

One the other hand, retrotransposons make up a large fraction of the host genome; for example, Ty retroelements account for 3.1% of the 12-Mb *Saccharomyces cerevisiae* genome (Kim et al., 1998). Since the movement of retrotransposons can potentially

mutate host genes or activate/repress adjacent genes under normal conditions, it is tightly controlled by host cells which have evolved a series of mechanisms to block retroelement replication. Significant progress toward identifying host genes that regulate retrotransposition has been made in the past few years, especially host genes that control Ty1 and Ty3 mobility. The two main strategies used to identify regulators of Ty1 and Ty3 retrotransposition were either to search for yeast mutants that affected retrotransposition of marked Ty1 elements placed under the control of the inducible *GAL1* promoter on a plasmid (Chapman and Boeke, 1991; Griffith et al., 2003) or to screen for mutations that increase transposition of a single, marked chromosomal Ty1 element (Conte et al., 1998; Lee et al., 1998; Scholes et al., 2001) (Figure 4). As a consequence, these two strategies led to the isolation of different types of Ty1 regulators. The former method screened for those mutants that could decrease retrotransposition, identifying genes that encode activators of Ty1 retromobility; the latter one exclusively identified mutations that could increase Ty1 mobility, identifying genes that encode repressors of Ty1 retrotransposition. These screens identified hundreds of host genes that influence every step of the Ty1 retrotransposition life cycle. These host genes participate in diverse cellular process such as transcription, RNA metabolism, translation, protein folding and modification, DNA repair, nuclear transport, etc.

As a complementary approach to the above genetic screening methods, I employed a proteomic method to identify and characterize additional host factors which allow us better understand the complexity of the host-retroelement relationship. Also, identification and characterization of additional host factors will offer novel potential targets for inhibition of retroviral replication.

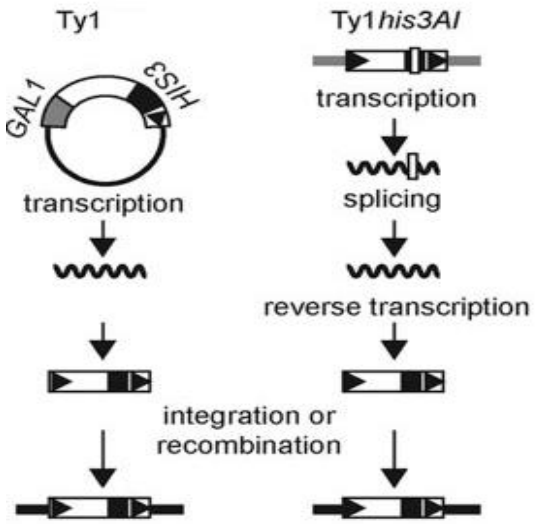


Figure 4. Ty1 mobility assays used in genetic screens for Ty1 regulators.

Two different experimental approaches used to measure Ty1 mobility in genetic screens.

Left column depicts a plasmid copy of Ty1 containing a *GAL1* promoter and a *HIS3* reporter gene. The resulting Ty*HIS3* cDNA product will be inserted into the genome through Ty IN mediated integration or host-mediated recombination with genomic Ty1 copies or LTRs. These integration/recombination events are detected by selecting for plasmid loss and the presence of a genomic copy of *HIS3*.

Right column depicts a genomic copy of a Ty1*his3AI* element in which a *HIS3* marker gene is disrupted by an artificial intron (AI). Following transcription of Ty1*his3AI,* the AI is spliced out of the Ty1*his3AI* RNA. Resulting Ty1*his3* cDNA molecules will then be inserted into the genome through the same mechanism as above. These integration/recombination events are detected by the presence of a genomic copy of *HIS3*.

(Maxwell PH and Curcio MJ (2007). "Host factors that control long terminal repeat retrotransposons in Saccharomyces cerevisiae: implications for regulation of mammalian retroviruses." Eukaryot Cell. 6(7):1069-80.)

Results

Ty1 VLP purification and identification

In order to identify additional host factors involved in Ty1 retrotransposition, I employed a proteomic approach: identifying host proteins that copurify with VLPs. One major step was taken to improve the purity and integrity of Ty1 VLPs: insertion of a short 1x FLAG tag (8 amino acids plus a spacer amino acid) at the N-terminal region of the TYA protein to facilitate the isolation of Ty1 VLPs. First, I inserted a 1x FLAG tag just downstream of Ty1 TYA start codon in the pAR100 plasmid. Purification using anti-FLAG agarose beads was unsuccessful (Data not shown). Based on immunological analysis of Ty1 VLP structure, two N-terminal regions of the TYA protein (amino acids 9-14 and 27-34) were mapped projecting from or at the surface of the proteinaceous shell of the VLP which are available to antibody (Brookman et al., 1995). The 1x FLAG tag was then added between amino acid 14 and 15. The position of 1x FLAG coding sequence in Ty1 RNA is upstream of the PBS, Box 0, Box 1 and Box 2.1 elements, so it should not interfere with interaction between Ty1 RNA and initiator $tRNA^{Met}$ (Figure 5A). Proper insertion of the coding sequence was verified by DNA sequencing. Pulldown assays with anti-FLAG agarose beads result in purification of FLAG-Gag from strains transformed with the FLAG-pAR100 plasmid, with very low levels of nonspecific purification (data not shown).

Immunoblots of proteins from individual sucrose gradient fractions showed that the highest concentration of CA and RT were in the lower layer of 30% sucrose fraction, although both of these proteins and their precursor forms were found throughout the gradient (Figure 6). Ty1 cDNA, the end product of reverse transcription, was found exclusively in the

lower layer of the 30% sucrose fraction (Figure 6). The fractionation pattern of Ty1 VLPs was a little different from that of Ty3 VLPs which showed Ty3 CA and RT proteins spread throughout the whole gradient and Ty3 cDNA spread throughout the whole 30% sucrose fraction (Karst et al., 1999). Ty1 VLPs showed good concentration at lower layer (2ml) of 30% sucrose fractions. Therefore, the lower 2 ml of 30% sucrose fractions were used for anti-FLAG VLP isolation.

Extracts from cells expressing both FLAG-tagged and endogenous Ty1 elements were run over a sucrose step gradient. Material from the dense fraction of the gradient that is known to contain Ty1 VLPs (30% sucrose fraction) was used for affinity purification of FLAG-tagged Gag complexes. As a negative control, a parallel sample was processed from cells expressing only the endogenous, untagged Ty1 elements. Affinity purified material was run on polyacrylamide gels and assessed by both silver staining and western blotting.

As shown in figure 7, there are dozens of bands visualized in a silver stained gel of purified Ty1 VLPs. Only one band corresponding to rabbit IgG light chain was visible in mock purification samples (Figure 7). The IgG comes from the anti-FLAG agarose beads. In order to further verify the purity and integrity of Ty1 VLPs, a portion of the eluate fractions was tested for Ty1 VLP CA, IN, RT proteins and Ty1 cDNA/RNA. Ty1 CA, IN, RT proteins and cDNA/RNA were exclusively identified in eluate from TMY188 cells transformed with FLAG-pAR100 cells and not in the negative control (TMY188 cells transformed with pRS316) (Figure 8).

A portion of each eluate (from tagged and untagged samples) was analyzed by mass spectrometry (multi-dimensional protein identification technology, MudPIT). Thousands of peptides were identified in the eluate from TMY188 cells transformed with

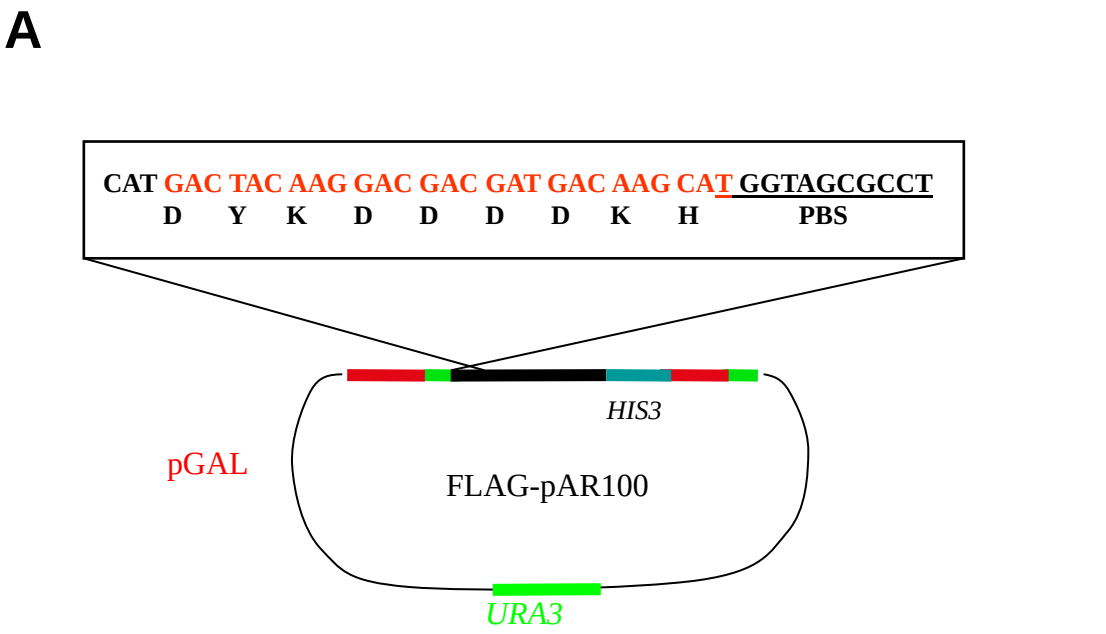


Figure 5. Schematic representation of the FLAG-pAR100 plasmid.

(A). The twenty-seven nucleotide sequence which encodes 1x FLAG peptide (9 amino acids) was inserted just upstream of the PBS sequence.

(B). Yeast cells transformed with FLAG-tagged Ty1 element (TMY679) and cells with untagged Ty1 element (TMY 684) were scraped from the SD plus 5-FOA plate, diluted to an OD600 of 1, and plated in twofold serial dilutions (from left to right). The SD-Histidine plate measures the level of retrotransposition in each strain. The SD complete medium serves as a control for cell titers.

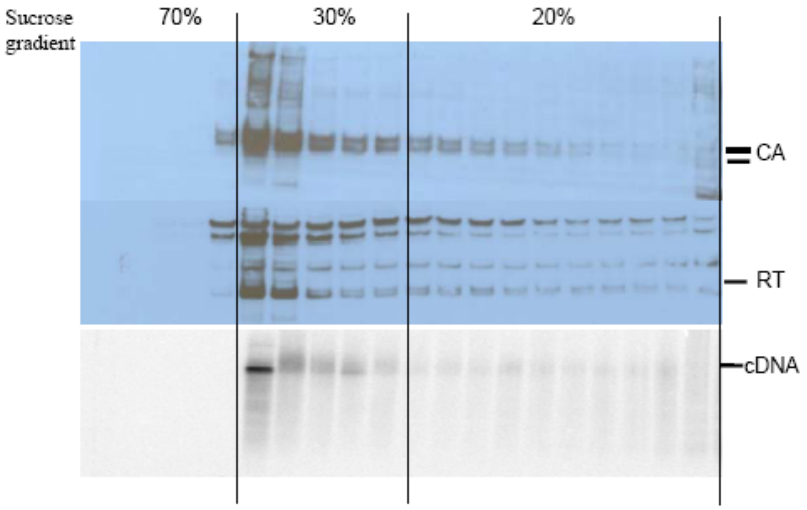


Figure 6. Analysis of Ty1 protein and cDNA fractionation patterns

Extract from a TMY188 + FLAG-pAR100 culture, induced to express Ty1 for 20 h at 24°C, was fractionated on a 70% /30% /20% sucrose step gradient and approximately one-milliliter fractions were collected. Immunoblots were performed using equivalent volumes (15ul) of individual fractions. In the anti-CA immunoblot, the 49-kDa primary translation product of Gag and a 45-kDa proteolytically processed product of this precursor can be seen. In the anti-RT immunoblot, the 63-kDa mature reverse transcriptase protein is seen, along with several higher bands which correspond to unprocessed Gag-Pol or Pol polyprotein.

For the Southern blot of Ty1 DNA, samples were hybridized with a probe of Ty1 DNA. DNA was extracted from individual fractions and equivalent volumes were loaded in each lane.

FLAG-pAR100 in addition to FLAG-CA peptide, which includes Ty1 RT, suggesting that Ty1 VLPs are part of the affinity-purified mixture. In contrast, few peptides were identified in the negative control eluate, which is consistent with results from the silver stained gel (Figure 7). Table 1 lists the proteins identified in TMY188 cells transformed with FLAG-pAR100 but not in negative control purifications.

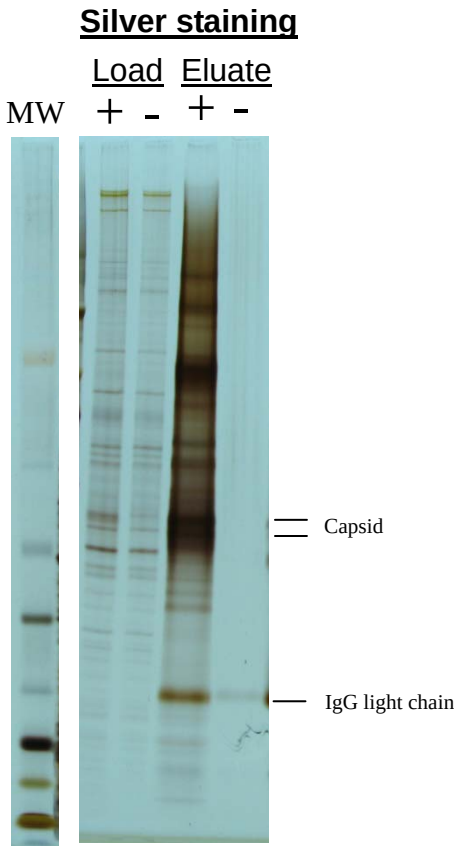


Figure 7. Purification of Ty1 VLP associated proteins

Proteins associated with Ty1 FLAG-Capsid (+) were purified with the anti-FLAG agarose beads from lower layers of the 30% sucrose fraction containing the appropriate tagged protein. Final eluates were concentrated and analyzed in a 4-12% SDS-PAGE gel. As a control, an aliquot of the eluate from cells transformed with pRS316 (-) was loaded. Protein bands were visualized by silver staining.

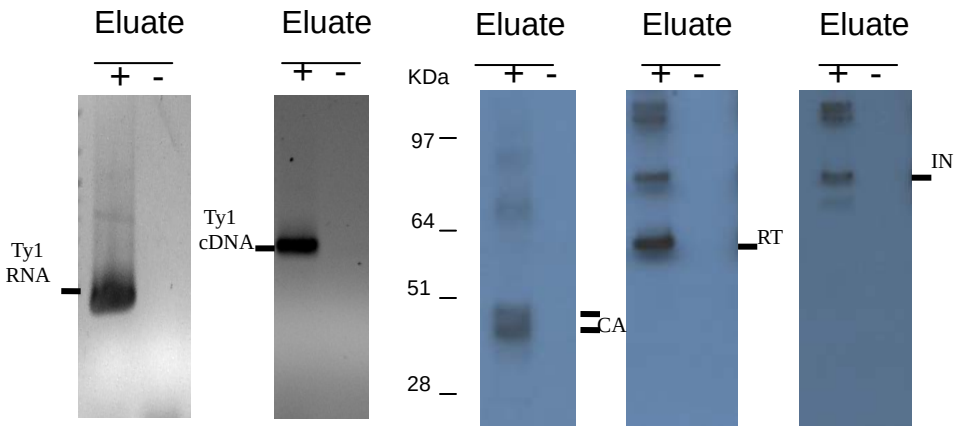


Figure 8. Analysis of Ty1 protein, RNA and cDNA from Ty1 VLP purifications.

Immunoblots of equivalent eluate fractions from TMY188 + FLAG-pAR100 (+) and TMY188 + pRS316 (-) anti-FLAG purification. In the anti-CA immunoblot, the 49-kDa primary translation product of Gag and a 45-kDa proteolytically processed product of this precursor can be seen. In the anti-RT and anti-IN immunoblots, the 63-kDa mature reverse transcriptase and 71-kDa integrase proteins are seen, along with several higher bands which correspond to unprocessed Gag-Pol or Pol polyprotein.

TABLE 1. Host factors associated with Ty1 VLPs by MudPIT analysis

Group/no. of proteins	Protein identified
Ribosomal proteins (large subunit) (22)	Rpl2Ap / Rpl2Bp, Rpl3p, Rpl4Ap / Rpl4Bp, Rpl6Ap / Rpl6Bp, Rpl7Ap / Rpl7Bp, Rpl8Ap / Rpl8Bp, Rpl10p, Rpl13Ap / Rpl13Bp, Rpl14Ap / Rpl14Bp, Rpl16Ap / Rpl16Bp, Rpl17Ap / Rpl17Bp, Rpl19Ap / Rpl19Bp, Rpl20Ap / Rpl20Bp, Rpl21Ap / Rpl21Bp, Rpl24Ap / Rpl24Bp, Rpl27Ap / Rpl27Bp, Rpl28p, Rpl31Ap / Rpl31Bp, Rpl32p, Rpl35Ap / Rpl35Bp, Rpl36Bp, Rpl40Ap / Rpl40Bp
Ribosomal proteins (small subunit) (7)	Rps1Bp, Rps2, Rps4Ap / Rps4Bp, Rps6Ap / Rps6Bp, Rps23Ap / Rps23Bp Rps27Ap / Rps27Bp, Rps31p
Other proteins (8)	Eft1p / Eft2p, Erj5p, Gpm1p, Hek2p (Khd1p), Puf6p, Pab1p, Sbp1p, Ssb1p

Puf6p and Khd1p as host factors of Ty1 transposition

The proteomic approach to identify Ty1 host factors described in this work is complementary to previous genetic screens. The working hypothesis guiding the research was that proteins associated with dense assemblies containing Ty1 CA would include Ty1 host factors that associate directly with Ty1 VLPs. Proteins identified could be inside VLPs or associated peripherally with VLPs. Some of the proteins may bind CA directly; others may be associated with other VLP components, including Ty1 RNA. In addition, some proteins identified may bind CA (or Gag/Gag-Pol) aggregates formed during steps prior to completion of VLP maturation or after the initiation of VLP breakdown.

Two RNA binding proteins, Puf6p and Khd1p, caught our special attention because: 1) *puf6* had been identified in a genetic screen for host mutants that exhibit decreased Ty1 transposition compared to a wild type strain (Griffith et al., 2003); 2) both Puf6p and Khd1p are RNA-binding proteins involved in the asymmetric localization of *ASH1* mRNA (Gu et al., 2004; Irie et al., 2002).

ASH1 mRNA localization is required for mating-type switching in *S. cerevisiae*. *ASH1* mRNA forms a ribonucleoprotein particle (RNP) containing Puf6p, Khd1p, She2p and She3p which recruits a cytoskeletal motor protein Myo4p (Chartrand et al., 2001; Kwon and Schnapp, 2001; Darzacq et al., 2003; Bohl et al., 2000) (Figure 9). The resulting RNP is then transported along polarized actin filaments to the bud tip of the emergent daughter cell (Takizawa et al., 1997; Long et al., 1997). Once at the bud tip, *ASH1* mRNA is locally translated where its proximity to the daughter cell nucleus facilitates its selective import there. In the daughter cell nucleus Ash1p binds to the HO

endonuclease promoter, represses its transcription and maintains the original mating type of the mother cell. During this process, *ASH1* mRNA translational repression occurs during transport to the bud tip to ensure that Ash1 protein expression only occurs in the appropriate site (Johnstone and Lasko, 2001; Shepard et al., 2003).

Because 1) Puf6p plays a role in Ty1 transposition based on previous genetic screen results and 2) both Puf6p and Khd1p have been identified as host proteins that physically interact with Ty1 VLP components, the roles of Puf6p and Khd1p in Ty1 transposition were studied further.

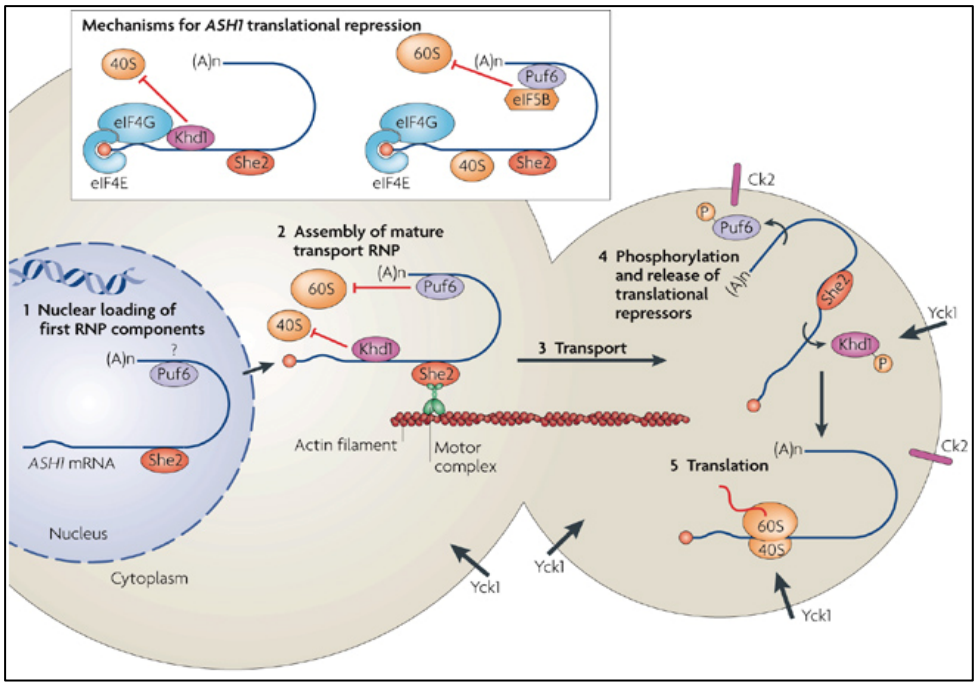


Figure 9. Spatial translational activation of *ASH1* mRNA in budding yeast.

Once transcribed, *ASH1* mRNA associates with She2p in the nucleus (step 1), In the cytoplasm, the She2p/*ASH1* mRNA complex associates with She3p and Myo4p forming a mature transport ribonucleoprotein particle (RNP) with further recruitment of translational repressors (Khd1p and Puf6p). During transport along actin filaments (step 3), *ASH1* mRNA translation initiation is blocked by two complementary mechanisms (inset). 1) assembly defect of the eukaryotic translation initiation factor-4F (eIF4F) complex and recruitment defect of the 40S ribosomal subunit (Khd1-mediated mechanism; left), 2) recruitment defect of the 60S ribosomal subunit (Puf6-mediated mechanism; right). After delivering *ASH1* mRNA to daughter cells, *ASH1* transport RNP contacts the membrane-associated kinases Yck1p (type I casein kinase) and casein kinase-II (Ck2) which will phosphorylate Khd1p and Puf6p, respectively (step 4). Then, *ASH1* mRNA is released from the transport RNP for translation (step 5).

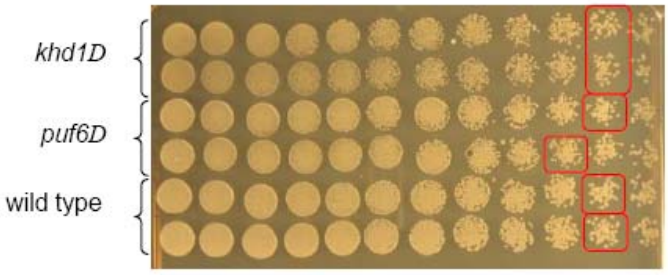
(Besse,F and Ephrussi,A. (2008). “Translational control of localized mRNAs: Restricting protein synthesis in space and time.” Nature Reviews Molecular Cell Biology 9, 971-980.)

PUF6 and *KHD1* genes are required for Ty1 retrotransposition

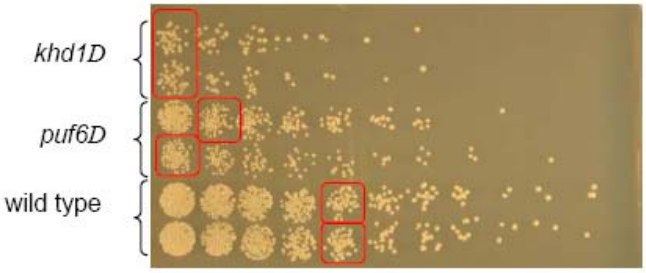
Our proteomic data showed that there are physical interactions between both Puf6p and Khd1p and dense aggregates containing Ty1 FLAG-Gag. As the first step to characterize the possible roles of *PUF6* and *KHD1* in Ty1 retrotransposition, we analyzed Ty1 transposition in *puf6* and *khd1* mutants.

The Ty1 transposition frequencies for these mutants were determined with Ty1 transposition dilution assays in order to quantitate these mutants' effects on transposition. The *puf6* mutant showed an 8-fold decrease in Ty1*his3* transposition compared to wild type, which is consistent with a previous report (Griffith et al., 2003). Although other screens did not report a Ty1 transposition defect in *khd1* mutants, our transposition dilution assay did show a 16-fold decrease in Ty1*his3* transposition for the *khd1* mutant compared to wild type.

A



complete medium (cell titers)



-histidine medium (selects for cells with Ty1 transposition events)

B

Summary:

Strain	transposition
Wild type	1x
puf6	8x↓
khd1	16x↓

Figure 10. Ty1 retrotransposition is defective in *puf6* and *khd1* mutants.

(A) *puf6, khd1*, and wild type cells were scraped from the SC plus 5-FOA plate, diluted to an OD600 of 1, and plated in twofold serial dilutions (from left to right). The SC-Histidine plate measures the level of retrotransposition in each strain. The SC medium serves as a control for cell titers. Red box means visibly equal yeast cells for each plate.

(B) Ty1 retrotransposition levels for *puf6 and kdh1* relative to wild type.

Physical interaction between Puf6p, Khd1p and Ty1 RNA

Since both Puf6p and Khd1p are RNA binding proteins and are found as host factors that associate with Ty1 Gag aggregates, we hypothesized that Puf6p and Khd1p bind to Ty1 RNA , have a role in Ty1 RNA localization, and ultimately get packaged into Ty1 VLPs along with Ty1 RNA.

Khd1p contains three KH-type RNA-binding domains like human heterogeneous nuclear ribonucleoprotein K (hnRNP K) and poly(C)-binding protein (PCBPs) (Makeyev and Liebhaber, 2002; Denisenko and Bomsztyk, 2002; Bomsztyk et al., 2004). Unlike the characterized KH domain proteins such as ZBP-1, Nova-1, and hnRNP K which bind sequences in the 3' -UTR of mRNAs, Khd1p preferentially associates with CNN repeats (where N is any nucleotide) in the coding regions of target mRNAs (Buckanovich and Darnell, 1997; Holcik and Liebhaber, 1997; Ostareck et al., 1997; Hasegawa et al., 2008). Therefore, Khd1p is an unusual member of this RNA binding protein (RBP) family, binding to coding sequences, a fact that may explain its role in interfering with translation.

A search of the Ty1 RNA sequence uncovered a possible Khd1p binding motif: eight CNN repeats (CNN_8) (caacaacacctgcttcatcagctg) that encode a peptide TTTPASSA in the Ty1 Gag protein coding sequence.

Puf proteins comprise a large and evolutionarily conserved protein family found in *Drosophila, C. elegans*, human and yeast (Zhang et al., 1997; Zamore et al., 1999). PUF proteins are defined by the presence of several (typically eight) conservative repeats of the Pumilo homology domain (Pum-HD), each consisting of 36 amino acids and conferring RNA binding activity (Zhang et al., 1997; Zamore et al., 19997; Wang et al.,

2002). *S. cerevisiae* has six PUF family proteins, Jsn1p (Puf1p), Ypr042c (Puf2p), Yll013c (Puf3p), Ygl014w (Puf4p), Mpt5p (Puf5p), and Ydr496c (Puf6p). Puf6p, directly interacting with *ASH1* mRNA, transiently regulates the translation of *ASH1* mRNA while it is transported to daughter cells (Gu et al., 2004; Zhang et al., 1997). The interaction and translational repression requires the binding of Puf6 protein with the consensus UUGU sequence in the 3' UTR of *ASH1* mRNA (Wang et al., 2002).

A search of the Ty1 RNA sequence identified one possible Puf6p binding site in the 3' UTR, a UUGU sequence.

To further determine whether Puf6p and Khd1p physically associate with Ty1 RNA *in vivo*, we immunoprecipitated the Puf6p-TAP or Khd1p-TAP complexes and tested them for the presence of Ty1 RNA by qRT–PCR. *ASH1* mRNA was used as a positive control, because it has been shown to interact with Puf6p and Khd1p (Munchow et al., 1999; Long et al., 2000; Gu et al., 2004). *BRO1* mRNA and *ACT1* mRNA were used as negative controls. Ty1 RNA, *ASH1*, *BRO1* and *ACT1* mRNA detected in the immunoprecipitates from an untagged strain were assigned a baseline enrichment factor of 1 to quantitate their enrichment in the immunoprecipitates from Puf6p-TAP and Khd1p-TAP strains.

As seen in figure 11, Ty1 RNA was enriched in the precipitate of Khd1p-TAP (enrichment of 22.6-fold compared to *BRO1* (4-fold) and *ACT1* mRNA (4.3-fold)). There was only slight enrichment of the Ty1 RNA and *ASH1* mRNA in the precipitate of Puf6p-TAP strain (enrichment of 8.2- and 7.1-fold respectively compared to *BRO1* (3.8-fold) and *ACT1* mRNA (4.6-fold)).

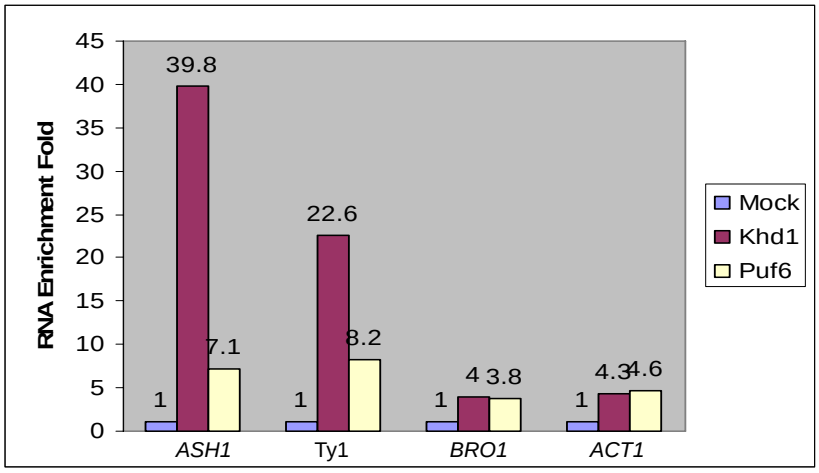


Figure 11. Both Khd1p and Puf6p associate with Ty1 RNA.

Ty1, *ASH1, BRO1* and *ACT1* mRNAs were detected by RT-qPCR in RNAs isolated from immunoprecipitates of Puf6p-TAP, Khd1p-TAP and mock (TMY188) affinity isolations. For comparison, Ty1, *ASH1, BRO1* and *ACT1* mRNAs amplified from the mock control immunoprecipitates were assigned a baseline enrichment factor of 1.

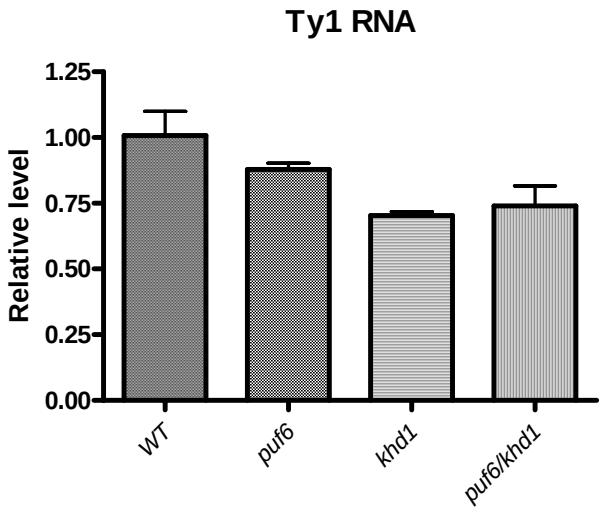
Defective Ty1 CA protein synthesis in *puf6* and *khd1* mutants

In order to further characterize the roles of *PUF6* and *KHD1* in Ty1 retrotransposition, Ty1 replication intermediates (Ty1 RNA, protein) were examined to determine in which step *PUF6* and *KHD1* play a role. First, Ty1 RNA levels in *puf6* and *khd1* mutants and wild type were compared. As seen in figure 12A, there is no difference in the Ty1 endogeneous RNA levels in *puf6, khd1* and wild-type strains.

Next, Ty1 CA protein levels were also examined in *puf6*, *khd1* and wild-type strains. As shown in figure 12B, a decreased amount of Ty1 CA was present in the *puf6* mutant compared to wild-type. In contrast, the *khd1* mutant contains an increased Ty1 CA level compared to wild-type. VLPs produced the same results as the total protein extracts (Figure 12B).

These results support the conclusion that Ty1 RNA translation is perturbed in *puf6* and *khd1* mutants.

A



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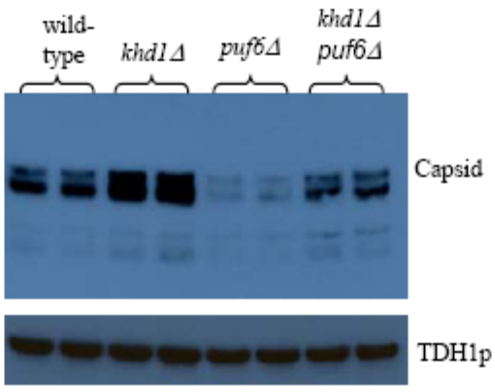


Figure 12. Ty1 RNA and Capsid protein levels in wild-type, *puf6* and *khd1* strains.

Total RNA and cellular protein were isolated from pairs of wild-type, *puf6* and *khd1* cultures. Incubations were done for 10 h at 24°C to early log-phase (OD600= 0.5-1).

(A) qRT-PCR analysis of Ty1 RNA levels using *ACT1* mRNA as internal control.
(B) Immunoblot analysis of total protein samples using anti-Ty1 VLP antibody.

She2p, She3p and Myo4p do not associate with Ty1 RNA *in vivo*

It is now clear that localizing *ASH1* mRNAs are packaged into RNPs that require cytoskeletal motors for directed transportation along cytoskeletal filaments (Figure 9) and ensure their translational silencing (Besse and Ephrussi, 2008). The molecular composition of these RNPs contain *cis*-regulatory elements that are present on the *ASH1* mRNA and specific *trans*-acting factors, including transporting proteins (She2p, She3p and Myo4p et al.) and anchoring proteins (Khd1p, Puf6p et al.).

In vivo and *in vitro* binding assays showed that She2p directly and specifically interacts with *ASH1* RNA through *cis*-acting localization elements E1, E2A/B and E3, although with apparently weak affinity. This complex formation of She2p and *ASH1* RNA was enhanced by She3p (Bohl et al., 2000; Darzacq et al., 2003; Long et al., 2000). Further experiments demonstrated that N-terminal domain of She3p binds to the myosin motor Myo4p, while its C-terminus involved in She2p binding. Since She2p–*ASH1* RNA complex associates with Myo4p heavy chain through bridging She3p, She3p is therefore considered as an adapter for the *ASH1* RNP. The molecular interactions among Myo4p, She3p, and She2p lead to the formation of a complex referred to as the locasome, which is essential for the proper localization of *ASH1* RNA (Bohl et al., 2000; Long et al., 2000; Munchow et al., 1999; Takizawa and Vale, 2000). Analysis of *ASH1* mRNA transportation in living cells revealed that Myo4p is capable of directly transporting the *ASH1* RNP along polarized actin cables (Beach et al., 1999; Bertrand et al., 1998; Munchow et al., 1999).

In addition to *ASH1* mRNA, over 30 additional mRNAs have been shown to

undergo active transportation to yeast daughter cells (Shepard et al., 2003; Takizawa et al., 2000). We hypothesized that Ty1 RNA along with RNA-binding proteins (transporting proteins) could also form Ty1 transporting RNPs which share similar locasome components as *ASH1* RNPs.

To address this, we investigated whether She2p, She3p and Myo4p physically associate with Ty1 RNA *in vivo*. Using the strategies described in previous experiments, we immunoprecipitated the She2p-13Myc, She3p-13Myc and Myo4p-13Myc complexes and tested immunoprecipitates for enrichment of Ty1 RNA by RT–qPCR. The results are shown in Figure 13. Although *ASH1* mRNA was enriched in the precipitates of She2p-13Myc, She3p-13Myc and Myo4-13Myc complexes (enrichment of ~ 47-, 13- and 21-fold respectively compared to *ACT1* mRNA) (Takizawa and Vale, 2000), there is no enrichment of Ty1 RNA in the precipitates of She2p-13Myc, She3p-13Myc and Myo4p-13Myc complexes (enrichment of ~1.5-, 1.3- and 1.96-fold, respectively, compared to *ACT1* mRNA).

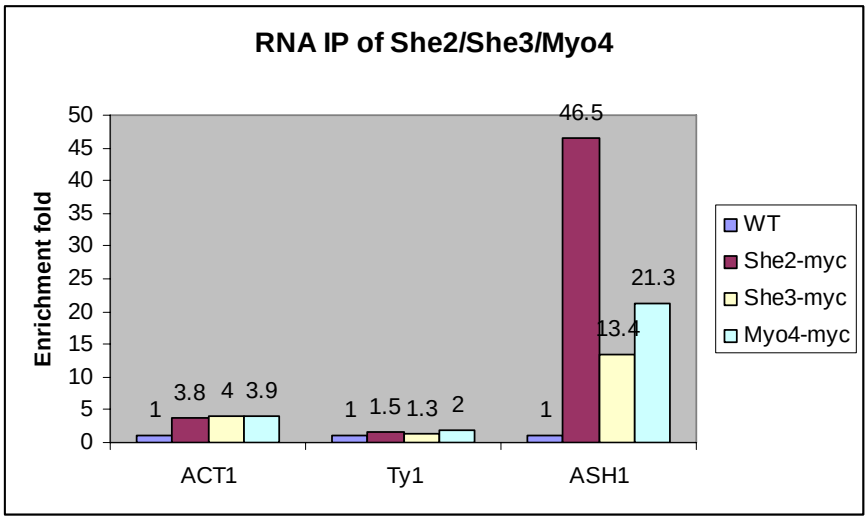


Figure 13. She2p, She3p and Myo4p do not associate with Ty1 RNA.

Ty1, *ASH1* and *ACT1* mRNAs were detected by RT-PCR in RNAs isolated from immunoprecipitates of She2p-13xMyc, She3p-13xMyc, Myo4p-13xMyc and WT (non-Myc-tagged strain). For comparison, Ty1, *ASH1* and *ACT1* mRNAs amplified from WT immunoprecipitates were assigned a baseline enrichment factor of 1.

Ribosomal proteins as host factors for Ty1 retrotransposition

The yeast ribosome is composed of a small 40S and a large 60S subunit and contains a total of four discrete rRNA species and 78 ribosomal proteins (RPs). In yeast, about 85% of RP genes are duplicated and encode nearly identical ribosomal proteins. Cells are viable with deletion of either paralog, but generally not both paralogs simultaneously (Steffen et al., 2008). Growth rates were further measured in RP gene knockouts to determine whether paralogous genes were functionally distinct. Strong correlation between fitness defects and expression levels of ribosomal proteins and the observation that overexpression of one ribosomal protein can rescue the growth defect from deletion of its paralog led to the conclusion that duplicated ribosomal proteins are functionally redundant and affect the cellular processes in proportion to their protein expression level (Rotenberg et al., 1988). More recently, experiments showed that a distinct subset of duplicated ribosomal proteins are required for translational regulation of *ASH1* mRNA, and that there is a direct correlation between RP genes required for *ASH1* mRNA localization and those required for bud-site selection. Further transcriptional and phenotypic profiling of cells lacking specific ribosomal protein genes have revealed differences between the functional roles of ribosomal protein paralogs and that ribosomal protein paralogs have disparate requirements for ribosomal localization and assembly, suggesting that ribosomal protein isoforms have become specialized for specific cellular processes (Komili et al., 2007).

In our analysis of proteins specifically associated with Ty1 Gag, twenty nine ribosomal proteins were identified (Table 1). Since most peptides derived from each pair

of ribosomal protein paralogs are components of both paralogs, mass spectrometry cannot distinguish between ribosomal proteins of a paralogous pair. Eight ribosomal genes were previously identified as Ty1 host factors in a genetic screen of the yeast knockout collection (Griffiths et al., 2003). Six of the eight were also identified in our proteomic screen (*RPL6A, RPL14A, RPL16B, RPL19B, RPL20B, RPL21B*). To determine whether duplicated ribosomal genes have distinct roles in Ty1 transposition, three pairs of ribosomal genes (*RPL6A/B, RPL14A/B and RPS10A/B*) were selected for further investigation. Previous results showed that Rpl6Ap and Rpl14Ap were present in cells at levels equivalent to their paralogs Rpl6Bp and Rpl14Bp (Ghaemmaghami et al., 2003). Rps10Ap was not identified as a host protein associated with Ty1 Gag but the *rps10A* mutant is defective for Ty1 retrotranspostion (Griffiths et al., 2003).

First, we wanted to assess Ty1 transposition in mutants knocked out for each gene of the paralogous pair (*rpl6A/B, rps10A/B* and *rpl14A/B*). As previously described, Ty1 transposition frequencies for the mutants were determined with Ty1 transposition dilution assays. The *rpl6A, rps10A* and *rpl14A* mutants showed 16-, 8- and 8-fold decreases, respectively, in Ty1 transposition compared to the paralogous mutants (*rpl6B, rps10B* and *rpl14B*), which have transposition levels similar to the wild-type strain. (Figure 14)

In order to characterize the limiting step for Ty1 transposition in the *rpl6A, rps10A,* and *rpl14A* mutants, the levels of Ty1 intermediates produced within these mutants were compared to those produced within the paralogous mutants (*rpl6B, rps10B* and *rpl14B*) and wild-type. First, quantitative RT-PCR (qRT-PCR) was performed to examine Ty1 RNA levels in *rpl6A/B, rps10A/B,* and *rpl14A/B* mutants and the wild-type strain. *ACT1* mRNA was used as the endogeneous control. There is no difference in Ty1

RNA levels between the two ribosomal paralog mutant pairs (*rpl6A*(0.91) Vs. *rpl6B* (0.77), and *rpl14A* (0.82) Vs. *rpl14B* (0.88)), when compared to the wild-type strain (1.0), but there is significant difference between *rps10A* (0.57) and *rps10B* (0.94) (Figure 15).

Next, Ty1 CA protein levels were examined in *rpl6A/B, rps10A/B* and *rpl14A/B* mutants. Total cellular protein was extracted and immunoblots were performed using anti-Ty1 VLP antiserum which detects Ty1 CA protein. The same nitrocellulose membranes used for detection of Ty1 CA levels were stripped and reprobed with anti-Tdh1 antibody. Tdh1p is glyceraldehyde 3-phosphate dehydrogenase (GAPDH) isozyme 1, a house keeping enzyme encoded by the *TDH1* gene whose levels are invariant. Tdh1p levels were used as internal loading controls to quantitate Ty1 CA levels. The results are shown in Figure 16. In contrast to wild-type Ty1 CA levels found in *rpl6B* and *rps10B* mutants, the *rpl6A* and *rps10A* mutants contain decreased amounts of Ty1 CA. There was no difference in Ty1 CA levels between the *rpl14A* and *rpl14B* mutants.

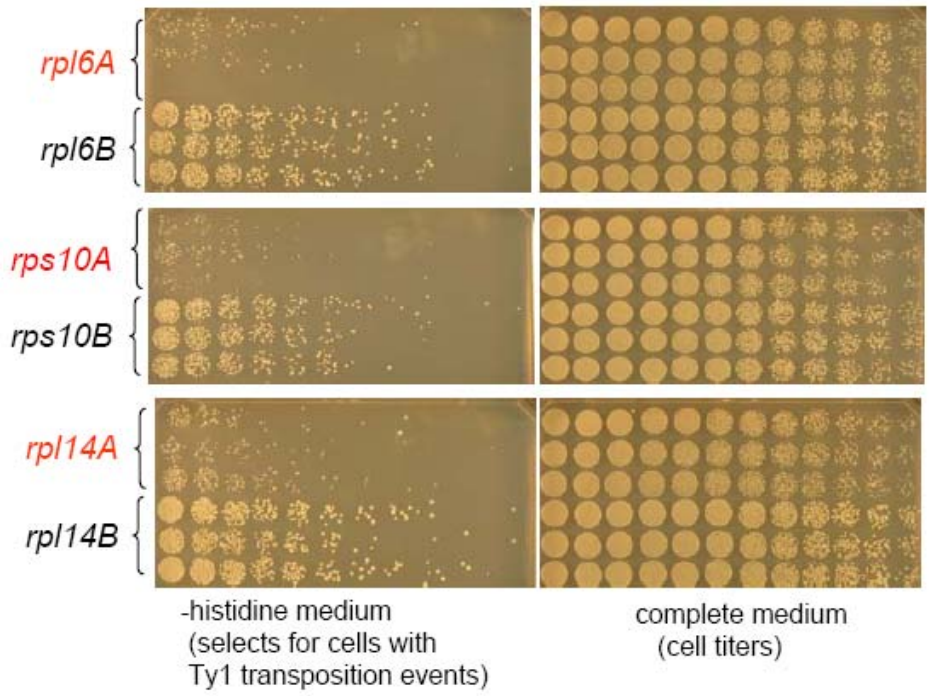


Figure 14. Ty1 retrotransposition is defective in *rpl6A, rps10A* and *rpl14A* but not in *rpl6B, rps10B* and *rpl14B* mutants.

Cells were scraped from the SC plus 5-FOA plate, diluted to an OD600 of 1, and plated in two-fold serial dilutions (from left to right). The SC-Histidine plate measures the level of retrotransposition in each strain. The SC medium serves as a control for cell titers. Ribosomal mutant strains in red exhibit decreased retrotransposition compared to their paralogous ribosomal mutants.

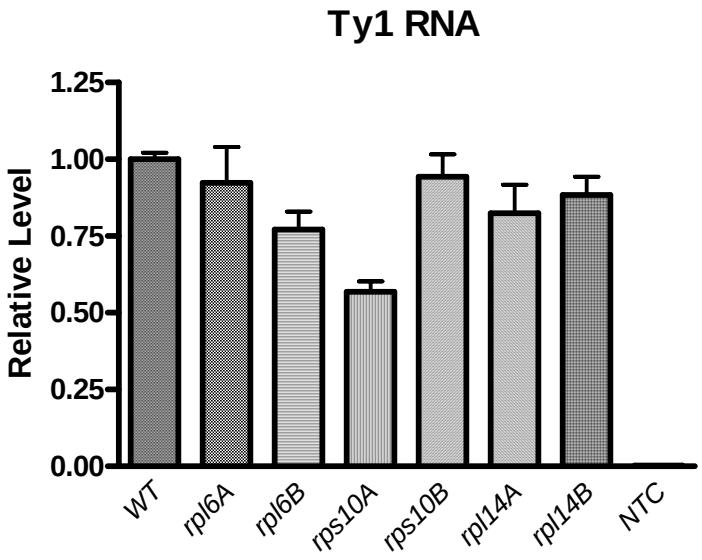


Figure 15. Ty1 RNA levels in ribosomal paralogous gene mutant strains.

Ty1 and *ACT1* mRNA were detected by qRT-PCR in total RNAs isolated from *rpl6A/B, rps10A/B* and *rpl14A/B* mutants and the wild-type strain. *ACT1* mRNA was used as the endogenous control. In parallel, a reaction with total RNA from the wild-type strain was set up without reverse transcriptase treatment to make sure there was no Ty1 DNA contamination in the total RNA sample (NTC group).

Further, equal amounts of total protein from each mutant were loaded on top of sucrose gradients. After ultracentrifugation, the lower layers of the 30% sucrose (2ml concentrated Ty1 VLPs) fraction were collected and 20 ul were immunoblotted with anti-CA, RT and IN and checked for Ty1 CA, IN and RT protein levels. Consistent with results for the total cellular protein samples above, *rpl6A* and *rps10A* mutants also showed decreased Ty1 CA, IN and RT protein levels compared to *rpl6B* and *rps10B* mutants in concentrated Ty1 VLP fractions (Figure 17). These results for total protein samples and concentrated VLPs suggest that the synthesis of Ty1 protein is reduced in *rpl6A* and *rps10A* mutants but not in *rpl6B* and *rps10B* mutants.

Although equivalent amounts of Ty1 CA protein were present in the total protein samples from *rpl14A* and *rpl14B* mutant cells, the *rpl14A* mutant has much higher Ty1 unprocessed Gag levels in Ty1 VLPs than the *rpl14B* mutant. Interestingly, the same immunoblots showed that Ty1 IN and RT protein levels were decreased in the *rpl14A* mutant compared to the *rpl14B* mutant, but unprocessed forms of these proteins were present at higher levels in *rpl14A* compared to *rpl14B*. This suggests that although there is no Ty1 protein synthesis defect in the *rpl14A* mutant, the Gag-Pol polyprotein processing step is defective in the *rpl14A* mutant compared to the *rpl14B* mutant.

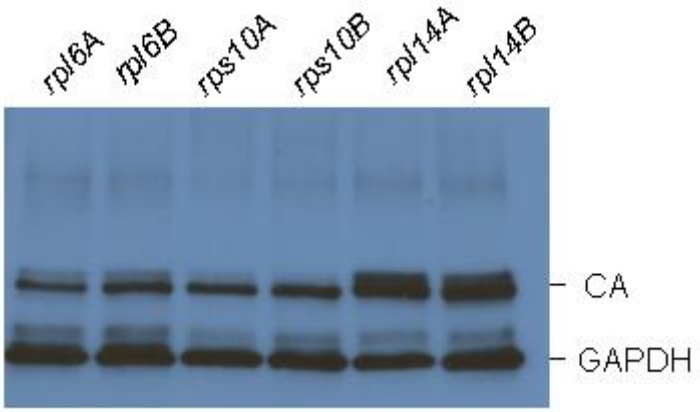


Figure 16. Ty1 total Capsid protein levels in *rpl6A/B, rps10A/B* and *rpl14A/B* mutants.

Cellular proteins were isolated from *rpl6A/B, rps10A/B* and *rpl14A/B* cultures. Incubations were done for 10 h at 24°C to early log-phase (OD600= 0.5-1). Immunoblot analysis of total protein samples was performed with anti-Ty1 VLP and anti-TDH1 antibodies.

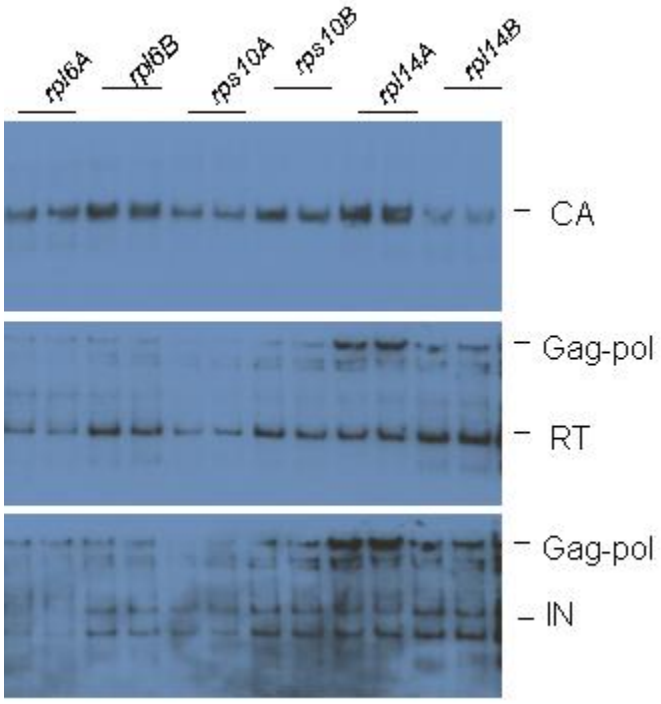


Figure 17. Gag-Pol polyprotein processing defect in *rpl14A* mutant strain.

Concentrated VLPs from *rpl6A/B, rps10A/B* and *rpl14A/B* mutants were immunoblotted with anti-CA, RT and IN and checked for Ty1 Capsid, Integrase and Reverse transcriptase protein levels.

Discussion

As a complementary approach to previous genetic screens and to broaden the identification of Ty1 host factors, we have employed a novel method to isolate Ty1 VLPs through two steps: 1) crude purification of Ty1 VLPs by ultracentrifugation of cellular extracts through sucrose step gradients; 2) specific purification of VLPs from the crudely purified material using anti-FLAG affinity purification. Since the cells express both endogenous (untagged) Capsid from their own genomic Ty1 copies and FLAG-tagged Capsid from the FLAG-pAR100 plasmid when grown in galactose, most VLPs from these cells should be mosaic VLPs composed of both endogenous and FLAG-tagged Capsid. Thus, VLPs captured by affinity purification are mosaic VLPs. In addition to mature Ty1 VLPs, it is likely that the final anti-FLAG eluate contains Capsid/FLAG-Capsid aggregates as well as partially formed VLPs. Host proteins associated with all of these different structures were expected to be recovered by the purification protocol.

An important step of retrotransposon is where and how the genomic RNA and Gag/Gag-Pol proteins are brought together for VLP assembly to allow orderly progression of the replication cycle. Once in the cytoplasm, full-length Ty1 RNAs can be used as translation templates for synthesis of Ty1 Gag and Gag-Pol polyproteins. Ty1 RNAs also serve as reverse transcription templates for Ty1 cDNA synthesis after they are packaged along with Gag and Gag-Pol proteins into Ty1 VLPs (Boeke et al., 1985; Garfinkel et al., 1985; Mellor et al., 1985; Roth, 2000). For some viruses, there is evidence that packaged (genomic) and translated (messenger) RNAs are segregated from early times. For others, it appears that genomic RNAs are translated and subsequently packaged (Butsch and Boris-Lawrie, 2002).

Previous results showed that retroviruses assemble at characteristic cytoplasmic or membranous locations (Coffin et al. 1997). For example, in the case of Mason-Pfizer monkey virus, a retrovirus, which assembles in pericentriolar clusters, specific chaperones have been implicated in the protein assembly process (Hong et al. 2001; Sfakianos et al. 2003) and in the case of human immunodeficiency virus, results showed that RNase L inhibitor (HP68 protein) is associated with HIV-1 Gag in a cell-free system to facilitate proper HIV-1 Capsid assembly along the host cell plasma membrane. (Zimmerman et al. 2002).

P-bodies are discrete cytoplasmic foci that contain messenger ribonucleoprotein complexes (mRNPs), which can be stored or degraded (Brengues et al., 2005; Parker and Sheth, 2007; Sheth and Parker, 2003; Teixeira et al., 2005). In *S. cerevisiae*, the decrease in translation efficiency by severe stress induces mRNA relocalization from ribosomes into P bodies. Interestingly, P bodies are not only sites of degradation but also can serve to store mRNAs, which may later reemerge and engage in translation (Brengues et al., 2005). P-bodies may be assembly sites for Brome mosaic virus (BMV), a positive-strand RNA plant virus. When BMV is expressed in *S. cerevisiae*, P-body components are required for BMV genomic RNA translation (Diez et al., 2000; Noueiry et al., 2003; Hushner et al., 2003) and P bodies sequester translationally repressed BMV RNA1 and RNA2 (Beckham et al., 2007). It is now clear that P-bodies are also Ty3 VLP assembly sites since they are required for retrotransposition, and Ty3 mRNA, proteins, and VLPs are found in association with P-body components (Aye et al., 2004; Beliakova et al., 2006). Although results showed that cytoplasmic P-body components in budding yeast facilitate the replication of Ty1 (Aye et al., 2004; Griffith et al., 2003; Irwin et al., 2005;

Beckham and Parker., 2008) and are required during assembly of Ty1 retrotransposition-competent VLPs, more recent fluorescent *in situ* hybridization (FISH) and immunofluorescence microscopy results suggest that Ty1 RNA/Gag foci (assembly sites) are distinct from P-bodies, co-localizing instead with an uncharacterized cytoplasmic granule referred to as the T-body (Malagon and Jensen, 2008).

Despite progress in the elucidation of the Ty1 VLP assembly process, relatively little is known about the possibility that host factors play a specific role in directing genomic Ty1 RNA into the assembly site rather than the translation pathway.

In the present study, we have shown that both Puf6p and Khd1p can physically interact with Ty1 Gag complexes and that Ty1 transposition is significantly decreased in *puf6* and *khd1* mutant cells. The role of the RNA binding proteins Puf6p and Khd1p have been well characterized in *ASH1* mRNA localization, in which Puf6p and Khd1p bind to *ASH1* mRNA 3' UTR and coding sequence, respectively. *ASH1* mRNA translation initiation is blocked by two complementary mechanisms: 1) assembly defect of the eukaryotic translation initiation factor-4F (eIF4F) complex and recruitment defect of the 40S ribosomal subunit (Khd1-mediated mechanism), 2) recruitment defect of the 60S ribosomal subunit (Puf6-mediated mechanism) (Figure 9). *ASH1* mRNA translational repression occurs while in transit to daughter cells, leading to spatially and temporally restricted activation of *ASH1* mRNA translation in daughter cells (Johnstone and Lasko, 2001; Shepard et al., 2003).

By analogy, we hypothesize that Puf6p and Khd1p participate in the localization of Ty1 RNA from the nuclear periphery to Ty1 VLP assembly sites (Ty1 RNA/Gag foci or T-bodies) in daughter or mother cells. We have shown that Khd1p associates with Ty1

RNA *in vivo*. In addition, a *khd1* knockout mutation caused increased Ty1 Capsid expression compared to wild-type cells, which did not result from an increased Ty1 RNA level, as the Ty1 RNA level was unchanged in the *khd1* mutant compared to wild-type. These data suggest that Khd1p functions as a translational repressor of Ty1 RNA.

Our experiments also showed less strong interaction of Ty1 RNA or *ASH1* mRNA with Puf6p, since only one Puf6p binding motif (UUGU) is present in Ty1 and *ASH1* mRNA 3' UTR sequences. One explanation for this is that the binding affinity between Puf6p and *ASH1* mRNA or Ty1 RNA is weak, making it sensitive to the methods of isolation and detection. This result was consistent with a previous report of a physical association between *ASH1* mRNA and Puf6p (Gu et al., 2004). In addition, we also showed that the Ty1 Capsid protein level was decreased in the *puf6* mutant compared to wild-type, which means Puf6p does not function as a translational repressor of Ty1 RNA.

Because 1) our data show that Khd1p functions as a translational repressor of Ty1 RNA and 2) Ty1 RNA/Gag foci co-localize with an uncharacterized cytoplasmic granule referred to as the T-body (Ty1 VLP assembly site), we hypothesized that, like the *ASH1* mRNA RNP, Ty1 RNA also forms a Ty1 RNP. This Ty1 RNP may direct genomic Ty1 RNA into the Ty1 VLP assembly site. One model for the Ty1 RNP complex, based on work with *ASH1,* is that Khd1p binds to genomic Ty1 RNA, inhibiting translation of Ty1 RNA, while other transporting proteins such as She2p, She3p, Myo4p form a locasome with Ty1 RNA. The resulting Ty1 RNP moves along actin microfilaments to the VLP assembly site, where Ty1 RNA is activated for translation and /or packing.

To test this model, two approaches were taken to check if Ty1 RNA could form a complex with the transporting proteins She2p, She3p and Myo4p. We collaborated with

Dr. Pascal Chartrand's lab (University of Montreal) to determine the intracellular distribution of the Ty1 RNA in *she2, she3, myo4* and wild-type strains by fluorescence in situ hybridization (FISH) analysis (P. Chartrand, personal communication). Three different Ty1 RNA distribution patterns were shown in wild-type cells (Figure 18A). Ty1 RNA either localizes to the budding (daughter) cell (57% of cells), the mother cell (20% of cells) or both the budding and mother cells (23% of cells) as Ty1 RNA/Gag aggregates (Ty1 bodies). In all of these situations, there is also Ty1 RNA present throughout the cytoplasm that is not localized to a Ty1 body (free Ty1 RNA). Compared with the wild-type strain, in which ~ 60% of daughter cells contain the Ty1 body, Chartrand observed a dramatic change in localization of Ty1 bodies in *she2, she3* and *myo4* mutant strains, where less than 5% of Ty1 bodies were localized to daughter cells. Instead, more than 80% of Ty1 bodies were localized to the mother cell in *she2, she3* and *myo4* mutant strains (Figure 18B). These results suggest that She2p, She3p and Myo4p play a role in localization of Ty1 bodies, possibly to a VLP assembly site.

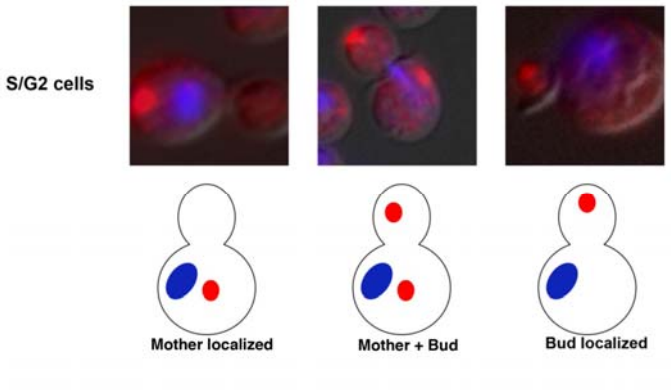
Molecular motors actively transport different mRNA along the cytoskeleton in a wide range of organisms. There are two classes of cytoskeletal elements, actin microfilaments and microtubules, that are involved in mRNA translocation (Kloc et al., 2002). While the disruption of astral microtubules in the *tub2-401* mutant or by nocodazole does not affect localization of *ASH1* mRNA, disruption of actin microfilaments by latrunculin A or inactivation of actin-dependent motor proteins, such as Myo4p, tropomyosin, profilin and actin, abolish *ASH1* mRNA localization to the bud and Ash1p asymmetry (Long et al., 1997; Takizawa et al., 1997). Chartrand has used the actin depolymerization agent latrunculin B (LATB) to show that actin filaments are

required for localization of Ty1 bodies. In cultures treated with LATB, less than 10% of daughter cells contain Ty1 bodies, compared to ~60% of daughter cells containing Ty1 bodies in untreated cultures (Figure 19). These results suggest that a Ty1 RNA locasome depends on actin microfilaments for transport to daughter cells.

A second approach used to test the locasome hypothesis was to investigate if there is a physical interaction between Ty1 RNA and She2p, She3p or Myo4p. For these experiments, Myc-tagged versions of She2p, She3p or Myo4p were used to affinity purify interacting RNAs, which were subsequently detected by RT-PCR. However, attempts to detect an enrichment of Ty1 RNA in purifications using the Myc tagged strains have so far been unsuccessful even though the enrichment of *ASH1* mRNA has been confirmed in the same experiments as positive control. While this result did not support the idea of a Ty1 locasome that involves a complex containing She2p, She3p and Myo4p, previous immunoprecipitation and DNA microarray data did show there was enrichment of Ty1 RNA in immunoprecipitates of She2p, She3p and Myo4p purification although the enrichment was low (2.0-, 1.6- and 3.5-fold, respectively) (Takizawa et al., 2000). These conflicting results using the same She2p, She3p and Myo4p 13x Myc-tagged proteins could be due to weak interaction between Ty1 RNA and the three proteins that are sensitive to experimental differences.

All the above data support the idea of a Ty1 RNP complex, but further work needs to be done to better characterize the components of Ty1 RNP complex. A novel technique called RNA-binding protein purification and identification (RaPID) that allows for the affinity purification of MS2 aptamer-tagged mRNAs and subsequent detection of bound RNA binding proteins (RBPs) and transcripts using mass spectometry and RT–PCR,

A



B

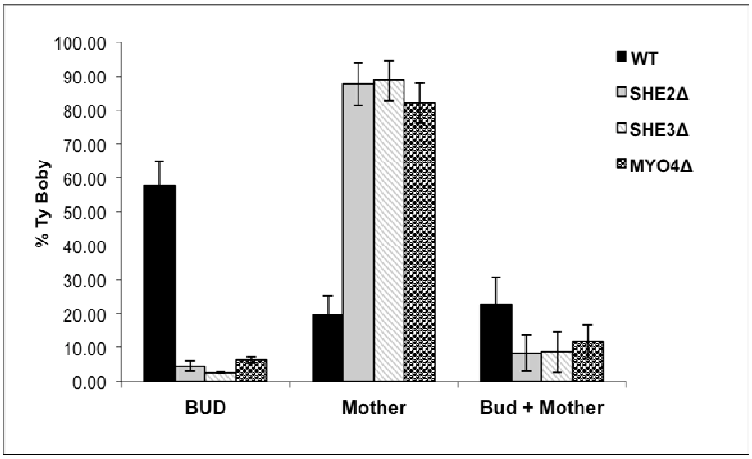


Figure 18. Distribution of Ty1 bodies in wild-type, *she2, she3,* and *myo4* cells.

(A) Fluorescent in situ hybridization of Ty1 RNA (red) in wild-type yeast (W303) cells at S/G2 stage of the cell cycle. Cells were stained with 4',6-Diamidino-2-phenylindol (DAPI) to visualize the nucleus (blue).

(B) Percentages of cells containing different distributions of Ty1 bodies in wild-type, *she2, she3,* and *myo4* cells.

(Pascal Chartrand, personal communication)

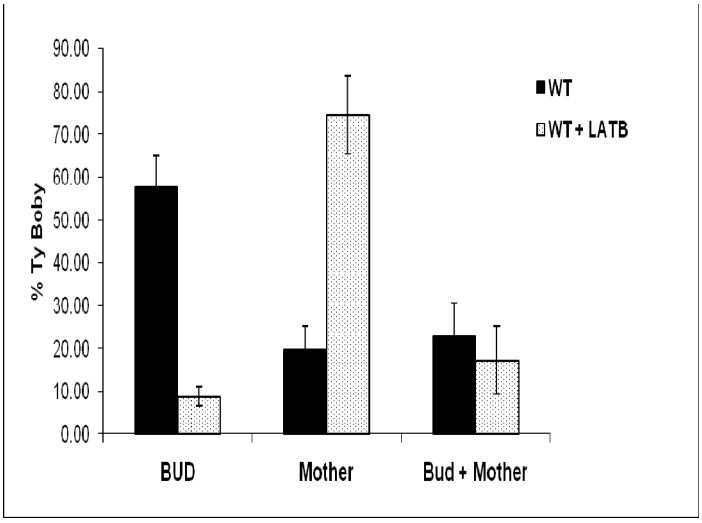


Figure 19. Localization of the Ty1 body is dependent on actin microfilaments.

Percentages of cells containing different distributions of Ty1 bodies. Wild-type yeast cells (W303) were treated with latrunculin B (LATB) to disrupt the actin cytoskeleton. Fluorescent in situ hybridization was used to detect Ty1 RNA in untreated cells or in cells treated with LATB. Cells were stained with (DAPI) to visualize the nucleus.

(Dr. Chartrand's Lab, personal communication)

respectively, may help us achieve this goal (Slobodin and Gerst, 2010).

One major protein category identified in our proteomic analysis of Ty1 VLP is ribosomal proteins. Due to their abundance in cells, they are normally considered as common contaminants in affinity purifications. For the past two decades, a broad idea that has been considered is that there may be special ribosomes for specialized circumstances (Komili et al., 2007; Dinman, 2009). Ribosomal proteins that are not deeply intertwined with rRNA such as non-integral ribosomal proteins can potentially dissociate from mature ribosomes and recent reports have demonstrated that this class of proteins can have ribosome-independent regulatory functions (Dinman, 2009). For example, phosphorylation of human ribosomal protein L13A, in response to interferon-γ, promotes its release from the large subunit, whereupon it silences translation of ceruloplasmin by binding to the 3'-untranslated region of its mRNA (Mazumder et al., 2003). In *S. cerevisiae*, where the majority of ribosomal proteins are encoded by paralogous pairs of genes, there are many examples where distinct mutant phenotypes are associated with each member of a pair. For example, mutants of one but not of the other ribosomal protein isogene produce the Mak$^-$ (maintenance of killer) phenotype, wherein the affected cells cannot maintain the double-stranded RNA killer virus (Wickner, 1996). Finally, the discovery that specific mRNAs are targeted for translation in specific areas of cells, *e.g.* localized translation of the GluR2 mRNA in axons, supports the notion of specialized ribosomes (Kelleher, 2008).

In addition to that, ribosomes found in neuronal dendrites and growth cones as well as ribosomal RNAs which are enriched at sites of integrin engagement and at spreading initiation centers presumably participate in localized translation of mRNAs at

those sites (Bramham and Wells, 2007; Chicurel et al., 1998; De Hoog et al., 2004; Lin and Holt, 2007). Intriguingly, studies showed that translation of the localized *ASH1* mRNA requires a specific subset of duplicated ribosomal protein paralogs, which may form "specialized" ribosomes and exhibit distinct features compared to the general translation machinery (Mili and Macara, 2009; Komili et al., 2007).

For Ty1 transposition, previous mutant screens and our proteomic analysis raise the issue of specific roles for ribosomal protein paralogs in Ty1 retrotransposition: Why does deletion of one ribosomal gene, but not of its paralog, result in a specific phenotype? To answer this question, we tested three ribosomal paralog pairs for paralog-specific roles in Ty1 transposition. For two paralogous pairs, mutation of one member of the pair resulted in decreased Ty1 capsid levels. Among these, the differences in Ty1 RNA levels for *rps10A* and *rps10B* correlate with differences in Gag/Capsid protein levels. This result is consistent with reduced levels of Ty1 RNA transcription or stability in the *rps10A* mutant compared to the *rps10B* mutant. However, the difference observed in Gag/Capsid levels for *rps6A* and *rps6B* appear to be due to translational differences, suggesting that Rpl6Ap may be a component of Ty1-specific specialized ribosomes. For the third paralogous pair (*rpl14A/rpl14B*), mutation of *rpl14A* resulted in higher Ty1 Gag and Gag-Pol levels along with decreased RT and IN levels compared with the paralogous mutant *rpl14B*. This result suggested that proteolytic processing to form mature Ty1 proteins is defective for the *rpl14A* mutant.

Our results also showed that unlike its role as a translational repressor in *ASH1* mRNA localization, Puf6p binds weakly to Ty1 RNA *in vivo*, but more importantly, the Ty1 Capsid level is dramatically decreased in *puf6* mutants. Puf6p has recently been

shown to play a role in 60S ribosomal subunit biogenesis (Lee et al., 2007). Since Puf6p and ribosomal proteins of the 60S subunit were found to associate with Ty1 Gag complexes in our proteomic analysis, it raises the possibility that Puf6p is involved in forming specialized Ty1 ribosomes which play roles in Ty1 retrotransposition distinct from general translation, such as translation at VLP assembly sites.

CHAPTER 2

ASSOCIATION OF THE DEBRANCHING ENZYME Dbr1p WITH VLPs OF THE YEAST RETROTRANSPOSON TY1

Introduction

Our previous work suggested that during reverse transcription Ty1 RNA forms a lariat, joining its 5'end with an internal 2'carbon near its 3' end. This lariat could promote the cDNA minus-strand transfer that occurs early during reverse transcription (Cheng and Menees, 2004), but subsequent cleavage of the 2'-5' bond by the debranching enzyme Dbr1p would be required to facilitate completion of reverse transcription. Although work by other labs has questioned the existence of the Ty1 RNA lariat (Coombes and Boeke, 2004., Pratico and Silverman, 2007), experiments in the Boeke and Menees labs have shown that there are capped and uncapped Ty1 mRNA populations inside Ty1 VLPs. The Menees lab previously hypothesized that uncapped Ty1 RNA could be created by debranching of a Ty1 RNA lariat.

There are several experiments that support the potential role of an uncapped Ty1 population in the Ty1 transposition cycle. First, genetic screens that identified large sets of putative regulators of Ty1 retrotransposition (Griffith et al., 2003; Scholes et al., 2001) identified several host factors involved in cap binding and decapping. Second, both Ty1 and Ty3 VLP components localize at cytoplasmic ribonucleoprotein complexes known as mRNA processing bodies (P-bodies), sites where mRNA decapping occurs. P body association is functionally significant because disruption of genes encoding P-body proteins decreases retrotransposition (Beliakova-Bethell et al., 2006; Scholes et al., 2001; Checkley et al., 2010).

Although the uncapped Ty1 RNA population may be created by debranching of a Ty1 RNA lariat, the role of Dbr1p in Ty1 transposition was investigated from a broader perspective that did not depend on the lariat hypothesis being correct. Specifically, we focused on 1) further establishing the relationship between Dbr1p's role in Ty1 transposition and its role in debranching RNA lariats and, 2) exploring possible physical interactions between Dbr1p and Ty1 VLPs.

Results

1. Copurification of Dbr1p with Ty1 Gag aggregates that contain VLPs.

a. Creation and expression of FLAG-tagged Ty1.

A hypothesis that is consistent with several pieces of information is that enzymatically active Dbr1p acts in Ty1 VLPs to promote Ty1 transposition. One piece of information is the fact the Dbr1p plays a role in promoting Ty1 cDNA accumulation, possibly by promoting reverse transcription (Karst et al., 2000; Griffiths et al., 2003). A second piece of information is that reverse transcription occurs in Ty1 VLPs. A third piece of information is that Dbr1p enzymatic activity is necessary for wild-type levels of Ty1 transposition (Ding et al., in preparation).

To test the hypothesis that enzymatically active Dbr1p acts in Ty1 VLPs to promote Ty1 transposition, the approach was taken to assess if Dbr1p associates with Ty1 VLPs. To carry out this approach, epitope-tagged VLPs were created, purified, and assessed for the presence of Dbr1p by western blotting. As described in chapter 1, DNA encoding the FLAG epitope was inserted near the 5' end of the Ty1 Gag coding region in order to create a FLAG-tagged capsid protein. The FLAG-tagged Ty1 element was tested for its ability to transpose, using the *HIS3* marker system described previously (Griffith et al., 2003). As shown in figure 5B, the FLAG-tagged Ty1 element transposes the *HIS3* marker at ~50% the level of the untagged Ty1 element in the same strain background. Although slightly defective for transposition, FLAG-tagged Ty1 appeared to be competent enough for us to proceed with our purification studies. The yeast strain used in these studies expresses endogenous Ty1 elements, so it was hypothesized that

coexpression of FLAG-tagged and untagged Ty1 elements would result in Ty1 VLPs containing a mixture of tagged and untagged capsid proteins, as described previously.

b. Copurification of Dbr1p-myc with Ty1 Gag complexes.

In order to determine if Dbr1p associates with Ty1 VLPs, extracts from cells coexpressing Dbr1p-13x myc along with FLAG-tagged and endogenous Ty1 elements were processed as described in chapter 1 to purify FLAG-tagged Gag complexes. Purified material was run on polyacrylamide gels and assessed by western blotting, probing for both Ty1 capsid and Dbr1-13x myc. As a negative control, a parallel sample was processed from cells expressing Dbr1p-13x myc and only the endogenous, untagged Ty1 elements. As shown in figure 20, Dbr1p-myc is copurified with FLAG-tagged Ty1 Gag complexes but not from extracts lacking FLAG-tagged Gag. Because Ty1 Gag complexes contain all the components of Ty1 VLPs, we conclude that Dbr1p associates with Ty1 VLPs.

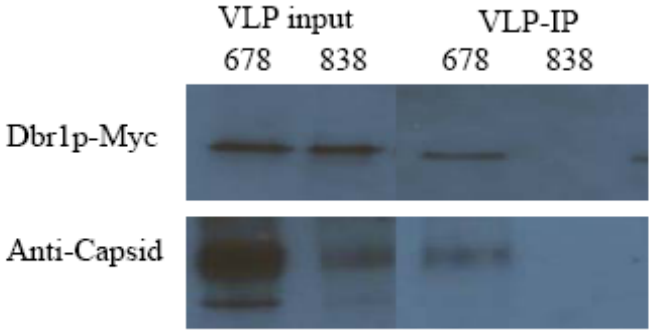


Figure 20. Copurification of Dbr1p-13x myc with Ty1 Gag complexes.

Chromosomal Dbr1p-13x myc tagged yeast cells were co-transformed with the Ty1 expression plasmid FLAG-pAR100 (TMY678) or a negative control plasmid pRS316 (TMY838). VLP-enriched fractions were immunoprecipitated with anti-FLAG agarose beads. Western blots of the immunoprecipitates were probed with anti-myc and anti-Capsid antibodies.

2. Inefficient copurification of Ty1 VLP proteins with Dbr1p-TAP.

a. Dbr1p-TAP does not associate with Ty1 VLP proteins as determined by IP-western.

To further investigate the association of Dbr1p with Ty1 VLPs, a complementary approach to the one described above was also undertaken. For this second approach, Dbr1p was purified and the copurifying proteins were assessed for the presence of Ty1 proteins. The outcome of these experiments was intended to further define the nature of the association of Dbr1p with Ty1 VLP components identified in the experiments using the FLAG-tagged Ty1 element described above. Specifically, robust copurification of Ty1 VLP components with Dbr1p-TAP would be inconsistent with Dbr1p being within VLPs. Conversely, poor copurification of Ty1 VLP components with Dbr1p-TAP would be consistent with Dbr1p being sequestered within VLPs.

To carry out this second approach, a yeast strain containing a TAP-tagged allele of *DBR1* was obtained (Open Biosystem). The *DBR1*-TAP allele encodes Dbr1p fused at its C terminus to an IgG binding domain (protein A) plus a calmodulin binding peptide domain. These domains are separated by a protease cut site for the tobacco etch virus (TEV) protease. This allele replaces the *DBR1* gene at the *DBR1* locus, and is expressed from the *DBR1* promoter. The *DBR1*-TAP allele produces a functional Dbr1 protein because yeast cells expressing Dbr1p-TAP as their only source of Dbr1p demonstrate wild-type levels of debranching *in vivo* (Ding et al., in preparation). A simplified IgG pulldown protocol was used for the affinity purification of Dbr1p-TAP and western blotting was used to show the purification worked as expected (Figure 21). Copurification of Ty1 VLP components was also assessed by western blotting, probing

material copurified with Dbr1p-TAP for Ty1 capsid proteins. As seen in figure 21, these experiments showed no evidence of copurification of Ty1 Capsid with Dbr1p-TAP.

b. Mass spectrometric analysis of proteins copurified with Dbr1p-TAP.

To more broadly survey the proteins that associate with Dbr1p-TAP, mass spectrometric analysis was performed on Dbr1p-TAP purification products. The purified Dbr1p-TAP sample was trypsinized and the peptides were analyzed by multidimensional protein information technology (MudPIT). For a negative control, the TAP purification procedure was performed on protein extracts from yeast cells lacking the *DBR1*-TAP allele. This negative control sample was also analyzed by MudPIT.

Several splicing factors and other RNA binding proteins were identified by mass spectrometric analysis of proteins that copurify with Dbr1p-TAP (Table 2). These data, along with the fact that Dbr1p-TAP produces a functional debranching enzyme *in vivo*, suggested that Dbr1p-TAP is capable of engaging in interactions like the wild-type Dbr1p. Furthermore, several Ty1 peptides were identified in the mass spectrometric analysis of proteins that copurify with Dbr1p-TAP. Peptides corresponding to all the different Ty1 proteins were identified in the Dbr1p-TAP sample: Gag, protease, integrase, and reverse transcriptase proteins. Association of Ty1 VLP components with Dbr1p-TAP is consistent with the association of Dbr1p-myc with FLAG-tagged Ty1 Gag complexes. The fact that the association of Ty1 proteins with Dbr1p-TAP is only evident by mass spectrometric analysis and not by IP western is consistent with Dbr1p lying within VLPs, where most of the TAP tag is inaccessible during the purification process.

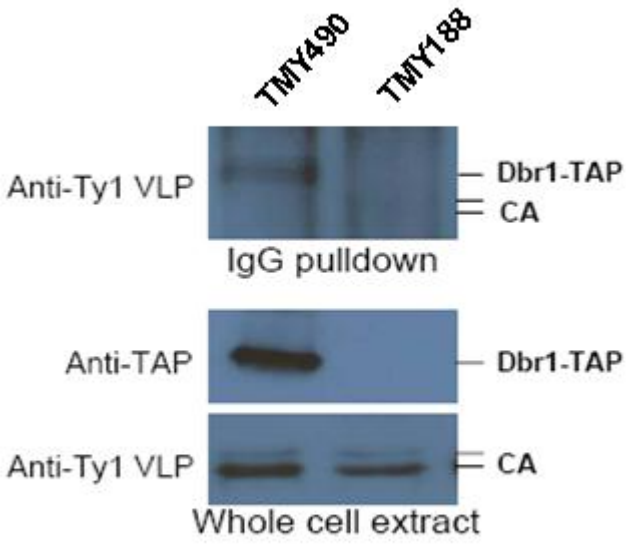


Figure 21. IgG pulldown of Dbr1-TAP.

Extracts from TAP tagged Dbr1p (TMY490) and untagged Dbr1p (TMY188) strains were incubated with IgG-sepharose. The bound fraction was analyzed by western using antibody against TAP tag and Ty1 anti-VLP. Since Dbr1-TAP tag contains a protein A sequence which is the IgG binding domain, Anti-Ty1 VLP antibody will detect both Ty1 Capsid and Dbr1-TAP.

TABLE 2. Host factors associated with Dbr1p by MudPIT analysis (partial results)

Group/no. of proteins	Protein identified
mRNA splicing	Mud2p, Sub2p, Snt309p, Msl5p, Prp19p
P-body proteins	Pat1p, Dcp2p, Edc3p
Ty1 proteins:	IN, RT, PR, CA

3. Ty1 VLP RNA is mixture of capped and decapped species.

a. Ty1 RNA 5' end mapping by RLM-RACE method.

Previous work in our lab had identified a novel, uncapped form of full-length Ty1 genomic RNA (Cheng and Menees, 2004). This was accomplished using RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). RLM-RACE is normally used to identify the *capped* 5' end of a specific mRNA. To identify capped 5'ends, RNA samples are treated with tobacco acid pyrophosphatase (TAP) to convert capped 5' ends into monophosphorylated 5' ends that can be ligated to a specific RNA oligonucleotide. An RNA must have a monophosphorylated 5' end in order to be ligated to the RNA oligonucleotide. Prior to TAP treatment, an RNA sample is pretreated with calf intestine phosphatase (CIP) to dephosphorylate any preexisting monophosphorylated 5' ends, which in most cases come from truncated mRNAs or non-mRNAs. This CIP pretreatment eliminates RLM-RACE products from such non-capped RNAs. Subsequent RT-PCR of RNA ligation products using gene-specific and RNA oligonucleotide-specific primers amplifies products from RNAs that were capped in the original RNA sample.

Control reactions for our RLM-RACE procedure were performed on WT total yeast RNA samples, looking for the *PDC1* mRNA 5' end (Figure 22). A single RNA sample is processed 2 ways: + and – CIP/TAP pretreatment. The one product from the +CIP/TAP reactions represents capped 5' ends of *PDC1* mRNA. As expected, no specific product was created from the –CIP/TAP reaction, indicating that no stable decapped species of *PDC1* RNA is present. Internal *PDC1* mRNA sequences were present equally in both + and – CIP/TAP reactions, confirming that equivalent amounts of RNA were processed through both reactions (data not shown).

Ty1 VLP RNA from wild-type cells was analyzed by RLM-RACE with + and – CIP/TAP treatment. The +CIP/TAP reaction produced one major product, which identifies nucleotide 241 of Ty1 as the capped 5' end (Figure 22). The 5' end of Ty1 RNA has been previously mapped to nucleotide 241 using primer extension. Surprisingly, the -CIP/TAP reaction with the same Ty1 VLP RNA sample generated the same product as the +CIP/TAP reaction and some lower bands which represent truncated Ty1 RNA (Figure 22).

Since the 5' end sequence of Ty1 and the 3' end sequence of the RNA oligonucleotide (Invitrogen) have the same 8 nt sequence, a cDNA created from the 5' end of Ty1 RNA could base pair with the 3' end of the RNA oligonucleotide. This base pairing would allow extension of the Ty1 cDNA to include sequences complementary to the RNA oligo. Such an extended cDNA would look like a cDNA created from a template created by ligation between the RNA oligonucleotide and Ty1 RNA. Therefore, artifactual RLM-RACE products can be created. In order to circumvent this, we have repeated this experiment using 3 different RNA oligonucleotides to eliminate the possibility of a RT-PCR artifact. We conclude that a substantial portion of the 5'end of Ty1 VLP RNA is uncapped. The existence of RNA cap binding and decapping factors as Ty1 host factors strengthens the hypothesis that decapping plays an important, previously unrecognized, positive role in Ty1 transposition.

b. Ty1 RNA 5' end mapping by primer extension.

The 5' end mapping of mRNA with RLM-RACE employs a PCR method which may cause artifacts and cannot provide quantitative information about the ratio of capped vs. decapped populations in the sample. As a complement to RLM-RACE, a primer

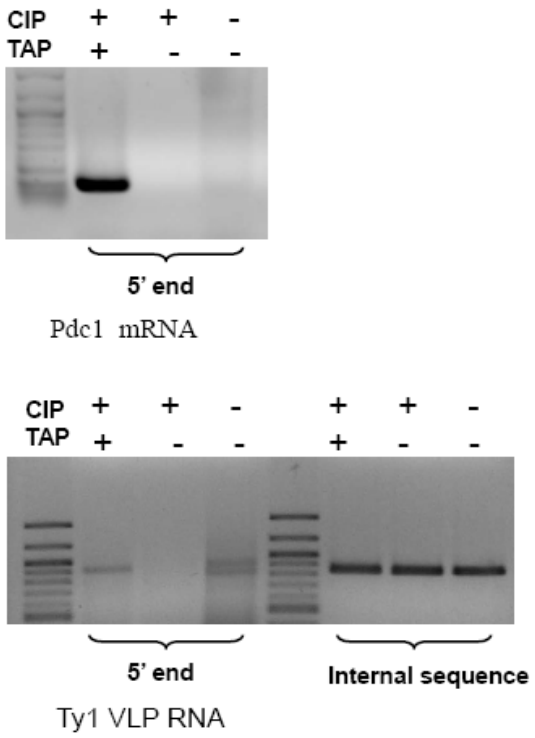


Figure 22. Mapping 5' ends of Ty1 RNA with RLM-RACE.

Agarose gel electrophoresis of RLM-RACE amplification products of Pdc1 mRNA and Ty1 VLP RNA 5' ends. Phosphatase (CIP) and pyrophosphatase (TAP) treatment prior to RLM-RACE is indicated for each lane.

extension assay was used to quantitatively analyze the cap status of Ty1 RNA in *dbr1* mutants cells. Previous results showed that the presence of a 5'cap structure of mRNA will template the addition of an extra 3' nucleotide to cDNA by reverse transcriptase; hence, the primer extension cDNA product formed on capped mRNA will be 1 nt longer than that synthesized on uncapped mRNA (Davison and Moss, 1989; Schwer et al., 1998).

In the experiment shown in Figure 23, a ^{32}P-labeled 20-mer oligonucleotide complementary to the translation start site of *PDC1* mRNA was annealed to total RNA and *PDC1* mRNA purified from wild-type cells and extended with reverse transcriptase. Without TAP treatment, two extension products, one nucleotide apart, were formed and mapped to the 5' end of *PDC1* mRNA that was reported previously (Zhang and Dietrich, 2005). The lower band product represents the decapped full length *PDC1* mRNA population, which is supposed to be a normal mRNA degradation product in cells. The upper band represents the capped *PDC1* mRNA which disappears after TAP treatment. The levels of both upper and lower band are equal, consistent with equivalent amounts of capped and decapped *PDC1* mRNA. For Ty1 VLP RNA or pure Ty1 RNA isolated from wild-type cells, there is only one band and it maps to Ty1 transcription start site (nt 241) in samples with or without TAP treatment (Elder et al., 1983; Mules et al.,1998). The most reasonable explanation is that there is no cap structure present at 5' ends of all of the Ty1 RNA detected in this experiment. Another possibility, consistent with the RLM-RACE results, is that there is a small population of capped Ty1 RNA, which is obscured by the band representing the uncapped Ty1 RNA.

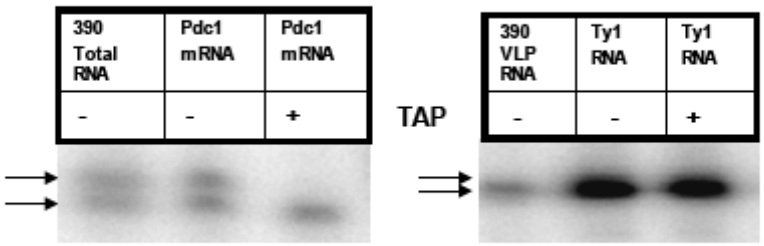


Figure 23. Primer extension analysis of the 5' end of Ty1 VLP RNA.

Primer extension reaction products were analyzed by 8% polyacrylamide sequencing gel. The cDNAs synthesized on RNA templates after TAP treatment are 1 nt shorter than cDNAs made on capped RNAs.

Discussion

All eukaryotic mRNAs contain a cap structure that consists of 7-methylguanosine joined to the 5’ end of the RNA transcript by a 5’-5’ triphosphate bridge. RNA capping occurs during transcription and is the first mRNA processing event. The message elongation phase follows capping, with RNA polymerase II (Pol2) transcribing the remainder of the mRNA. RNA capping therefore is a key event in the transition from transcription initiation to elongation. The cotranscriptional nature of the capping process means that no uncapped, full-length pre-mRNA is ever produced. Retroviral RNAs also acquire a 5’ cap, consistent with the fact that Pol2 transcribes retroviral RNA.

Eventually, cellular mRNAs become decapped during their degradation, most often after removal of the 3’ polyA tail. In yeast, the main decapping enzyme (Dcp1p-Dcp2p) is associated with cytoplasmic processing bodies (“P bodies”), which also contain 5’ exonucleases that act immediately to degrade the decapped mRNAs. Thus, full-length decapped mRNAs are never observed as a discrete species.

We employed two different methods, RLM-RACE and primer extension, to map the 5’ ends of Ty1 VLP RNAs. Both results suggest that a large fraction of Ty1 RNA within VLPs lacks a 5’ cap and is monophosphorylated on its 5’ end.

Dbr1p is a 2'-5' phosphodiesterase that cleaves intron RNA lariat branch points after splicing, facilitating ribonucleotide turnover (Chapman and Boeke, 1991; Vijayraghavan et al., 1989). Mature mRNA is produced in *dbr1* mutant cells with accumulation of intron lariats (Salem et al., 2003; Chapman and Boeke, 1991). Mutations in *DBR1* inhibit both Ty1 transposition and cDNA formation (Griffith et al., 2003).

Previously, Ye et al showed that siRNA knockdown of hDbr1 blocked HIV-1 replication during reverse transcription at the stage of cDNA minus-strand transfer, a result that is consistent with our model for the role of Dbr1p in Ty1 replication (Ye and Camerini, 2005). Recently, others have reported that siRNA knockdown of the message for the human debranching enzyme (hDbr1) within human cells results in decreased HIV-1 replication (Bushman et al., 2009), suggesting that the branch may be widely conserved among LTR retroelements, including retroviruses.

A correlation between Dbr1p's roles in RNA lariat debranching and Ty1 transposition was determined previously (Salem et al. 2003) using a set of *dbr1* point mutants generated by random mutagenesis. However, besides testing only a small number of *dbr1* mutants, the study only analyzed mutants already known to be defective for Ty1 transposition. To more broadly analyze the relationship between Dbr1p's roles in Ty1 transposition and RNA lariat debranching, a set of forty-three additional yeast *dbr1* point mutants was studied in collaboration with Beate Schwer (Weill-Cornell Medical College) (Ding et al, in preparation). Each mutation in this set of forty-three mutants resulted in the replacement of a conserved amino acid residue within Dbr1p with alanine.

The results of this experiment indicated a nearly complete correlation between Ty1 transposition and the debranching activity of Dbr1p. The *dbr1* mutants that retained normal debranching activity also exhibited wild-type levels of Ty1 transposition. The mutants with decreased debranching activity exhibited decreased Ty1 transposition. The simplest explanation for these results is that active Dbr1p plays a positive role in Ty1 transposition. This leads to the conclusion that there is one or more RNA substrates that Dbr1p acts on to promote Ty1 transposition. Since Dbr1p plays a role in reverse

transcription, this RNA substrate for Dbr1p may lie within VLPs, the site of reverse transcription.

In order to test this hypothesis, we checked if there is physical interaction between Dbr1p and Ty1 VLPs. FLAG-CA immunoprecipitation assays show that Dbr1p copurifies with Ty1 Gag complexes. Reciprocal immunoprecipitation of Dbr1p was not highly effective in pulling down Ty1 VLPs. However, peptides corresponding to Ty1 VLP proteins were identified in the mass spectrometric analysis of proteins that copurify with Dbr1p. These data support the idea of Dbr1p lying within VLPs, where the Dbr1p that is relevant to Ty1 as a host factor is inaccessible during the purification process.

Further, we wanted to test whether Dbr1p directly interacts with an RNA within Ty1 VLPs. That RNA could be Ty1 RNA. However, the inability to detect Ty1 RNA in Dbr1p pulldowns suggests that Dbr1p does not associate with Ty1 RNA outside of VLPs. This would occur if Ty1 RNA forms a lariat after packaging.

In vitro and *in vivo* debranching analyses showed that mutation of His86 to alanine in Dbr1p greatly diminishes its debranching activity on natural lariat RNAs and synthetic branched RNAs (Khalid et al., 2005). Based on structural studies of λ phosphatase and Mre11 (Voegtli et al., 2000; Hopfner et al., 2001), His86 participates in catalysis through direct contact with the scissile 2'-5' phosphodiester bond of the branched RNA (Khalid et al., 2005). Our collaborator, Beate Schwer, has shown that Dbr1p-*H86A* interacts with the RNA branch point without cutting the 2'-5' phosphodiester bond (B. Schwer, personal communication). Therefore, I have used this mutant to assess the possible interaction between Dbr1p and Ty1 RNA. As expected, results showed that Dbr1p-*H86A* protein could pull down short RNA lariats such as *ACT1*

lariat RNA but not *ACT1* linear mRNA. However, no Ty1 RNA was enriched in precipitates of Dbr1p-*H86A*-TAP. Possible explanations are (1) there is no branch in Ty1 RNA, (2) the binding affinity between the Dbr1p-*H86A* protein and the putative Ty1 branch is weak, or (3) the Dbr1p-*H86A*-TAP that is bound to Ty1 RNA lies within VLPs, where it is inaccessible. This last possibility is supported by the data for the Dbr1p-TAP pull down experiment described in this chapter.

Materials and Methods

Yeast strains

The *S. cerevisiae* strain TMY188 (*MAT***a** *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) was used for the transposition assays, TAP purifications, and Ty1 VLP isolations. Strain TMY 248 (*MAT***a** *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 dbr1Δ::kan*^{R}) was used for *dbr1-H86A* IgG pulldown assay. Strain W303 (*MAT*a *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*) was used for She2p-13myc, She3p-13myc, and Myo4p-13myc RNA immunoprecipitation.

Plasmid, media, and general procedures

pCR4-TOPO (Invitrogen) was used for cloning PCR products by the topoisomerase cloning method (Shuman, 1994). pAR100 contains p*GAL1*-Ty1::*HIS3* on a *URA3*-marked, low-copy (*CEN* origin of replication) plasmid (Griffith et al., 2003). pRS316 was used as negative control for pAR100, which does not contain Ty1::*HIS3* sequence (Sikorski and Hieter, 1989). YNB/CAA medium (6.7g yeast nitrogen base without arnino acids + 20g casamino acids/lL media), supplemented with adenine but lacking uracil, trytophan, or both depending on the plasmid selection(s) required in specific experiments was used as selective media, with 2% raffinose as the nonrepressing carbon source. Induction of galactose-inducible Ty reporter elements was performed in all cases by direct addition of galactose at a final concentration of 2% to the cultures. Rich medium (YPD) and minimal. sporulation medium were prepared as described (Kaiser et al., 1994), as were all plates described below. Synthetic dextrose minimal (SD) plates and SD+FOA plates (plates containing 5-fluoro-orotic acid, or 5-FOA) were

supplemented with the necessary amino acids dependent on the strain and transformant. Synthetic galactose minimal (SG) plates contain 2% galactose in place of the 2% glucose in SD plates. Bacterial cultures were grown in LB media (l0g tryptone + 5g yeast extract + l0g sodium chloride/lL media) + 100 µg/ml ampicillin.

Plasmid DNA was isolated from yeast cells using standard methods (Ausubel et al., 1997). Isolation of plasmid DNA from bacterial cells was performed with the Qiagen miniprep kit.

Yeast transformations were done by the TRAFO methods developed in the Gietz laboratory (Agatep et al., 1998; Gietz, 1994). The Quick and Easy TRAFO protocol was used for all transformations. Bacterial transformations were done by electroporation or chemoporation (Ausubel et al., 1997).

VLP preparation

Yeast cells were grown overnight at 30^0C as 20 ml cultures in YNB/CAA medium (– uracil) containing 2% D (+) raffinose as the carbon source. Cells were then transferred to 300 ml of the same medium containing 2% Galactose with starting OD600 = 0.1 and grown for 16-20 h at 22-24^0C to an OD600 = 1.0 (mid-log phase). Cells were harvested by centrifugation, washed once with water, and resuspended in 5ml cell breakage buffer B (15mM KCl, 10mM HEPES/KOH pH 7.8, 10mM EDTA, 3mM DTT, 40ul Sigma protease inhibitor mix, 40U/ml Invitrogen RNase out). Cells were broken open by vortexing in the presence of glass beads. Clarified cell extracts were quantitated by Bio-Rad Bradford assay and eauivalent amounts of whole cell extracts were loaded onto sucrose step gradients (20 ml of 20%, 5 ml of 30%, 5 ml of 70%). Following

centrifugation at 25000 rpm for 3h at 4^0C, the bottom 2 ml of the 30% sucrose layer was collected as the VLP-enriched fraction containing mostly mature VLPs.

Affinity purification of Ty1 Gag complexes

After galactose induction, the lower layer (2ml) of 30% sucrose fractions containing FLAG-VLPs, endogenous VLPs or mosaic VLPs (Containing both FLAG-Gag and untagged Gag proteins) from TMY188 cells transformed with FLAG-pAR100 were collected. 200 ul VLPs were mixed with 700ul IP buffer (50 mM HEPES/KOH pH 7.8, 1mM EDTA, 50mM NaCl, 10ul sigma protease inhibitor mix), and 100ul anti-FLAG agarose beads (Sigma). The mixture was incubated for 2-4 h at 4°C. The immunoprecipitates were then washed in ice-cold IP buffer with 300mM NaCl. Ty1 FLAG-Gag complexes and mosaic Gag complexes were eluted with 8M urea. One third of the eluate was trichloroacetic acid (TCA) precipitated and fractionated on 4-12% SDS-PAGE gel and visualized by silver staining (Figure 7). Another one third was sent for MudPIT analysis. Anti-FLAG eluate from TMY188 cells transformed with pRS316 (mock purification) was used as negative control since it only contains endogenous Ty1 VLPs that should not be isolated with anti-FLAG agarose beads.

Yeast RNA preparation

Total yeast RNA samples were prepared from 10 ml cultures grown to early-logarithmic phase (OD600=0.5-1.0) using the RNeasy system from Qiagen (Valencia, California, USA). For this procedure, cells were broken open by vortexing with acid washed 0.5 mm glass beads (BioSpec; Bartlesville, Oklahoma, USA). After DNase I treatment to remove DNA contamination, the final RNA concentration was determined by

OD_{260}. RNA was also assessed by 1% TAE agarose gel electrophoresis and staining with ethidium bromide. The presence of sharp bands for the 25S and 18S ribosomal RNAs in the gel is an indication of minimal RNA degradation in a sample.

RNA from Ty1 virus-like particles (VLPs) was prepared as described previously (Karst and Menees, 1999). Briefly, yeast cells were grown overnight as 10 ml cultures in YNB/CAA medium (– uracil) containing 2% D (+) raffinose as the carbon source. Cells were subcultured in the same medium except the carbon source was 2% D (+) galactose. When cultures were in log phase, cells were transferred to 300 ml of the same galactose-containing medium and grown for 16-20 h to an OD600 = 1 (mid-logarithmic phase). In some cases galactose was not added until the final culture. Cells were harvested by centrifugation, washed once with water, and resuspended in a cell breakage buffer. Cells were broken open by vortexing in the presence of glass beads and clarified extracts were loaded onto sucrose step gradients (16 ml of 20%, 5 ml of 30%, 5 ml of 70%). Following centrifugation and isolation of the VLP-enriched fraction, nucleic acids were extracted with phenol/chloroform and precipitated with ethanol. Following resuspension of the nucleic acids in nuclease free water, DNA was degraded by treatment with RQ1 DNase I (Fisher; USA) using conditions recommended by the supplier. RNA concentration was then determined by OD_{260}.

Transposition dilution assay

Single colonies of each mutant strain as well as a wild-type strain were patched onto SD-Ura plates to begin the retrotransposition assay. Patches were replica plated onto SG-Ura medium containing 2% galactose and then incubated for 4 days at room

temperature (24^0C) to induce retrotransposition. After 4 days of incubation, the SG-Ura plates were replica plated sequentially to: (i) SD-Ura medium containing glucose, (ii) yeast peptone dextrose (YPD) medium, (iii) SC medium containing glucose and 1.2 g/liter 5-fluoroorotic acid (SD+FOA plates). Cells were scraped from the SD+FOA patches into water and diluted to an OD600 of 1. Two-fold dilutions were prepared in 96-well microtiter dishes and then plated on SD and SD-His plates using a multichannel pipettor. The SD plate served as a control for adjusting the cells to an OD600 of 1. The number of cells growing at each dilution on the SC-His plate (e.g. for a *KHD1* strain) was compared to similar dilutions prepared from the wild-type strain and the fold change was estimated (rounding to the nearest twofold dilution) (Griffith et al., 2003).

Southern Hybridization

All Southern blot hybridizations were done using standard methods (Ausubel et al., 1997). For Ty1 Southern blots, a 1.2 kb fragment of the Ty1 sequence encoding the CA protein was used as a probe.

Immunoblot Analysis

Proteins from whole cell extracts, VLP preparations, or VLP IPs were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 2% nonfat dry milk in TBS-T for 1 h and subsequently incubated with the relevant primary antibody against Ty1 proteins CA, RT, IN and Tdh1p for 1 h, washed in TBS-T, and incubated for 1 h with peroxidase conjugated secondary antibody. The membranes were washed in TBS-T and developed with enhanced

chemiluminescence (ECL) Plus (Amersham), followed by exposure to film. For some experiments, the same nitrocellulose membranes used for detection of Ty1 CA were then stripped and reprobed with anti-Tdh1 antibody. Tdh1p levels were used as internal loading control to quantitate Ty1 CA level. FLAG-Gag was detected using either anti-FLAG mAb or anti-VLP antiserum.

Immunoprecipitation

One hundred milliliters of yeast cells were grown to early log phase (OD_{600} ~ 1) at 24°C or 30°C in YPD medium. For She2p-13myc, She3p-13myc and Myo4p-13myc immunoprecipitation, formaldehyde was added to a final concentration of 1%, and cells were incubated at room temperature (RT) for 20 min. Then, glycine was added to a final concentration of 300 mM. Cells were washed twice in cold 1× PBS, harvested by centrifugation, and resuspended at an OD_{600} of 100 in the extraction buffer (25 mM HEPES-KOH, pH 7.5, 150 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 87.5 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 ul/ml Sigma protease inhibitor mix, 40U/ml Invitrogen RNaseOUT). The cells were broken with glass beads, vortexed six times for 30 seconds each on ice with a 1-min pause between each vortex. After centrifugation, the supernatant was used for immunoprecipitation and Western blot. For the immunoprecipitation of She2p-13myc, She3p-13myc, Myo4p-13myc, 10 μg of anti-myc antibody (Covance 9E10 mAb) was added to 500 μl of supernatant and incubated at 4°C with agitation for 1 h; 50 μl of protein A-Sepharose beads were then added, and the incubation at 4°C was continued for 2 h. For immunoprecipitation of tandem affinity purification (TAP)-tagged proteins, 50 μl of IgG-agarose beads was added to 500 μl of supernatant. The beads were washed four times for 3 min at 4°C with a wash buffer (25

mM HEPES-KOH, pH 7.5, 150 mM KCl, and 2 mM $MgCl_2$). The RNA was eluted from the beads with 200 µl of elution buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, and 1% SDS) by incubating 10 min at 65°C, followed by phenol-chloroform extraction and ethanol precipitation.

Reverse Transcription and qPCR

For the reverse transcription of IP RNAs, 2 µl of RNA was incubated at 70°C for 5 min in the presence of 0.5 µg of random primer or specific primers. For the reverse transcription of total or VLP RNAs, 1 ug RNA was incubated at 70°C for 5 min in the presence of 0.1 µg of random primer or specific primers and quickly chilled on ice. The reverse transcription reaction was performed in a 20ul volume containing 1× buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$, and 10 mM dithiothreitol), 10 mM dNTPs and 20 U of RNAseOUT, with 40 U reverse transcriptase for 1 h at 50°C. The cDNAs were then amplified by PCR using primers base pairing to *ASH1, BRO1, ACT1* and Ty1 sequences using standard conditions recommended in the Sybrgreen qPCR kit from BioRad.

Isolation of Ty1 and *PDC1* mRNA

0.5-1.0 mg of total RNA from TMY 490 was brought to a final volume of 500 µl, and incubated at 65°C for 10 minutes. 3 µl of the Biotinylated-Ty1/*PDC1* RNase-free DNA oligonucleotides and 13 µl of 20X SSC was added to the above total RNA and incubated at 65°C until completely cooled to RT by using PCR touchdown program (decrease 1°C/1 min); 100 µl of Streptavidin-Paramagnet Particles (SA-PMPs) were then added, and the incubation at RT was continued for 10 minutes with gentle mixing by

inverting every 1-2 minutes. Magnetic particles were washed four times for 3 minutes at RT with 0.1X SSC. The Ty1/*PDC1* mRNA was eluted from beads with 100 µl of the RNase-Free water by incubating 3 minutes at 65°C.

Primer extension assay

The deoxyoligonucleotides 198 and 376, which served as primers for reverse transcription of Ty1 RNA and *PDC1* mRNA respectively, were labeled at their 5′-ends by incubation with T4 polynucleotide kinase and [γ-^{32}P] ATP (3000 Ci/mmol; PerkinElmer, Inc). These primers are complementary to 5' ends of Ty1 RNA and *PDC1* mRNA. The ^{32}P-labeled primer was added to VLP RNA or Ty1/*PDC1* mRNA, and incubated for 5 min at 65°C followed by incubation on ice for 2 min in primer extension buffer (40 mM Tris-HCl (pH 7.5) and 0.2 mM EDTA) with 20 U of RNAseOUT. Primer extensions were initiated by adding 20 U Cloned Avian Myeloblastosis Virus reverse transcriptase (AMV-RT, Invitrogen) and incubated at 50°C for 30 min. followed by phenol-chloroform extraction and ethanol precipitation. RNA gel loading buffer was added to primer extension products, and incubate at 95^{o}C for 3 minutes before loading on 8% polyacrylamide sequencing gels. For reference, RNA sequence ladders were generated by primer extension with dideoxynucleotides. Autoradiograms of the sequencing gels were exposed to phosphor screens, and subsequently read on a phosphor imager (Molecular Dynamics).

Tandem affinity purification (TAP) of protein complexes

TAP purifications were carried out by growing six liters of cells to an OD600 ≈ 1-1.5. Cells were washed once with ice-cold water and extracted by glass bead disruption in

extraction buffer (40 mM HEPES-KOH, pH 7.5, 10% glycerol, 350 mM NaCl, 0.1% Nonidet P-40, 1 mM PMSF, 10 µl/ml Sigma protease inhibitor mix, and 0.5 mM DTT). After centrifugation, clear supernatants (around 10 ml) were incubated with 200 µl IgG-sepharose (Amersham). The mixture was incubated at 4 °C, rotating for 2 h, and washed 3 times with extraction buffer and 1 time with TEV protease cleavage buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 10 µl/ml Sigma protease inhibitor mix, 0.5 mM EDTA, 1 mM DTT). The IgG Sepharose was then resuspended in 300 µl of TEV protease cleavage buffer with 100 U of TEV protease (Invitrogen) and incubated at RT for 2h. The TEV cleavage elute was added to calmodulin beads (Stratagene) in calmodulin binding buffer (10 mM Tris-HCl, pH 8, 10% glycerol, 150 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% Nonidet P-40, 1 mM PMSF, 10 µl/ml Sigma protease inhibitor mix, and 0.5 mM DTT). This mixture was then incubated for 2 h at 4 °C with rotating. After incubation, the beads were washed 3 times in calmodulin binding buffer and proteins were eluted by incubating calmodulin beads with calmodulin elution buffer (10 mM Tris-HCl, pH 8, 10% glycerol, 0.15 M NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM EGTA, 0.1% Nonidet P-40, 1 mM PMSF, 10 µl/ml Sigma protease inhibitor mix, and 0.5 mM DTT). The eluate was then trichloroacetic acid (TCA) precipitated, and the pellet was washed twice with ice cold acetone.

IgG beads Pulldown

Extracts prepared from TAP tagged and untagged control strains were prepared in extraction buffer (Eberharter et al., 1998) supplemented with 2 mM $CaCl_2$, 1 mM Mg acetate, and 1 mM imidazole and mixed with IgG sepharose. The resin was washed 3

times in PD buffer (50 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM PMSF and 0.5 mM DTT) supplemented with 2 mM $CaCl_2$, 1 mM Mg acetate, and 1 mM imidazole. The resin was then suspended in SDS-sample buffer, run on an SDS-gel and analyzed by western blot with antibody directed against calmodulin and Ty1 CA. For *dbr1-H86A* IgG pulldown assay, the *dbr1-H86A* mutant allele fused to a TAP (Protein A-HA-6x His) tag was carried on low-copy (*CEN*) plasmids and expressed via the native *DBR1* promoter. The *dbr1-H86A*-TAP plasmid was introduced into a yeast strain in which the endogenous copy of *DBR1* had been deleted, so the mutant was the only source of Dbr1p. A *DBR1* deletion strain containing a *dbr1-H86A* without a TAP tag was used as negative control.

RLM-RACE

5' ends of RNAs were determined by RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) (Volloch and Rits, 1994; Maruyama and Sugano, 1994; Schaefer, 1995) using the GeneRacer system from Invitrogen and Firstchoice system from Ambion, and synthesized RNA oligonucleotide from IDT company. Briefly, RNA samples (1 μg) were treated with calf intestine phosphatase (CIP) (60 min at 50^{o}C) followed by phenol/chloroform extraction and ethanol precipitation. RNA samples were then treated with tobacco acid pyrophosphatase (TAP) (60 min at 37^{o}C) followed by phenol/chloroform extraction and ethanol precipitation. An RNA oligonucleotide was then ligated to the 5' ends of monophosphorylated RNAs using T4 RNA ligase (37^{o}C for 60 min) followed by phenol/chloroform extraction and ethanol precipitation. Ligated RNA samples were reverse transcribed for 60 min at 50^{o}C using AMV reverse

transcriptase and either a gene-specific primer or random primers. The cDNAs produced by reverse transcription were amplified by PCR using *Taq* DNA polymerase plus a gene-specific primer and the RNA-oligonucleotide-specific primer. PCR products were subjected to agarose gel electrophoresis and the separated products were visualized in long-wave-length UV light. PCR products were excised from the gel, purified, and cloned into the pCR4-TOPO vector using the topoisomerase cloning method. Clones were sequenced using either T3 or T7 primers, which have binding sites flanking the cloning site of pCR4-TOPO. RLM-RACE conditions varied for the specific targets.

Table 3. Oligonucleotides

primer	sequence	position
146	cactctcccataacctccta	*ACT1* intron nt 100-119
148	ccaagacactggcctgaaac	Ty1 nt 2469-2488
149	gatccgttagacgtttcagc	Ty1 nt 384-403
152	gaggagaacttctagtatat	Ty1 nt 241-994
198	catttatgaacgcttactcagg	*DBR1* nt 269-247
215	ctcaaaccaagaagaaaaagaa	*ACT1* nt -128 – -107
216	tgataccttggtgtcttggtct	*ACT1* nt 130-109
224	atagatgggaagacagcacga	*ACT1* nt 409-389
363	gcaagcgctagaacatacttag	*ACT1* intron nt 18-1, 265-262
401	agtcaatagccataccaccaaa	*DBR1* nt 152-173
417	ttgcgaattgctgtacaagg	*DBR1* nt 10-29
418	caagtcatgaatttagagataaatgc	*DBR1* nt 1217-1192
429	tttgcatgtcaggttcatgg	*DBR1* nt 693-712
430	aacccggggatccgtcgacc	TAP tag reverse primer
467	aattgggtgaatgttgagataa	Ty1 nt 330-309
472	gactacaaggacgacgatgacaaggaatcccaacaattatctcaa	Flag sequ plus Ty1 nt 298-319
473	cttgtcatcgtcgtccttgtagtccattgttgataaaggctataa	Flag sequ plus Ty1 nt 297-276
476	gatgatggatactgcggaacctg	Ty1 nt 700-678
496	ggcacgacaggtttcccgac	pRS316 nt 2572-2592
517	cattcttctgttttggaagctg	Ty1 nt 446-424
534	ctgcagcgaggagccgtaat	kanB oligo
680	aggaaaaagaaaaagtctcacccta	nt -322- -297 upstream of *KHD1* ORF
681	gataatcgatgagatatgggaattg	*KHD1* nt 516-491
682	tgaattgaaatgtgaaccaactcaa	nt -355- -330 upstream of *PUF6* ORF

683	ttttggctaatttcttttctgaatg	*PUF6* nt 79-54
689	tctaagagaccggagcgccc	*ASH1* nt 456-476
690	cggggaatggaacttggacgacc	*ASH1* nt 762-739
694	tcccaagatcgaaaatttactgaat	nt -30 - -6 upstream of *ACT1* ORF
695	tttacacataccagaaccgttatcaat	*ACT1* nt 54-28
13A	gcagtgcatgatgacccaaa	Ty1 nt 554-573
13B	gtgtccgtaaaatgaccaacca	Ty1 nt 611-590

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