DOI: 10.1534/genetics.104.028126

Host Factors That Affect Ty3 Retrotransposition in *Saccharomyces cerevisiae*

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Manuscript received March 3, 2004
Accepted for publication June 18, 2004

ABSTRACT

The retrovirus-like element Ty3 of *Saccharomyces cerevisiae* integrates at the transcription initiation region of RNA polymerase III. To identify host genes that affect transposition, a collection of insertion mutants was screened using a genetic assay in which insertion of Ty3 activates expression of a tRNA suppressor. Fifty-three loci were identified in this screen. Corresponding knockout mutants were tested for the ability to mobilize a galactose-inducible Ty3, marked with the *HIS3* gene. Of 42 mutants tested, 22 had phenotypes similar to those displayed in the original assay. The proteins encoded by the defective genes are involved in chromatin dynamics, transcription, RNA processing, protein modification, cell cycle regulation, nuclear import, and unknown functions. These mutants were induced for Ty3 expression and assayed for Gag3p protein, integrase, cDNA, and Ty3 integration upstream of chromosomal $tDNA^{Val(AAC)}$ genes. Most mutants displayed differences from the wild type in one or more intermediates, although these were typically not as severe as the genetic defect. Because a relatively large number of genes affecting retrotransposition can be identified in yeast and because the majority of these genes have mammalian homologs, this approach provides an avenue for the identification of potential antiviral targets.

RETROVIRUSES and their endogenous counterparts, the LTR retrotransposons, have evolved complex relationships with their eukaryotic hosts. These retroelements rely on host cells for provision of the basic functions related to gene expression, including transcription, RNA processing, and translation (Coffin 1996; Boeke and Stoye 1997). In addition, in more specific steps of the retroid life cycle, retroviruses and retrotransposons are affected by host factors and conditions in the host cell. Life-cycle steps where host factors have been shown to play positive and negative roles include assembly and budding, reverse transcription, uncoating, nuclear entry, and integration (Goff 2001; Greene and Peterlin 2002; Pornillos *et al.* 2002; Dvorin and Malim 2003).

Assembly of retroviruses and LTR retrotransposons depends upon correct proportions of structural and catalytic proteins. Most retroviruses and retrotransposons use frameshifting as a mechanism to achieve proper stochiometry of structural and catalytic proteins. It requires not only programmed frameshifting sites in the RNA but also specific tRNA species in correct abundance (Farabaugh 1995). Despite the fact that assembly of retrovirus particles can occur *in vitro* (Scarlata and Carter 2003), chaperones are likely to play an important role in the assembly of at least some retroviruses (Zimmerman *et al.* 2002) and possibly of retrotransposons. Stability of Ty3 virus-like particles (VLPs) is sensitive to ubiquitination during the stress response (Menees and Sandmeyer 1994; Karst *et al.* 1999). Host proteins and RNAs have been shown to be packaged within retroviral particles (Ott 2002). Such proteins include cyclophilin A (Braaten *et al.* 1996), which functions by antagonizing host restriction factors (Best *et al.* 1997; Bieniasz 2003; Towers *et al.* 2003) and CEM15/APOBEC3G, which deaminates cytidine in nascent viral cDNA (Harris *et al.* 2003). Deletion of nucleotide excision repair genes elevates Ty1 cDNA, suggesting that these factors normally mediate its turnover (Lee *et al.* 1998; Garfinkel and Bailis 2002).

In the case of retrotransposons, assembly is followed by protein processing and reverse transcription. Reverse transcription of retrotransposons is sensitive to the cell cycle (Menees and Sandmeyer 1994) and divalent cation availability (Bolton *et al.* 2002). Large-scale screens by Scholes *et al.* (2001) and Griffith *et al.* (2003) have recently shown that large numbers of host genes can affect cDNA levels and transposition of the *Saccharomyces cerevisiae* element Ty1.

Retroviruses, which infect nondividing cells, must translocate the preintegration complexes through intact nuclei. This is also the case for retroelements in yeast, where the nuclear membrane does not break down during mitosis. In the case of HIV, for which nuclear import has been studied extensively, domains in MA, IN, and Vpr and the cDNA flap have been suggested to make contri-

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butions, and Vpr has been shown to interact directly with nucleoporins (Dvorin and Malim 2003). In the case of Ty1 (Moore *et al.* 1998) and Ty3 (Lin *et al.* 2001), nuclear translocation sequences have been described in the carboxyl-terminal domain of the respective IN species. In the case of Tf1, a *Schizosaccharomyces pombe* retroelement, a short domain in Gag (Dang and Levin 2000) and nucleoporin Nup124p (Balasundaram *et al.* 1999) are required for import.

It is likely that host proteins also influence intranuclear delivery of preintegration complexes to the chromosomes. Ty1 (Voytas and Boeke 2002) and Ty3 (Sandmeyer *et al.* 2001) integrate upstream of pol III-transcribed genes, and Ty5 (Voytas and Boeke 2002) integrates into heterochromatic DNA. In the case of Tf1, integration occurs preferentially into intergenic regions (Behrens *et al.* 2000; Singleton and Levin 2002). Chromatin factors have been identified genetically and shown to affect the frequency or distribution of Ty1 integration sites (Kang *et al.* 1992; Liebman and Newnam 1993; Qian *et al.* 1998). Although retroviruses do not display the same degree of integration preference as the yeast elements, several studies have now shown that there is preferential insertion of some retroviruses into transcribed regions (Schroder *et al.* 2002; Wu *et al.* 2003). In addition, several DNA-binding proteins enhance or direct *in vitro* integration reaction. These include Ini1 (Kalpana *et al.* 1994), HMG proteins (Farnet and Bushman 1997; Hindmarsh *et al.* 1999), BAF1 (Suzuki and Craigie 2002), and LEDGF (Maertens *et al.* 2003). Although strand transfer is mediated by IN, complete repair of the integration site is presumed to require the function of host repair proteins. DNA-PK and Ku proteins have been implicated in repair of the integration intermediates in animal cells and Ty elements in yeast (Daniel *et al.* 1999; Downs and Jackson 1999).

In this study, we sought to identify host factors in *S. cerevisiae* that affect retrotransposition of Ty3. A collection of 27,000 mini-Tn3 (mTn3) insertion mutants was screened for a change in frequency of Ty3 transposition into a divergent tRNA gene target, and 63 mutants of interest were identified. The insertion sites included 10 genes that were recovered twice, leaving a total of 53 loci. Six of the disruptions were within the coding regions of essential genes, producing viable mutants with truncation alleles. Approximately one-third of the disruptions were located in ORFs of unknown function. The genes identified in our study include cell cycle regulators, transcription and chromatin factors, protein kinases, nuclear pore proteins, RNA-binding proteins, and factors involved in stability and degradation of proteins. Biochemical and molecular analyses of the Ty3 intermediates in these mutant strains showed that many of them display moderate biochemical differences from wild-type cells in Ty3 intermediates. These included differences in precursor polyprotein processing, accumulation of reverse transcripts, and integration into chromosomal DNA.

MATERIALS AND METHODS

Yeast strains and culture methods: Media and standard techniques for *S. cerevisiae* were as previously described (Sherman *et al.* 1986). The haploid yeast strain YPH500 (*MAT*α *ade2-101*$_{oc}$, *lys2-801*$_{am}$, *his3-Δ200*, *trp1-Δ63*, *leu2-Δ1*, *ura3-Δ52*) was used to generate mutants for a pilot screen of 3000 transformants. Because the mTn3 was marked with the *LEU2* gene, mutagenized YPH500 cells are Leu$^+$. A *LEU2* derivative of YPH500, yMA1235 (Aye and Sandmeyer 2003), was constructed to serve as the control strain for transposition in the pilot screen. The haploid yeast strain yMA1322 (*MAT*α *ade2*$_{oc}$, *lys2*$_{oc}$, *his3*, *trp1*, *leu2*, *ura3*) was derived from YPH500 (Aye *et al.* 2001). yMA1322, which contains ochre alleles of both *ADE2* and *LYS2*, gives reduced background in the genetic assay for transposition and was used as the parental strain for subsequent shuttle mutagenesis and for large-scale mutant screening. The *LEU2* version of this strain, yMA1342, was generated by transformation with a DNA fragment containing the wild-type *LEU2* gene and was used as the wild-type control for transposition.

Plasmid constructions: pNB2142, a high-copy, *URA3*-marked plasmid containing a galactose-inducible, *HIS3*-marked Ty3 element (Ty3-*HIS3*), was derived from pGAL-Ty3 m*his3*AI (Sadeghi *et al.* 2001). The latter plasmid was used to induce Ty3 transposition. In the course of the transposition Ty3 RNA was produced from which the synthetic intron in the HIS3AI was spliced. This was reverse transcribed and the cDNA product recombined with the starting plasmid in a gene conversion resulting in pNB2142. Plasmid pNB2143 was generated by replacing the *Kpn*I fragment from the pNB2142 with the corresponding *Kpn*I fragment from the pGAL-Ty3 m*his3*AI IN mutant (Sadeghi *et al.* 2001), containing Ty3 IN with mutations in catalytic residues.

Yeast mutagenesis: Disruption mutagenesis of yeast to obtain candidate transposition mutants was performed as previously described (Burns *et al.* 1994). Briefly, DNA was prepared from 14 library pools separately mutagenized with mTn3 (kindly provided by M. Snyder, Yale University). Library DNA was isolated from each pool, cleaved with *Not*I, and transformed using the lithium acetate procedure (Ito *et al.* 1983) into either the YPH500 or the yMA1322 strain.

Genetic assays for Ty3 retrotransposition (target plasmid): A genetic assay for Ty3 position-specific transposition (Aye *et al.* 2001) was used to screen for Ty3 transposition mutants. The parental strains for the pilot (YPH500), the large-scale screen (yMA1322), and the wild-type control strains were transformed with pTM45, a low-copy plasmid marked with *TRP1* and carrying a galactose-inducible Ty3 element (Menees and Sandmeyer 1994), and pPK689, a low-copy plasmid carrying a divergent tRNA gene target for Ty3 insertion (Kinsey and Sandmeyer 1995). Integration of Ty3 upstream of the *sup2b*$_o$ (tyrosine-inserting, nonsense suppressor tRNA) in this plasmid results in activation of its expression. Cells in which transposition has occurred can be selected on medium requiring nonsense suppressors for growth.

Individual colonies representing Leu$^+$ mutant transformants and wild-type strains, yMA1235 or yMA1342 (carrying pTM45 and pPK689), were patched onto synthetic medium containing glucose as the carbon source, SC-His-Trp-Leu. After incubation for 24 hr at 30°, each plate was replicated to a medium with galactose, SC(gal)-His-Trp-Leu, for induction of Ty3 expression. After 48 hr of growth at 30° on this medium, cells were replica plated to minimal medium with glucose, supplemented with uracil and lysine or with uracil alone, for

YPH500 (*ade2o*) and yMA1322 (*ade2o, lys2o*) transformants, respectively, for detection of retrotransposition events. Replicated cultures were incubated at 30° for 5 days, and the number of papillae within each patch was compared. Negative control cultures grown on SC(glu)-His-Trp-Leu medium and lacking Ty3 expression were replicated to selective medium after 1 day at 30°. Growth on the selective medium occurred when expression of the $SUP2b_o$ tRNA gene on the target plasmid was activated by insertion of Ty3. The number of papillae per patch reflects the frequency of individual transposition events in that patch.

A more quantitative assay was performed for strains of particular interest (Aye *et al.* 2001). Yeast cells carrying pTM45 and pPK689 plasmids were grown in SC(raffinose). Log-phase cultures (A_{600} ~0.4) were adjusted for the cell density, and ~10^6 cells were plated in triplicate to SC(glu) and SC(gal). After approximately the same number of cell divisions (29 hr growth on glucose and 48 hr on galactose medium), each plate was replicated to the selective medium described above. Following 5 days of incubation at 30°, the number of colonies on these plates was determined, and the average taken from three plates. The transposition frequency was calculated as the number of cells grown on the selective medium divided by the total number of cells plated, which was determined from the YPD plates.

Genetic assay for Ty3 retrotransposition (*HIS3*-marked Ty3 assay): BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and its derivatives were transformed with pNB2142, a high-copy, *URA3*-marked plasmid containing Ty3-*HIS3*. At least three transformants for each strain were patched to SC-Ura plates, and incubated at 30° for 2 days. Confluent patches were replicated to SC(gal)-ura plates and incubated at room temperature for 3 days to induce Ty3 expression. Cells expressing Ty3-*HIS3* were scraped off the plates and resuspended in 1 ml of sterile water, and density was adjusted to A_{600} ~1. The suspension was serially diluted and plated to SC-Ura for the number of Ura^+ cells and to YPD plates to allow for the loss of plasmid. After 1 day at 30°, YPD plates were replicated to SC-His plates containing 5-FOA to select for cells with genomic integration events. As a negative control, transformants with pNB2143, carrying the catalytic site mutant of Ty3 IN, were assayed. The transposition frequency was calculated as the number of 5-FOA^r His^+ cells divided by the number of Ura^+ cells plated and expressed as the percentage of wild-type transposition.

Identification of genomic loci disrupted by mTn3: Chromosomal loci disrupted in the mutant strains were identified by one of two methods. In the pilot screen, each mutant of interest was transformed with *Pvu*I-linearized, YIp5 plasmid to integrate an *Escherichia coli* origin of replication into the mTn3 construct (Aye and Sandmeyer 2003). Genomic DNA prepared from each Ura^+ Leu^+ transformant was cleaved with *Nsi*I or *Bgl*II and treated with T4 DNA ligase to circularize DNA fragments. The ligation mixture was transformed into the *E. coli* strain HB101, and transformants with *LEU2*-marked plasmids were selected. (HB101 is a *leuB* mutant that could be complemented by the yeast *LEU2* gene for leucine prototrophy.) Plasmids with the characteristic restriction pattern of the mTn3 construct were sequenced using M13 (−40) primer (New England Biolabs, Beverly, MA), which anneals to the proximal *lacZ* sequence.

Loci disrupted by mTn3 insertions in the large-scale screen were amplified by vectorette PCR and identified by sequence analysis (Aye and Sandmeyer 2003). Briefly, total yeast DNA prepared from the mutant strain was digested with *Rsa*I or *Dra*I and ligated with preannealed anchor bubble primers (C. Friddle, Stanford University, personal communication). One-tenth of the ligation reaction was used as the template DNA for amplification of the mTn3-genomic DNA junction. PCR products were separated on an agarose gel, and each product was excised. DNA was extracted and sequenced. Each sequence generated was compared to complete *S. cerevisiae* genomic DNA using a BLASTn search of the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces) to identify the position of the insertion.

Reconstruction of deletion and truncation mutants: To confirm that the characterized disruption was linked to the observed change in the Ty3 transposition phenotype and to test the phenotype using other measures of transposition, each mutant was reconstructed in one or two additional strain backgrounds. Deletion of specific genes in strain yTM443 (*MAT***a** *ura3-52 trp1-H3 his3Δ200 ade2-101 lys2-1 leu1-12 can1-100 bar1::hisG* Ty3 null), TMY43 (*MAT***a** *ura3-52 trp1-H3 his3Δ200 ade2-101 lys2-1 leu1-12 can1-100 bar1::hisG spt3-Δ202* Ty3 null), or BY4741 was performed as described (Wach *et al.* 1994). Pairs of oligonucleotides, which contained 50 nucleotides of homology to the target gene at the 5′ end and 20 nucleotides of complementarity to the KanMX4 cassette (Wach *et al.* 1994) at the 3′ end, were designed. (The sequences of these oligonucleotides are available upon request.) These oligonucleotides were used to prime PCR synthesis of DNA fragments, which were transformed into yeast. In the case of nonessential genes, the oligonucleotide primers were designed to delete the entire ORF. For essential genes, the oligonucleotide primers were designed to truncate the ORF downstream of the mTn3 insertion site. Transformants were selected on YPD medium containing 200 μg/ml geneticin (G418). Disruptions were screened for single insertions of the KanMX4 cassette into a restriction fragment of the predicted size. At least three transformants were tested for transposition.

To test the transposition phenotypes in complete knockout mutants, the collection of deletion strains in the haploid BY4741 background was purchased (Research Genetics, Huntsville, AL). The effect of mutations in nonessential genes was tested directly using knockout mutants from the collection. Genes that were not represented in the collection (*nup157*, *nup159*, and *kic1*) were disrupted in BY4741 using oligonucleotide primer pairs as described above.

Immunoblot analysis: BY4741 cells containing pTM45 or pDLC201 (pEGTy3-1) were grown under noninducing [SC(raf)] or inducing [SC(gal)] conditions for 6 hr. Whole-cell extracts (WCEs) were prepared from 10-ml cultures by vortexing with glass beads in the presence of denaturing buffer [9 M urea, 5 mM EDTA as previously described (Continental Labs, Carlsbad, CA]. WCEs or concentrated VLP proteins were fractionated by electrophoresis in a SDS-10% polyacryamide gel. Proteins were transferred to Immobilon-P (Millipore, Bedford, MA) and were incubated with rabbit polyclonal anti-Ty3 CA or anti-Ty3 IN antibodies diluted 1:20,000 and 1:2000, respectively (Menees and Sandmeyer 1994). Rabbit IgG was visualized using HRP-conjugated secondary antibody and ECL + Plus (Amersham, Buckinghamshire, UK) according to manufacturer's instructions.

Southern blot analysis: BY4741 cells were grown as described above for immunoblot analysis. Cells were suspended in extraction buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 2% Triton X-100, 1% SDS] and vortexed vigorously with glass beads in the presence of phenol:chloroform. Nucleic acid was precipitated from the aqueous phase with ethanol, redissolved, and digested with RNase (Hoffman and Winston 1987). RNA-free DNA (0.5 μg) was digested with *Eco*RI, fractionated by electrophoresis in a 0.8% agarose gel, transferred to Duralon UV membrane (Stratagene, La Jolla, CA), and immobilized by UV crosslinking in Stratalinker 1800 (Stratagene). Hybridization was performed with a ^{32}P-labeled, internal *Bgl*II fragment of Ty3, which hybridizes with the full-length 5.4-kbp Ty3 cDNA, as well as Ty3 donor plasmid and chromo-

somal Ty3 elements. The filters were washed and exposed to a phosphorimager screen, and hybridization signals were quantitated using Quantity One software (Bio-Rad, Richmond, CA). To test for proper gene disruptions, Southern analysis was performed using a probe against the *kan*^{R} gene.

PCR assay for Ty3 transposition *in vivo*: For detection of Ty3 integration upstream of 14 genomic valine tRNA (AAC) [tDNA$^{Val(AAC)}$] genes, 25 ng of BY4741 DNA produced from induced cells as described above was used as the template for PCR with primers 278 (Ty3) and 676 [for 14 tDNA$^{Val(AAC)}$]. The PCR conditions and primers were essentially the same as previously described (Aye and Sandmeyer 2003), except that the number of PCR cycles was reduced from 40 to 32.

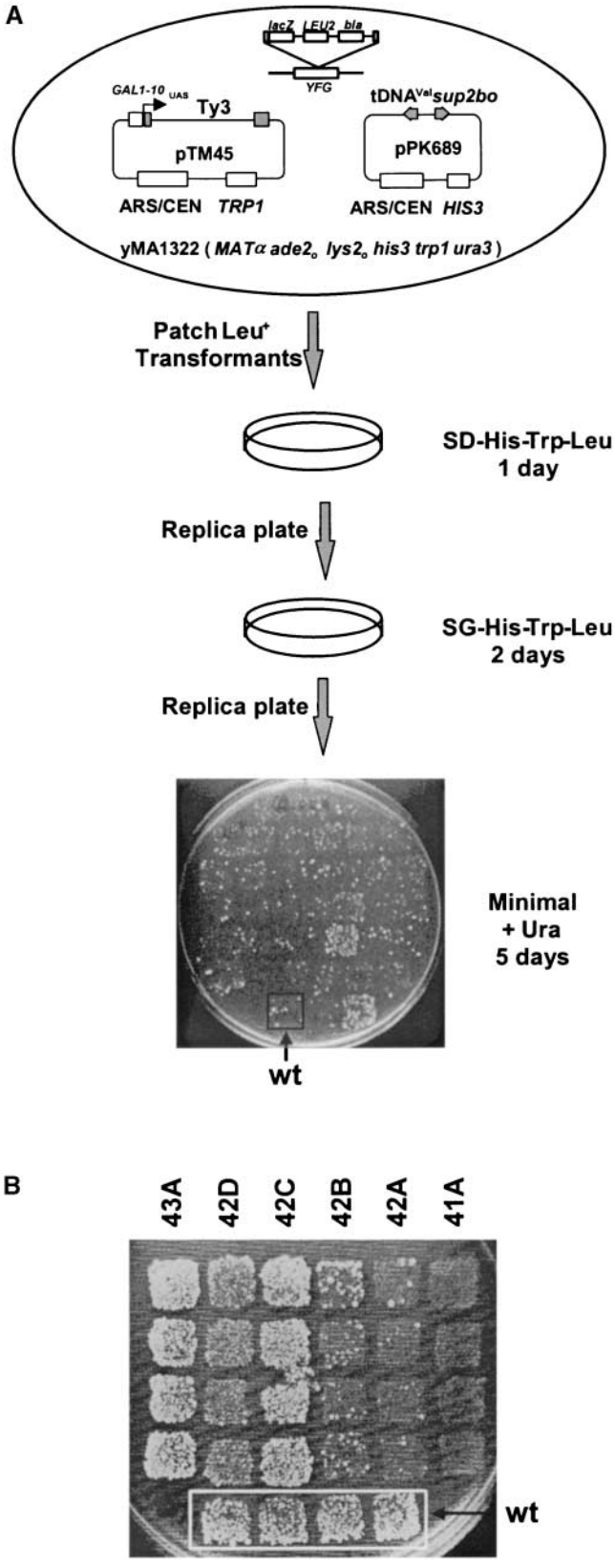

Figure 1.—(A) Outline of the genetic assay for Ty3 retrotransposition screen. Leu^{+} transformants resulting from insertion mutagenesis were patched onto SD-His-Trp-Leu media and replica plated onto galactose-containing medium to induce expression of Ty3. These cultures were replicated onto appropriate selective medium (lacking adenine and/or lysine) to select for colonies in which suppressor activation by Ty3 insertion into the target plasmid allowed growth. (B) Patch assay of mutants isolated from the primary screen. Mutants were retested in replica patches (columns), and the wild type control is shown in the bottom row.

RESULTS

To identify a spectrum of host factors influencing Ty3 retrotransposition, a wild-type yeast strain was mutagenized by integrative transformation with a library of yeast DNA fragments disrupted by mTn3 insertion, and yeast mutants were screened for Ty3 transposition phenotype. The transposition assay used for screening mutants measured Ty3 transposition into the transcription initiation site of plasmid-borne, divergent tRNA genes and so identified mutants with changes in Ty3 replication or properties of integration specificity.

Identification of mutants defective in transposition: The screen for Ty3 transposition mutants was performed on a relatively small scale and then on a large scale using a modified version of the original screening method. The parental YPH500 strain was transformed with a low-copy plasmid, pTM45, containing a galactose-inducible Ty3 element. and a low-copy, divergent tRNA gene target plasmid, pPK689 (Kinsey and Sandmeyer 1995). In the pilot screen, YPH500 carrying pTM45 and pPK689 was subjected to shuttle mutagenesis (Burns *et al.* 1994) by integrative transformation with a library of yeast DNA disrupted by random insertions of mTn3, containing *lacZ*, β-lactamase, and yeast *LEU2* genes (Seifert *et al.* 1986).

To screen for mutants with Ty3 phenotypes, transformants were first grown on SC-His-Trp-Leu medium and replicated to SC (gal)-His-Trp-Leu medium to induce Ty3 expression. Cells expressing Ty3 were then replicated to minimal medium supplemented with appropriate amino acids to select for cells, in which transposition into the target plasmid had occurred (Figure 1A). The *HIS3*-marked Ty3 target plasmid contains two divergent tRNA genes, a tDNA$^{Val(AAC)}$ and a suppressor tDNA^{Tyr}, *sup2bo*, separated by a 19-bp synthetic sequence. Expression of these tRNA genes is almost undetectable due to the physical constraint created by overlapping promoter regions and unfavorable nucleotide sequence

upstream of the gene for the suppressor. Ty3 integration into the divergent promoter region alleviates interference between the two tRNA genes and changes the sequence composition upstream of the suppressor, thereby activating its expression (Kinsey and Sandmeyer 1995). Cells in which Ty3 transposition has occurred are identified by their ability to suppress one or two ochre nonsense alleles in the strains used for the screen.

In the initial screen, ~3000 Leu$^+$ transformants were tested for transposition using a semiquantitative patch assay. Four mutants were identified: one isolate had a hypotransposition phenotype and the remaining isolates had hypertransposition phenotypes (Figure 1 and data not shown). Southern blot analysis using a *lacZ* probe for sequences within the mTn3 insertion showed that each contained a single integrated mTn3 (data not shown). DNA from these four strains was extracted and the sequence flanking the mTn3 insertion was determined as described in materials and methods. The resulting sequences were searched in the *Saccharomyces* Genome Database to identify genomic insertion sites. The hypotransposition mutant was disrupted in *LHP1* (Aye and Sandmeyer 2003), and the three hypertransposition mutants were disrupted in *NUP159*, *SKI2*, and *YHR209w*. The *lhp1* mutant had an ~8-fold lower frequency of transposition than the wild type. The frequency in hypertransposition mutants ranged from 5- to 14-fold of wild type. These genes and the mTn3 insertion site are listed in Table 1.

For the large-scale screen, the starting strain was modified by the addition of a second nonsense allele, *lys2o*, to reduce background growth on the selective medium. The modified strain was designated yMA1322. A Leu$^+$ version of that strain, yMA1342, was constructed and used as the control comparison for cells carrying mTn3 insertions containing *LEU2*. YMA1322 was transformed with mTn3-mutagenized DNA and 24,000 Leu$^+$ transformants were generated. These mutants were tested for transposition using the same patch assay as that described above except that the final selection for cells, in which Ty3 had undergone transposition, was on medium lacking both adenine and lysine.

Screening of 24,000 Leu$^+$ transformants yielded 780 mutants that showed non-wild-type numbers of papillations in the patch assay (Figure 1A and data not shown). Retesting of these mutants showed a consistent phenotype for 280 mutants (Figure 1B). Transformants that grew poorly on the dropout medium with glucose or galactose were not further analyzed. The 280 isolates would have also included mutants with defects in metabolism required for growth on the selective medium. To eliminate these, each mutant was transformed with pLY1850, the pPK689 target plasmid with an activating Ty3 insertion between the tRNA genes (L. Yieh, unpublished results). Of the 280 mutants, 63 were able to grow on the selective medium when transformed with the pLY1850 plasmid (data not shown). Mutant strains that failed to exhibit suppression were considered to be potentially defective in cellular processes unrelated to transposition and were not analyzed further (Figure 2).

Sequences flanking the mTn3 insertions in the 63 mutants from the large-scale screen were determined by vectorette PCR as described in materials and methods. ORFs and inter-ORF regions disrupted by mTn3 insertions are listed in Table 1. Although most loci identified were recovered only once, two or more independent insertions were identified at 10 loci (Table 1). Including the initial screen, 53 loci were identified as the sites of mTn3 insertions in strains with altered transposition. Among these, six were essential genes with the mTn3 insertion in the downstream end of the coding region (*CDC39*, *CYR1*, *DML1*, *NUP159*, *RGR1*, *TFC1*), resulting in truncation alleles. Although most of the 63 insertions occurred within coding regions, 6 were within 180 bp upstream of the 5′ ends of coding regions, either in the promoter or in the 5′ untranslated region. Two insertions each were located in the rDNA locus, in regions lacking annotated ORFs and in regions of overlapping ORFs. Of the 53 genes identified in the two screens, only *nup159*, *rdn37* (rDNA), *ski2*, and *yhr209w* mutants showed elevated transposition frequencies; the remaining mutants exhibited decreased transposition frequency (data not shown). Mutants with insertions in rDNA and in intergenic regions were not analyzed further, resulting in a collection of 50 genes associated with the Ty3 phenotype.

Two of the mutants with hypotransposition phenotypes, *tfc1* (Aye *et al.* 2001) and *lhp1* (Aye and Sandmeyer 2003), were studied in detail and have been reported previously. In each case, transformation of the mutant strain with a plasmid containing a copy of the corresponding wild-type gene rescued the Ty3 transposition phenotype. In the case of the *tfc1* mutant, orientation of Ty3 integration into the divergent target plasmid was anomalous, causing the observed decrease in Ty3 transposition-dependent papillations. In the *lhp1* mutant, Ty3 cDNA accumulation was reduced. These two mutants are included for completeness, but are not discussed further. Quantitative suppressor transposition assays were performed for some, but not all, of the mutants. These suppressor assays showed that phenotypes ranging from 3- to 40-fold decreases were detected. Mutants with increased transposition phenotypes ranged from 2- to 14-fold of the wild-type frequency (data not shown).

The next step in characterization of these mutants was determination of the null phenotype in an independently derived set of mutants. The yeast knockout collection in strain BY4741 was used for this purpose (Research Genetics, now available from Invitrogen, San Diego). Because characterization of the *tfc1* mutant suggested that some effects might be specific for the synthetic target plasmid used in the first screen, a genetic assay, which uses a *HIS3*-marked Ty3 element (Ty3-*HIS3*) to detect genomic transposition events, was used to determine the frequency of Ty3 mobilization in the null mutants (Figure 3; Table 1). For mutants with mTn3

TABLE 1

Mutants isolated from the genetic screen

Gene/ORF	ORF	Cellular function/similarity[a]	mTn3 insertion[b]
	A. Mutated genes that produced similar phenotypes in suppressor and Ty3-H assays		
AMS1	YGL156w	Vacuolar α-mannosidase	(−46)
BRO1[c]	YPL084w	Vacuolar protein sorting, ubiquitin-dependent protein degradation	456/844
CKA1	YIL035c	Catalytic (α) subunit of CKII, required for efficient pol III transcription	276/372
ECM17/MET5	YJR137c	NADPH-dependent, sulfite reductase (putative)	156/1442
FUN30	YAL019w	Homolog of Snf2, overexpression affects chromosome stability	190/1131
FYV10	YIL097w	Protein of unknown function	368/516
GTR1	YML121w	Small GTPase, negative regulator for Ran/TC4 GTPase cycle	27/310
KSP1	YHR082c	Serine/threonine kinase similar to casein kinase II	(−127)
NCL1[c]	YBL024w	tRNA:m5C-methyltransferase	602/684
NUP157[c]	YER105c	Nonessential nuclear pore protein, affects both nuclear import and export	85/1391
NUP159[d,e]	YIL115c	Essential nuclear pore protein (cytoplasmic face)	1432/1460
PPA1	YHR026w	Proteolipid, vacuolar ATPase	134/213
RIM13[c]	YMR154c	Cysteine protease, involves in proteolytic processing of Rim1p	444/727
SHM1 and *YBR262c*	YBR263w and 262c	*SHM1* encodes mitochondrial serine hydroxymethyltransferase	(−45), 15/106
SIF2	YBR103w	Interacts with and targets Sir4p from telemeres to other sites, Set3 complex	3/535
SIN4[c]	YNL236w	Pol II mediator complex	826/974
STE7	YDL159w	Kinase involved in pheromone-induced signal transduction pathway	(−95)
SWD1	YAR003w	Set1 complex	421/426
VHS3 and *YOR055w*	YOR054c and 055w	Protein of unknown function	116/674, 10/144
YBR284w	YBR284w	Similarity to AMP deaminase	13/797
YBR293w	YBR293w	Similarity to multi-drug resistant protein	(−84)
YPL150w	YPL150w	Serine/threonine protein kinase of unknown function	683/901
	B. Mutated genes not tested for effects on Ty3-H mobilization		
CDC39/NOT1[e]	YCR093w	mRNA degradation: negatively regulates basal transcription	1272/2108
CYR1[e]	YJL005w	cAMP synthetase: required for START	215/2026
DML1[e]	YMR211w	Weak similarity to β-tubulins	160/475
LHP1[d]	YDL051w	RNA chaperone for pol III transcripts	149/275
NUP116	YMR047c	Nuclear pore protein: affects both nuclear import and export	52/1113
RDN37/TAR1[c]	NA	rDNA or *TAR1* gene	
REG1	YDR028c	Protein phosphatase type 1 activity: involved in transcription regulation	70/1014
RGR1[e]	YLR071c	Pol II mediator complex	810/1082
SSY1[c]	YDR160w	Regulation of amino acid metabolism	66/852
TFC1[e]	YBR123c	τ95-subunit of TFIIIC: binds to A box and interacts with τ55-subunit	526/649
Intergenic	YLR126c and 125w	NA	NA
Intergenic	YHR210c and 211w	NA	NA
YEL077c	YEL077c	Protein of unknown function	693/1277
	C. Mutated genes that did not produce consistent phenotypes in suppressor and Ty3-H assays		
CDH1	YGL003c	*CDC20* homolog; ubiquitin-dependent protein degradation	536/566
DAP1	YPL170w	Protein of unknown function	31/152
ECM30	YLR436c	Extracellular mutant, hypersensitivity to calcofluor white	423/1274
FRE3	YOR381w	Ferric reductase	(−179)
HSL1	YKL101w	G_2/M transition of mitotic cell cycle, negative regulator of Swe1 kinase	218/518
LEO1	YOR123c	Transcription elongation factor (Cdc73/Paf1) complex	20/464
NRK1	YHR102w	N-rich protein kinase(S/T): interacts with Cdc31p	219/1080
PRR1	YKL116c	Protein (S/T) kinase involved in pheromone signaling	165/518

(*continued*)

TABLE 1

(Continued)

Gene/ORF	ORF	Cellular function/similarity[a]	mTn3 insertion[b]
SKI2[d]	YLR398c	RNA helicase, translational repression, suppressor of yeast killer dsRNA	12/1287
UBP13	YBL067c	Ubiquitin carboxyl-terminal hydrolase, similar to Ubp9p	12/688
VPS51/67[c]	YKR020w	Protein vacuolar targeting, GARP complex	35/164
YAL053w[c]	YAL053w	Protein of unknown function	56/783
YCR079w	YCR079w	Weak similarity to protein phosphatase 2C	20/442
YDR128w	YDR128w	Protein of unknown function	841/1148
YHR045w	YHR045w	Protein of unknown function	135/560
YHR209w[d]	YHR209w	Putative SAM-dependent methyl transferase	76/291
YOR291w[c]	YOR291w	Has cation translocating ATPase motif, 10-transmembrane span motifs	100/1472
YPL225w	YPL225w	Protein of unknown function	32/146

[a] From *Saccharomyces* Genome Database and Yeast Protein Database.
[b] Number of intact/total amino acids or, in parentheses, of nucleotides upstream of the coding region.
[c] Isolated more than once.
[d] Identified from the pilot screen.
[e] Essential gene.

insertions in regions of overlapping ORFs, deletions of each complete ORF were tested. *Nup159*, *nup157*, and *kic1* mutants were reconstructed by inserting a KanMX4 cassette at the position of the original insertion mutation. Other essential genes were not further examined in this study nor were three genes (*NUP116*, *YEL077c*, and *SSY1*) that were absent in the collection and were particularly difficult to reconstruct (Table 1B). In the genetic assay performed on the null and reconstructed mutants, mobilization of Ty3-*HIS3* was monitored for three independent transformants. Cells were induced for Ty3 expression by growth on galactose-containing medium, replica plated to YPD to allow loss of the donor plasmid, and finally replica plated to SD-His medium containing 5-FOA. The last step selected for cells, which had acquired a chromosomal Ty3-*HIS3* and lost the *URA3*-marked plasmid bearing Ty3-*HIS3*. The frequency of transposition was quantitated as described in the MATERIALS AND METHODS (Figure 4). Of the 42 mutants tested, 25 showed a difference in transposition frequency compared to the parental strain ($P \leq 0.05$). In 20 cases, the frequency was less than half that of the wild type (Table 1A). In 7 of these, the decrease was 10-fold or more. In the case of *nup159*, the frequency of Ty3 transposition was 4-fold higher than that in the wild type. This was consistent with the increased frequency observed in the original suppressor assay. In 18 cases, mutants that displayed a phenotype in the suppressor activation assay did not show a comparable phenotype in the Ty3-*HIS3* assay (Table 1C).

Analysis of Ty3 proteins in the mutant strains: Twenty-one mutant loci were selected for further study on the basis of the consistent transposition phenotype in both genetic tests and having a >40% decrease in transposition frequency in the Ty3-*HIS3* assay. In addition, *nup159*, which was consistently increased in transposition, was examined, for a total of 22 mutants (Table 2). The major Ty3 structural polyprotein is Gag3p. Assembly of Ty3 VLPs is followed by Gag3p processing and cDNA synthesis in wild-type cells. These processes were examined in the 22 mutants to better understand the basis of the mutant phenotype. Wild-type and mutant strains transformed with a galactose-inducible, high-copy Ty3 plasmid pDLC201 (pEGTy3-1) were grown in a galactose-containing liquid medium to induce Ty3 expression. Two independent transformants were examined for each mutant, except for *fun30*, for which the Ty3 plasmid was apparently lost from one transformant. Cells were induced for 6 hr and then extracted for protein and DNA as described in MATERIALS AND METHODS. A 6-hr induction was chosen because precursor and mature protein species are present in wild-type cells at this time. Ty3 cDNA levels increase from 3 to 6 hr (MENEES and SANDMEYER 1994; data not shown) and at 6 hr transposition can be measured using a PCR assay to physically correlate transposition and processing phenotypes. Therefore 6 hr was a time when it was possible to observe the effects of disruptions on the rate of Gag3p processing as well as on cDNA and transposition levels.

The amounts of Ty3 Gag3p, 31 kD and 26 kD (CA), for each mutant were determined by immunoblot analysis with anti-CA antibody (Figure 5). IN is produced from the Gag3-Pol3p polyprotein by the Ty3 protease. The levels of IN and some intermediate species were monitored for each mutant using anti-IN antibodies. In both cases, the amount of protein used was such that the Ty3 species would be measured in the linear range of exposure (data not shown). The majority of mutants

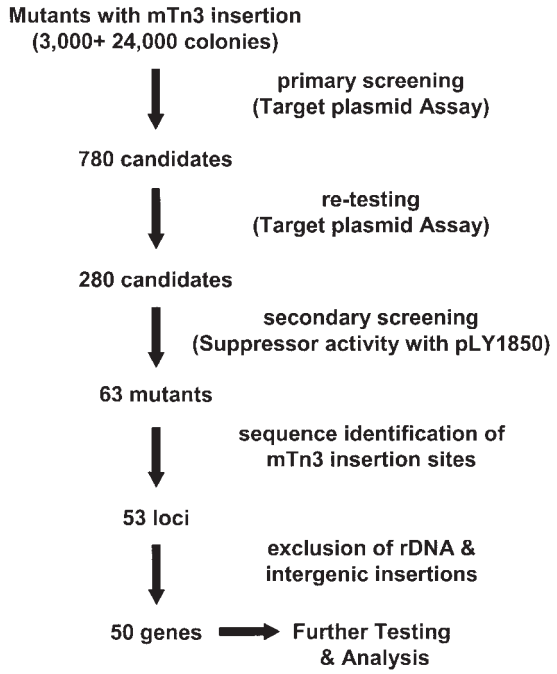

FIGURE 2.—Genetic screen outline. Identification of host factors for Ty3. The number of candidate genes at each stage in the screen for host factors is shown. For details, see RESULTS.

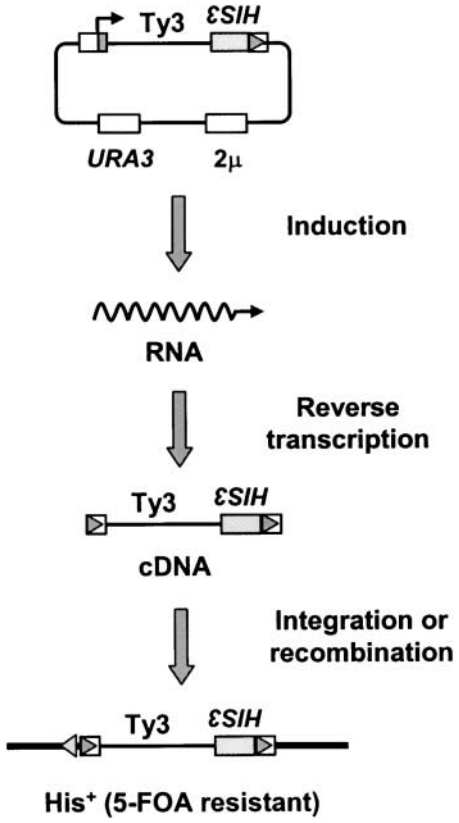

FIGURE 3.—Genetic assay using the *HIS3*-marked Ty3 element. Expression of the Ty3-*HIS3* RNA (wavy line with arrow) was induced by growth on galactose-containing medium. Cells in the assay were replicated as described in MATERIALS AND METHODS. Following reverse transcription, Ty3-*HIS3* cDNA is integrated or recombined into the genome upstream of a tDNA (shaded triangle), leading to histidine prototrophy in the absence of Ty3 donor plasmid. Note that the *HIS3* gene is antisense to Ty3.

expressed the Gag3p and Gag3-Pol3p precursor polyproteins and processed them to levels of mature protein comparable to levels in wild-type cells. Of the 22 mutants, 13 displayed some differences from wild type for Ty3 protein patterns (Table 2). Some variability was also observed in wild-type samples (Figure 5; data not shown). The *sin4* and *ppa1* mutants had reduced levels of Gag3p species, but only *ppa1* appeared to also have reduced amounts of IN. Mutants also had some qualitative differences in the relative representation of processed species. For example, *sin4*, *ste7*, *fyv10*, *shm1*, and *rim13* mutants had elevated levels of CA relative to Gag3p. This could reflect either changes in turnover with the processed form being more stable, particularly in the case of *sin4* where total Gag3p appears to be decreased, or actual changes in processing efficiency. The pattern of proteins reacting with the anti-IN antibody was more complex. As mentioned above, the amount of IN was reduced in the *ppa1* mutant. In addition to mature IN, three higher molecular weight species, which are assumed to be partially processed Gag3-Pol3p species, were observed in most samples (Figure 5). This pattern differed from wild type in the cases of the *fun30*, *sin4*, *nup157*, *nup159*, *ams1*, *bro1*, *ppa1*, *ybr262c*, and *ybr284w* mutants, although the significance of this variation was not known.

Analysis of Ty3 cDNA in mutant strains: Subsequent to maturation of particle proteins, Ty3 RNA is reverse transcribed to form cDNA (HANSEN *et al.* 1992). Aliquots of the same cultures of independent transformants induced for 6 hr and examined for Ty3 proteins were also examined for Ty3 cDNA. DNA was cleaved with *Eco*RI and visualized using a probe specific for the internal region of Ty3 (Figure 6A). In addition to Ty3 cDNA, this probe hybridizes to chromosomal Ty3 elements and the plasmid-borne Ty3. The hybridization signal for each sample was quantified using Quantity One software (Bio-Rad). Amounts of cDNA from the two different transformants were normalized to amounts of the Ty3 plasmid to allow comparison of the relative amounts of cDNA per Ty3 plasmid copy and were averaged (Figure 6B). In the wild-type strain, the ratio of plasmid to cDNA was ~5:1.

With the exception of *ncl1*, which did not maintain the plasmid (Figure 6A), mutants had detectable amounts of cDNA. However, many mutants had reduced amounts of the cDNA compared to wild-type cells (Figure 6B). Three mutants (*fun30*, *nup157*, and *ste7*) displayed less than half of the wild-type level of cDNA (Figure 6). These three mutants were among the 13 mutants with differences from wild-type cells in Ty3 protein pro-

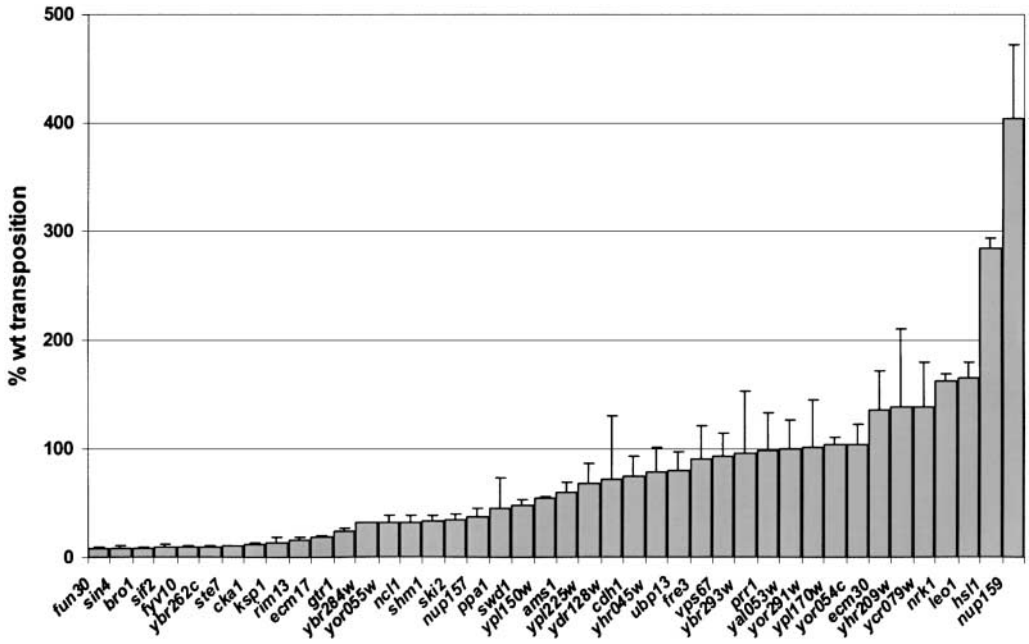

FIGURE 4.—Relative frequency of Ty3 transposition with the *HIS3*-marked Ty3 element. The frequency of Ty3-*HIS3* transposition in deletion and reconstructed mutants was determined as described in MATERIALS AND METHODS and expressed as the percentage of wild-type transposition (*y*-axis). The error bars represent standard deviation for each strain of three independent transformants.

cessing. *Ybr284w*, *rim13*, *ppa1*, and *bro1* had reductions in cDNA of 43–49%.

Transposition into chromosomal tDNA$^{Val(AAC)}$ genes: A PCR assay was used to monitor integration at 6 hr into a family of 14 chromosomal tRNA$^{Val(AAC)}$ genes to correlate the results of biochemical assays with transposition for the 22 mutant strains. The tDNA$^{Val(AAC)}$ transposition assay is similar to the Ty3 PCR assay previously described (AYE and SANDMEYER 2003; Figure 7A). In this case, amplification of a diagnostic fragment relies on primers that anneal to the Ty3 and to a conserved sequence in this family of tDNAs. The tDNA$^{Val(AAC)}$ family was chosen because it has multiple members, it is the same gene that provided the dominant target in the original genetic assay, and it has low background in the PCR assay in the absence of transposition. PCR products were fractionated by gel electrophoresis and stained with ethidium bromide. Different amounts of DNA samples from wild-type cells expressing Ty3 were used to determine the linear range of PCR products (Figure 7B, top). As a control, the *RAD52* locus was amplified using the same amount of genomic DNA. Since the *RAD52* locus is represented in every genome, the linear range was achieved with fewer PCR cycles (Figure 7B, bottom). Using the defined range, PCR was performed for the wild-type and mutant strains (Figure 7C; data not shown). Each gel was scanned, and PCR products were quantitated using Quantity One software. The ratio of the tDNA$^{Val(AAC)}$ PCR product to the *RAD52* locus for each mutant was compared to the ratio determined for wild-type cells. Of 22 mutants tested, 5 showed less than half of the wild-type ratio and 7 others showed smaller decreases. The one mutant (*nup159*) with increased transposition showed more than a twofold increase in frequency of integration. Seven mutants had frequencies close to or just exceeding the wild type. This could reflect different usage of tDNA families or 5S *vs.* tDNA among different mutants, or discrepancies caused by differences in the genetic and physical assays for transposition: for example, the length of induction. Tests of 3 other mutants were inconclusive: 1 was tested for only one transformant (*fun30*), *ecm17* showed discrepant ratios for the two transformants tested, and *ncl1* did not maintain the Ty3 plasmid. Each of the 3 mutants with >50% reduction in cDNA (*fun30*, *nup157*, and *ste7*) showed decreases in transposition into the chromosomal tDNA. Interestingly, 2 mutants (*fyv10* and *shm1*), which appeared to have relatively elevated levels of cDNA, were reduced for transposition into the chromosomal tDNA, consistent with the genetic phenotype.

DISCUSSION

Due to their limited coding capacity, retrotransposons and retroviruses rely heavily upon the host factors for most processes in their life cycle. Some of these factors were identified by biochemical approaches whereas others were implicated by genetic data. Genetic approaches in simple organisms such as yeast are a powerful tool for dissecting complex processes. By employing a large-scale genetic screen, we have identified a panel of yeast genes that affect the transposition of Ty3 into a target plasmid. Determination of the level of Ty3 intermediates in each mutant background indicated that some mutants were defective in protein expression, proteolytic processing, or in reverse transcription. In addition, a PCR assay for Ty3 integration revealed that some mu-

TABLE 2

Summary of Ty3 phenotypes for host mutants

Mutant	Group	Gene/ORF	Function	Transposition		Protein/ processing Ty3 poteins	cDNA[b] (%)	Integration PCR (%)
				Suppressor	Ty3-*HIS3*[a]			
1	Chromatin	*fun30*	Chromosome stability	D	8.2 ± 0.9	HMW	<50	<60
2		*sif2*	A subunit of SET3 complex	D	9.0 ± 2.9			
3		*sin4*	Pol II mediator complex	D	8.2 ± 2.8	Low Gag; CA > Gag; HMW		<50
4		*swd1*	A subunit of SET1 complex involved in histone methylation	D	47.4 ± 5.5			
5	Nuclear pore	*nup157*[c]	Nuclear pore complex protein	D	37.9 ± 7.0	HMW	<50	<50
6		*nup159*[c]	Nuclear pore complex protein	I	404.5 ± 67.6	HMW		>200
7	Kinase	*cka1*	A subunit of casein kinase II	D	11.4 ± 1.7			
8		*ksp1*	A nuclear protein kinase	D	13.9 ± 4.4			<50
9		*ste7*	MAPKK component of pheromone signal transduction pathway	D	10.3 ± 0.6	CA > Gag	<50	<50
10		*YPL150w*	Protein kinase of unknown function	D	54.8 ± 1.2			
11	Vacuolar	*ams1*	α-Mannosidase (vacuolar membrane)	D	60.0 ± 8.6	HMW		
12		*bro1*	Class E protein involved in vacuolar protein trafficking	D	8.5 ± 1.1	HMW	<60	
13		*ppa1*	H^+ ATPase involved in vacuolar acidification	D	44.8 ± 28.1	Low Gag; low IN, HMW	<60	
14	Miscellaneous	*ecm17*	Sulfite reductase (NADPH) involved in cell wall biogenesis	D	19.0 ± 1.2			
15		*fyv10*	Proteasome-dependent, glucose-induced catabolite degradation	D	9.2 ± 1.9	CA > Gag	>150	<61
16		*gtr1*	Small GTPase involved in phosphate transport	D	23.4 ± 2.7		>150	
17		*ncl1*[d]	tRNA (cytosine-5) methyl transferase	D	32.1 ± 6.9	Very low Gag	Not detected	<10
18		*rim13*	Cysteine-type endopeptidase	D	15.9 ± 2.5	CA > Gag	<60	
19		*shm1*	Glycine hydroxymethyl transferase	D	33.9 ± 5.1	CA > Gag	>140	<50
20	Unknown	*YBR262c*	Unknown function	D	9.6 ± 1.6	HMW		
21		*YBR284w*	Unknown function	D	31.5 ± 0.9	HMW	<60	
22		*YOR055w*	Unknown function	D	32.0 ± 7.1			

D and I, decreased and increased transposition relative to wild type. HMW, altered pattern of high-molecular-weight IN-related species.

[a] Percentage of wild-type transposition ± standard deviation.

[b] Percentage of wild type (shown if <61% or >140% of wild type)

[c] Mutation reconstructed in BY4741 background.

[d] Mutant with reduced level of Ty3 plasmid.

tants were defective in postreplication aspects of the Ty3 life cycle.

Genetic selection of transposition mutants: In the current study, a genetic screen of 27,000 insertion mutants was undertaken to identify genes that affect the frequency of Ty3 activation of a target suppressor tRNA gene. The sites of the gene disruptions in these mutants were determined by sequence analysis. Two of these

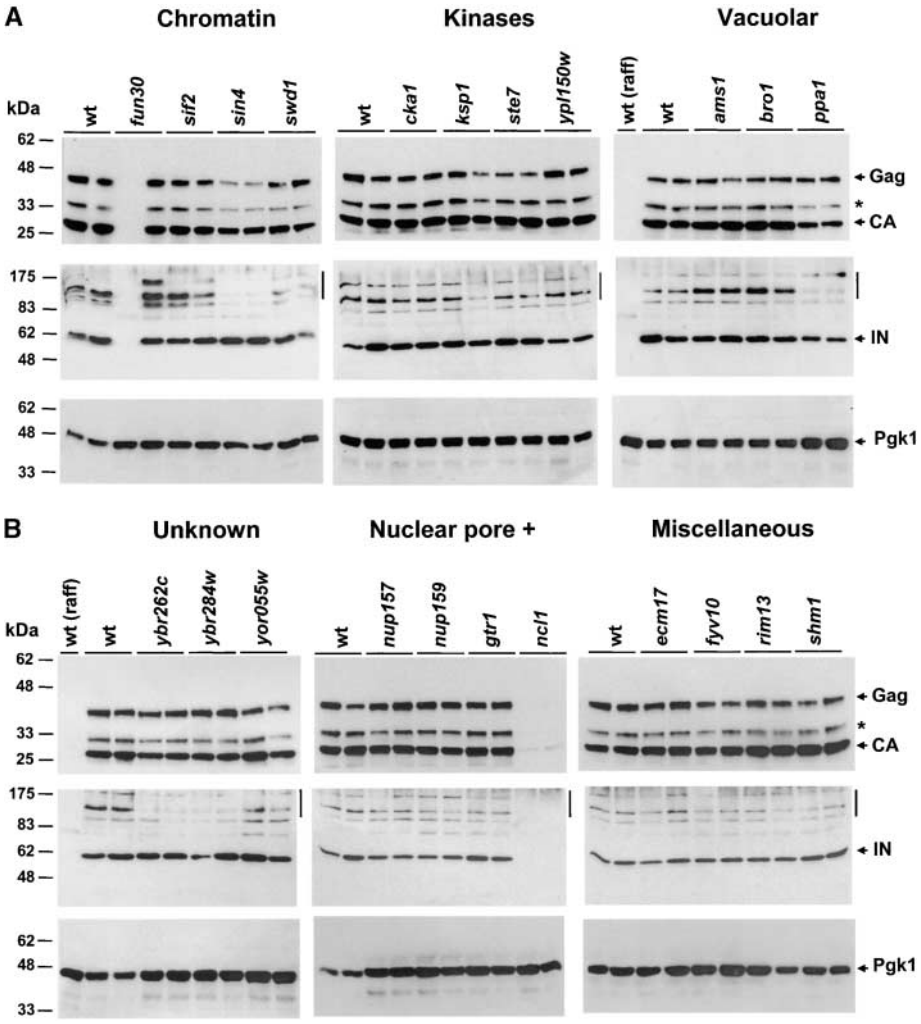

FIGURE 5.—Immunoblot analysis of Ty3 CA and IN proteins in BY4741 mutants. (A and B) Mutants that exhibit the Ty3 phenotype with two different genetic assays. Immunoblots with antibodies to Ty3 CA or IN proteins were performed on yeast extracts prepared from cultures induced for Ty3 expression. Two independent transformants were monitored for each mutant. Antibodies to CA recognize mature CA protein (26 kD) and precursor Gag3p (38 kD) as well as p31 (asterisk). Antibodies to IN recognize mature IN (61 kD) and three larger species (vertical line). These larger species have sizes consistent with Gag3-Pol3, Pol3, and RT-IN, but have not been definitely assigned. For gel-loading control, filters for IN immunoblots were reprobed with anti-Pgk1 polyclonal antibodies (Molecular Probes, Eugene, OR). A wild-type culture that was not induced for Ty3 expression [wt (raff)] was used as the negative control.

mutants, one containing an insertion in *TFC1* and one containing an insertion in *LHP1*, were examined in detail and have been reported previously (AYE *et al.* 2001; AYE and SANDMEYER 2003). Although our original assay had the advantage of selecting for position-specific transposition, the properties of the *tfc1* and *lhp1* mutants suggested that the selection might have been so specific that the collection included a number of mutants that were affected primarily for transposition into the synthetic target. Mutants were therefore rescreened with a galactose-inducible Ty3-*HIS3* to identify those that displayed aberrant genomic mobilization of Ty3. Because the insertion mutagenesis strategy could be associated with a significant frequency of unlinked mutations (ROSS-MACDONALD *et al.* 1999), and because of the relatively large number of genes involved, the second assay was employed using null mutants in the yeast knockout collection (WINZELER *et al.* 1999). Among 42 mutants examined using the Ty3-*HIS3* assay, 25 showed a difference from wild type in Ty3 mobilization ($P \leq 0.05$); 22 of these were further analyzed using additional assays. Because of the different genetic backgrounds and different mutant alleles (insertion *vs.* null) used in the first and second transposition assays, mutants that were not tested further in the current study (Table 1, B and C) are not necessarily uninteresting with respect to transposition. Quantitative comparison of a subset of mutants, including *nup157* and *nup159*, which were reconstructed as truncation mutants, suggested that the suppressor assay might be more sensitive to changes in transposition frequency than the Ty3-H assay (data not shown).

Analysis of Gag3p production and processing, cDNA, and transposition into chromosomal tRNA^{Val} genes: The 22 mutants were investigated further for protein processing, cDNA production, and insertion into the tDNA$^{Val(AAC)}$ family of chromosomal tRNA genes after 6 hr of Ty3 expression. Although this investigation did not indicate how directly the host function related to the Ty3 phenotype, our results support a relatively consistent pattern of effects of gene deletions at different stages of the Ty3 life cycle (Table 2). Only 2 mutants (*sin4* and *ppa1*) showed an overall reduction in Gag3p-derived protein. However, 13 mutants showed qualitative differences from wild-type patterns of Gag3p-derived protein or IN. Among those, 3 mutants (*nup157*, *ste7*, and *fun30*) showed a >50% decrease in cDNA. Five mutants (*nup157*, *ste7*,

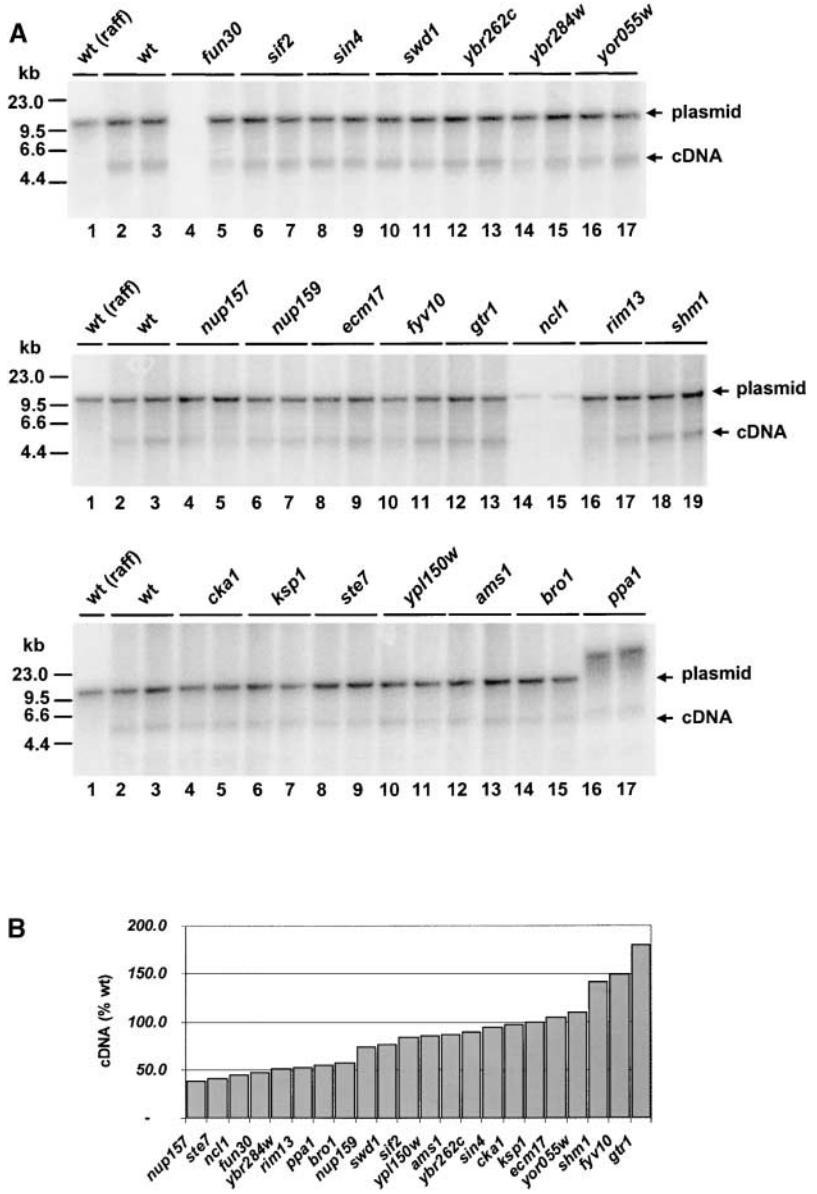

FIGURE 6.—cDNA production in BY4741 mutants expressing Ty3. (A) Southern blot analysis of Ty3 cDNA. Cells were induced for 6 hr for Ty3 expression and total DNA was extracted and cleaved with *Eco*RI. Southern blot analysis was performed with ^{32}P-labeled, Ty3-specific probe, which hybridizes to full-length cDNA of 5.4 kbp as well as to Ty3 donor plasmid. Two independent transformants were monitored for each mutant. (B) Relative amount of Ty3 cDNA in transposition mutants. Hybridization signals from Southern blots were quantitated using Quantity One software (Bio-Rad). Ty3 cDNA normalized to the plasmid was expressed as the percentge of wild type (*y*-axis). The bars represent the average of two independent transformants for each strain except *fun30* for which one mutant showed no plasmid.

sin4, *ksp1*, and *shm1*) had less than half of the frequency observed in wild-type cells for Ty3 transposition into the chromosomal tDNA$^{Val(AAC)}$ gene family.

Overall, dramatic biochemical phenotypes were not observed at 6 hr, and the extent of the genetic defect frequently failed to correlate with a particular biochemical defect. Similar apparent discordance between biochemical and genetic phenotypes has been noted for large-scale screens for mutants affected in Ty1 transposition (SCHOLES *et al.* 2001; GRIFFITH *et al.* 2003). We attribute this apparent lack of correlation to a combination of factors, including accumulation of a large number of inactive particles, which disguise biochemical deficiencies in a small population of active particles; failure of some biochemical restrictions to impinge directly on limiting steps in transposition; and inability of certain types of effects to be detected in any of our assays. For example, changes in protein modification or alterations in the cell cycle that are deleterious to transposition may not generate detectable consequences in our assays.

Distribution of gene functions affecting transposition and potential roles in the Ty3 life cycle: The initial collection of 50 genes included eight transcription or chromatin factors, seven protein kinases, four cell cycle regulators, three nucleoporins, four protein trafficking and vacuole related, three involved in RNA processing, two related to ubiquitin transfer, and other miscellaneous proteins. Twelve of the genes were of unknown function. The 22 genes, mutations in which showed consistent and significant effects on transposition in two genetic assays, encode proteins including four transcription or chromatin factors, four kinases, three protein trafficking or vacuole related proteins, two nucleoporins, and four proteins of unknown function. The decrease in representation of kinases and transcription-related proteins between the first and second genetic assays sug-

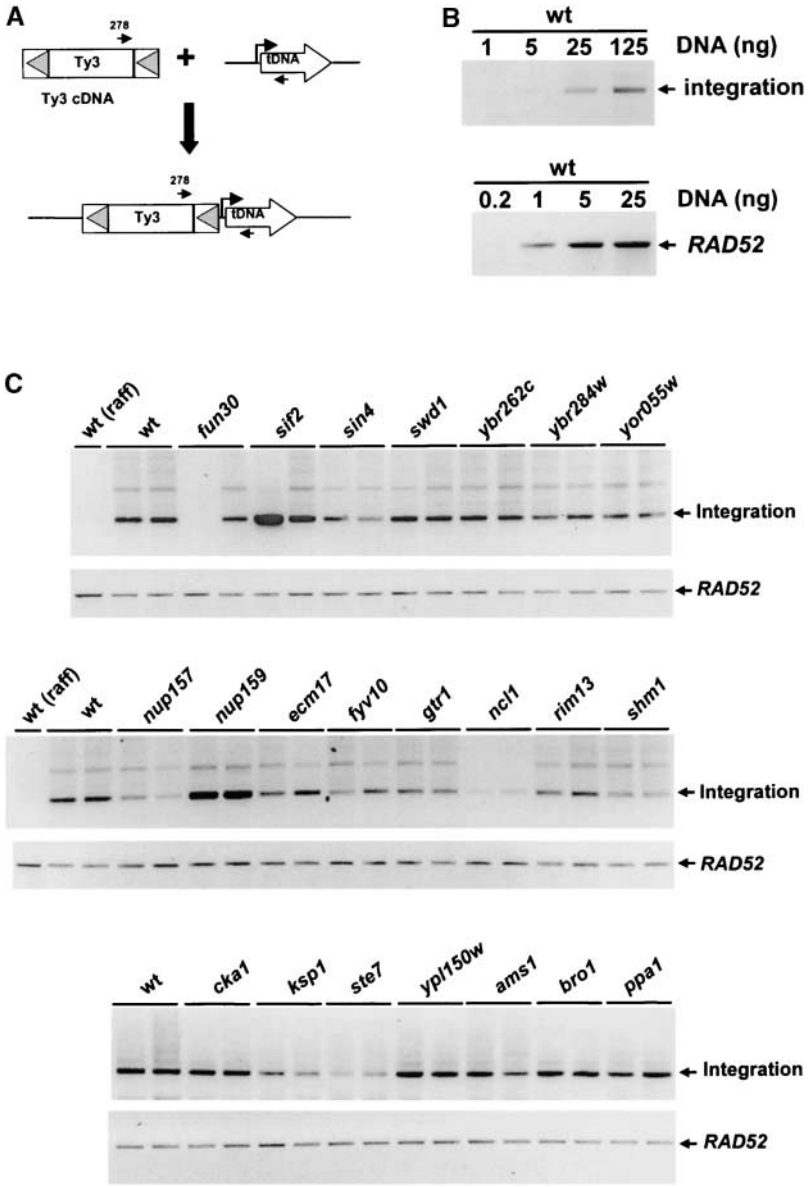

FIGURE 7.—PCR analysis of Ty3 integration upstream of genomic tDNA$^{Val(AAC)}$ genes. (A) Strategy for PCR detection of Ty3 integration into a target. Primers are specific to Ty3 (278) and to the 14 tDNA sequences. After insertion of Ty3, a diagnostic junction fragment can be amplified in PCR from the two primers. (B) Ty3 transposition into chromosomal tDNA$^{Val(AAC)}$ loci. Total DNA was extracted from cultures induced for Ty3 expression for 6 hr, and different amounts of genomic DNA were used to establish the linear range of PCR detection. A total of 1–125 ng of DNA was used as the template for PCR. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. PCR for integration (32 cycles, top); PCR templated by *RAD52* to quantify starting DNA (20 cycles, bottom). (C) PCR analysis of Ty3 integration in mutant strains. PCR was performed as described for B using 25 and 5 ng of DNA for integration and *RAD52*, respectively. Two independent transformants were tested for each mutant. Data for 22 mutants are shown. For the negative control, 25 ng DNA was used from an uninduced culture (raff).

gests that some of those were specific to transposition into the divergent tDNA target.

Mutants that affected transposition in both genetic assays are discussed according to the observed effects on Ty3 intermediates. The *sin4* and *ppa1* deletion mutants displayed reduced levels of Gag3p protein. The *ppa1* mutant also had reduced levels of cDNA. The reduction in Gag3p could be due to reduction in mRNA level, translation, or stability of Gag3p. In the case of *sin4*, reduction in mRNA would be consistent with the role of Sin4p in the mediator complex (Boube *et al.* 2002). However, relatively wild-type levels of IN and cDNA were observed at 6 hr. This suggests that Sin4p could also act indirectly or could act at a late step, such as integration, to influence the target site.

The *fun30*, *ste7*, *nup157*, and *ppa1* mutants and, to a lesser extent, the *ybr284w*, *rim13*, and *bro1* mutants all showed differences in the patterns of protein processing and reduced amounts of cDNA compared to wild type. Fun30p is a homolog of Snf2p with no known function (Clark *et al.* 1992). Ste7p is a mitogen-activated protein kinase kinase in the pheromone response pathway (Errede *et al.* 1995). The MAP kinase Fus3p, which occurs downstream of Ste7p in the same pathway, has been previously shown to have a negative effect on Ty1 transposition through activation of filamentation and destabilization of Ty1 proteins (Conte and Curcio 2000). However, in the case of Ty3, expression is induced by the pheromone response (Van Arsdell *et al.* 1987; Clark *et al.* 1988). Thus, Ste7p may have evolved to have a positive role in the Ty3 transposition cycle.

Ppa1, *bro1*, and *rim 13* mutants were all affected in protein processing and cDNA. Ppa1p encodes a subunit of the vacuolar H^+ ATPase (Graham *et al.* 2000) and Bro1p is a class E gene required for multivesicular body formation (Odorizzi *et al.* 2003). Rim13p is a cysteine

proteinase and interacts with Rim20p, a homolog of Bro1p (Xu and Mitchell 2001). Rim20p interacts with Snf7p (Ito *et al.* 2001), which is a member of the ESCRTIII complex involved in endosome-to-vacuole trafficking (Katzmann *et al.* 2002). *Bro1* and *rim13* showed relatively subtle differences from wild type, with both mutants having increased levels of high-molecular-weight IN-containing species. Not only is IN processed from Gag3-Pol3p, but also it exists as a RT-IN fusion protein, and reverse transcription is sensitive to mutations in IN (Sandmeyer *et al.* 2001). Thus changes in Gag3-Pol3p processing could directly affect cDNA levels. Although the mechanism through which Rim13p and Bro1p would affect protein processing is by no means clear, it is interesting that AIP1/Alix, the mammalian homolog of Bro1p, has been shown to play an important role in retroviral budding mediated by the (class E) ESCRT I–III complexes (Strack *et al.* 2003; Von Schwedler *et al.* 2003).

NUP157 and *NUP159* (Rout *et al.* 2000; Allen *et al.* 2001) are two nucleoporin genes identified in our screen. Both had modest changes in Gag3-Pol3 protein processing, and Nup157 exhibited decreased cDNA and transposition. Nup159p had roughly wild-type levels of cDNA and had increased transposition in all three assays. Ty3 VLPs accumulate in a perinuclear position by 6 hr (Hansen *et al.* 1992). Because Ty3 VLPs are too large to pass through nuclear pores, uncoating and reverse transcription may occur in proximity to the nucleus. Hence, it is possible that nucleoporins influence this process. If the loss of Nup157p, a major nucleoporin is directly related to reduced cDNA levels, it would indicate significant linkage between Ty3 replication and nuclear import. The loss of 36 aa from the C terminus of Nup159, an essential protein, results in increased transposition. Interestingly, Nup159p is one of the few proteins distributed asymmetrically in the yeast nuclear pore (Rout *et al.* 2000). This filamentous protein extends outward into the cytoplasm. Thus, it will be interesting to determine whether the deletion of 36 aa in this mutant has exposed an interaction surface that facilitates translocation of the preintegration complex.

Ksp1 and *shm1* mutants had wild type or greater amounts of cDNA but were both <50% of wild-type transposition into the tDNAVal$^{(AAC)}$ target. Ksp1p is a S/T kinase and was isolated as a suppressor of mutations in the Prp20p, the guanine exchange factor for Ran/Gsp1 (Fleischmann *et al.* 1996). Shm1p encodes a mitochondrial serine hydroxymethyltransferase (Kastanos *et al.* 1997). The *SHM1* gene deletion also affected the overlapping ORF ybr262c, but deletion of that ORF did not cause a tDNA$^{Val(AAC)}$ phenotype. The *sin4* mutant had reduced amounts of Gag3p, but produced wild-type amounts of IN and cDNA. It was also significantly reduced for tDNA$^{Val(AAC)}$ integration, consistent with a late phenotype as might be expected for effects at the target.

A group of mutants including *sif2*, *swd1*, *cka1*, *ypl150w*, *yor055w*, *gtr1*, and *ecm17* showed no biochemical defect and were deficient in both genetic assays, but were <39% decreased in tDNA$^{Val(AAC)}$ transposition. This could be attributable to differences between the performance of tDNA$^{Val(AAC)}$ in the divergent plasmid target and chromosomal assay or to the different times and growth conditions of the genetic and physical assays. A subset of these genes is potentially interesting because they affect chromatin and might be expected to have a late phenotype and affect targets differentially. Swd1p is a member of the Paf1 complex and the SET1 methyltransferase complex, and mutants have defects in telomeric and ribosomal silencing (Miller *et al.* 2001; Roguev *et al.* 2001). *Leo1*, which is a component of Paf1 complex (Krogan *et al.* 2003), was also identified in the original screen. Histone methylation has been implicated in Tf1 targeting to pol II promoters (H. Levin, personal communication) and several elongator proteins were identified as mutants defective in Ty1 transposition (Griffith *et al.* 2003). Sif2p is a member of the complex associated with the Set3 complex, which has NAD-dependent and -independent deacetylation functions (Pijnappel *et al.* 2001). Sif2p genetically interacts with Sir4p and antagonizes Sir4p activity at telomeres by recruiting it to other locations (Cockell *et al.* 1998).

The implication of chromatin factors in Ty3 targeting is of interest since tRNA genes could be repressive for pol II-transcribed genes (Kinsey and Sandmeyer 1991; Hull *et al.* 1994), but relatively little is known about the chromatin stucture of tRNA genes. Targeting of Ty5 is determined by the components of silent chromatin (Zou and Voytas 1997), particularly Sir4p (Xie *et al.* 2001). Ty1 elements exhibit preferential integration upstream of pol III-transcribed genes, possibly depending on the configuration of chromatin at those genes (Devine and Boeke 1996). The selection of Ty1 integration sites, at least at pol II-transcribed genes, is influenced by chromatin factors such as Rad6p (Kang *et al.* 1992; Liebman and Newnam 1993) and chromatin assembly factor I in conjunction with the level of histone proteins (Qian *et al.* 1998). Although targeting of Ty3 is determined by the transcription preinitiation complex of pol III (Kirchner *et al.* 1995; Yieh *et al.* 2000), binding of these factors or access to the target could be affected by local chromatin structure. Cka1p is a subunit of casein kinase II (CKII), and phosphorylation of the TATA-binding protein by CKII activates pol III transcription (Ghavidel and Schultz 1997). With the exception of Cka1p and Tfc1p, proteins known to affect pol III transcription were not isolated in our screen. This is most likely because components of the preinitiation complex are essential genes and because mutants incapable of expressing the suppressor tRNA were excluded from further analyses.

Gtr1p is a negative regulator of the Ran GTPase (Nakashima *et al.* 1999), which is required for nuclear import and export. Although this factor may not act

directly on Ty3 transposition, it could impinge on a downstream point in the life cycle, such as nuclear entry, consistent with a lack of observable biochemical defects.

Disruptions of five genes affecting the cell cycle (*cyr1*, *hsl1*, *kic1*, *cdc39*, and *cdh1*) decreased transposition into the divergent target in the original genetic assay. *CYR1* (Matsumoto *et al.* 1982) and *CDC39* (Collart and Struhl 1993) are essential genes; *KIC1* was not available in the knockout collection and was reconstructed as the original disruption. However, both *kic1* and *hsl1* mutants, in contrast to decreased transposition in the suppressor assay, showed increased Ty3 mobilization in the BY4741 background and so were not examined further in the present study.

Ty3 has reduced cDNA synthesis in G_1-arrested cells (Menees and Sandmeyer 1994), analogous to what is observed for retroviruses in G_0 (Fritsch and Temin 1977a,b; Varmus *et al.* 1977; Harel *et al.* 1981; Zack *et al.* 1990). In the case of retroviruses, exogenous nucleosides can promote the completion of DNA synthesis in G_0-arrested fibroblasts (Goulaouic *et al.* 1994; Kootstra *et al.* 2000). Thus it is interesting to speculate that extended G_1 periods in mutants such as *cyr1* and *cdc39* could have decreased transposition relative to wild-type cells. Cyr1p is a cyclic AMP synthetase that influences the level of cyclic nucleotides in the cell (Matsumoto *et al.* 1982). Cdc39p is an essential protein that negatively regulates gene expression (Collart and Struhl 1993) and is involved in mRNA degradation (Tucker *et al.* 2002).

CDH1, a homolog of Cdc20p, is an activator of the ubiquitin ligase, anaphase-promoting complex (Visintin *et al.* 1997; Irniger 2002). The *cdh1* mutant showed a consistent phenotype, but the decrease in the Ty3-*HIS3* mobilization assay was modest, and it was not pursued further in this study. Interestingly, the degradation of Hsl1p, which showed allele and assay-dependent transposition phenotypes, is mediated by Cdh1p and Cdc20p (Burton and Solomon 2001). Analysis of these mutants may shed some light on the specific requirements for the completion of Ty3 reverse transcription and the life cycle.

Recovery of anticipated mutants and those related to Ty1: The *tfc1* and *lhp1* mutants recovered in our screen and analyzed in detail were confirmed as *bona fide* disruptors of Ty3 transposition. In addition, a significant fraction of the mutants identified in our original screen were confirmed to affect Ty3 transposition in a different background with an independent assay. Nevertheless, it is clear that this screen did not saturate the search for yeast genes, which affect Ty3 transposition. Only 10 genes were recovered two or more times, suggesting that additional genes could have been recovered using the same screen. Although we used multiple pools of disrupted genomic fragments, it is also possible that some genes are not represented the disruption pools. For example, Dbr1p (Chapman and Boeke 1991; Karst *et al.* 2000), Pmr1p (Bolton *et al.* 2002), and Ku proteins (Downs and Jackson 1999) are known to affect Ty1 and Ty3 (L. Yieh, unpublished work), but were not identified in our screen. Preliminary results from systematic screening of the yeast knockout collection using Ty3-*HIS3* mobilization (B. Irwin, personal communication) also suggests that there will be very little overlap between the results of that screen and the one reported here. We conclude that the nature of the target gene used in the initial assay contributed to a highly selective collection of mutants.

Two large-scale screens were recently performed for mutants affected by Ty1. The first group (Scholes *et al.* 2001) used chromosomal Ty1 under its native promoter and tagged with *HIS3AI* to screen a disruption collection in a different genetic background. The second group (Griffith *et al.* 2003) used *GAL1*-Ty1, marked with *HIS3* on a low-copy plasmid, to screen the diploid yeast knockout collection. Scholes *et al.* (2001) found that most of the identified disruptions yielded elevated transposition and were in DNA checkpoint, repair, and telomere maintenance genes. The screen by Griffith *et al.* (2003) recovered predominantly reduced transposition mutants and identified a number of genes that function in a variety of cellular processes: transcription regulation, RNA metabolism, nucleocytoplasmic transport, and protein modification. The latter is more similar to the distribution of genes recovered in our screen. These screens showed relatively little overlap with each other and with the results of our screen. *SIN4* was one exception as it was identified in our screen and in the Griffith *et al.* (2003) screen. An ongoing screen of the knockout collection using Ty3-*HIS3*, which is technically more analogous to the screen by Griffith *et al.* (2003), shows more overlapping genes, but also many nonoverlapping genes. These results emphasize the contribution of the role played by the screening approach itself in filtering the genes that are identified among an apparently large number of potential candidates.

Ty3 and Ty1 differ structurally, in mode of expression, and in position specificity and therefore have probably evolved fundamentally different interactions with their yeast host. For example, Ty3 is expressed predominantly in a rapid induction in mating cells and inserts at initiation sites of genes transcribed by pol III (Chalker and Sandmeyer 1992; Bilanchone *et al.* 1993); Ty1 is constitutively highly expressed and inserts upstream of genes transcribed by pol III at distances from −50 to −750 bp (Kim *et al.* 1998), as well as into pol II-transcribed genes and promoters (Boeke and Sandmeyer 1991). Thus it is likely that biological differences between Ty1 and Ty3 also contribute to the sparse overlap in host factors. These comparative findings serve to underscore the complexity of the search for host factors.

Summary: Since Ty3 is similar to retroviruses in its genome organization and gene products, it is conceivable that the mammalian orthologs of some of the Ty3

host factors may play a similar role in retroviral life cycle. A relatively small number of host factors that have specific interactions in the retrovirus life cycle have now been identified. However, given the large number of factors discovered in a limited number of screens in yeast, it seems likely that many retrovirus-interacting genes remain to be discovered. The initial results from three large-scale screens in yeast suggest that host factors for retroelements may be relatively specific and that their identification is dependent on the assay utilized. Comparison of the genes identified in this study with the sequences in GenBank yielded at least 27 mammalian homologs with a *P*-value of $\leq 10^{-10}$ (data not shown). The search of host factors therefore serves as a starting point into the investigation of the roles of their homologs in retroviral life cycle.

We thank M. Snyder for providing the mTn3::*lacZ/LEU2* library; C. Friddle for vectorette PCR protocol; J. A. Claypool for his help with the genetic screen; L. Yieh for providing plasmids; and T. M. Menees for providing plasmids and for helpful discussions. This work was supported by Synthesis and Structure of Biological Macromolecules training grant GM07311-24 (M.A.) and Public Health Service grants GM33281 to S.B.S. and AI45405 to Wesley I. Sundquist (University of Utah).

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Communicating editor: D. Voytas