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Research

Retrotransposon profiling of RNA polymerase III initiation sites

Xiaojie Qi,^{1,4} Kenneth Daily,^{2,4,5} Kim Nguyen,^{1,4,6} Haoyi Wang,^{3,7} David Mayhew,³ Paul Rigor,² Sholeh Forouzan,² Mark Johnston,^{3,8} Robi David Mitra,³ Pierre Baldi,^{1,2} and Suzanne Sandmeyer^{1,9}

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Although retroviruses are relatively promiscuous in choice of integration sites, retrotransposons can display marked integration specificity. In yeast and slime mold, some retrotransposons are associated with tRNA genes (tDNAs). In the *Saccharomyces cerevisiae* genome, the long terminal repeat retrotransposon Ty3 is found at RNA polymerase III (Pol III) transcription start sites of tDNAs. Ty1, 2, and 4 elements also cluster in the upstream regions of these genes. To determine the extent to which other Pol III-transcribed genes serve as genomic targets for Ty3, a set of 10,000 Ty3 genomic retrotranspositions were mapped using high-throughput DNA sequencing. Integrations occurred at all known tDNAs, two tDNA relics (*iYGR033c* and *ZOD1*), and six non-tDNA, Pol III-transcribed types of genes (*RDN5*, *SNR6*, *SNR52*, *RPRI*, *RNA170*, and *SCR1*). Previous work in vitro demonstrated that the Pol III transcription factor (TF) IIIIB is important for Ty3 targeting. However, seven loci that bind the TFIIB loader, TFIIC, were not targeted, underscoring the unexplained absence of TFIIB at those sites. Ty3 integrations also occurred in two open reading frames not previously associated with Pol III transcription, suggesting the existence of a small number of additional sites in the yeast genome that interact with Pol III transcription complexes.

[Supplemental material is available for this article.]

The genomic patterns of retrovirus and retrotransposon integration sites provide useful insights into host factors that influence retroelement proliferation as well as the potential impact of these integrations on host cell biology. Despite the relatively low selectivity of retroviruses for integration into complex genomes, high-throughput sequencing has revealed influences of DNA sequence and structure, transcription factors, and epigenetic modifications of DNA and histones on retrovirus integration site preferences (e.g., Berry et al. 2006). Analysis of integration patterns of non-native transposable elements has also provided insights into chromatin structure and mechanisms of integration, for example, the targeting of nucleosome-free chromatin by the *Hermes* cut-and-paste DNA element expressed in *Saccharomyces cerevisiae* (Gangadharan et al. 2010). In some contrast to these systems, some retrotransposons in single-celled organisms have evolved efficient targeting mechanisms. These include the global targeting of RNA polymerase II (Pol II) promoters in *Schizosaccharomyces pombe* (e.g., Guo and Levin 2010), the targeting of heterochromatin in *S. cerevisiae* (Baller et al. 2011), and the targeting of Pol III promoters in *S. cerevisiae* and *Dictyostelium*.

In *S. cerevisiae*, long terminal repeat (LTR) retrotransposons Ty1-4 and isolated LTR remnants cluster upstream of tRNA genes

(tDNAs). Ty1 and Ty3, the most extensively studied, both require active Pol III promoters, but associate in distinct patterns (Sandmeyer et al. 2002; Voytas and Boeke 2002). Ty1 clusters from ~60 bp to 750 bp upstream of the tDNA transcription start site (TSS). Integration frequency decreases with distance from the target with an 80-bp periodicity (Bachman et al. 2004). Consistent with the interpretation that a particular aspect of nucleosome structure is favored, Ty1 insertion patterns are sensitive to chromatin organization. For example, truncation of the Bdp1 component of TFIIB or loss of Isw2, a chromatin-remodeling factor that maps to tDNAs, reduces the amplitude of the characteristic periodicity (Bachman et al. 2005; Gelbart et al. 2005). Other long-range factors including the local concentration of tDNAs and isolated LTRs also influence Ty1 integration patterns (Bachman et al. 2004).

Other retrotransposons target the Pol III transcription complex more directly. In *Dictyostelium*, non-LTR retrotransposons TRE5 and TRE3 cluster ~44 to 54 bp upstream and 40 to 150 bp downstream of tDNA-coding sequences, respectively (Winckler et al. 2005). Genomic Ty3 and LTR remnants occur within a few base pairs of the tDNA TSS (Sandmeyer et al. 2002). Although genomic copies of Ty3 are associated with tDNAs, studies using plasmid-borne Pol III-transcribed genes indicate that other genes associated with TFIIB including 5S (*RDN5*) and U6 (*SNR6*) genes are also targets.

Two general types of promoters are recognized by Pol III in the yeast genome (Kassavetis and Geiduschek 2006; Dieci et al. 2007). For most of these genes, the promoter elements are short sequences inside the transcribed region, and terminators are tracks of five or more T's on the template strand (Allison and Hall 1985). Type 1 promoters require factors TFIIA, TFIIC, and TFIIB. TFIIA loads onto internal box C and box A promoter elements and mediates binding of TFIIC, which loads TFIIB upstream of the initiation site (Taylor and Segall 1985; Braun et al. 1989). TFIIB is the initiation factor proper comprised of Brf1, Tbp1, and Bdp1

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(Kassavetis et al. 1990). *RDN5* genes are the sole representatives of Type 1 promoters. They are present in 100–200 copies alternating with repeats of *RDN37*, the gene for the 35S rRNA, on chromosome XII (Johnston et al. 1997).

Pol III Type 2 promoters have box A and box B elements downstream from the TSS. TFIIC binds Type 2 promoters through interactions with box A and box B sequences and, similar to its activity on Type 1 promoters, mediates binding of TFIIB upstream of the TSS. For a subset of genes, there is an upstream TATA element that enhances binding of TFIIB (Eschenlauer et al. 1993; Giuliodori et al. 2003). Informatic analysis and genome-wide chromatin immunoprecipitation (ChIP) have identified more than 280 Type 2 genes (Lowe and Eddy 1997; Percudani et al. 1997; Hani and Feldmann 1998; Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004). These include 274 tDNAs and one pseudo-tDNA; *SCR1*, encoding the RNA component of the signal recognition particle (Dieci et al. 2007); *SNR52*, encoding a C/D box snoRNA guide for rRNA processing; *RPR1*, encoding the RNA component of the RNase P1-processing complex; *SNR6*, encoding the U6 spliceosomal RNA; and three genes of unknown function. These are *iYGR033C* and *ZOD1*, which encode ancient tDNAs for tRNA^{Arg} and tRNA^{Ile}, respectively (Guffanti et al. 2006), and *RNA170* (Olivas et al. 1997). For the most part, Type 2 genes are occupied by TFIIB, TFIIC, and Pol III in logarithmically growing cells (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004). These loci vary with respect to expression, upstream TATA elements, separation of box A and box B and position of box B relative to transcribed sequence.

Recent studies have implicated Pol III transcription factors in functions in addition to Pol III transcription. A small but intriguing set of loci in the *S. cerevisiae* genome binds TFIIC, but low or undetectable amounts of TFIIB. These were dubbed Extra Three C (ETC) (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004) and include *RNA170/ETC5* and *ZOD1*, which are weakly transcribed, and *iYGR033c*, *ETC1-4*, and *ETC6-8*, which are not detectably transcribed (Olivas et al. 1997; Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004). About 400 condensin binding sites in *S. cerevisiae* are associated with TFIIC, and only a subset of these are tDNAs (D'Ambrosio et al. 2008). Roles for Pol III promoter elements in simple eukaryote chromatin structure have been also observed in *S. pombe* (Noma et al. 2006). In addition to structural roles for ETCs, tDNAs establish heterochromatic boundaries (Donze and Kamakaka 2001), phase nucleosomes (Morse et al. 1992), bind condensins (D'Ambrosio et al. 2008; Haeusler et al. 2008; Gard et al. 2009), repress transcription of neighboring Pol II-transcribed genes (Kinsey and Sandmeyer 1991; Hull et al. 1994), undergo ectopic recombination (Munz et al. 1982), and disrupt progression of downstream replication forks (Deshpande and Newlon 1996).

Ty3 elements target Pol III TSS (Chalker and Sandmeyer 1990, 1992;

Sandmeyer et al. 2002). In vitro, recombinant TFIIB subunits Tbp1 and Brf1 are sufficient to target strand-transfer of Ty3 cDNA from virus-like particles (Kirchner et al. 1995; Yieh et al. 2000). However, TFIIC is implicated by in vivo experiments in which truncation of Tfc1, a subunit of TFIIC, restricts orientation of Ty3 insertions (Aye et al. 2001). Nonetheless, genomic Ty3 elements have only been observed in association with tDNAs. The present study was undertaken to saturate de novo Ty3 genomic transposition targets and determine the overlap with genes transcribed by Pol III. The results show that Ty3 integrates with high specificity at all known Pol III-transcribed genes and at additional sites, two of which were confirmed to be dependent on the presence of a consensus box B (Olivas et al. 1997; Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004; Guffanti et al. 2006; Marck et al. 2006). ETC loci did not support Ty3 integration.

Results

Ty3 retrotransposition is predominantly mediated by integration rather than homologous recombination

S. cerevisiae LTR retrotransposons reverse-transcribe genomic RNA into a full-length cDNA copy, which transposes into the host genome via Rad52-dependent homologous recombination or integrase (IN)-dependent strand-transfer reactions. The impact of homologous recombination on Ty3 transposition was assessed using S288C-related YMA1322 and its *rad52Δ* derivative, YMA1356 (Table 1A). YMA1322 and YMA1356 were transformed with a low-copy plasmid marked with *URA3* and bearing a galactose-inducible Ty3 modified by the insertion downstream from a Ty3 protein-coding sequence of a copy of the *HIS3* gene flanked by unique

Table 1. Yeast strains and plasmids used in this study

(A) Yeast strains		
Strain	Genotype	Reference
YPH500	<i>MATα ura3-52 lys2-801-amber ade2-101-ochre trp1-Δ63 his3-Δ200 leu2-Δ1</i>	Sikorski and Hieter 1989
YMA1322	<i>MATα ura3-52 trp1-Δ63 his3-Δ200 ade2-101 lys2_o leu2-Δ1</i>	Aye et al. 2001
YMA1356	<i>YMA1322, rad52Δ</i>	Aye et al. 2001
S288C	<i>MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6</i>	Mortimer and Johnston 1986
YKN1692	<i>YMA1322, BRF1::BRF1-3HA-loxP-HIS3-loxP</i>	This study
(B) Plasmids		
Plasmid	Description	Reference
pKN3050	Ty3- <i>HIS3</i> -ppt (galactose-inducible Ty3 tagged downstream from <i>POL3</i> with a <i>HIS3</i> gene flanked by 60-bp unique sequences and followed by an extra copy of ppt and LTR sequences present in the <i>POL3</i> open reading frame)	This study
pKN3097	Ty3-ppt (as above but lacking the <i>HIS3</i> insertion and one of two unique 60-bp tag sequences)	This study
pXP622	Shuttle vector, Amp ^R , 2μ, <i>loxP-LEU2-loxP</i> , used to construct plasmid-borne targets	Fang et al. 2011
pFA6a-3HA-KanMX6	Template for 3HA tag	Bähler et al. 1998
pKN3481	pXP622 with <i>loxP-LEU2-loxP</i> replaced by <i>loxP-HIS3-loxP</i>	This study
pCR2.1	TA-cloning vector	Invitrogen

60-bp sequence tags (Ty3-ppt *HIS3* on plasmid pKN3050) (Table 1B; Supplemental Table S1). Transformants were induced for Ty3 expression by growth in medium containing galactose. Cells that had lost the plasmid but acquired a chromosomal Ty3-ppt *HIS3* were selected by growth on medium selecting against the donor plasmid, and for the presence of the *HIS3* marker gene. A quantitative version of this assay showed that the frequency of transposition in the wild-type strain was threefold that of the *rad52* strain (data not shown). This was similar to the twofold difference between wild-type and *rad52* derivative previously reported (Sadeghi et al. 2001). Enrichment for cells that had undergone transposition before production of genomic libraries for sequencing was necessitated by the low percentage of transformants (<1%) that undergo transposition and by the diversity of those events. Approximately 10,000 His⁺ colonies were collected and pooled for each strain. Ty3 insertion junctions were enriched by an inverse PCR (iPCR) strategy using primers inside Ty3-ppt *HIS3* (Fig. 1; Supplemental Table S2; Ochman et al. 1988; Wang et al. 2008; Methods).

Pooled iPCR products were sequenced using Illumina GAIIX technology (Illumina; Tpn-seq). Sequencing primers annealed so that sequence began with the terminal 17 nt of the Ty3 LTR U5 and read into ~19 nt of flanking genomic DNA (Fig. 2A). Sequences were aligned with the yeast reference sequence from the UCSC Genome Browser (build sacCer2, June 2008) (Rhead et al. 2010) using Bowtie (Langmead et al. 2009). Including data from both

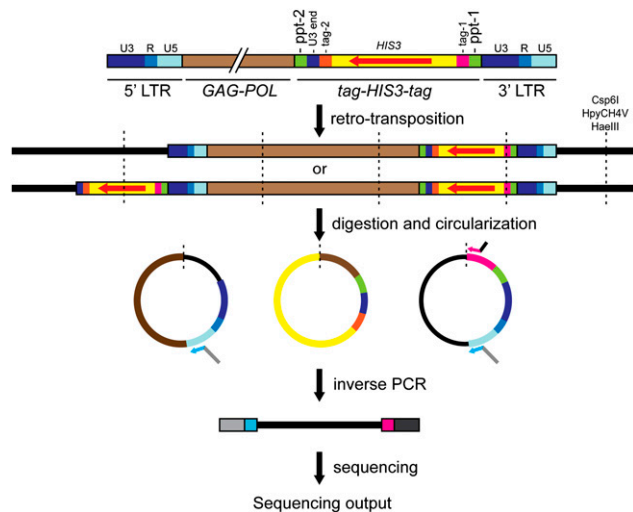


Figure 1. High-throughput sequencing of de novo Ty3 integration sites. Step 1: Expression of Ty3-ppt-*HIS3* containing two unique 60-bp tags for amplification of integration joints. Step 2: Alternative reverse transcription products, dependent on position of ppt. Step 3: Restriction of genomic DNA with one of three restriction enzymes (Csp6I, HpyCH4V, and HaeIII) and circularization. DNA fragments flanking de novo Ty3 integration sites were selectively amplified by inverse PCR (iPCR) using primers that annealed to the Ty3 U5-end LTR and Ty3 tag-1. Primers contained Illumina adaptor sequences and the Ty3 LTR primer contained the Illumina sequencing primer sequence. Step 4: The iPCR products from separate reactions were combined and processed for sequencing using the Illumina GAIIX system. Ty3 transcription begins at the 5' edge of R and continues through U5 into the internal domain and the 3' U3 and R regions. Reverse transcription regenerates the complete U3-R-U5 LTR at each end of the cDNA. (Vertical dashed lines) Digestion/ligation sites; (red arrow) *HIS3* ORF; (bent arrows) iPCR primers. Arrows indicate primers: (light blue) Ty3 U5-end LTR; (pink) tag-1; (gray) upstream Illumina adaptor with sequencing primer; (black) downstream Illumina adaptor.

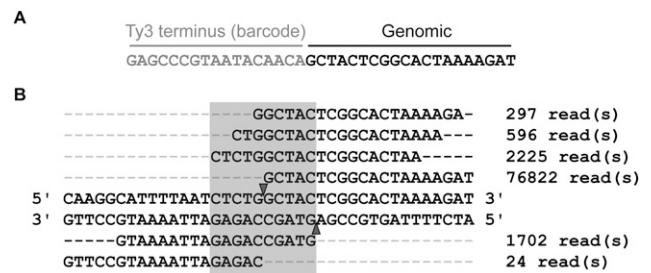


Figure 2. Alignment of sequencing reads to yeast genomic sequence. (A) Sequence of Ty3 integration joint. High-throughput sequencing reads began with 17 nt from the U5 end of the Ty3 LTR and continued into genomic sequence. Informative reads aligned uniquely with ~19 nt of target site DNA in the reference genome. Each unique junction defined an integration event. A sequencing read representing a collection of events at *tK(CUU)F* is shown. (B) Analysis of target loci. Reads captured by events separated by 10 nt or less were combined into clusters (shaded box). The distribution of reads (hits) among events clustered at *tK(CUU)F* is shown. (Dashed lines) Ty3 LTR sequences. Depending on the orientation in which Ty3 integrated, sequencing reads aligned with either the Watson or Crick strand of the genomic sequence of yeast reference strain S288C. (Right column) The numbers of reads for the same integration joint (event densities). The most-sequenced junctions in the two strands were offset by the 5-bp stagger characteristic of Ty3 insertion sites (triangles).

experiments, a total of 12.4×10^6 reads were aligned to the Ty3 LTR sequence (Table 2; Supplemental Table S3; NCBI Gene Expression Omnibus, <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31820>). Of these, 6.1×10^6 reads aligned to the genome with no mismatches and were analyzed further. A total of 0.37×10^6 reads aligned, but not uniquely (“multiples”), and were separately evaluated. Individual Ty3 insertion sites (“events”) separated by 10 bp or less were clustered, yielding a total of 296 clusters. The number of reads per cluster was defined as the “cluster density” (see example, Fig. 2B), and this value was normalized to total density per experiment. The average density per cluster between the two experiments ranged from ~100,000 reads per [*tT(UGU)P*] to six reads per [*tV(AAC)L*]. Because of the precision of Ty3 targeting, multiple independent transpositions were likely to map to the same insertion site. Therefore, the number of events is likely to underestimate the number of independent insertions (Supplemental Table S3; Chalker and Sandmeyer 1992; Kinsey and Sandmeyer 1995; data not shown).

Comparison of normalized cluster densities for *RAD52* and *rad52Δ* strains showed very similar results (Fig. 3). In particular, the plots of Ty3-ppt *HIS3* transpositions to loci associated with Ty3 LTRs showed only a slight reduction in slope compared with the plot for loci not associated with Ty3 LTRs (slope = 1.0556; $R^2 = 0.9327$ vs. slope = 0.8861; $R^2 = 0.871$, respectively), indicating that recombining at previously occupied sites is not a major determinant of de novo Ty3 integration.

Ty3 retrotransposition targets the complete Pol III transcriptome

The 296 Ty3 integration clusters were evaluated for proximity to annotated Pol III-transcribed genes (Table 3). This comparison showed that 262 of 274 identified tDNAs were associated with Ty3 integration clusters. In only one case was the cluster closest to the tDNA not within a few bases of a presumptive site of transcription initiation. In that case, *tW(CCA)G1*, integration mapped near the

Table 2. Distribution of aligned reads

Plasmid	Strain	Raw ^a	LTR tag ^b	Singly aligned ^c	Not aligned ^d	Multiply aligned ^e
pKN3050	YMA1322	7,130,751	6,809,596	3,034,624	3,589,582	185,390
pKN3050	YMA1356	5,995,101	5,596,236	3,057,512	2,350,228	188,496

^aRaw reads are the output of the sequencing run.

^bCorrect reads are those beginning with the LTR sequence.

^cSingly aligned reads aligned perfectly and uniquely to the reference genome.

^dNot aligned reads had one or more mismatches with the reference sequence.

^eMultiply aligned reads aligned with more than one locus in the reference genome.

presumptive initiation site, but also 45 bp downstream from the tRNA-coding sequence.

The 13 tDNAs not represented among Ty3 integration clusters were examined individually (summarized in Table 3). We considered four explanations for their absence: (1) Short regions of conserved sequence immediately upstream of the tRNA-coding sequence resulted in alignment of clusters to multiple genomic loci, and exclusion from the uniquely aligned data set. (2) Sequence differences between YMA1322/YMA1356 and the reference S288C strain within the sequenced region led to imperfect alignment and exclusion from further analysis. (3) The tDNA was missing from the YMA1322/YMA1356 strain background. (4) A subset of tDNAs was not targeted because members do not bind factors required by Ty3 or reside in a chromosomal region that excludes access. The data set containing non-unique sequences (“multiples”) (Supplemental Table S3B) was inspected for matches to Pol III–transcribed genes. It contained an insertion junction aligning with two *tL(CAA)* genes on chromosomes VII and XIII. Thus, either or both of these absent tDNAs were targets of Ty3 integration. The multiples set also included junctions inside four identical *tR(UCU)* genes. There are four tandem tR-tD genes in the *S. cerevisiae* genome. The *tR(UCU)* sequence lies immediately upstream of *tD(GUC)* on chromosomes II and IV, and two sites on X. Thus, both the upstream member of the pair, *tR(UCU)*, and the downstream member, *tD(GUC)*, were targeted by Ty3. In these cases, it was not possible to assign insertion joints to specific loci. However, given the broad use of other tDNAs and the similar target sequence context, we think it likely that the insertions were distributed among the loci that failed to map uniquely.

We next considered that tDNAs not found as Ty3 targets in the unique or multiple sequence reads [*tM(CAU)J3*, *tP(UGG)O1*, *tY(GUA)J2*, *tD(GUC)N*, *tD(GUC)L2*, *tK(CUU)C*, and *tM(CAU)C*] (Supplemental Table S3B) occurred in sequences of the host strain that diverged from the sequence of the S288C reference strain. To directly query the representation of these sequences in the host genome used in our experiments, the respective regions from the YMA1322 genome were assayed by PCR using primers flanking the reference sequence. In the first four cases, amplification was successful, and the amplicons were sequenced. This analysis revealed mismatches between the sequence from YMA1322 and the reference *S. cerevisiae* genome S288C for *tM(CAU)J3*, *tP(UGG)O1*, and *tY(GUA)J2*. Our reference genome sequence was modified to contain these variant sequences and queries of the modified reference sequence identified clusters at each of these loci. The pseudogene *tD(GUC)N* was also amplified from YMA1322, and this amplicon was identical in sequence to the S288C reference sequence. For the remaining three loci [*tK(CUU)C*, *tM(CAU)C*, and *tD(GUC)L2*], a fragment of the predicted size could not be amplified from the YMA1322 genomic DNA template. Inspection of the reference genome showed that *tK(CUU)C* and *tM(CAU)C* were flanked by direct repeats of

Ty1 LTRs so that recombination could have deleted both tDNAs, thus accounting for failure to recover those loci by sequencing or by PCR. Sequence analysis of BY4741 indicated that these genes, consistent with previous observations (Harismendy et al. 2003), were also not present in that S288C-related strain (T. Najdi, pers. comm., data not shown). The *tD(GUC)L2* locus could not be amplified. However, the S288C reference genome has a single sigma LTR upstream of this gene. Thus, this gene appears to be absent in YMA1322 but shows evidence of being a Ty3 integration target in S288C.

Pol III–transcribed loci that are not tDNAs were also targeted by Ty3 integration (Fig. 4; Table 3). *RDN5* is present in the highest copy number. The *RDN5-1* copies are repeated within the ~100–200-copy *RDN37* repeat, and *RDN5-2* is located in the last repeat and could not be distinguished (Johnston et al. 1997). Four additional orphan copies designated *RDN5-3* to -6 flank the rDNA repeat. These orphan genes are expressed (Piper et al. 1984). However, the sequence context diverges 2 bp upstream of the native TSS, the terminal 4 bp differ, and the poly(T) termination tract is shortened. Ty3 insertion clusters were identified in the multiples data set representing *RDN5-1* and 2, but not the orphan variant *RDN5-3* to 6 genes (Supplemental Table S3B).

All six non-tDNA, Type 2 genes were associated with Ty3 insertions. Similar to tDNAs, *SCR1* (Dieci et al. 2002; Guffanti et al. 2006) contains promoter box A and box B elements within the mature RNA-coding sequence. *RPR1* and *SNR52* (Ishiguro and Kassavetis 2003; Guffanti et al. 2006) contain box A and box B sequences downstream from the TSS and within the transcribed region, but upstream of the mature coding sequence. *SNR6* has an upstream TATA element and an internal box A, but terminates upstream of box B (Eschenlauer et al. 1993). Ty3 clusters overlapped the TSS of these diverse Pol III–transcribed genes (Table 3; Supplemental Table S3A).

In addition to Pol III–transcribed genes of known function, genome-wide ChIP experiments using epitope-tagged subunits of

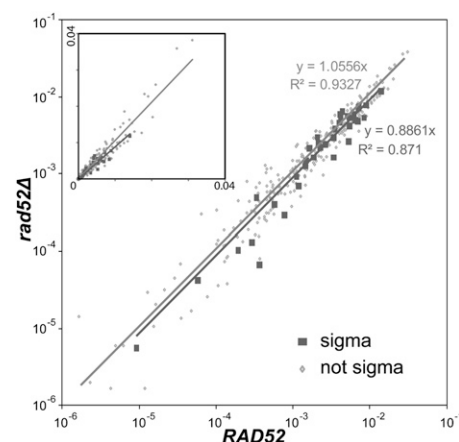


Figure 3. Targets with preexisting Ty3 LTRs were used at a slightly greater frequency in the presence of *RAD52*. Cluster densities determined in *RAD52* (x-axis) and *rad52Δ* (y-axis) strains are shown as the fraction of total cluster densities per experiment in log scale (inset in linear scale). Clusters at targets with preexisting Ty3 LTRs (sigma; solid squares, lower line and description) and clusters at unoccupied sites (not sigma; dots, upper line and description) were plotted.

Table 3. De novo Ty3 integration sites identified in HTS

Target sites	Genome alignment	Number	Proximal genes (cluster ID #)	Notes	
tRNA	Single	262			
	Multiple	2	<i>tL(CAA)G2</i> <i>tL(CAA)M</i>		
	Not in original data set	11	<i>tD(GUC)B</i> <i>tD(GUC)D</i> <i>tD(GUC)J2</i> <i>tD(GUC)J3</i> <i>tM(CAU)J3</i> <i>tP(UGG)O1</i> <i>tY(GUA)J2</i> <i>tD(GUC)N</i> <i>tD(GUC)L2</i> <i>tK(CUU)C</i> <i>tM(CAU)C</i>	Part of tD-tR tandem pairs, originally assigned to tR Sequence mismatch, corrected then aligned Pseudogene Absent Absent Absent	
	Non-tRNA Pol III	Total	275		
		Single	7	<i>iYGR033C</i> <i>RNA170</i> <i>RPR1</i> <i>SCR1</i> <i>SNR52</i> <i>SNR6</i> <i>ZOD1</i>	
		Multiple	2	<i>RDNS-1</i> and <i>2</i>	
		None	4	<i>RDNS-3, 4, 5,</i> and <i>6</i>	
	Ty3 LTR	Total	13		
		Single	18		iPCR artifact
	Atypical integration sites	Single	5	<i>YIL100W</i> (#72) <i>SCS3</i> (#98) <i>YJL206C</i> (#49) <i>tW(CCA)G1</i> (#99) <i>YCG1</i> (#172)	Verified Verified Not verified Not tested Not tested

TFIIIC, TFIIIB, and Pol III identified sites in the genome that bind small amounts of TFIIIC comparable to that bound by tDNAs, but low or undetectable amounts of TFIIIB or Pol III subunits (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004). These include *ZOD1* and *RNA170*, which are weakly transcribed, and *iYGR033C* and *ETC1-4* and *ETC6-8*, for which transcription has not been detected (Olivas et al. 1997; Guffanti et al. 2006). Ty3 integrated close to the TSS of *ZOD1* and *iYGR033C*. Transcription of *RNA170* initiates atypically close to the proposed box A (7–15 nt upstream as opposed to ~20 nt upstream) and terminates downstream from box B, but upstream of a poly(T) tract (Olivas et al. 1997). Surprisingly, Ty3 integrated within the Pol III-transcribed *RNA170* sequence between the predicted box A and box B promoter elements. ETC sites (*ETC1-4, 6-8*) (Olivas et al. 1997; Moqtaderi and Struhl 2004; Guffanti et al. 2006) did not act as Ty3 targets. Thus, ETC sites are neither occupied by significant amounts of TFIIIB and Pol III nor targeted by Ty3.

Our analysis used a *HIS3*-tagged Ty3 and involved growth of transformants that had undergone transposition on selective medium. While this introduced bias against integrations that affect viability or growth rate, integrations upstream of *tS(CGA)C* and *SNR6*, the two essential Pol III-transcribed targets of which we are aware, were recovered. The additional possibility of failure to recover integrations into essential non-Pol III targets cannot be formally excluded.

Correlation of genomic features with Ty3 target activity

To estimate the range in targeting activity among tDNAs, targets were ranked according to cluster density, and a subset was re-

evaluated using a quantitative (q)PCR assay for transposition. Five targets from the top [*tK(CUU)F*, *tG(GCC)G2*, *tA(UGC)E*, *tT(UGU)G1*, and *tG(CCC)D*] and bottom [*tG(GCC)F2*, *tV(AAC)G1*, *tD(GUC)K*, *tQ(UUG)D1*, *tV(AAC)G3*] deciles (Fig. 5A; Supplemental Table S3A) were tested. The *ETC1* locus was used as a negative control. Naive and Ty3 insertion-bearing versions of the loci were cloned and used as standards. Cells were transformed with pKN3097 bearing an unmarked galactose-inducible Ty3-ppt and induced in triplicate for Ty3 expression by growth in SGal-Ura for 24 h. DNA was extracted and subjected to qPCR using primers specific to sequences flanking the 10 genomic target sites and to either the U3 or the U5 end of the Ty3 element. The assay was standardized using a dilution series of the respective cloned Ty3-associated targets, and outcomes were expressed as integrants normalized to an *ACT1* control (Fig. 5B). Comparison of the overall pattern of target usage showed that seven targets were recovered with insertions represented at 8×10^{-6} to 16×10^{-6} . The remaining three were represented at 2×10^{-6} to 4×10^{-6} . The seven targets with activity in the upper range included all five of the targets in the top decile and two [*tV(AAC)G1* and *tV(AAC)G3*] from

the bottom decile of Tpn-seq reads. *ETC1* was not used. Despite a relative consistency in that top targets in Tpn-seq were more highly used in the qPCR assay, the difference between the different classes of targets was much reduced using the quantitative assay. This result indicated that the sequencing reads provided a sensitive record of positions of integration rather than a quantitative measure of target utilization.



Figure 4. Ty3 integration targets known Pol III-transcribed genes. Ty3 integration sites (filled triangles); Pol III promoter elements, TBP binding site (TATA), box A (unfilled), box B (dark gray), and box C (light gray). TSS are shown only for genes that are transcribed as precursors (bent arrow). TSS is not available for *iYGR033C*.

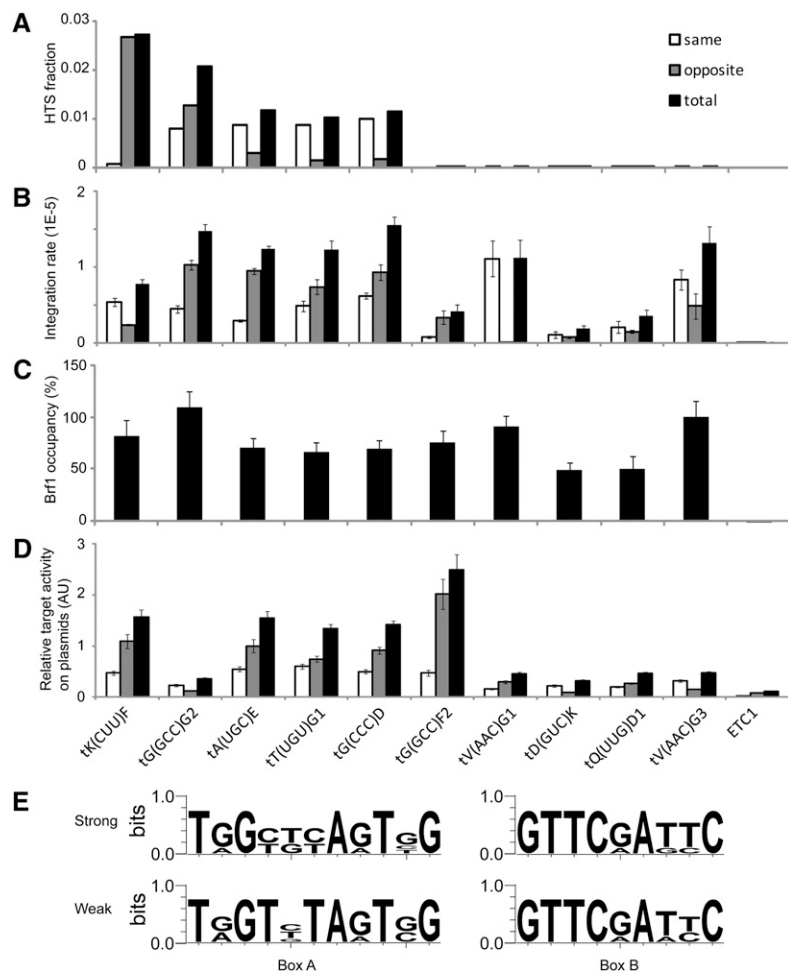


Figure 5. Quantitative Ty3 transposition at tDNAs with high and low DNA sequencing reads. (A) Distribution of sequencing reads from five top decile tDNA clusters and five bottom decile tDNA clusters. Yeast strains YMA1322 and YMA1356 transformed with Ty3-*HIS3* expression plasmid pKN3050 were induced for Ty3 expression for 24 h. Insertions into tDNA target clusters were ranked by number of reads, and example top and bottom decile genes (shown) were selected. *ETC1* was not detected by sequencing and was used as a negative test case. Bars show reads in which Ty3 and target are transcribed in the same (open) and divergent (gray) orientations and the total (black) is shown. (B) YMA1322 transformed with Ty3-ppt expression plasmid pKN3097 was induced for Ty3-ppt expression as described in A, and Ty3 integrations at reporter target loci were measured using qPCR (Methods). Measurements were within the linear range as standardized using plasmid versions of the Ty3 insertions at each locus. Bars represent technical triplicates of biological duplicates. (C) TFIIB occupancy of reporter Ty3 target loci. Yeast strain YKN1692 expressing TFIIB subunit Brf1 tagged with 3HA and transformed with empty *URA3* vector plasmid YCplac33 was grown under inducing conditions for 24 h, and Brf1 occupancy at reporter loci was measured using ChIP. ChIP values were determined relative to an *ACT1* control. The fold of enrichment for tDNA reporter *tV(AAC)G3* to *ACT1* was 73:1. The data are expressed relative to the fold enrichment at *tV(AAC)G3* set at 100%. (D) Ty3 integration frequencies at tDNA reporter loci and flanking sequence. YMA1322 transformed with Ty3-ppt plasmid pKN3097 and vector pXP622 containing reporter target loci and flanking sequence (120 bp upstream and downstream of target gene) was induced to express Ty3 for 36 h, and integrations were quantified by qPCR. Data are expressed as described in B. (E) Based on target activity (D), promoter elements in strong target loci [*tK(CUU)F*, *tA(UGC)E*, *tT(UGU)G1*, *tG(CCC)D*, and *tG(GCC)F2*] and weak target loci [*tG(GCC)G2*, *tV(AAC)G1*, *tD(GUC)K*, *tQ(UUG)D1*, and *tV(AAC)G3*] were analyzed for consensus using logo sequence analysis software (<http://weblogo.berkeley.edu>).

Based on in vitro assays, TFIIB occupancy is the major determinant of Ty3 targeting. ChIP was therefore performed in order to assess the correspondence between TFIIB occupancy and reporter targeting activity (Fig. 5C). YMA1322 was modified as previously described (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004) to express a 3HA-tagged Brf1 (Brf1-3HA; YKN1692). This strain was induced for Ty3 retrotransposition,

and cells were processed for qPCR-ChIP analysis. Eight genes, including the six genes with highest Ty3 targeting by qPCR, showed relatively high Brf1 occupancy; as reported previously (Moqtaderi and Struhl 2004), *ETC1* did not show significant TFIIB association.

To test the contribution of sequences in the immediate vicinity of the reporter tDNAs to targeting, the 10 reporter genes and flanking upstream and downstream 120 bp were subcloned and retested for Ty3 transposition (Fig. 5D). QPCR analysis was performed using primers that annealed within the Ty3 LTR U3 or U5 sequence and within the vector sequence downstream from the target. The signal for plasmid-borne tDNA was adjusted for plasmid copy number and normalized to the signal of the chromosomal *tF(GAA)* family. This family gives a robust qPCR integration signal after induction of Ty3-ppt (data not shown). The comparison showed that five of the genes, including three used at higher frequency as chromosomal targets, were used at levels threefold to sixfold higher than the other five targets. Only *tD(GUC)K* and *tQ(UUG)D1* were consistently low in Ty3 targeting in chromosomal and plasmid contexts and in Brf1 occupancy. Targeting to the chromosome and plasmid did not correlate ($R^2 = 0.29$), suggesting that local sequence played a greater role in plasmid-based targeting. Box B and box A motifs were generated from high- and low-frequency targets (Crooks et al. 2004; <http://weblogo.berkeley.edu>). The resulting box B motif conformed to the consensus (Fig. 5E), whereas more active and less active targets showed distinct versions of the consensus box A sequence (TRGYYYAGTNG vs. TRGTNTAGTNG, respectively). If this sample is representative, these findings indicate that chromosomal context and local sequence contribute to tDNA targeting, but targets bind similar amounts of TFIIB and overall differ by less than eightfold in activity.

Insertions at targets not known to be Pol III-transcribed genes

Previous efforts to identify Pol III-transcribed genes have used computational approaches to identify tDNA structures (Lowe and Eddy 1997), scans for box A and box B sequences and transcripts (Olivas et al. 1997), and ChIP-chip of inter-ORF regions (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004). As described above, excluding one tRNA pseudogene, 266 of the 296 clusters identified in our study corresponded to 262 uniquely aligning tDNAs [*tK(UUU)O*, *tN(GUU)P*, *tS(AGA)J*, *tY(GUA)M1* have two up-

stream clusters each]. Seven single Pol III–transcribed non-tDNA loci aligned uniquely with genome sequence. Eighteen other sequence clusters, including one at an unannotated Ty3 LTR at *RHB1*, were excluded as PCR artifacts generated from preexisting solo Ty3 LTRs. Five clusters identified unique sites in the genome but did not correspond to known Pol III–transcribed genes. Additional PCR using subcloned *YJL206C* failed to verify it as a reproducible target (Supplemental Table S4). Two were true targets (*SCS3* and *YIL100W*), and two were not cloned [*W(CCA)G1* downstream insertion and *YCG1*] (Supplemental Table S4).

Novel Ty3 clusters neither associated with Pol III transcription nor containing known Ty3 LTRs were verified inside ORFs *YIL100* (*W* and *C-A*) and *SCS3*. *YIL100W* and *YIL100C-A* designate a bi-directional ~100-codon ORF classified as dubious (<http://www.yeastgenome.org/cgi-bin/locus.fpl?locus=YIL100C>; henceforth, *YIL100W*) (Fig. 6A,B). *SCS3* encodes a gene required for inositol prototrophy (Hosaka et al. 1994). Regions of ~500 bp flanking insertion sites inside *YIL100W* and *SCS3* (*iYIL100W* and *iSCS3*, respectively) were cloned. YMA1322 transformants containing Ty3-ppt donor plasmid pKN3097 and each of the target plasmids were assayed for Ty3 transposition. De novo Ty3 insertions were

detected using combinations of Ty3- and plasmid-specific primers. Insertions were detected for *iYIL100W* and *iSCS3* in induced, but not uninduced cells. PCR products were cloned and sequenced. Insertions occurred at both cloned targets (Fig. 6B,C). We examined *YIL100W* and *SCS3* sequences for box A and box B promoter elements. In the case of Ty3 insertions upstream of tDNAs, the Ty3 junction is ~20 bp upstream of box A. Scanning of genomic sequence flanking the *iYIL100W* cluster revealed a box A–box B pair separated by 29 bp (Fig. 6A). The Ty3 cluster mapped 33 nt upstream of the 5' end of the box A sequence (Fig. 6A). To test whether, similar to other Ty3 insertion sites, targeting was dependent on active Pol III promoter elements, conserved bases in the box B consensus were mutated to disrupt Pol III promoter activity (Fig. 6A; Marck et al. 2006). YMA1322 transformants containing the Ty3 donor plasmid and wild-type or mutated *iYIL100W* plasmid were induced for transposition, and samples were assayed by PCR using Ty3- and target plasmid–specific primers. PCR products were generated from insertions upstream of *iYIL100W* in the wild type but not in the box B mutant clone (Fig. 6B).

The Ty3 insertions into *SCS3* detected by Tpn-seq were clustered centrally within the ORF. Inspection of the sequence surrounding these insertions showed that they were flanked by inverted box A–box B motifs (Fig. 6A,C). Ty3 insertion into plasmid-borne *SCS3* was investigated using an approach similar to that described for *YIL100W*. De novo Ty3 insertions clustered close to the position identified in Tpn-seq. The two box B motifs in *SCS3* were individually mutated (Fig. 6A,C), and the two resulting plasmid-borne targets were retested. Mutations in the rightward box B had no effect. In contrast, mutations in the leftward box B eliminated the majority of Ty3 integration-dependent PCR products. To verify localization of the targeting activity, the left and right halves of *SCS3* were subcloned and retested for targeting. As predicted, targeting activity was retained in the clone of the left half of *SCS3* but not the right half. This result was consistent with the existence of a Pol III promoter targeting Ty3 integration into the *SCS3* ORF (Fig. 6C). These results suggest that there are a limited number of sites in the yeast genome that are not annotated as Pol III transcription units but that may interact with Pol III initiation complexes or Pol III itself.

Discussion

Ty3 accesses the Pol III transcriptome

Before this study, chromosomal Ty3 elements and LTRs had been observed at ~15% of tDNAs (Chalker and Sandmeyer 1990; Goffeau et al. 1996). This study showed that Ty3 integrates close to the known or predicted TSS known Pol III–transcribed chromosomal loci. The targets include 274 tDNAs, as well as *SNR52*, *SNR6*, *RPR1*, *RDN5-1/2*, and *SCR1*. In addition, Ty3 integrated upstream of two loci, *ZOD1* and *RNA170*, predominantly occupied by TFIIC (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004), at which transcription is enhanced under conditions of nucleosome depletion (Olivas et al. 1997; Guffanti et al. 2006), and one locus, *iYGR033C*, for which transcription has not been observed (Guffanti et al. 2006).

Because of its distinctive targeting, Ty3 potentially offers an independent marker for sites of Pol III initiation complex assembly. Several arguments support the inference that Ty3 integration and TSS are tightly linked. First, where known for tDNAs, as well as *SNR6* and *RDN5* (Chalker and Sandmeyer 1992), the gene-proximal strand transfer of Ty3 cDNA coincides with the Pol III TSS and the

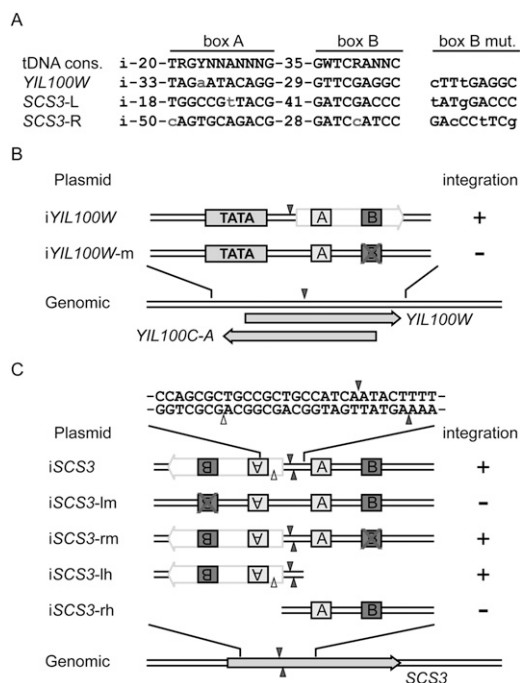


Figure 6. Ty3 targeting of novel loci requires Pol III promoter element. (A) Box A–box B pairs at *iSCS3* and *iYIL100W* target loci. (Top row) Approximate 20-nt spacing between integration sites (i) and box A and the consensus box A and box B sequence of fungal tDNAs, with an average spacing of 29 bp (Marck et al. 2006). Lowercase indicates divergence from consensus. (Right column, box B mut.) Box B mutations described in B and C. (B) Target activity of novel Ty3 target *iYIL100W*. YMA1322 transformed with pKN3050 and plasmids containing 500-bp *iYIL100W*-flanking sequence and a box B mutant derivative was induced to express Ty3 for 36 h. Integration was monitored by PCR (+/–). (Triangles) Insertion sites; (double line) chromosomal DNA; (TATA) upstream TATA-box consensus; (m) mutated box B (mutation sequence listed in A). (C) Target activity of *iSCS3*. As described in B, 500-bp *iSCS3*-flanking sequence and variants were tested for targeting. (Filled triangles) Insertion sites determined from sequence analysis; (empty triangles) those recovered from target plasmids; (lm and rm) mutated box B in loci shown; (lh and rh) half truncation as shown.

gene-distal strand transfer is 5 nt upstream. In our study, only 21% of cluster widths exceeded 11 bp, and within these clusters, integrations showed peaks offset by 5 nt on the two strands (e.g., Fig. 2; Supplemental Table S3; GEO <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31820>). Second, in vivo mutations in the transcription template that affect TSS selection similarly affect Ty3 integration site selection (Chalker and Sandmeyer 1992, 1993; Kirchner et al. 1995). Third, Brf1 and TBP, which support transcription initiation in vitro on templates with relaxed requirements for promoter opening (Kassavetis et al. 1999), also constitute the minimum Ty3 integration target (Yieh et al. 2000).

Ty3 integrations occurred at a small number of sites not anticipated to be Pol III TSS. These included sites at Pol III-transcribed *RNA170*, *tR(UCU)-tD(GUC)*, and *tW(CCA)G1*. It is possible that these insertions represent experimental artifacts, transient atypical interference by nucleosomes, or initiation factor occupancy not predicted by transcript mapping. In the case of *RNA170* and *tR(UCU)-tD(GUC)*, loci contain multiple potential matches to box A and box B consensus sequences. The 5' end of the *RNA170* transcript was mapped by primer extension to positions 7 and 15 nt upstream of box A; the minor 3' end mapped at the poly(T) tract, as is typical, but the major 3' end mapped upstream of both box B and the poly(T) tract (Olivas et al. 1997). Ty3 insertions mapped between the putative box A and a box B motif implicated by mutagenesis experiments (Olivas et al. 1997). We consider two possible explanations of the seemingly exceptional Ty3 integration site in *RNA170*. First, alternative positions of nucleosomes in the region of *RNA170* affect positioning of TFIIC and consequently TFIIB. Consistent with an effect of nucleosome positioning, *RNA170* transcripts are observed at low levels but are distinct from some other Pol III promoters in that they change significantly under conditions of nucleosome depletion (Guffanti et al. 2006). Second, alternative box A and box B promoter elements are associated with *RNA170*. Inspection of the sequence in the vicinity of the Ty3 insertion site shows a candidate box A–box B pair in the opposite orientation to those promoting *RNA170*. Thirty-nine nucleotides downstream from the Ty3 insertion site is a perfect box A (TGGTATATAAG), 28 nt after which is an imperfect box B (GTTCAGATC; the nucleotide for g is A in consensus). Thus, the Ty3 pre-integration complex could associate with TFIIB docked via conventional interactions with TFIIC bound to alternative promoter elements.

A second example of Ty3 integration at a site not associated with initiation is provided by the four *tR(UCU)-tD(GUC)* genes. A single dimeric pre-tRNA from this locus is observed in vitro (Kjellin-Straby et al. 1984; Engelke et al. 1985). In addition, the tR and tD members of a pair are closely spaced so that simultaneous occupancy of both genes by TFIIC and TFIIB would be predicted to be disrupted by steric interference. Surprisingly, integrations occurred associated with both *tR(UCU)* and *tD(GUC)* box A motifs. We conclude that it is likely that the downstream *tD(GUC)* actually binds the initiation factor TFIIB and is therefore competent to initiate transcription at some frequency.

Ty3 integrations reflect the general activity of Pol III-transcribed genes in yeast

Three ChIP-chip studies in *S. cerevisiae* (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004) showed that all but approximately a dozen annotated tDNAs bind TFIIC, TFIIB, and Pol III (Lowe and Eddy 1997; Olivas et al. 1997; Percudani et al. 1997; Hani and Feldmann 1998). In the Moqtaderi and Struhl

(2004) study, quantitative comparison of complexes at nine test tDNA genes using ChIP-qPCR showed robust binding averaging 60% of maximum for the reporter set. Even in the most extreme cases, factors bound with only fivefold to sevenfold differences in efficiency within the test gene set. Similarly, we observed that all Pol III-transcribed candidate genes were used by Ty3, and qPCR sampling of target usage in our study showed an eightfold range in targeting and twofold range in Brf1 occupancy.

Despite significant conservation of the Pol III transcription apparatus between single-celled and metazoan genomes (Huang and Maraia 2001), there is evidence for intriguing differences as well. As noted above, most Pol III genes in yeast are transcriptionally active. This contrasts with the situation in cultured human cells where recent studies showed that only about half of annotated tRNA genes bind TFIIB, TFIIC, and Pol III (Canella et al. 2010; Moqtaderi et al. 2010; Oler et al. 2010). Furthermore, in yeast, Pol III-transcribed genes (Hull et al. 1994) are repressive to local Pol II promoters (Kinsey and Sandmeyer 1991; D'Ambrosio et al. 2008; Haeusler et al. 2008), and condensin association, which also occurs at ETCs, is implicated in this repression (D'Ambrosio et al. 2008; Haeusler et al. 2008). In human cells, active Pol III genes and active Pol II promoters are associated to a significant extent (Moqtaderi et al. 2010; Oler et al. 2010), as are ETC and sequences with properties of enhancers (Moqtaderi et al. 2010; Oler et al. 2010).

Discrimination of ETC loci

Although TFIIC loading of TFIIB has been studied extensively in vitro, the process is less well understood in vivo. The observation of Ty3 integration at annotated Pol III-transcribed loci for which transcription factor binding and transcription have barely been detected suggests that it represents a very sensitive probe for bona fide Pol III-transcribed genes. The *S. cerevisiae* genome has approximately 3700 box A and box B pairs within 100 bp of one another (i.e., similar to *SNR6* box A and box B spacing) with a normalized log odds score (NLOD) >0.83 (above the similarity of many tDNAs to consensus) (Xie et al. 2009; data not shown). It was therefore striking that Ty3 integrated virtually exclusively at known Pol III transcription units. What discriminates transcribed loci from ETC and ETC from box A and box B pairs? One possibility is that requirements are compensatory so that the threshold is determined by the synergy of several features. Filtering the 3700 box A/B pairs for those with spacing of 35 bp or less, which is closer to the average distance separating tDNA box A and box B motifs, reduces the number to approximately 1700. With further filtering for a minimum NLOD score of 0.9, the number of pairs drops to about 360, more consistent with the number of Pol III-transcribed genes. One interesting possibility is that a significant subset of these sequences actually are associated with TFIIC. In examination of just over 400 sites of condensin binding in the yeast genome, D'Ambrosio et al. (2008) found that most contained box B motifs and were associated with at least a low level of TFIIC (less than found at tDNAs, for example). Interestingly, they also showed that box B alone was sufficient to mediate a low level of TFIIC association. Moqtaderi et al. (2010) observed an extended box B motif at yeast ETC loci but did not report box A sequences. Inspection of the eight yeast ETC loci shows that few have optimal box A and box B motifs within 35 bp. These observations raise the possibility that box A, which is proximal to the initiation site, also plays a role in the activity of TFIIC as a TFIIB loader. Consistent with that, the tDNA pseudogene *tD(GUC)N* with a single base

change in the box A consensus binds only TFIIC (Harismendy et al. 2003) and is not a Ty3 target. Although we examined only a small number of Ty3 targets by qPCR, weak targets shared a distinct box A motif, potentially supporting a role for the strength and position of box A elements in determining TFIIB loading by TFIIC.

Functional significance of association of retrotransposons and Pol III-transcribed genes

Ty3 targeted all genes known or predicted to be transcribed by Pol III. Ty3 clusters were also identified upstream of box A–box B pairs in *iYIL100W* and *iSCS3*. Although the transcripts predicted for Pol III transcription from these loci were not detectable by Northern blot analysis (data not shown), based on the overall specificity of Ty3 for Pol III-transcribed genes, we speculate that these loci are occupied at low levels by Pol III initiation complexes. Although *iYIL100W* is a dubious ORF, *iSCS3* is transcribed into poly(A) RNA (Nagalakshmi et al. 2008). These findings raise the possibility that Pol III transcription factor binding sites in Pol II-transcribed regions provide a mechanism for regulatory cross talk. An explicit example of this occurs at *ETC6*, which was recently implicated in auto-regulation of transcription of the gene encoding a TFIIC subunit, Tfc6 (Kleinschmidt et al. 2011).

In addition to highlighting potentially novel transcription initiation sites, the comprehensive association we observed between the Ty3 retrotransposon and Pol III-transcribed genes extends the intriguing relationship between retroelements and Pol III-transcribed genes. It was previously proposed that Pol III-transcribed genes could be dispersed by retrotransposition (Weiner et al. 1986). Since that time, LINEs (long interspersed elements) have been demonstrated to mobilize SINEs (short interspersed elements) (Dewannieux et al. 2003). Retrovirus particles, which are analogous to Ty3 virus-like particles, can, depending on the virus, be enriched for 7SL, U6, and 5S Pol III transcripts in addition to primer tRNAs (for review, see Onafuwa-Nuga et al. 2005). The pattern of transcripts hybridizing to Ty3 LTR probes indicates that readthrough from Ty3 into adjacent genomic sequence occurs (Clark et al. 1988). Thus, readthrough transcription from Pol III-specific retrotransposons into downstream genes is a plausible additional mechanism for inclusion of Pol III-transcribed sequences in virus-like particles. If these are reverse-transcribed, they would provide a pool of cDNAs that could promote dissemination or homogenization of Pol III-transcribed gene families.

Summary

In this study, we show that Ty3 provides a highly sensitive and irreversible probe for Pol III transcription initiation complexes. The absence of Ty3 integration into ETC sites underscores the question of what properties distinguish DNA-bound and condensin-associated TFIIC that loads TFIIB and allows Ty3 targeting from that which does not. The specificity of Ty3 association with Pol III-transcribed genes make Ty3 a useful tool for future exploration of the association of retrotransposons and Pol III-transcribed genes.

Methods

Bacterial and yeast strains and culture conditions

Yeast strains are described in Table 1A. Strain S288C derivative YPH500 (Johnston and Davis 1984; Sikorski and Hieter 1989) was modified, generating YMA1322 and its *rad52Δ* derivative YMA1356 (Aye et al. 2001).

Plasmids (Table 1B) were prepared from DH5 α [$F^- \phi 80lacZ\Delta M15 \Delta$ (*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17*($r_K^- m_K^+$) *phoA supE44 thi-1 gyrA96 relA1 \lambda^-] (Invitrogen) transformants.*

For ChIP, YMA1322 was modified by epitope tagging Brf1 with three copies of the hemagglutinin epitope (3HA). The 3HA coding sequence was introduced in-frame at the downstream end of *BRF1* in YMA1322 (Longtine et al. 1998) to create yeast strain YKN1692. Brf1 was previously tagged using a similar strategy and was shown not to affect growth of the tagged strain (Harismendy et al. 2003; Roberts et al. 2003; Moqtaderi and Struhl 2004). Correct integrants were confirmed by PCR using Brf1-specific primers KN2485 and KN2411 and verified by sequencing with KN2485. Western blot analysis was performed using α -3HA to verify expression of the tagged protein (data not shown).

Yeast and bacterial culture methods were as previously described (Amberg et al. 2005; Ausubel et al. 2007). Synthetic complete medium lacking His, Leu, or Ura (0.67% yeast nitrogen base, 0.2% amino acid drop-out mix) was supplemented to 2% glucose (SD), 2% galactose (SGal), or 1% raffinose, 2% (v/v) glycerol, 2% (v/v) lactic acid (SR) (Burke et al. 2000). Rich medium was 1% yeast extract 2% peptone, 2% dextrose (YPD).

Plasmid constructions

Standard cloning procedures were used throughout (Ausubel et al. 2007). Ty3-ppt-*HIS3* was expressed under control of the *GAL1-10* UAS from a *URA3*-marked low-copy plasmid (pKN3050). Ty3 derived from pNB2361 and containing an antisense insertion of *HIS3* (Beliakova-Bethell et al. 2009) at the downstream end of *POL3* was recloned into YCplac33 (ATCC), and unique 60-bp sequence tags were introduced flanking *HIS3*. Because Ty3 *POL3* extends into the downstream LTR, insertion of the *HIS3* gene resulted in an unfavorably long downstream LTR. The LTR sequence was reconstituted downstream from *HIS3*. This Ty3 expression plasmid was designated pKN3050. Plasmid pKN3050 was cleaved with *Afl*III and *Sbf*I and religated, thereby removing one tag and the *HIS3* gene and creating pKN3097 (Table 1A). Positive clones were verified by DNA sequencing. This and other sequence analysis were performed at Genewiz Inc. Details of this construction are provided in the Supplemental Methods.

Testing integrations at specific loci

To test specific loci for target activity, 300–600-bp DNA fragments containing the loci were amplified in PCR reactions. To construct the plasmids used as qPCR standards for measurement of integration at specific chromosomal loci, tDNAs with a segment of flanking Ty3 insertion were amplified and cloned into the pCR2.1 vector (Invitrogen). Details of the constructions are provided in the Supplemental Methods. Oligonucleotide primers are listed in Supplemental Table S2. Product plasmids are listed in Supplemental Table S1.

Ty3 transposition

For HTS analysis of Ty3-*HIS3* insertion sites, YMA1322 and YMA1356 transformed with the Ty3-*HIS3* expression plasmid pKN3050 were grown to log phase in SR–Ura, –His at 24°C (RT). From each culture, 2×10^6 transformants were transferred to each of 10 100-mm plates of SGal–Ura, –His medium or on SD–Ura, –His medium as negative control and maintained for 3 d to allow Ty3 expression. Subsequent steps were performed for both tested conditions. Cultures were replica-plated onto YPD and incubated for 24 h at 30°C. Colonies were replica-plated onto SD–His medium supplemented with 0.1% 5-fluoro-orotic acid (5FOA) (Boeke

et al. 1984) to select for cells that had lost the *URA3*-marked donor plasmid but contained Ty3-*HIS3* chromosomal integrants. On average, there were $\sim 10^3$ integrants per plate for Ty3-induced samples and 20–40 colonies for negative-control samples. Approximately 10,000 colonies each for YMA1322 and YMA1356 cultures were scraped from plates. Harvested cells were inoculated into 10 mL of SD–His and incubated for 24 h at 30°C on a rotating wheel. Genomic DNA was extracted according to standard procedures (Ausubel et al. 2007).

For quantitative comparison of transposition into different chromosomal target loci, yMA1322 transformed with Ty3-ppt donor plasmid pKN3097 was grown to log phase in SR–Ura medium, diluted into 25 mL of SGal–Ura medium to a final concentration of 0.2 OD₆₀₀, and grown with shaking at 180 rpm, for 24 h at room temperature. As negative control, cultures were diluted into 5 mL of SD–Ura medium to 0.2 OD₆₀₀ and allowed to grow for 24 h at room temperature. Quantitative comparison of transposition into plasmid-borne targets was performed similarly except that –Ura –Leu medium was used to select for donor and target plasmids. Slower growth necessitated extending the SGal –Ura –Leu incubation from 24 h to 36 h.

Analysis of Ty3 transposition using Illumina GAIx sequencing (Tpn-seq)

Ty3–host junctions were identified using an inverse (i) PCR strategy (Fig. 1; Ochman et al. 1988; Wang et al. 2008). Samples were processed as follows: (1) gDNA was digested independently with three different 4-bp cutters (Csp6I, HaeIII, and HpyCH4V). (2) Cleaved DNA was circularized in a ligation reaction. (3) DNA containing Ty3 U5 end joint with genomic DNA was amplified by iPCR using primers within the Ty3 U5 and tag1. (4) Samples were submitted to the Genetics Department Sequence Facility, Washington University (St. Louis, MO) for analysis using the Illumina GAIx. Details are provided in the Supplemental Methods.

Chromatin immunoprecipitation

For quantitative comparison of Brf1 occupancy of target loci, YKN1692 transformed with plasmid YCplac33 was grown under conditions described for Ty3 induction. Samples were processed for ChIP analysis essentially as previously described (Strahl-Bolsinger et al. 1997). Details of the processing are provided in the Supplemental Methods.

Quantitative PCR

Nucleic acid was extracted according to standard procedures (Ausubel et al. 2007). Samples were treated with RNase A (0.2 μg/μL), and DNA was purified using Microspin-30 columns (Bio-Rad). DNA concentrations were measured on a NanoDrop-1000 (Thermo Scientific Inc., NanoDrop products).

QPCR was conducted in an iCycler (Bio-Rad). The total volume of 25-μL reactions contained 1× iQ SYBR Green Super Mix (Bio-Rad), 0.4 μM each primer, and various amounts of DNA template. Serial dilutions of standard plasmids of known concentration were used to construct a standard curve. All reactions were performed in triplicate. Thermal cycling included 40 cycles of extension (30 sec at 95°C, 30 sec at 55°C, 30 sec at 72°C). Fluorescence was captured after each extension step. PCR products were analyzed by melt curves and electrophoresis.

QPCR of DNA samples containing integrations at chromosomal loci were standardized using plasmid templates constructed as described above to contain the U5 or U3 end of the Ty3 LTR juxtaposed to each specific target. QPCR primers are described

in Supplemental Table S2. Chromosomal *ACT1* copy number determined by qPCR using forward primer XQ3563 and reverse primer XQ3564 was used to normalize the integrations determined in different samples.

For plasmid target experiments, standard curves were used to determine the copy numbers of: (A) Ty3 integrations at the target plasmids using primers XQ2603/XQ3313 for U5 orientation, and using 3135/3582 for U3 orientation; (B) total plasmids using primer pair JY3435/3436; (C) Ty3 integrations at chromosomal tF(GAA) family members using primer pair XQ2603/3621; and (D) total chromosomal DNA, indicated by *ACT1* copy number, using primer pair XQ3563/XQ3564. Relative integration was calculated as the ratio on plasmids (A/B) normalized with the integration ratio on chromosome (C/D): (A/B)/(C/D). Duplicate samples were used, and the mean of relative integrations was plotted.

For quantification of Brf1 binding, 2 μL of ChIP DNA sample containing 1–5 ng of DNA was used in a 25-μL qPCR reaction. QPCR was conducted in the linear range for each set of primers determined using a serial dilution of template DNA. Primers are listed in Supplemental Table S2. Occupancy values (in arbitrary units) were calculated as described previously (Moqtaderi and Struhl 2004). The apparent immunoprecipitation (IP) efficiency (i.e., the amount of PCR product in the IP sample divided by the amount of PCR product in the input sample) was divided by the apparent cross-linking efficiency of the *ACT1* control DNA segment in each sample. The resulting relative IP efficiency of *ACT1*, 1.0, was then subtracted from all relative values to yield a background of zero.

Computational analysis

HTS image files were converted to fastq files using Illumina pipeline software. Each sequence began with the last 17 nt of the Ty3 LTR, and HTS reads that did not initiate with this sequence were discarded. For remaining sequences, the LTR barcode was truncated, and the genomic sequence was extracted. Sequence reads with <19 nt of genomic sequence were also excluded from further analysis. Retained reads were mapped to the yeast genome sequence downloaded from the UCSC Genome Browser (build sacCer2 June 2008) (Rhead et al. 2010) using Bowtie (v 0.9.8.1) (Langmead et al. 2009). Only reads with no mismatches were kept for further analysis. Reads that matched multiple genomic loci were tagged for separate analysis.

Mapped sequence reads were converted to insertion sites defined by chromosome number, strand (Watson or Crick), and coordinate. The read density of an insertion site was defined as the number of reads that mapped to that site. Insertion sites with densities less than 5 in either data set were excluded from further analysis. Neighboring insertion sites were linked into clusters by aggregating any two sites that were no more than 10 nt apart, regardless of strandedness. Thus, a cluster is defined by a genomic interval, delimited by two addresses. This procedure yielded a total of 296 clusters using only reads mapping to unique genomic locations. The read density of a cluster is the sum of the read densities of the insertion sites in the cluster. The event density of a cluster is the number of unique insertion sites associated with the cluster. Clusters are described in Supplemental Table S3.

Each of the cluster intervals was used to find nearby genomic features based on publicly available genome-wide annotations. The locations of Pol III–transcribed genes, ORFs, ARSs, and LTRs were downloaded from the *Saccharomyces* Genome Database (<http://www.yeastgenome.org/>, Dec 2009). Box A and box B sequences associated with Pol III–transcribed genes were from Marck et al. (2006).

Sequence processing and annotation were conducted using a variety of tools. In particular, the MotifMap pipeline (Xie et al.

2009) and the open source Python package Pygr (Alekseyenko and Lee 2007) were used. All data processing and manipulation were done with Python (van Rossum and de Boer 1991), and statistical analyses were computed with R (R Core Development Team 2009).

Feature density for Pol III–transcribed genes, LTRs, and ARS sequences represents the average number of occurrences of a given feature within a sliding window from 7 kb upstream to 7 kb downstream of the cluster center point.

Data access

The original fastq files are available at the NCBI Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under accession no. GSE31820.

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