

The pheromone response pathway activates transcription of Ty5 retrotransposons located within silent chromatin of *Saccharomyces cerevisiae*

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The *Saccharomyces* retrotransposon Ty5 integrates preferentially into transcriptionally inactive regions (silent chromatin) at the *HM* loci and telomeres. We found that silent chromatin represses basal Ty5 transcription, indicating that these elements are encompassed by silent chromatin in their native genomic context. Because transcription is a requirement for transposition, integration into silent chromatin would appear to prevent subsequent rounds of replication. Using plasmid-borne Ty5-*lacZ* constructs, we found that Ty5 expression is haploid specific and is repressed 10-fold in diploid strains. Ty5 transcription is also regulated by the pheromone response pathway and is induced ~20-fold upon pheromone treatment. Deletion analysis of the Ty5 LTR promoter revealed that a 33 bp region with three perfect matches to the pheromone response element is responsible for both mating pheromone and cell-type regulation. Transcriptional repression of Ty5 by silent chromatin can be reversed by pheromone treatment, which leads to transcription and transposition. Ty5 replication, therefore, is normally repressed by silent chromatin and appears to be induced during mating. This is the first example of transcriptional activation of a gene that naturally resides within silent chromatin.

Keywords: pheromone response pathway/
retrotransposon/silent chromatin/telomere/Ty5

Introduction

Heterochromatin is the darkly staining, highly condensed regions of chromosomes that are observed during interphase near the centromeres and telomeres. A widely studied feature of heterochromatin is the repressive effect it has on gene expression (e.g. see Weiler and Wakimoto, 1995). For example, when euchromatic genes are placed in or near regions of heterochromatin, their expression is often silenced. This silencing is sometimes unstable and may result in variegated phenotypes among genetically identical cells (referred to as position effect variegation). Besides transcriptional repression, heterochromatin is characteristically late replicating and is often associated with the nuclear periphery.

Heterochromatin is typically composed of satellite sequences and middle repetitive DNA, the latter of which

includes transposable elements. For example, transposable elements are structural components of *Drosophila melanogaster* heterochromatin (Pimpinelli *et al.*, 1995), are enriched in terminal or paracentric heterochromatin in some plants (Pelissier *et al.*, 1996; Brandes *et al.*, 1997) and are often found, along with endogenous retroviruses, in heterochromatic Y chromosomes (Steinmann and Steinmann, 1992; Kjellman *et al.*, 1995). Transposable elements may accumulate in heterochromatin because they are not eliminated by recombination, which is typically lower in heterochromatic regions. Alternatively, this accumulation could be explained if transposable elements preferentially integrate into heterochromatic sites. Such targeted integration may be an adaptive feature of transposable elements; heterochromatin typically contains fewer genes than euchromatin, and therefore integration is likely to be less deleterious to the host. Targeting to telomeric regions has been demonstrated for the *D.melanogaster* *HeT-A* and *TART* elements, and the yeast transposable element Ty5 (Biessmann *et al.*, 1990; Levis *et al.*, 1993; Zou *et al.*, 1996a).

Transposable elements also accumulate at other chromosomal regions, which like heterochromatin have a low gene density and repress genes transcribed by RNA polymerase II. For example, many maize transposable elements are clustered in highly methylated intergenic regions, which are characterized by low transcriptional activity (Bennetzen *et al.*, 1994; SanMiguel *et al.*, 1996). The *Saccharomyces cerevisiae* Ty1 and Ty3 retrotransposons integrate preferentially upstream of genes transcribed by RNA polymerase III (e.g. tRNA and 5S rDNA genes) (Chalker and Sandmeyer, 1992; Devine and Boeke, 1996). Transcription of tRNA genes can inhibit transcription of flanking RNA polymerase II promoters (Kinsey and Sandmeyer, 1991; Hull *et al.*, 1994), and genes in rDNA arrays are transcriptionally silenced (Bryk *et al.*, 1997; Smith and Boeke, 1997). The association of transposable elements with regions of transcriptional repression, therefore, appears to be a widespread phenomenon.

Ty5 is one of five retrotransposon families in *S.cerevisiae* (Boeke and Sandmeyer, 1991; Zou *et al.*, 1996a). Like all retroelements, Ty5 replicates through an RNA intermediate. Its life cycle begins with transcription, which generates a genomic mRNA. After translation of this message, a virus-like particle is formed in which a cDNA copy of the element is synthesized by reverse transcription. This cDNA is then integrated into the chromosome by an element-encoded integrase. Studies from our laboratory have demonstrated that Ty5 preferentially integrates into regions of silent chromatin at the telomeres and silent mating loci (Zou *et al.*, 1996a,b; Zou and Voytas, 1997). These regions share features with heterochromatin of other eukaryotes in that they are transcriptionally silent, late replicating and associated with the nuclear membrane

(Gottschling *et al.*, 1990; Laurenson and Rine, 1992). The transcriptional repression characteristic of Ty5 targets presents a dilemma for this element: how can Ty5 initiate additional rounds of replication while embedded in repressive chromatin? We explored the transcriptional regulation of Ty5 to understand better how this retrotransposon has adapted to its chromosomal context.

Results

Transcription of Ty5 is repressed by silent chromatin

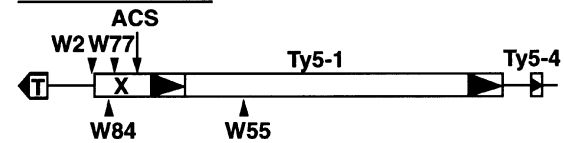
Saccharomyces cerevisiae does not have functional Ty5 elements, so we developed a Ty5 transposition assay using a transposition-competent Ty5 element from the related species *Saccharomyces paradoxus* (Zou *et al.*, 1996a,b; Zou and Voytas, 1997). We found that Ty5 primarily integrates into transcriptionally inactive regions of silent chromatin at the telomeres and *HM* loci. To determine whether silent chromatin affects Ty5 transcription, expression of chromosomal Ty5 insertions was measured using a reverse transcription-mediated polymerase chain reaction (RT-PCR) strategy. Endogenous *S. cerevisiae* Ty5 elements have an integrase deletion, so integrase-specific primers were designed that only detect transcripts from previously mapped *de novo* Ty5 insertions (Zou *et al.*, 1996a,b). Transcription was measured for four insertions at *HMR-E* (W9, W113, W76 and W66) and four at the chromosome III left telomere (W2, W77, W84 and W55) (Figure 1). A plasmid-borne Ty5 (W200) and a Ty5 insertion at an internal region of chromosome XI (W3) were used as controls. The expected ~1.1 kb PCR product was observed for the controls, indicating that these elements are expressed (Figure 2A). Restriction enzyme digestion confirmed that the major PCR product originated from Ty5 (data not shown). No PCR products were observed for the eight Ty5 insertions in silent chromatin. Transcripts specific for the transcription factor *SNF6* were amplified from each reaction to serve as an internal control (Estruch and Carlson, 1990), and a band of similar intensity was observed for all reactions (Figure 2B).

Ty5 expression is under pheromone and cell-type regulation

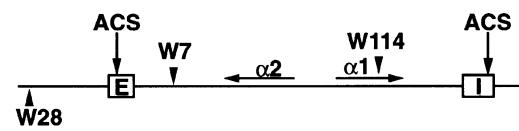
Although Ty5 transcription is normally repressed by silent chromatin, it may be regulated by internal or external stimuli that result in transcription and, ultimately, transposition. Analysis of the Ty5 LTR revealed three perfect matches (although in opposite orientation) to the 7 bp pheromone response element [PRE; (A)TGAAACA; Figure 3]. PRE sequences are normally found in the promoters of pheromone response genes, which encode proteins involved in mating (Sprague and Thorner, 1992). Yeast has three cell types, haploid *a* and α cells and diploid *a*/ α cells, which are determined by the gene products encoded by the *MAT* locus. During mating, the binding of yeast pheromones (*a*- or α -factor) to receptors on haploid cells of the opposite mating type triggers a signal transduction cascade that culminates in the activation of Ste12p, a transcription factor that binds PRE sequences and activates transcription of pheromone response genes (Figure 4). Using a plasmid-borne Ty5-*lacZ* fusion construct as a reporter, we found that

Chromosome III insertions:

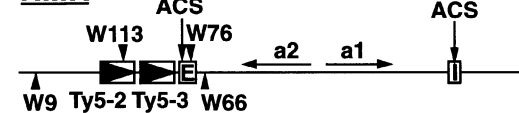
Left telomere



HML

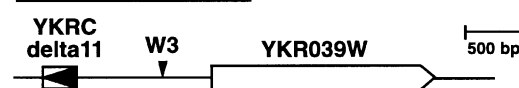


HMR



Controls:

Chromosome XI



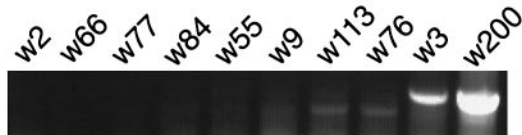
Ty5 on a 2μ plasmid: W200

Fig. 1. Ty5 insertions used in this study. Locations of Ty5 insertions are indicated by short vertical arrows and strain designations. Arrows pointing down indicate Ty5 insertions in the same orientation as the chromosome sequence, while arrows pointing up indicate Ty5 insertions in the opposite orientation. T at the left telomere designates the telomeric TG₁₋₃ repeats; X designates the subtelomeric X repeat; E and I at *HML* and *HMR* designate the flanking transcriptional silencers. The ARS consensus sequences (ACSs) in the X repeat and E and I silencers are indicated by the long vertical arrows. Ty5-1 to Ty5-4 are endogenous Ty5 insertions. On chromosome XI, YKRCdelta11 is a Ty1 insertion and YKR039W is an anonymous open reading frame.

β -galactosidase activity in *a* cells treated with α -factor was induced 19.3-fold compared with the control (Table I). This induction required genes in the pheromone response pathway, because strains with mutations in three components of the signal transduction cascade (*STE7*, *STE11* and *STE12*) failed to induce Ty5 transcription in response to α -factor (Table II). The levels of induction for wild-type cells were not as high as those given in Table I, probably due to differences in strain background and culture growth between experiments. Strains with the three *STE* mutations also showed a decrease in levels of uninduced Ty5 transcription, suggesting that basal activity of the pheromone response pathway may play a role in basal Ty5 transcription.

Ty5 expression was also found to be regulated by cell type. While comparable levels of β -galactosidase activities were observed in haploid *a* and α cells, activity was ~10-fold lower in the diploid strain (Table I). This repression seems to be mediated by the co-expression of both mating type information, because repression was observed in

A RT-PCR using Ty5-specific primers



B RT-PCR using SNF6-specific primers

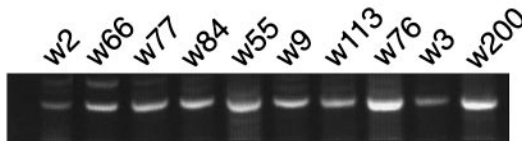


Fig. 2. Ty5 transcription is repressed by silent chromatin. RT-PCR analysis was conducted to determine the expression of Ty5 insertions at the chromosome III left telomere and *HMR* in *S.cerevisiae* strain W303-1A. A Ty5 insertion at an internal site on chromosome XI (W3) and a plasmid copy of Ty5 (W200) were used as controls. The PCR amplification products are shown for Ty5 integrase-specific primers (A) and for *SNF6*-specific primers (B), which served as an internal control. Authenticity of the major amplification products was confirmed by restriction enzyme analysis (data not shown).

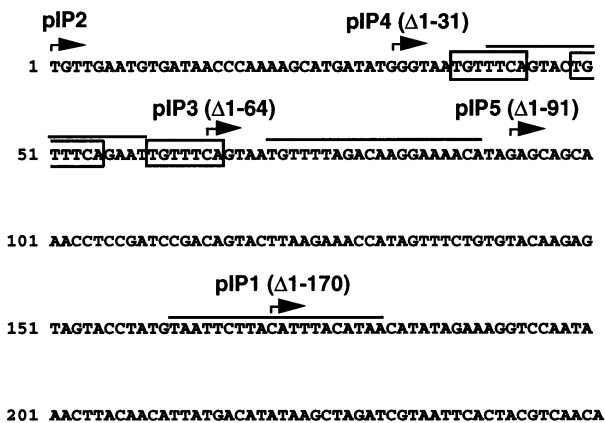


Fig. 3. Analysis of Ty5 LTR sequences. The 5' Ty5 LTR sequence is shown. Boxes denote sequences that have similarities to PRE sequences. Lines above the sequence denote regions of similarity to the consensus a1-α2 binding site. The labeled arrows indicate the positions of deletions in the various LTR promoter constructs.

MATα cells in which *HMRa* was derepressed by an *HMR-E* silencer deletion (Table I). Three proteins, *Mata1p* (encoded by *MATa*), *Matα1p* and *Matα2p* (encoded by *MATα*) determine cell-type regulation (Herskowitz *et al.*, 1992). When all three proteins are expressed, such as in diploid cells, the *Mata1p*-*Matα2p* complex represses transcription of haploid-specific genes by binding to a1-α2 binding sites in their promoters (Miller *et al.*, 1985; Siliciano and Tatchell, 1986; Company and Errede, 1988; Bilanchone *et al.*, 1993). Three sequences within the Ty5 LTR are similar to the consensus a1-α2 binding sites and may mediate cell-type control (nt 161-181, 80% similarity; nt 71-90, 75% similarity; nt 41-60, 60% similarity) (Figure 3).

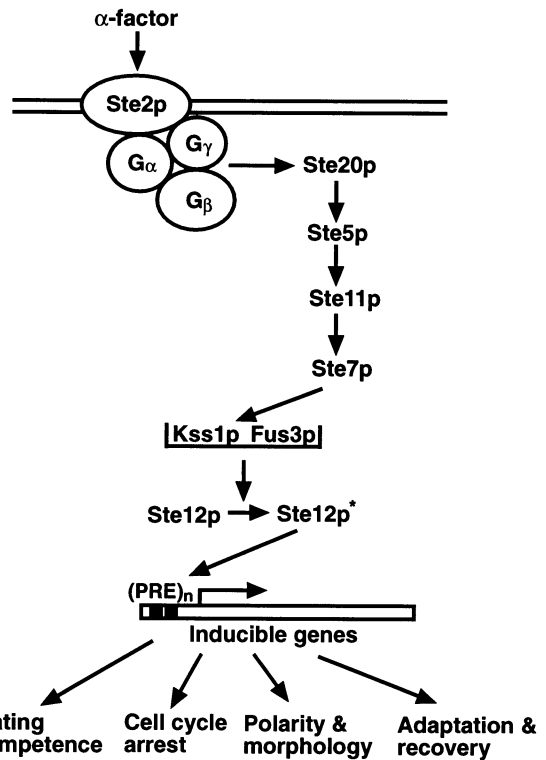


Fig. 4. The pheromone response pathway. The binding of α-factor to its receptor (Ste2p) initiates the pheromone response signal transduction cascade in *MATa* cells. The receptor then interacts with the heteromeric G protein, which in turn activates components of the protein kinase cascade (Ste20p, Ste5p, Ste11p, Ste7p, Kss1p and Fus3p). Activated Ste12p (indicated with an asterisk) binds PRE sequences and induces transcription of genes involved in mating. The figure is adapted from Sprague and Thorner (1992).

Table I. Transcription of Ty5-*lacZ* fusions is pheromone- and cell-type-regulated

Strain	β-galactosidase activity (SD)	Fold difference compared with <i>MATa</i>
<i>MATa</i> / <i>MATα</i>	0.607 (0.136)	0.1
<i>MATa</i>	7.05 (1.66)	1.0
<i>MATα</i>	6.60 (2.19)	0.9
<i>MATa</i> + α-factor	136.1 (44.1)	19.3
<i>MATα</i> <i>HMR-E</i> (ΔΔΔEΔB)	0.658 (0.290)	0.1

β-galactosidase activity for three independent transformants was calculated as described in Materials and methods. Activity units were standardized with respect to reaction time and cell number. SD, standard deviation.

Cis-acting sequences that mediate pheromone and cell-type regulation

Deletion analysis of the Ty5 LTR was conducted to identify the *cis*-acting sequences that mediate pheromone and cell-type regulation. Four LTR deletion constructs were made by PCR-based mutagenesis, which removed 31 bp (Δ1-31), 64 bp (Δ1-64), 91 bp (Δ1-91) or 170 bp (Δ1-170) from the 5' end of the LTR (Figure 3). Δ1-170 was used as a negative control, since base 176 marks the transcription start site for Ty5 (Zou *et al.*, 1996a). The LTR deletions and 361 bp of internal sequences were then fused in-frame to *lacZ* and introduced into various yeast strains to measure transcription.

LTR deletions up to nt 91 did not have much effect on

Table II. Pheromone-induced transcription of Ty5 requires genes in the pheromone response pathway

Strain	Genotype	β -galactosidase activity			
		Basal	Basal relative to wild type	Induced	Fold induction ^a
EG123	wild type	129 \pm 5	1.0	504 \pm 32	3.9
DC40	EG123 (<i>ste11</i> - Δ 1)	19.4 \pm 4.6	0.2	19.2 \pm 3.8	1.0
L5528	wild type	88.1 \pm 3.1	1.0	547 \pm 37	6.2
L5559	L5528 (<i>ste7</i>)	23.5 \pm 2.0	0.3	25.1 \pm 1.9	1.1
L5573	L5528 (<i>ste12</i>)	20.9 \pm 0.5	0.2	21.5 \pm 2.3	1.0

β -galactosidase activity was calculated as described in Materials and methods. The values shown are the average of three independent experiments. Note that two strain backgrounds were used for the experiments.

^aFold induction was calculated by dividing the induced activity by the basal activity.

Table III. *Cis*-acting sequences mediating pheromone induced Ty5 transcription

Construct (in W303-1A)	β -galactosidase activity			
	Basal	Basal relative to full-length LTR	Induced	Fold induction ^a
pIP2 (LTR)	20.3 \pm 11.1	1.0	386.7 \pm 77.2	19.0
pIP4 (Δ 1–31)	24.6 \pm 2.7	1.2	428.9 \pm 35.8	17.4
pIP3 (Δ 1–64)	20.7 \pm 7.3	1.0	50.1 \pm 7.2	2.4
pIP5 (Δ 1–91)	27.5 \pm 2.1	1.4	42.0 \pm 6.4	1.5
pIP1 (Δ 1–170)	1.62 \pm 1.60	0.1	1.67 \pm 1.47	NA
YEp356R	-0.11 \pm 0.52	NA	0.84 \pm 1.95	NA

β -galactosidase activity was calculated as described in Materials and methods. The values shown are the average of three independent experiments. NA, not applicable.

^aFold induction was calculated by dividing the induced activity by the basal activity.

Table IV. The effect of LTR deletions on cell-type-regulated Ty5 transcription

Construct	β -galactosidase activity			Fold repression ^a
	<i>MATa</i>	<i>MATα</i>	<i>MATa</i> / α	
pIP2 (LTR)	33.8 \pm 8.1	41.7 \pm 2.3	4.49 \pm 1.34	7.5 (9.3)
pIP4 (Δ 1–31)	76.2 \pm 5.4	55.9 \pm 2.9	7.77 \pm 1.30	9.8 (7.2)
pIP3 (Δ 1–64)	34.2 \pm 1.6	35.1 \pm 5.3	18.1 \pm 4.1	1.9 (1.9)
pIP5 (Δ 1–91)	41.4 \pm 1.5	47.1 \pm 12.7	15.9 \pm 2.4	2.6 (3.0)
pIP1 (Δ 1–170)	1.03 \pm 0.24	0.61 \pm 0.83	0.46 \pm 0.15	NA
YEp356R	-0.11 \pm 0.52	NA	0.84 \pm 1.95	NA

β -galactosidase activity was calculated as described in Materials and methods. The values shown are the averages of three independent experiments. NA, not applicable.

^aFold repression was calculated by dividing the average basal activity in haploid *a* strains or α strains (in parentheses) by the activity in the diploid strains.

the basal levels of β -galactosidase activity (Table III). However, the negative control (Δ 1–170) abolished basal transcription completely. This suggests that the Ty5 core promoter is between nt 91–170. When treated with α -factor, β -galactosidase activities for constructs with the full LTR and Δ 1–31 were induced 19.0- and 17.4-fold respectively, while Δ 1–64 and Δ 1–91 were induced only 2.4- and 1.5-fold respectively. This indicates that the *cis*-acting sequences mediating α -factor induction are between nt 31 and 64, corresponding well with the location of the putative PRE sequences.

β -galactosidase activities were also determined for the LTR deletion constructs in strains of different cell type (Table IV). Consistent with the data in Table III, the levels of activities for deletions up to nt 91 were very similar in the haploid strains. In diploid strains, however, the repres-

sion decreased from 7.5- and 9.8-fold for the full-length LTR and Δ 1–31 constructs, to 1.9- and 2.6-fold for the Δ 1–64 and Δ 1–91 constructs. The *cis*-acting sequences mediating cell-type control, therefore, also reside between nt 31 and 64.

α -Factor induces transcription of Ty5 elements in silent chromatin

Although the Ty5 promoter is responsive to pheromone controls, most chromosomal Ty5 elements are located within silent chromatin, which typically occludes the transcription machinery. To determine whether pheromone-induced transcription can overcome this barrier, transcription was evaluated for representative Ty5 elements at *HMR* (W76) and the chromosome III left telomere (W77). The chromosome XI insertion (W3) and the

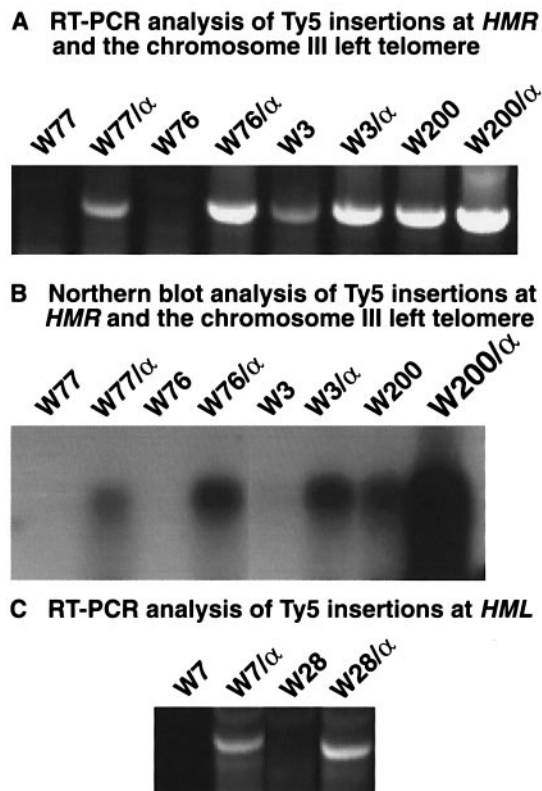


Fig. 5. Transcription of Ty5 insertions in silent chromatin is induced by α -factor. RT-PCR and Northern blot analysis were conducted to determine whether α -factor can induce Ty5 transcription within silent chromatin. (A) RT-PCR amplification products are shown for Ty5 insertions at *HMR* (W76) and the chromosome III left telomere (W77) that have been treated with or without α -factor. A Ty5 insertion on chromosome XI (W3) and a plasmid copy of Ty5 (W200) were used as controls. (B) Northern blot analysis with the same strains as in (A). (C) RT-PCR amplification products are shown for Ty5 insertions at *HML* (W7 and W28).

plasmid Ty5 element (W200) were used as controls. No basal transcription was observed for Ty5 elements located in silent chromatin, whereas a distinct PCR product was detected for the induced cells (Figure 5A). This demonstrates that even in silent chromatin, Ty5 transcription can be induced by α -factor. Similar results were obtained by Northern blot analysis (Figure 5B). In an independent experiment, transcription of two representative Ty5 insertions at *HML* (W7 and W28) was measured with or without α -factor treatment by RT-PCR (Figure 5C). As with *HMR* and telomeric insertions, transcription of Ty5 at *HML* is repressed by silent chromatin and induced by α -factor.

Ty5 transposition is induced by α -factor

Because transcription is the first step in the life cycle of retroelements, the transcriptional induction of Ty5 insertions within silent chromatin by α -factor suggests that pheromone exposure may also induce transposition. To test this, the *HIS3* marker genes carried by chromosomal Ty5 insertions at *HML*, *HMR* and the chromosome III left telomere were replaced with a *HIS3* gene interrupted by an artificial intron (*his3AI*) (Curcio and Garfinkel, 1991). This marker makes it possible to select new transposition events genetically, because the *HIS3* gene is only reconstituted after transcription, splicing of the intron, and

Table V. α -Factor-induced transposition of Ty5

Strain	Insertion (location)	Number of His ⁺ cells	
		+ α -factor	- α -factor
YNK562	W114 (<i>HML</i>)	0	35
YNK566	W9 (<i>HMR</i>)	1	22
YNK567	W2 (telomere)	0	7
YNK568	W77 (telomere)	0	20
YNK570	W3 (internal)	15	27

Equivalent numbers of cells (1 ml of culture, OD₆₀₀ = 3.0) were plated on to SC-H plates. His⁺ colonies were scored after 2.5 days' growth at 30°C.

reverse transcription of the Ty5 mRNA. The chromosome XI insertion (W3) was used as a positive control. Consistent with the transcription data, no transposition events (except one for W9) were detected in untreated strains that had Ty5 elements in silent chromatin (Table V). In contrast, the constitutively expressed W3 control had a relatively high transposition frequency even in uninduced conditions, which was increased two-fold by pheromone treatment. For strains with elements in silent chromatin, transposition was significantly induced by α -factor, suggesting that pheromones released during mating initiate the transposition process.

Discussion

Retroelement replication may have detrimental effects on the host due, in part, to integration into coding sequences. As a consequence, retroelement expression is often regulated at the transcriptional, translational and post-translational levels, as well as through targeted integration (Boeke and Sandmeyer, 1991; Farabaugh, 1995). Targeting is a salient feature of the Ty5 life cycle. Ty5 elements avoid most coding regions by integrating preferentially into regions of silent chromatin at the telomeres and silent mating loci (Zou *et al.*, 1996a,b). We have found that silent chromatin at Ty5 integration sites regulates Ty5 by repressing basal transcription. This appears to be somewhat suicidal for an element that requires transcription to replicate. Ty5 elements in silent chromatin, however, can be transcriptionally induced by the signal transduction pathway triggered by mating pheromones. This transcriptional induction is uniquely immune to the repressive effects of silent chromatin and limits transposition to discrete points in the yeast life cycle.

Silent chromatin and Ty5 transcription

Transcriptional silencing at the telomeres and *HM* loci is initiated from *cis*-acting silencers (e.g. *HMR-E* or telomere repeat sequences) and propagated outwards along the chromosome (Gottschling *et al.*, 1990; Renaud *et al.*, 1993; Loo and Rine, 1994). The repressive effect of silencers on adjacent gene expression is dependent on both the strength of the gene's promoter and the distance of the promoter from the silencers (Renaud *et al.*, 1993). For example, *URA3* is silenced when placed 2–3 kb away from the telomere, but not when placed more than 3.5 kb away. Promoter strength is also important in that deletion of the *URA3* transcriptional activator *PPR1* weakens *URA3*

promoter activity and results in repression of a *URA3* gene located 11–12 kb away from the telomere.

Ty5 insertions at the telomeres and *HM* loci provide an ideal system for studying the effect of silent chromatin on gene expression. Independent insertions can be readily recovered at different distances and orientations from the silencers. We observed that basal transcription was repressed for all 10 independent insertions analyzed in silent chromatin, even though the LTR promoters for several insertions (e.g. W55, W84, W113 and W66) were more than 6 kb away from the nearest silencer. In addition, we did not detect any transcription from telomeric insertions, despite the variegated expression ascribed to telomeric silencing (Gottschling *et al.*, 1990). Recent work by Vega-Palas *et al.* (1997) suggests that Ty5 is repressed by previously characterized components of silent chromatin, since mutations in *SIR3* and histone H4 derepress the endogenous *S.cerevisiae* Ty5-1 element. The effectiveness of Ty5 silencing could be due to the relatively weak Ty5 promoter, or in the case of the telomeric insertions, to the presence of other sequences (e.g. X repeats) that facilitate repression and were not present in previous studies of telomeric silencing. Although we have observed that all Ty5 insertions are silenced, it is possible that there are subtle differences in expression for insertions at different distances from the silencers that could not be detected by our assays.

Most Ty5 insertions moved the *HML α* gene 6.5 kb from the *HML-E* or *HML-I* silencer. In qualitative mating assays conducted with these strains, we found that they mated at wild-type frequencies, indicating that *HML* silencing was largely intact (our unpublished data). The silent mating loci, therefore, appear to be safe havens for Ty5 integration, since insertions at these loci typically do not affect the ability of yeast to mate. Furthermore, Ty5 transcription is repressed upon integration, which prevents additional rounds of potentially deleterious transposition. The lack of disruption in silencing observed at *HML* could be because the *HML-E* and *HML-I* silencers are redundant, and it has previously been shown that deletion of either silencer does not derepress *HML* (Mahoney and Broach, 1989). Alternatively, a single Ty5 insertion may be sufficiently small such that silent chromatin can extend from the silencer through the element to silence *HML*. Consistent with this hypothesis, single Ty1 or solo Ty1 LTR insertions at *HML* do not disrupt the silencing of *HML α* , whereas silencing is disrupted by insertion of tandem Ty1 arrays (Weinstock *et al.*, 1990; Mastrangelo *et al.*, 1992).

Mating pheromone and cell-type regulation of Ty5 transcription

Ty5 transcription is induced by pheromone treatment. This induction requires genes in the pheromone response pathway (*STE7*, *STE11* and *STE12*). The pheromone signaling cascade ultimately results in the activation of Ste12p, which binds PRE sequences in the promoters of pheromone-regulated genes and induces their transcription (Sprague and Thorner, 1992). Ty5 transcription is also haploid specific and is repressed in diploid cells. Cell type regulation is normally mediated by the binding of the $\alpha 1$ – $\alpha 2$ heterodimer to sites in the promoter regions of target genes (Miller *et al.*, 1985; Bilanchone *et al.*, 1993). We have identified a region in the Ty5 LTR that is

responsible for both pheromone induction and cell-type regulation. This region contains three perfect matches to PRE elements and one degenerate $\alpha 1$ – $\alpha 2$ binding site (60% similarity). We demonstrated through deletion analysis that the PRE elements are responsible for pheromone-induced transcription. However, because the PRE elements overlap with the putative $\alpha 1$ – $\alpha 2$ binding site, we could not determine whether the latter sequence was responsible for cell-type regulation. It is possible that the PRE elements are also responsible for the lower levels of Ty5 expression observed in diploids, due to the reduced expression in diploid cells of Ste12p (Fields and Herskowitz, 1987).

α -Factor-induced transcription of Ty5 elements in silent chromatin

Silent chromatin restricts the access of some protein factors to DNA. *In vivo*, the *HM* loci and telomeres are resistant to HO endonuclease cleavage and methyltransferase activity, respectively (Strathern *et al.*, 1982; Gottschling, 1992). In isolated nuclei, the *HMR* locus is resistant to restriction enzyme digestion (Loo and Rine, 1994). Despite this inaccessibility, transcription of Ty5 in silent chromatin is still responsive to α -factor, suggesting that Ste12p can negotiate silent chromatin to bind the Ty5 LTR. This indicates that repression by silent chromatin is not static, and competition between activators and repressors can reset the transcriptional status. Transcriptional activation of genes within silent chromatin has been previously reported: silencing of a telomeric *URA3* gene can be reversed by over-expression of its transactivator Ppr1p (Aparicio and Gottschling, 1994); when *HMR* is placed adjacent to *HSP82*, transcriptional silencing of *HSP82* is lost upon heat stress (Lee and Gross, 1993).

The accessibility of silent chromatin to protein factors may be cell cycle-dependent (Fox and Rine, 1996). In studies with a telomeric *URA3* gene, Aparicio and Gottschling (1994) demonstrated that Ppr1p can only activate *URA3* expression in G₂ and not in G₁, G₀ or early S phase. The authors suggested that after DNA synthesis, when silent chromatin is disassembled, Ppr1p competes for the *URA3* promoter with protein factors responsible for assembling silent chromatin, such as the SIR proteins. Once established, either an active or silent state is maintained. Consistent with the above observation, it was shown that repair enzymes can only gain access to *HML α* during G₂ (Terleth *et al.*, 1990). It will be interesting to determine whether there is a G₂-dependence for Ty5 transcriptional induction.

Comparison of Ty5 transcriptional regulation with other Ty elements

Ty1, Ty2, Ty3 and Ty5 are all cell-type-regulated (Errede *et al.*, 1985; Company and Errede, 1988; Fulton *et al.*, 1988; Bilanchone *et al.*, 1993; this study). Because most wild yeast cells are diploid, repression of retrotransposon transcription and transposition in diploids is likely important to minimize the detrimental effects of element mobility. For Ty1 and Ty2, the $\alpha 1$ – $\alpha 2$ binding sites are located within the coding region (Errede *et al.*, 1985; Company and Errede, 1988; Fulton *et al.*, 1988), whereas this site is located in the LTR of Ty3 (Bilanchone *et al.*, 1993). Although diploid repression is also mediated by the Ty5 LTR, the mechanism may be different. We could not

distinguish whether this repression is mediated by the degenerate $\alpha 1$ - $\alpha 2$ binding site, or by the PRE sequences as a consequence of reduced levels of Ste12p in diploid cells (Fields and Herskowitz, 1987).

Transcription of both Ty3 and Ty5 is regulated by the pheromone response pathway and is induced about 20-fold by the mating pheromones (Bilanchone *et al.*, 1993; this study). For both elements, this induction is mediated by the PREs in the LTRs. Studies with Ty1, Ty2 and Ty4 failed to reveal similar transcriptional controls, even though two putative PRE elements are found within the Ty4 coding region, and a Ste12p response element is found within the Ty1 coding region (Company and Errede, 1988; Errede and Ammerer, 1989; Hug and Feldmann, 1996).

Repression of transcription by flanking sequences is not specific to Ty5. Endogenous Ty1–Ty4 elements are preferentially associated with genes transcribed by RNA polymerase III, such as tRNA genes and 5S rDNA. For Ty1 and Ty3, this genomic organization is due to targeted integration to sites of pol III transcription (Chalker and Sandmeyer, 1992; Devine and Boeke, 1996). Transcription of tRNA genes represses adjacent pol II promoters, including the Ty3 promoter (Kinsey and Sandmeyer, 1991; Hull *et al.*, 1994). Recently, it was shown that Ty1 integrates efficiently into rDNA arrays, which, in turn, repress Ty1 transcription (Bryk *et al.*, 1997; Smith and Boeke, 1997). Therefore, the transcriptional repression of Ty elements by flanking sequences may be a widespread phenomenon.

The association of transposable elements with silent or heterochromatic regions of the genome is not limited to the yeast model. Examples include highly methylated transposable elements in plants and protists, telomeric and paracentromeric elements in *D.melanogaster*, and elements associated with heterochromatic Y chromosomes (Rothnie *et al.*, 1991; Kjellman *et al.*, 1995; Pimpinelli *et al.*, 1995; Pelissier *et al.*, 1996; SanMiguel *et al.*, 1996; Brandes *et al.*, 1997). Some of these transposable elements, such as the telomeric *HeT-A* elements, are transcribed within heterochromatin (Danilevskaya *et al.*, 1997). Many of these elements, however, are probably transcriptionally repressed and, like Ty5, must escape this repression if they are to replicate. Transcriptional activation that overcomes the silencing effect of heterochromatin could be achieved by the variety of cellular pathways that perceive and transduce internal or external stimuli. The adaptation of elements to proliferate within specific chromosomal contexts is one of an increasing number of examples of the finely tuned interrelationship between transposable elements and their hosts.

Materials and methods

Strains

Yeast strains used in this study were W303-1A (*MATa ade2-1 can1-100 his3-11 leu2-3 trp1-1 ura3-1*), W303-1B (*MAT α*), W303-1 (diploid) and their isogenic derivatives. The strain with the *HMR-E* mutation was a kind gift of R.Sternglanz (YAB61) (Brand *et al.*, 1987). This strain contains deletions of the three *cis*-acting sequences required for *HMR-E* silencer function ($\Delta A\Delta B\Delta E$). Non-W303 strains with mutations in the pheromone response pathway include EG123 (*MATa his4-519 leu2 trp1 ura3 can1-101*), DC40 (EG123 *ste11- Δ*), L5528 (*MATa his3::hisG ura3-52*), L5559 (L5528 *ste7::LEU2 leu2::hisG*) and L5573 (L5528 *ste12::LEU2 leu2::hisG*) (kindly provided by L.Pillus) (Stone and Pillus, 1996). The *Escherichia coli* strain XL1-Blue (Stratagene) was used

for recombinant DNA manipulations. *E.coli* and yeast strains were transformed by electroporation as described by Ausubel *et al.* (1987).

Plasmids

A transposition-competent Ty5 element (Ty5-6p) from *S.paradoxus* was used for all plasmid constructs (Zou *et al.*, 1996a). The Ty5-*lacZ* translational fusion (pNK125) was constructed by inserting a 1.6 kb *Bam*HI fragment from a Ty5-6p subclone, pNK301, into the *Bam*HI site of YEp356R (Myers *et al.*, 1986). The 1.6 kb fragment includes ~1 kb of the Ty5-6p 5' flanking sequence, 251 bp of the Ty5-6p LTR and 361 bp of internal element sequence. LTR deletion constructs were generated by PCR-based mutagenesis (Ausubel *et al.*, 1987). An *Xho*I site was introduced at various positions within the Ty5 LTR by amplifying pNK301 with a mutagenic primer and the reverse primer by PCR. The PCR fragments were then digested with *Xho*I and *Bam*HI, cloned into the *Sall*I and *Bam*HI sites of YEp356R and sequenced. Mutagenic primers included DVO278 (5'-CCGCTCGAGTGTGAATGTGATAACCCA-3') for the full-length LTR construct pIP2, DVO182 (5'-GGGTAATGTTT-CAGT-3') for the $\Delta 1$ -31 construct pIP4, DVO279 (5'-CCGCTCG-AGTAATGTTTTAGACAAG-3') for the $\Delta 1$ -64 construct pIP3, DVO183 (5'-CCTCGAGCAGCAAACCTCCGAT-3') for the $\Delta 1$ -91 construct pIP5 and DVO214 (5'-CCCTCGAGCATTTACATAACATATAGAAAG-3') for the $\Delta 1$ -170 construct pIP1.

Strain W200 was used as a control in the Northern blot and RT-PCR analyses. This strain carries pNK335, which contains the wild-type Ty5-6p element with the *his3AI* selectable marker. It was constructed by replacing the *Xho*I-*Bam*HI fragment of pSZ152 with the corresponding fragment of pNK318 (Zou *et al.*, 1996a). This replaced the *GALI*-Ty5 LTR promoter with the Ty5 LTR promoter. pNK422 was used to replace the *HIS3* marker in the Ty5 insertions with *his3AI*. It was constructed by cloning the *Clal* fragment with the *his3AI* marker gene from pSZ153 into the corresponding site of the integration plasmid pRS406 (Sikorski and Hieter, 1989).

β -galactosidase assays

Plasmids containing the Ty5-*lacZ* fusions (described above) were introduced into the yeast strains W303-1, W303-1A and W303-1B by electroporation. Three Ura⁺ transformants were selected for each construct and β -galactosidase activities were measured as described by Ausubel *et al.* (1987). For α -factor induction, W303-1A strains with different Ty5-*lacZ* fusion constructs were grown to log phase (OD₆₀₀ = 0.4–0.6) in synthetic complete medium without uracil (SC-U medium) at 30°C. Cells were then treated with or without 3.5 μ M α -factor (Sigma) for an additional 60–90 min before assaying β -galactosidase activity.

To determine whether pheromone-induced Ty5 transcription is dependent on the pheromone pathway, the Ty5-*lacZ* construct (pIP2) was introduced into strains EG123, DC40, L5528, L5559 and L5573. Overnight cultures were diluted 10-fold into fresh medium, and allowed to grow for 3 h before α -factor was added. Cells were grown for an additional 90 min before β -galactosidase activities were measured.

Ty5 transcription analysis

An RT-PCR strategy was used to determine the expression of different chromosomal Ty5 insertions. The chromosomal locations of Ty5 insertions in the 10 strains tested are shown in Figure 1 (Zou *et al.*, 1996a). W3 has a Ty5 insertion in the middle of chromosome XI (Zou *et al.*, 1996b); W200 has Ty5 on a 2 μ plasmid (pNK335). Strains with chromosomal Ty5 insertions were grown overnight in 10 ml of synthetic complete medium without histidine (SC-H) at 30°C. W200 was grown in 10 ml SC-U medium. RNA was made by the hot phenol method and resuspended in 20 μ l ddH₂O (Ausubel *et al.*, 1987). Contaminating yeast DNA was removed by DNase I treatment at 37°C for 35 min in the presence of 2 μ l 10 \times DNase I buffer (400 mM Tris-Cl, 100 mM NaCl, 60 mM MgCl₂, 100 mM CaCl₂) and 0.4 μ l DNase I (98.1 units/ml) (Sigma). DNase I was removed by extracting with phenol and chloroform, precipitating with ethanol and resuspending the pellet in 20 μ l ddH₂O.

Reverse transcription was carried out with equal amounts of RNA using MLV reverse transcriptase according to the manufacturer's specifications (Gibco BRL). Primers DVO309 (5'-ATCGATGAACGGCTCATTGG-AATAA-3') (specific to *SNF6*) and DVO265 (5'-CTTGCTACAGTATG-ATTGTATTGGACACC-3') (specific to Ty5 integrase) were used for first-strand cDNA synthesis. PCR amplifications were carried out with 1 μ l of the reverse transcription reactions and primers DVO265 and DVO238 (5'-GTCGGATCTGTTCGTGCAGCAATGGTACAG-AATT-3') to measure Ty5 expression, and primers DVO308 (5'-AGAT-CTCACCATGGAAAGGCTTCG-3') and DVO309 to measure *SNF6* expression.

α -Factor induction of Ty5 expression was tested among strains carrying Ty5 insertions at different chromosome locations (W77, W76, W7 and W28; see Figure 1). W3 and W200 were used as controls. The strains were grown in SC-H medium to log phase ($OD_{600} = 0.4-0.6$) and then treated with or without 3.5 μ M α -factor for 80 min. RNA was prepared and RT-PCR was conducted using the strategy described above. Northern blot analysis was also conducted for comparison with the RT-PCR results (Ausubel *et al.*, 1987). Filters were probed with 32 P-labeled Ty5 sequences corresponding to integrase (a 0.5 kb *SphI-HpaI* fragment).

α -Factor-induced transposition

To determine whether α -factor can induce transposition of chromosomal Ty5 insertions, the functional *HIS3* genes carried by these elements were replaced with the *his3AI* marker by the transplacement method (Scherer and Davis, 1979). Plasmid pNK422 containing *his3AI* was digested with *NheI* and transformed into strains with Ty5 insertions (W114, W9, W2, W77 and W3) by the lithium acetate method (Ausubel *et al.*, 1987). Ura⁺ transformants from each transformation were picked, patched on to YPD plates and allowed to grow for 24 h. The patches were then replica-plated on to synthetic complete medium with 5-fluoroorotic acid (SC/5-FOA) to select for recombination between the two *HIS3* genes; 5-FOA-resistant colonies from each strain were then patched on to SC-H/5-FOA and allowed to grow for 48 h at 30°C. Cells that failed to grow were subjected to PCR analysis using a primer specific to the artificial intron DVO235 (5'-CTGTTAATAAATAATACCATTG-3') and a primer specific to Ty5 DVO187 (5'-CGGTACCTATATACCAC-3'). This confirmed the structure of the transplanted elements.

For those strains with Ty5 insertions in which the *HIS3* marker was replaced by the *his3AI* marker, transposition assays were conducted in the presence or absence of α -factor. A 0.5 ml culture of each strain was grown in YPD medium overnight and diluted 10-fold into 5 ml of fresh YPD and allowed to grow for ~4.5 h ($OD_{600} = 0.8$). The culture was then divided, with α -factor (3.5 μ M) added to 2 ml of the culture and no α -factor added to the remainder. The cultures were allowed to grow at room temperature for 3 h before harvesting. Cells were resuspended in an equal volume of YPD medium and allowed to grow for another 3 h at room temperature. One milliliter of each culture ($OD_{600} = 3.0$) was then plated on to SC-H plates. The number of His⁺ colonies was scored after 2.5 days growth at 30°C.

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