Transcriptional silencing of Ty1 elements in the *RDN1* locus of yeast

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We demonstrate that in *Saccharomyces cerevisiae*, the tandem array of ribosomal RNA genes (*RDN1*) is a target for integration of the Ty1 retrotransposon that results in silencing of Ty1 transcription and transposition. Ty1 elements transpose into random rDNA repeat units and are mitotically stable. In addition, we have found that mutation of several putative modifiers of *RDN1* chromatin structure abolishes silencing of Ty1 elements in the rDNA array. Disruption of *SIR2*, which elevates recombination in *RDN1*, or *TOP1*, which increases psoralen accessibility in rDNA, or *HTA1-HTB1*, which reduces histone H2A-H2B levels and causes localized chromatin perturbations, abolishes transcriptional silencing of Ty1 elements in *RDN1*. Furthermore, deletion of the gene for the ubiquitin conjugating enzyme Ubc2p, which ubiquitinates histones in vitro, derepresses not only Ty1 transcription but also mitotic recombination in *RDN1*. On the basis of these results, we propose that a specialized chromatin structure exists in *RDN1* that silences transcription of the Ty1 retrotransposon.

[Key Words: Ty1; rDNA; chromatin; transcriptional silencing; *UBC2*; histones H2A-H2B]

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The expression of retroviral proviruses and related families of retrotransposons may be affected by their position of integration (Feinstein et al. 1982; Conklin and Groudine 1986; Akroyd et al. 1987; Curcio and Garfinkel 1991a). Typically, the effect observed is silencing of the expression of certain integrated proviruses or retrotransposons. This phenomenon is poorly understood but it is reminiscent of the position-dependent silencing of transcription units in heterochromatic regions of yeast (Laurenson and Rine 1992), *Drosophila* (Weiler and Wakimoto 1995), and mouse (Bedell et al. 1996) genomes, a process known as position effect. In *Saccharomyces cerevisiae*, there are at least two regions of repeated DNA sequences where positional silencing occurs, the homothallic mating loci (*HM*) and telomeres. Several trans-acting modifiers of position effect have been identified, including products of the *SIR2* (*MAR1*), *SIR3* (*MAR2*), *SIR4*, *RAP1*, and *ABF1* genes, histones H3 and H4, and the origin recognition complex (for review, see Rivier and Pillus 1994; Shore 1994; Dillin and Rine 1995). The essential role of histones in silencing (Thompson et al. 1994) and the inaccessibility of silenced domains to DNA-modifying enzymes (Park and Szostak 1990; Gottschling 1992; Singh and Klar 1992; Wright et al. 1992; Loo and Rine 1994) have led to the hypothesis that

a heterochromatin-like conformation in these domains is responsible for transcriptional silencing.

The *RDN1* locus, which encodes a large tandem array of ribosomal RNA genes, has characteristics of heterochromatin. First, the *RDN1* locus consists of tandemly repeated DNA sequences, a situation analogous to that of sequences in heterochromatic domains of higher eukaryotes (Lohe and Hilliker 1995). Second, each copy of the rRNA gene repeat contains DNA sequences that have been implicated in transcriptional silencing at telomeres and the *HM* loci, including a Rap1p-binding site and an autonomously replicating sequence (ARS; Dillin and Rine 1995). Third, recombination is repressed at *RDN1*, a characteristic of silenced regions of the *Drosophila* and *Schizosaccharomyces pombe* genomes (Petes 1980; Szauter 1984; Christman et al. 1988; Thon et al. 1994). Significantly, *SIR2*, which is required for transcriptional silencing at the *HM* loci and telomeres, is also required for suppressing recombination in *RDN1* (Gottlieb and Esposito 1989). In contrast to the other heterochromatin-like regions in yeast, however, genes in *RDN1* are transcribed actively. This includes not only the RNA polymerase (Pol) I-transcribed 35S rRNA gene and Pol III-transcribed 5S rRNA gene present in each rDNA repeat, but also Pol II-transcribed genes *URA3* and *LEU2* introduced into *RDN1* (Szostak and Wu 1980; Gottlieb and Esposito 1989).

This study was undertaken to determine whether the

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chromosomal position of a retroelement insertion can influence its expression. The Ty1 element of yeast is a long terminal repeat (LTR)-containing retrotransposon present in ~25–35 copies per haploid genome. A characteristic of Ty1 biology that facilitates the identification of transposition events whose expression is affected by adjacent chromatin is that the amount of RNA produced from an individual Ty1 element determines its rate of transposition. The total level of Ty1 proteins, rather than RNA, limits transposition; consequently, competition between Ty1 RNA for association with the Ty1 proteins that mediate cDNA synthesis determines the level of transposition of each element (for review, see Farabaugh 1995). Furthermore, the functional state of proteins encoded by a particular Ty1 element does not affect its rate of transposition, as Ty1 proteins act efficiently in *trans*. In contrast, the transcript of a Ty1 element is *cis*-acting in its role as template for reverse transcription; therefore, changes in the transcription of an individual element that do not affect total Ty1 RNA levels can affect the transposition of that individual element (Curcio and Garfinkel 1994). Individual Ty1 elements have been demonstrated to express varied levels of RNA and differ as much as 50-fold in their rate of transposition (Curcio and Garfinkel 1991b).

Although Ty1 elements have been shown to transpose into numerous selectable target loci, studies of unselected transposition events have shown that integration is not random. Like that of retroviruses, Ty1 integration is not sequence specific (Natsoulis et al. 1989; Ji et al. 1993), but is influenced by the structural or functional state of chromatin (for review, see Curcio and Morse 1996). Ty1 elements frequently integrate into the vicinity of tRNA genes (Ji et al. 1993), and recent work by Devine and Boeke (1996) has established that the presence of an actively transcribed Pol III gene, including tRNA, 5S rRNA, or snRNA genes, stimulates Ty1 integration into target plasmids. Ty1 elements have also been found associated with 5S rRNA genes in the *RDN1* locus (Piper et al. 1984; Vincent and Petes 1986); in general, however, Ty1 elements are underrepresented or absent in the rDNA array (Oyen and Gabrielsen 1983; Rustchenko and Sherman 1994). Interestingly, the 5S rRNA gene located in the rDNA array is also a poor target for the Ty3 retrotransposon in comparison to a plasmid-borne 5S rRNA gene (Chalker and Sandmeyer 1992).

To determine whether the position of integration of a Ty1 retrotransposon could influence its expression, we induced transposition of a Ty1 element and screened for strains that have unselected *de novo* Ty1 insertions marked with the *his3AI* retrotransposition indicator gene (Curcio and Garfinkel 1991b). Several Ty1*his3AI* insertions, which had very low levels of transposition and transcription, were identified. We show that most of the inactive elements integrated into different rDNA repeats on chromosome XII, suggesting that transcriptional silencing was a consequence of their position of integration.

To understand the mechanism of Ty1*his3AI* element

silencing in *RDN1*, we examined several genes whose products potentially alter chromatin structure and the biology of *RDN1*. One potential modifier of chromatin assembly that was analyzed was *UBC2*(*RAD6*), a DNA repair gene that encodes an E2 ubiquitin-conjugating enzyme that participates in several diverse cellular processes. A role for *UBC2*(*RAD6*) as a modifier of localized chromatin domains is suggested by the observation that Ty1 integration specificity into target genes is altered significantly in *ubc2Δ* mutants (Kang et al. 1992; Liebman and Newnam 1993). Moreover, a recent report suggests that ubiquitination of histones or chromatin-associated proteins provides a means to remodel chromatin in yeast (Moazed and Johnson 1996). To establish a direct connection between chromatin structure and regulation of Ty1 elements in *RDN1*, we have also investigated the effect of deleting the *HTA1-HTB1* locus, one of two loci that encodes the histones H2A and H2B. The H2A–H2B dimer is a component of the nucleosome core whose depletion causes nucleosome instability, local perturbations in chromatin structure, altered positioning of nucleosomes at specific promoters, altered transcription of specific genes in yeast (Han et al. 1987; Clark-Adams et al. 1988; Han and Grunstein 1988; Norris et al. 1988; Hirschhorn et al. 1992), and suppression of position-effect variegation in *Drosophila* (Moore et al. 1983).

Here we identify *UBC2*, *SIR2*, *TOP1*, and *HTA1-HTB1* as modifiers of Ty1 element silencing in the rDNA array. Our findings reveal an unprecedented role for *UBC2* in transcriptional silencing and repression of recombination in *RDN1*. The participation of the histone dimer H2A–H2B and topoisomerase I in transcriptional silencing indicates that a specialized chromatin structure in *RDN1* regulates expression of Ty1 elements. The requirement for the chromatin modifier encoded by *SIR2* suggests that the mechanism of silencing in *RDN1* is related to the *SIR2*-dependent suppression of rDNA repeat recombination and to position effect at telomeres and the *HM* loci. We discuss models whereby these modifiers facilitate the formation of a chromatin structure in *RDN1* that represses Ty1 transcription and recombination between rDNA repeats.

Results

De novo Ty1*his3AI* integrants differ in transpositional activity

Colonies that undergo transposition-induction by expression of plasmid pGTy1–H3m*his3AI* sustain an average of 1.7 marked transposition events per genome, and most of the insertions retain the retrotransposition indicator gene *his3AI* because of inefficient splicing of the artificial intron (AI; Curcio and Garfinkel 1991b). The *his3AI* retrotransposition indicator gene, which consists of an AI cloned in antisense orientation into the coding sequences of the *HIS3* gene, confers a His[−] phenotype (Fig. 1). The marker gene can be converted into a functional *HIS3* allele in the process of Ty1 retrotransposi-

Figure 1. Transposition of a genomic *his3AI*-marked Ty1 element. A chromosomal Ty1 element is marked with a *HIS3* gene (open rectangle) in the opposite transcriptional orientation to Ty1. Coding sequences of the *HIS3* gene are interrupted by an artificial intron (AI) in the antisense orientation relative to the *HIS3* coding sequence. The AI is not spliceable from transcripts that initiate from the *HIS3* promoter; however, it is in the correct orientation to be spliced from Ty1 transcripts. When a spliced transcript from the Ty1*his3AI* element is used as template for reverse transcription and integration, the newly transposed element will contain a functional copy of *HIS3* created by precise removal of the AI, rendering the cell phenotypically His⁺. The Ty1*HIS3* transposition event is shown on the same chromosome for simplicity. (Tripartite stippled and open boxes) LTRs; (bold lines) chromosomes; (open circles) centromeres; (wavy lines) RNA transcripts.

tion, and in doing so, renders the cell phenotypically His⁺. The rate of transposition of Ty1*his3AI* elements is directly proportional to the rate of formation of histidine prototrophs in *his3Δ200* strains (Curcio and Garfinkel 1991b).

To investigate the role of transcriptional silencing in the variability of genomic Ty1 element expression, we screened transposition-induced colonies by Southern hybridization for yeast strains that contained a single de novo Ty1*his3AI* transposition event. The transposition activity of Ty1*his3AI* elements was determined initially in a test for His⁺ prototroph formation at 20°C in a small patch of cells. Active Ty1*his3AI* elements, which have rates of His⁺ prototroph formation of 10^{-6} to 10^{-7} per generation (Curcio and Garfinkel 1991b, 1992), typically represent ~75% of the de novo transposition events after induction of pGTy1-H3m*his3AI* expression. However, seven Ty1*his3AI*-containing strains were found that had a relatively low frequency of His⁺ prototrophy. The rate of His⁺ prototroph formation was determined for each of the seven strains as well as strains JC242 and JC273, which harbor active Ty1*his3AI* elements (Table 1). The seven transpositionally inactive strains had rates of His⁺ prototroph formation ranging from $<1.2 \times 10^{-9}$ to 2.5×10^{-8} . In contrast, active elements Ty1*his3AI*-242 and Ty1*his3AI*-273 produced $\sim 1.5 \times 10^{-7}$ His⁺ proto-

trophs per generation. The data show that the transposition rates of inactive Ty1*his3AI* elements can be at least 100-fold lower than that of typical active elements (Table 1).

Mapping inactive Ty1*his3AI* elements to *RDN1*

To determine whether the seven genomic Ty1*his3AI* elements exhibiting low levels of transposition share a common location in the yeast genome, chromosomes from strains with inactive Ty1*his3AI* elements (JC234, JC236, JC272, JC815, JC816, JC820, and JC821), and active Ty1*his3AI* elements (JC242 and JC273) were separated by contour-clamped homogenous electric field (CHEF) gel electrophoresis, and the Ty1*his3AI* elements were mapped by hybridization to a *HIS3* probe (Fig. 2A). Five inactive Ty1*his3AI* elements were located on chromosome XII, the identity of which was confirmed by rehybridizing the CHEF blot with a chromosome XII-specific probe (data not shown). In contrast, the other two inactive Ty1*his3AI* elements and the two active elements were located on four different chromosomes. Ty1*his3AI*-815 and Ty1*his3AI*-821 mapped to chromosomes X and XVI, respectively. The transpositionally active element Ty1*his3AI*-273 was located on chromosome II (data not shown) and Ty1*his3AI*-242 was located on chromosome XII, demonstrating that location on this chromosome did not in itself result in low levels of transposition.

Southern analysis revealed that the inactive Ty1*his3AI* elements that mapped to chromosome XII were on a common-sized *Sfi*I fragment (data not shown). Therefore, a DNA fragment containing the junction between Ty1*his3AI* and genomic sequences downstream of the 3' LTR was isolated from each strain by integration and eviction of pGEM-HIS3, a plasmid containing the *HIS3* gene on an *Escherichia coli* vector. The sequence of the Ty1*his3AI* insertion junctions was determined (Fig. 2B). The five inactive Ty1*his3AI* insertions on chromosome XII had all integrated into an rDNA repeat (Fig.

Table 1. Transposition of genomic Ty1*his3AI* elements

Strain	Location of Ty1 <i>his3AI</i> element	Transposition rate ^a		Ratio of rates (<i>ubc2Δ</i> / <i>UBC2</i>)
		<i>UBC2</i>	<i>ubc2Δ</i>	
JC234	<i>RDN1</i>	3.4×10^{-9}	3.7×10^{-8}	11
JC236	<i>RDN1</i>	1.1×10^{-8}	1.9×10^{-7}	17
JC272	<i>RDN1</i>	$<1.2 \times 10^{-9}$	8.7×10^{-8}	>73
JC816	<i>RDN1</i>	$<1.3 \times 10^{-9}$	6.6×10^{-8}	>50
JC820	<i>RDN1</i>	$<1.8 \times 10^{-9}$	1.3×10^{-7}	>70
JC815	Chr X	2.5×10^{-8}	$<5.9 \times 10^{-9}$	<0.2
JC821	Chr XVI	$<1.8 \times 10^{-9}$	$<1.7 \times 10^{-9}$	—
JC242	Chr XII ^b	1.6×10^{-7}	1.4×10^{-7}	0.9
JC273	Chr II	1.5×10^{-7}	1.1×10^{-7}	0.7

^aThe rate of His⁺ prototroph formation per cell per generation, as determined by the maximum likelihood method of Lea and Coulson (1949).

^bOutside *RDN1*.

Figure 2. Determination of *Ty1his3AI* integration sites. (A) Agarose-embedded DNA was prepared from the strains listed above the lanes, and individual chromosomes were separated by CHEF gel electrophoresis, transferred to a nylon membrane, and hybridized to a *HIS3* riboprobe. Labels 234 through 821 indicate the number of the strain and the *Ty1his3AI* insertion. The locations of chromosomes XII, XVI, and X are indicated. (B) Sequence of *Ty1his3AI*-flanking genomic DNA junctions. *Ty1* 3' LTR is in lowercase lettering; genomic sequences downstream of the 3' LTR are in uppercase lettering. Labels, as in A. (C) Schematic representation of two rDNA repeats depicting the locations of five inactive *Ty1his3AI* elements. (Hatched boxes) rRNA genes, 35S (18S, 5.8S, and 25S) and 5S. (Bold vertical lines) Position of the *Ty1his3AI* insertion. (Small arrows) Direction of transcription. (E) Enhancer of RNA Pol I; (I) Initiator of RNA Pol I; (NTS) nontranscribed spacer. Insertion sites of *Ty1his3AI* are as follows: -272, -234, and -816 at 120, 360, and 841 bp upstream of the 5S rRNA transcription initiation site, respectively; -236 at 701 bp downstream of the 5S rRNA transcription termination site; -820, in 18S rRNA gene, at 844 bp downstream of the 35S rRNA initiation site.

2C). There are ~100–200 copies of the 9.1-kb rDNA repeat in yeast, which are found in a tandem array in the *RDN1* locus. Each repeat contains the transcription unit for the 35S precursor to 25S, 18S, and 5.8S rRNA, transcribed by Pol I. In addition, there is a 2.5-kb nontranscribed spacer (NTS) in each repeat. The NTS consists of

promoter elements for the 35S rRNA gene flanking the 120-bp 5S rRNA gene, which is transcribed in the opposite orientation by Pol III. In strains JC234, JC236, JC272, and JC816, *Ty1his3AI* insertions were found in the NTS on either side of the 5S rRNA gene. The *Ty1his3AI* element in strain JC820 was inserted in the 35S rRNA gene (Fig. 2C).

The junction between *Ty1his3AI* and adjacent genomic sequences was also isolated from the two strains with transpositionally active *Ty1his3AI* elements. The integration site of *Ty1his3AI*-242 at position 1060533 of chromosome XII was in an open reading frame (ORF) just outside of the *RDN1* locus. The inactive element *Ty1his3AI*-815 at position 354470 of chromosome X was flanked by 70 nucleotides from the middle of a *Ty1* LTR in the same orientation, followed by 150 nucleotides of a *Ty2* LTR in the opposite orientation. In summary, although both inactive and active *Ty1* elements mapped to random locations outside of *RDN1*, most (five of seven) inactive *Ty1his3AI* elements were found in *RDN1*.

Ty1his3AI elements are located in dispersed rDNA repeats

The number and location of *Ty1his3AI* elements in *RDN1* were determined by CHEF gel analysis of chromosomal DNA digested with *Bam*HI. No *Bam*HI sites are present in *RDN1*, therefore, digestion with *Bam*HI leaves the 1- to 2-Mb *RDN1* locus intact. However, a single *Bam*HI site is present in *Ty1his3AI*; therefore, insertion into *RDN1* will result in *Bam*HI-generated subfragments of *RDN1*.

Undigested and *Bam*HI-digested chromosomal DNA from strains containing *Ty1his3AI* insertions was separated on CHEF gels and hybridized to an 18S rRNA probe and a *HIS3* probe (Fig. 3A,B). The sizes of the *Bam*HI fragments hybridizing to the 18S rRNA probe were determined by comparison to size standards. The length of *RDN1* is highly variable, ranging from ~1000 to 1900 kb among the insertion-containing strains (Fig. 3). These lengths correspond to 110–210 copies of the 9.1-kb rDNA repeat, respectively.

In strains JC242 and JC273, a single large *Bam*HI fragment was detected with the 18S rRNA probe, consistent with there being no *Ty1his3AI* element in *RDN1* in these strains. In contrast, two *Bam*HI fragments hybridized to the 18S rRNA probe in DNA from strains JC234, JC236, and JC272, one of which also hybridized to *HIS3* (Fig. 3A,B). *Bam*HI digestion of chromosomal DNA from strains JC816 and JC820 generated three fragments that hybridized to the 18S rRNA probe (Fig. 3A), but only a 1025-kb band in JC816 DNA and a 900-kb band in JC820 DNA hybridized to *HIS3* (Fig. 3B). CHEF analysis of single-colony isolates of JC816 and JC820 revealed that both strains contain mixed populations of cells with at least two different sized *RDN1* loci that have consistently a single *Bam*HI site (data not shown). The presence of only one *Bam*HI fragment in DNA from strains JC234, JC236, JC272, JC816, and JC820 that hybridized

Figure 3. Location of Ty1his3AI elements in the rDNA array. (A) Chromosomal DNA from strains containing Ty1his3AI elements was incubated in the absence (-) or presence (+) of *Bam*HI and separated by CHEF gel electrophoresis. The DNA was transferred to a nylon membrane and hybridized to a ³²P-labeled 18S rRNA riboprobe. The location of chromosome XII and fragment sizes (kb) are indicated. Undigested chromosome XII often bands as a doublet because of its large size. (B) The Southern blot from A was hybridized with a *HIS3* DNA probe. Labels, as in A. (C) Schematic representation of *RDN1* depicting the location and orientation of Ty1his3AI insertions. The data from A and B were used to determine the relative location and orientation of each Ty1his3AI element in *RDN1*. The *Bam*HI fragment that hybridized to *HIS3* contains the upstream portion of the Ty1his3AI element. This information and the orientation of the Ty1 element (Fig. 2C) was used to determine the location of the Ty1 insertion (solid arrowhead) in *RDN1*. For strains JC816 and JC820, the smallest *Bam*HI fragment (370 and 120 kb, respectively) was added to that of the *HIS3*-hybridizing fragment (1025 and 900 kb, respectively). Only the length of *RDN1* (open rectangles) is drawn to scale. (○) Centromere; (Mb) megabase.

to both probes indicates that a single Ty1his3AI element inserted into *RDN1*. These mapping data, together with the known orientation of the Ty1his3AI element within an rDNA repeat (see Fig. 2C), were used to determine the position of the marked Ty1 element within *RDN1*, which is shown schematically in Figure 3C. The results suggest that rDNA repeats located throughout *RDN1*, rather than in a specific location in *RDN1*, serve as targets for Ty1 element insertion.

To determine the frequency of Ty1 insertion into the NTS of the rDNA repeats, strains that sustained Ty1 transposition events marked with *HIS3* or *ADE2* were collected after induction of transposition in strain GRF167, and a PCR assay was used to detect Ty1 insertions in a 2.5-kb region encompassing the NTS. (Neither the *HIS3* nor the *ADE2* marker within a Ty1 element is silenced when transposition into rDNA occurs; see below.) We found that 12 of 119 (10%) Ty1 insertions analyzed had integrated into an NTS. The data demonstrate that the NTS, whose 100–200 copies represent ~2%–4% of the yeast genome, is an efficient target for de novo integration of Ty1.

Ty1his3AI elements in rDNA are regulated by *UBC2*

In a screen of major genes within the DNA repair epistasis groups for their effect on Ty1 transposition, we noticed that the *UBC2* gene had a differential effect on transpositionally inactive versus active Ty1his3AI insertions. To test whether *UBC2* was involved in silencing of Ty1 elements in *RDN1*, *UBC2* was deleted from nine strains containing individual Ty1his3AI elements. When the transposition rate in *ubc2Δ* mutants was compared to that of isogenic wild-type strains, two distinct classes of elements emerged (Table 1). Ty1his3AI elements located in *RDN1* in strains JC234, JC236, JC272, JC816, and JC820 had low rates of His⁺ prototroph formation ranging from $<1.2 \times 10^{-9}$ to 1.1×10^{-8} in wild-type strains and increases of 11-fold to >70-fold in isogenic *ubc2Δ* mutants. A second class of elements located outside *RDN1* in strains JC242, JC273, JC815, and JC821 showed the same or slightly lower rates of His⁺ prototrophy in *ubc2Δ* strains relative to the isogenic wild-type strain. This class was comprised of two elements that transposed at relatively high rates and two that transposed at low rates. These data show that the yeast genome contains one class of Ty1 elements located in *RDN1* that is repressed transpositionally by a *UBC2*-dependent mechanism and another class located at random positions that is not regulated by *UBC2*.

The finding that only a subset of Ty1his3AI insertions in yeast cells is regulated by *UBC2* suggests that Ty1his3AI expression is affected at the level of transcription, as the Ty1his3AI RNA is *cis*-acting in its role as template for cDNA synthesis. To verify this, relative levels of Ty1his3AI RNA in *UBC2* and isogenic *ubc2Δ* strains were determined in Northern hybridization analysis (Fig. 4). The five strains that contain Ty1 elements in *RDN1* whose transposition is repressed by *UBC2*, JC234, JC236, JC272, JC816, and JC820, had low

Figure 4. Effect of *ubc2Δ* mutation on *Ty1his3AI* and total *Ty1* RNA levels. Total RNA from isogenic *UBC2* (+) and *ubc2Δ* (-) strains containing single *Ty1his3AI* insertions was hybridized to a ³²P-labeled sense-strand *HIS3* riboprobe to detect *Ty1his3AI* RNA (top), an antisense *Ty1* riboprobe to detect total *Ty1* RNA (middle), and an antisense *PYK1* riboprobe as a loading control (bottom). GRF167 is the parental strain used to generate derivative strains containing *Ty1his3AI* transposition events. Strains with *Ty1his3AI* insertions in *RDN1* are marked with a superscript o. The *ubc2Δ/UBC2* ratio of *Ty1his3AI* RNA normalized to *PYK1* RNA, as determined by scanning densitometry is JC234°, 8.1; JC242, 0.45; JC236°, 24.3; JC272°, 472.3; JC273, 3.5; JC820°, 33.0; JC815, >0.48; JC816°, >339.2; JC821, 1.1.

or undetectable levels of *Ty1his3AI* RNA relative to the levels of a control transcript *PYK1* RNA. In contrast, *Ty1his3AI* RNA was increased significantly in isogenic *ubc2Δ* derivatives. For example, the *ubc2Δ* mutation increased *Ty1his3AI* RNA levels in strain JC816 by at least 339-fold (Fig. 4). The data demonstrate that *UBC2*-mediated repression of *Ty1his3AI* transposition is attributable to regulation of *Ty1his3AI* element RNA levels. On the other hand, the four strains containing *Ty1his3AI* elements whose transposition was not regulated by *UBC2* (Table 1) also showed no significant change in *Ty1his3AI* RNA levels as a result of *ubc2* deletion (Fig. 4). The transpositionally active *Ty1his3AI* elements in strains JC242 and JC273 were well transcribed in both *UBC2* and *ubc2Δ* strains. The *Ty1his3AI* element in JC821 was also highly transcribed in both the *UBC2* and *ubc2Δ* strains although it transposes at a very low rate, indicating that it may contain a *cis*-acting mutation required for formation of *Ty1HIS3* transposition events. The transpositionally inactive *Ty1his3AI* element in strain JC815 had no detectable *Ty1his3AI* RNA in the presence or absence of *UBC2*. In summary, the data demonstrate that *UBC2* is uniquely required for repressing transcription of *Ty1his3AI* elements located in *RDN1*.

Despite derepression of transcriptionally silenced *Ty1* elements in *ubc2* mutants, total *Ty1* RNA was not increased significantly in any of the *ubc2Δ* derivatives of *Ty1his3AI*-harboring strains or the parental strain GRF167 (Fig. 4). This result has been obtained previously in other yeast strains (Picologlou et al. 1990) and can be explained by the presence of low numbers of *Ty1* elements in *RDN1*.

In addition to a role in repressing transcription of *Ty1*

elements in *RDN1*, the product of the *UBC2* gene has been shown to participate in N-end rule protein degradation, postreplicative DNA repair, induced mutagenesis, and sporulation (Prakash et al. 1993; Lawrence 1994). To determine whether the N-end rule protein degradation function of *UBC2* is linked to repression of *Ty1his3AI* elements in *RDN1*, partial activity alleles of *UBC2* were tested for complementation of the hypertransposition defect of *ubc2Δ* mutants. The plasmid-borne *ubc2* alleles are completely (*ubc2_{Δ1-9}* *ubc2-149*) or partially (*ubc2-153*) defective for N-end rule protein degradation (Madura et al. 1993; Watkins et al. 1993). The results in Table 2 show that the CEN vector alone and the *ubc2_{Δ1-9}* allele did not complement the derepression of *Ty1his3AI* transposition, whereas the full-length *UBC2*, *ubc2-149*, and *ubc2-153* alleles repressed transposition of rDNA-*Ty1his3AI* elements in *ubc2Δ* mutants. Hence, transcriptional silencing of *Ty1* elements in *RDN1* is not correlated with loss of N-end rule protein degradation, but must involve another function of *UBC2* that is defective in the *ubc2_{Δ1-9}* allele. Consistent with these findings, a disruption of *UBR1*, the recognition component of the N-end rule degradation pathway (Dohmen et al. 1991), also did not affect transposition of *Ty1his3AI* elements in or outside of *RDN1* (data not shown). The results indicate that *UBC2*-mediated N-end rule protein degradation is not required for silencing of *Ty1* elements in *RDN1*.

SIR2 is a regulator of *Ty1his3AI* element silencing in *RDN1*

The *SIR2* gene product is required for the maintenance of a repressed chromatin structure that silences transcription units at the *HM* loci and telomeres and represses recombination between rDNA repeats. Therefore, if transcriptional silencing of *Ty1his3AI* elements in

Table 2. Complementation of *ubc2Δ* strains with partial-activity *ubc2* alleles

Strain + <i>ubc2</i> allele ^a	Rate of <i>Ty1his3AI</i> element transposition ^b	Transposition rate relative to <i>UBC2</i>
JC236 <i>ubc2Δ</i> + <i>UBC2</i>	2.8×10^{-8}	1
JC236 <i>ubc2Δ</i> + vector	3.4×10^{-7}	12.1
JC236 <i>ubc2Δ</i> + <i>ubc2_{Δ1-9}</i>	1.5×10^{-7}	5.4
JC236 <i>ubc2Δ</i> + <i>ubc2-149</i>	3.3×10^{-8}	1.2
JC236 <i>ubc2Δ</i> + <i>ubc2-153</i>	1.3×10^{-8}	0.5
JC272 <i>ubc2Δ</i> + <i>UBC2</i>	1.1×10^{-8}	1
JC272 <i>ubc2Δ</i> + vector	1.5×10^{-7}	13.6
JC272 <i>ubc2Δ</i> + <i>ubc2_{Δ1-9}</i>	1.6×10^{-7}	14.5
JC272 <i>ubc2Δ</i> + <i>ubc2-149</i>	1.3×10^{-8}	1.2
JC272 <i>ubc2Δ</i> + <i>ubc2-153</i>	1.1×10^{-8}	1

^aContained on the *URA3*-marked plasmid YCp50.

^bThe rate of His⁺ Ura⁺ prototroph formation per Ura⁺ cell per generation, as determined by the method of Lea and Coulson (1949).

RDN1 is the result of their location in repressed chromatin, disruption of *SIR2* might be expected to relieve positional silencing of Ty1 elements. To investigate this, *SIR2* was disrupted in strains JC234 and JC272, which contain Ty1*his3AI* elements in *RDN1*, and in strains JC242 and JC273, which have Ty1*his3AI* elements outside *RDN1* and the rates of transposition of Ty1*his3AI* elements were assessed (Table 3). Disruption of *SIR2* permits expression of the silent *HML* and *HMR* cassettes, resulting in haploid strains that are phenotypically *a/α* (Klar et al. 1979; Shore et al. 1984). This alteration of mating type is significant because Ty1 RNA is decreased ~20-fold in *a/α* cells compared to *a* or *α* cells (Elder et al. 1981). In accord with *a/α* control of Ty1 expression, the transposition rates of Ty1*his3AI* elements in strains containing a marked Ty1 element outside *RDN1* were decreased 10-fold (Table 3; JC242 and JC273). In contrast, for strains containing a marked Ty1 element in *RDN1*, the rates of transposition of Ty1*his3AI* increased 8- and >129-fold (Table 3; JC234 and JC272, respectively), despite the repressive effect of the *a/α* phenotype. Thus, the results indicate that the product of the *SIR2* gene suppresses transposition of Ty1 elements in *RDN1*.

To differentiate between a direct influence of *SIR2* disruption or an indirect influence by *a/α* regulation, isogenic MAT *a/α* and MAT *a* haploid derivatives of strains JC234, JC242, JC272, and JC273 were constructed (see Materials and Methods). In comparison to *a* or *α* haploids, Ty1*his3AI* elements outside *RDN1* transposed at a reduced level in *a/α* haploids, equivalent to that observed in the isogenic *sir2::URA3* strains (data not shown). However, strains with marked Ty1 elements in *RDN1* showed repressed levels of His⁺ prototrophy in *a*, *α*, and *a/α* derivatives, in contrast to the elevated formation of His⁺ prototrophs seen in *sir2::URA3* strains. In

addition, we disrupted *SIR3*, another silencing gene required for repression of the *HM* loci and telomeres, in strains containing Ty1*his3AI* elements in or outside *RDN1*. As in *sir2::URA3* strains, transposition of Ty1 elements outside *RDN1* was reduced in *sir3Δ* strains, as a result of the derepression of the *HM* loci. However, in contrast to *sir2* mutants, no increase in transposition of Ty1 elements in *RDN1* was observed (data not shown). Thus, derepression of rDNA–Ty1*his3AI* transposition in *sir2* mutants is not merely a consequence of the *a/α* phenotype of these strains.

To determine whether the changes in transposition rates observed in *sir2* mutants reflected alterations in the levels of Ty1*his3AI* RNA, Northern blot analysis was performed. Total RNA from eight strains was analyzed with probes for Ty1, *PYK1*, and sense-strand *HIS3*, to detect Ty1*his3AI* RNA (Fig. 5). Total Ty1 RNA levels in the four *sir2::URA3* mutants were significantly decreased relative to levels present in the four *SIR2* strains analyzed (Fig. 5). Similarly, the levels of Ty1*his3AI* RNA in strains with elements outside *RDN1* were also reduced in *sir2::URA3* mutants (Fig. 5; JC242 and JC273). In contrast, disruption of *SIR2* resulted in considerably increased levels of Ty1*his3AI* RNA in strains with elements in *RDN1* that are normally silenced transcriptionally (Fig. 5; JC234 and JC272). Thus, the effects of *SIR2* disruption on transcript levels of Ty1*his3AI* elements located inside or outside *RDN1* mirrored the changes in transposition rates, indicating that *SIR2* functions in transcriptional silencing of Ty1 elements in *RDN1*.

Mitotic stability of Ty1 insertions in RDN1 is dependent on SIR2 and UBC2

Previous work has suggested that Ty1 elements are un-

Table 3. Derepression of transposition of Ty1*his3AI* elements in *RDN1*

Strain	Ty1 <i>his3AI</i> in <i>RDN1</i>	Transposition rate ^a		Ratio of rates { <i>sir2</i> / <i>SIR2</i> }
		<i>SIR2</i>	<i>sir2</i>	
JC234	yes	3.5×10^{-9}	2.9×10^{-8}	8
JC272	yes	$<5.9 \times 10^{-10}$	7.6×10^{-8}	>129
JC242	no	1.5×10^{-7}	1.7×10^{-8}	0.1
JC273	no	1.1×10^{-7}	6.4×10^{-9}	0.1
		<i>HTA1-HTB1</i>	<i>hta1-htb1Δ</i>	{ <i>hta1-htb1Δ</i> / <i>HTA1-HTB1</i> }
JC236	yes	4.0×10^{-9}	5.9×10^{-8}	15
JC272	yes	2.3×10^{-9}	7.0×10^{-8}	30
JC816	yes	$<2.0 \times 10^{-10}$	4.8×10^{-8}	>240
JC242	no	3.6×10^{-8}	1.6×10^{-7}	4
JC273	no	8.0×10^{-8}	4.9×10^{-7}	6
		<i>TOP1</i>	<i>top1Δ</i>	{ <i>top1Δ</i> / <i>TOP1</i> }
JC234	yes	6.0×10^{-9}	2.4×10^{-8}	4
JC236	yes	$<4.7 \times 10^{-9}$	4.7×10^{-8}	>10
JC242	no	1.5×10^{-7}	1.9×10^{-7}	1

^aThe rate of His⁺ prototroph formation per cell per generation, as determined by the method of Lea and Coulson (1949).

Figure 5. Effect of *SIR2* disruption on *Ty1his3AI* and total *Ty1* RNA levels. Total RNA from isogenic *SIR2* (+) and *sir2::URA3* (–) strains containing single *Ty1his3AI* insertions was subjected to Northern blot analysis. The blot was hybridized to a sense-strand *HIS3* riboprobe to detect *Ty1his3AI* transcript (top), an antisense *Ty1* riboprobe to detect total *Ty1* transcript (middle), and an antisense *PYK1* riboprobe as a loading control (bottom). Strains with *Ty1his3AI* insertions in *RDN1* are marked with a superscript o. The *sir2/SIR2* ratio of *Ty1his3AI* RNA normalized to *PYK1* RNA, as determined by scanning densitometry is JC234^o, 89.0; JC242, 0.16; JC272^o, 78.9; JC273, 0.04.

derrepresented and may be mitotically unstable in *RDN1* (Petes 1980), which could explain their low levels of transposition. To test the mitotic stability of de novo *Ty1* insertions in *RDN1*, a *HIS3* allele was introduced into strains containing *Ty1his3AI*-234, -236, -242, -272, and -820 by gene disruption of the *Ty1his3AI* element. We then tested for loss of the *HIS3* marker in the *Ty1* element in each strain, after growth for ~120 generations in nonselective YPD medium. A *His*[–] phenotype could result from loss of the *HIS3* marker by recombination between rDNA repeats, recombination between LTRs,

by ectopic recombination with an unmarked *Ty1* element, or from transcriptional silencing of the *HIS3* gene. Interestingly, no more than 3.8×10^{-4} *His*[–] colonies per generation arose in strains containing *RDN1* elements *Ty1HIS3*-234, -236, -272, and -820 (Table 4). This rate of loss of the *His*⁺ phenotype was equivalent to that of the element *Ty1HIS3*-242, located outside *RDN1* on chromosome XII. The stability of the *HIS3* marker in these strains was not attributable to a selective growth advantage of *His*⁺ cells in YPD broth, as mixed cultures of the *His*[–] strain harboring *Ty1his3AI*-242 and the isogenic *His*⁺ strain containing *Ty1HIS3*-242 had only a slight decrease in the fraction of *His*[–] cells over 120 generations of growth (Table 4). In addition, we found that three *Ty1ADE2* insertions in the NTS and three control *Ty1ADE2* insertions outside *RDN1* had equivalent frequencies of loss of the *Ade*⁺ phenotype after nonselective growth for ~100 generations (data not shown). Hence, the mitotic stability of *Ty1* insertions in *RDN1* indicates that their low level of transcription is not attributable to rapid loss of the *Ty1* insertion from the rDNA array. Furthermore, mitotic instability of *Ty1* elements probably does not explain their underrepresentation in *RDN1* in typical yeast strains. Significantly, these findings also indicate that *HIS3* and *ADE2* marker genes in *Ty1* are not subject to position effect in *RDN1*, even after prolonged nonselective growth.

To determine whether the mitotic stability of *Ty1* insertions is altered in *sir2* mutants, we measured the rate of loss of *Ty1HIS3* elements within (-234 or -272) or outside (-242) *RDN1* in *sir2* strains (Table 4). Loss of the *HIS3* marker from *RDN1* was increased 7.8-fold (*Ty1HIS3*-234) and 12.6-fold (*Ty1HIS3*-272) in isogenic *sir2* derivatives compared to *SIR2* strains. However, the mitotic stability of *Ty1HIS3*-242, located outside *RDN1*,

Table 4. Mitotic stability of *Ty1HIS3* elements in *RDN1*

<i>Ty1</i> element (in <i>RDN1</i>)	Relevant strain genotype	Number of <i>His</i> [–] auxotrophs/ total number of cells analyzed (fraction) ^a	<i>HIS3</i> loss/ generation	Loss relative to WT
<i>Ty1HIS3</i> -234 (yes)	WT	102/2324 (0.044) ^b	3.7×10^{-4}	—
	<i>sir2</i>	176/498 (0.353)	2.9×10^{-3}	7.8
	<i>ubc2Δ</i>	281/1550 (0.181)	1.5×10^{-3}	4
<i>Ty1HIS3</i> -236 (yes)	WT	0/296 (<0.003)	$<2.8 \times 10^{-5}$	—
<i>Ty1HIS3</i> -272 (yes)	WT	127/2749 (0.046) ^b	3.8×10^{-4}	—
	<i>sir2</i>	204/355 (0.575)	4.8×10^{-3}	12.6
	<i>ubc2Δ</i>	334/913 (0.366)	3.0×10^{-3}	7.9
<i>Ty1HIS3</i> -820 (yes)	WT	1/66 (0.015)	1.3×10^{-4}	—
<i>Ty1HIS3</i> -242 (no)	WT	124/2760 (0.045) ^b	3.7×10^{-4}	—
	<i>sir2</i>	10/189 (0.053)	4.4×10^{-4}	1.2
	<i>ubc2Δ</i>	85/1305 (0.065)	5.4×10^{-4}	1.5
<i>Ty1his3AI</i> -242 + <i>Ty1HIS3</i> -242 ^c (no)	WT	1024/1838 (0.557) ^b	N.A.	—

(WT) Wild type. (N.A.) Not applicable.

^aDetermined after 120 generations of nonselective growth in YPD broth.

^bAverage of three independent cultures.

^cThree mixed cultures of isogenic *His*[–] (*Ty1his3AI*-242) and *His*⁺ (*Ty1HIS3*-242) strains were analyzed to determine whether *His*⁺ cells had a growth advantage in nonselective medium. The *Ty1his3AI*-242 + *Ty1HIS3*-242 cultures contained a fraction of 1008/1705 (0.591) *His*[–] cells prior to growth on YPD broth.

was unaltered in a *sir2* derivative. Significantly, the results in Table 4 demonstrate that like *SIR2*, *UBC2* also suppresses mitotic recombination in *RDN1*. In *ubc2Δ* strains, the rate of loss of the *HIS3* marker in *RDN1* increased 4-fold (Ty1*HIS3*-234) and 7.9-fold (Ty1*HIS3*-272), whereas loss of the *HIS3* marker outside *RDN1* (Ty1*HIS3*-242) was not altered significantly. To summarize, our results are consistent with the previous report that *SIR2* regulates mitotic recombination at *RDN1* (Gottlieb and Esposito 1989). Moreover, these results uncover a second novel function for *UBC2* in suppression of mitotic recombination at *RDN1*.

Depletion of histones H2A–H2B abolishes silencing of Ty1his3AI elements in RDN1

If positional silencing of Ty1 elements in *RDN1* is occurring through the effect of a repressive chromatin structure, then perturbations in nucleosome structure might be expected to disrupt silencing. Therefore, deletion of *HTA1–HTB1*, one of two loci that encodes histones H2A and H2B, might reveal a role for chromatin structure in silencing of Ty1 transcription in *RDN1*. Significantly, histones H2A and H2B have also been shown to be targets for the *UBC2* ubiquitin-conjugating activity in vitro (Sung et al. 1988).

The *HTA1–HTB1* locus was deleted in strains containing Ty1*his3AI* elements both in and outside *RDN1*. Transposition of Ty1*his3AI* elements outside *RDN1* increased four- to sixfold in *hta1–htb1Δ* derivatives of strains JC242 and JC273. In contrast, the transposition rates of Ty1*his3AI* elements in *RDN1* increased dramatically from 15-fold (strain JC236) to >240-fold (strain JC816) in the isogenic *hta1–htb1Δ* derivatives (Table 3). Thus, alterations in the levels of histones H2A and H2B cause a modest increase in transposition of Ty1*his3AI* elements located outside *RDN1*, but a far greater enhancement of transposition is seen for Ty1*his3AI* elements in *RDN1*. The higher transposition rates in *hta1–htb1Δ* mutants are accompanied by increases in Ty1*his3AI* and total Ty1 RNA transcript levels (data not shown). In summary, these results suggest that silencing of Ty1 elements in *RDN1* is the result of a specialized chromatin structure at *RDN1*, which may involve packaging of the DNA into a stable nucleosome structure.

Topoisomerase I mediates silencing of Ty1 elements in RDN1

Topoisomerase I has been shown to suppress mitotic recombination between rDNA repeats and to maintain the chromatin structure of *RDN1* (Christman et al. 1988, 1993; Cavalli et al. 1996). We were interested to determine whether the function of topoisomerase I at *RDN1* influenced expression of Ty1*his3AI* elements in *RDN1*. Therefore, the *TOP1* gene was deleted from strains with Ty1*his3AI* insertions in and outside of *RDN1*. As shown in Table 3, the transposition rates of Ty1*his3AI* elements in *RDN1* increased 4-fold (strain JC234) to 10-fold (strain JC236) in *top1Δ* derivatives, whereas the transpo-

sition rate of the element outside *RDN1* stayed the same (strain JC242). The higher transposition rates in *top1Δ* mutants are the result of an increase in Ty1*his3AI* transcript levels from elements in *RDN1* (data not shown). Our results suggest that the role of *TOP1* in maintaining the chromatin structure of the rDNA array is required for transcriptional silencing of Ty1 elements and further support the model that the *RDN1* locus has a unique chromatin structure that is repressive to both mitotic recombination and Ty1 transcription.

Discussion

We have demonstrated transcriptional silencing of five different Ty1 retrotransposons that integrated in varied positions in *RDN1*, resulting in repression of their transcription and therefore, transposition. Moreover, a unique set of *trans*-acting modifiers, *UBC2*, *SIR2*, *HTA1–HTB1*, and *TOP1*, silence transcription of Ty1 elements in *RDN1* but do not affect significantly Ty1 elements outside *RDN1*, supporting a model whereby silencing results from a specialized chromatin structure at *RDN1*. Our results reveal novel features of position-dependent silencing at *RDN1* and differentiate the mechanism of *RDN1* silencing from other types of position effect seen at the *HM* loci and telomeres.

Ty1his3AI elements in rDNA are silenced by a specialized chromatin structure

Ty1*his3AI* elements in rDNA have rates of transposition ranging from 10- to >100-fold lower than a typical active Ty1*his3AI* element located outside *RDN1*, such as the one in strain JC242 (Table 1). The variation in silencing may be attributable to differences in the position of Ty1 elements in the rDNA repeat (Fig. 2C) or in the rDNA array (Fig. 3C), although no simple correlation was observed. Alternatively, inherent *cis*-acting differences in the individual Ty1*his3AI* elements themselves could account for their different transposition rates. Interestingly, however, the magnitude of transcriptional derepression seen in *ubc2Δ*, *sir2*, *hta1–htb1Δ*, and *top1Δ* mutants is related to the strength of silencing for each individual element in the rDNA array. For example, Ty1*his3AI*-234 transposition is derepressed 11-, 8-, and 4-fold in *ubc2Δ*, *sir2*, and *top1Δ* mutants, respectively, whereas the more strongly silenced element Ty1*his3AI*-272 is derepressed >73-, >129-, and 30-fold in *ubc2Δ*, *sir2*, and *hta1–htb1Δ* mutants, respectively (Tables 1 and 3). Thus, the data support a model in which *UBC2*, *SIR2*, *HTA1–HTB1*, and *TOP1* participate in a common mechanism to mediate transcriptional silencing of Ty1 elements in *RDN1*.

The *UBC2*, *SIR2*, *HTA1–HTB1*, and *TOP1* gene products have all been implicated previously in other modifications of chromatin or DNA structure, and their role in transcriptional silencing in *RDN1* is likely to be related to these other functions. *SIR2* is required for positional silencing of repeated regions in the yeast genome, a process that entails formation of a heterochromatin-

like DNA structure. Overexpression of Sir2p has been shown to lead to deacetylation of histones and as nucleosomes at the *HM* loci are hypoacetylated, the proposal has been made that Sir2p is associated with hypoacetylation of silent regions (Braunstein et al. 1993, 1996). In addition, *SIR2* and *TOP1* repress recombination in *RDN1* (Christman et al. 1988; Gottlieb and Esposito 1989) and in this work, we show that *UBC2* has the same effect (Table 4). *TOP1* is required to maintain the normal physical structure of the *RDN1* locus (Christman et al. 1993), and *top1* mutants show increased psoralen accessibility in transcribed rDNA (Cavalli et al. 1996).

A definitive role for chromatin structure in silencing of Ty1 transcription was revealed by a dramatic increase in rDNA-Ty1*his3AI* transposition in *hta1-htb1Δ* mutants (Table 3). Deletion of *HTA1-HTB1* results in depletion of the H2A-H2B dimer from nucleosomes, which is proposed to destabilize nucleosomes by weakening the contacts between histones and DNA (Lee et al. 1993). Hence, transcription is thought to be altered in *hta1-htb1Δ* mutants at loci where positioned nucleosomes are components of transcriptional regulation. For instance, nucleosome positioning in *hta1-htb1Δ* mutants is not altered at *HIS4* and Ty1 LTRs, but is changed dramatically at centromeres *CYH2* and *UBI4*, indicating that nucleosome integrity can play a critical role in regulation of transcription of certain genes (Norris et al. 1988). Smith and Boeke (this issue) show in an accompanying manuscript that a silenced *mURA3* marker within an rDNA repeat is no longer subject to transcriptional silencing when the rDNA repeat is placed outside of the *RDN1* locus. Their data argue against the interpretation that rDNA contains a *cis*-acting silencer of Pol II genes. Hence, the data strongly suggest that the rDNA array adopts a specific chromatin structure that is repressive to certain Pol II genes, including Ty1 elements.

Several features of the *RDN1* locus are analogous to domains of heterochromatin, including the presence of tandemly repeated DNA sequences containing ARS elements, which have been shown to be involved in maintenance of position effect in yeast. Furthermore, suppression of recombination is a common feature of heterochromatin domains in other organisms. The involvement of *SIR2* suggests that the mechanism of transcriptional repression at *RDN1* shares some aspects of position effect at the telomeres and *HM* loci. On the other hand, differences between heterochromatic regions in yeast and the *RDN1* locus suggest that there are different pathways of establishing chromatin structures that are repressive to transcriptional and recombinational processes. At telomeres and the *HM* loci, Sir4p is known to interact with Sir2p, Sir3p and the amino-terminal tails of the histone H3 and H4. However at *RDN1*, transcriptional silencing of Ty1 requires the Sir2 protein and histones H2A and H2B, but does not require Sir3p. Furthermore, Smith and Boeke (1997) show that silencing of other Pol II genes in the rDNA array does not require Sir1p, Sir3p, or Sir4p. In addition, chromatin at telomeres and the *HM* loci has been shown to be inaccessible to DNA-modifying enzymes, whereas the

RDN1 locus is partially accessible to *dam* methylase in wild-type strains (M. Bryk and M.J. Curcio, unpubl.). These results agree with the partial accessibility of rDNA to psoralen cross-linking (Cavalli et al. 1996; Smith and Boeke 1997). Perhaps the need for transcription by Pol I and Pol III necessitates formation of a more accessible type of chromatin at *RDN1* that is repressive to Pol II transcription. One possibility is that the locus is packaged in stabilized nucleosomes that can be repositioned by Pol I and Pol III transcription factors but restrict access preferentially to Pol II transcription factors and mediators of recombination.

The UBC2 protein is a regulator of chromatin structure in RDN1

The *UBC2* gene encodes an E2-ubiquitin-conjugating enzyme that is involved in many cellular processes including DNA repair, damage-induced mutagenesis, N-end rule protein degradation, growth control, sporulation, and Ty1 integration specificity. Ubc2p has been shown to form a specific complex with the E3 protein Ubr1p to mediate N-end rule protein degradation (Madura et al. 1993) and a second complex with the single-stranded DNA-binding protein Rad18p, which is thought to function in DNA repair (Bailly et al. 1994).

Theoretically, the role of *UBC2* in N-end rule degradation could explain the observed transcriptional repression if there was a limiting factor for a cryptic Pol II enhancer in *RDN1* whose concentration is increased in *ubc2Δ* mutants. However, rDNA-Ty1*his3AI* transposition is repressed in strains containing *ubc2-149* and *ubc2-153* alleles and in *ubr1* mutants, all of which are defective in N-end rule degradation (Table 2; data not shown). Hence, N-end rule degradation is not involved in transcriptional silencing of Ty1 elements in rDNA. Furthermore, the existence of a limiting factor required for Ty1 transcription is not supported by our findings that *UBC2* is involved in two silencing processes at *RDN1*, transcriptional repression of de novo Ty1 insertions (Table 1; Figure 4) and suppression of mitotic recombination (Table 4).

There are at least two basic models for the involvement of *UBC2* in formation of a chromatin conformation at *RDN1* that is repressive to Pol II transcription and recombination. First, Ubc2p may alter the chromatin structure of *RDN1* indirectly through its ability to ubiquitinate cellular proteins. Ubiquitination of target proteins by Ubc2p may facilitate the formation of higher-order chromatin structure at *RDN1* or alter the repressive effect of individual nucleosomes. Potential substrates for ubiquitination by Ubc2p include the histones H2A and H2B, as Ubc2p has been shown to ubiquitinate these histone proteins in vitro (Jentsch et al. 1987; Sung et al. 1988). The function of ubiquitination of H2A and H2B in yeast has not been established, as deletion of a potential ubiquitination site that is conserved in higher eukaryotes results in none of the DNA repair, mutagenesis, growth, or sporulation defects associated with *ubc2Δ* mutants (Swerdlow et al. 1990). How-

ever, alteration of chromatin in *RDN1* would result in a subtle phenotype that probably would not have been detected. By examining the transposition phenotype of Ty1 elements in *RDN1* in strains with an *hta1-htb1* allele lacking the proposed ubiquitination site, we will be able to test this model. Alternatively, Ubc2p may ubiquitinate a non-nucleosomal protein that mediates the formation of a repressive chromatin structure, as has been suggested for the deubiquitinating protein Ubp3p, which interacts with Sir4p (Moazed and Johnson 1996).

A model in which the *UBC2* protein modifies a component of chromatin in *RDN1* can explain both the positional silencing of Ty1 elements and the suppression of rDNA recombination. Several other observations also implicate the ubiquitin-conjugating activity of *UBC2* in the modification of chromatin function. First, mutations in *ubc2* have been shown to increase Ty1 integration into Pol II ORFs and to randomize its target specificity (Picologlou et al. 1990; Lieberman and Newnam 1993). This has been interpreted as indicative of a change in chromatin assembly in Pol II ORFs, and our results support this paradigm. As observed previously, total Ty1 RNA levels are not increased in *ubc2Δ* mutants (Fig. 4), and no increase in Ty1*his3AI* transposition is observed unless it is accompanied by an increase in transcription of a specific silenced element (Table 1). Thus, increased targeting to ORFs is not the result of more efficient transposition per Ty1 RNA in *ubc2* mutants. Instead, local alterations in chromatin structure may allow ORFs, which are normally poor integration targets, to be used more frequently in *ubc2* mutants. Second, a recent paper has reported that mice with a disruption in a *UBC2* homolog exhibit defects in condensation of chromatin during spermatogenesis (Roest et al. 1996). Interestingly, the sporulation defects of *ubc2* mutants have also been proposed to result from improper chromatin condensation (Lawrence 1994). The involvement of *UBC2* and its mouse homolog in these processes suggests a role for *UBC2* in chromatin remodeling. Third, Moazed and Johnson (1996) have shown that Ubp3p, an enzyme that deubiquitinates target proteins, is an inhibitor of position-dependent transcriptional silencing at the *HM* loci and telomeres. Their results indicate that ubiquitination can enhance silencing in yeast and support a role for ubiquitination in regulation of chromatin structure.

A second model to explain the role of *UBC2* in a repressive chromatin structure in *RDN1* is that Ubc2p interacts directly with DNA in the *RDN1* locus. *UBC2* functions with *RAD9*, *RAD18*, and other genes in an "error-free" DNA repair pathway, which is involved in repairing single-stranded regions formed by incomplete DNA replication or certain recombination events (Lawrence 1994). Domains of single-stranded DNA may be common in *RDN1*, given the high occurrence of sister-chromatid exchange and gene conversion. In the absence of *UBC2*, repair at *RDN1* may be slowed and/or replacement repair functions may be recruited, resulting in a block to reformation of the specialized chromatin structure in *RDN1*. There is a precedent for disruption of

chromatin structure during defective damage repair, as expression of a silenced telomeric *URA3* gene is stimulated in the absence of *RAD7*, which encodes an accessory for the nucleotide-excision repair pathway (Paetkau et al. 1994). This second model can be tested by analyzing the effect of other genes implicated in the error-free DNA repair pathway on transcriptional silencing and suppression of recombination in *RDN1*.

A role for integration into RDN1 in the regulation of Ty1 transposition

The expression of Ty1 appears to be more sensitive to the effects of chromatin structure in *RDN1* than other Pol II-transcribed genes. Expression of Ty1*his3AI* elements in *RDN1* is barely detectable in our Northern analysis and transposition assays. Interestingly, however, there appears to be no repression of the marker gene. That is, when the *his3AI* gene within the Ty1 element is replaced with *HIS3*, it is expressed, even after 120 generations of nonselective growth. Similarly, no evidence of variegated expression of an *ADE2* marker gene was observed in colonies after 100 generations of nonselective growth of strains containing Ty1*ADE2* insertions in *RDN1*. The stability of marker genes in *RDN1* that are outside the context of Ty1 has been documented previously (Szostak and Wu 1980; Gottlieb and Esposito 1989). Consistent with the absence of an upstream activating sequence (UAS) in the Ty1 promoter, the potency of position effect at *RDN1* may reflect promoter strength. Indeed, the degree of silencing of expression near telomeres is determined primarily by the strength of a gene's promoter (Renauld et al. 1993). Smith and Boeke (1997) have shown that a *URA3* gene with a minimal *trp1* promoter (*mURA3*), the *MET15* gene, and the *LEU2* gene are also subject to position effect in *RDN1*, which is relieved by disruption of *SIR2*.

The fact that the *RDN1* locus is an efficient target for Ty1 integration could play an important role in the regulation of Ty1 transposition, as Ty1 insertions in *RDN1* are phenotypically silent. Because Ty1*his3AI* insertions are present in a single copy of the rDNA repeat (Fig. 3), their presence is unlikely to have any deleterious consequences. Moreover, they are significantly more likely to be lost rather than amplified by the unequal recombination processes that predominate in *RDN1*. Ty1 elements in *RDN1* are also very poorly expressed and therefore, do not contribute significantly to the overall rate of transposition in the cell. Studies with Ty1 elements fused to the *GAL1* promoter have established that a modest increase in Ty1 RNA can overcome a post-translational block that is responsible for maintaining transpositional dormancy (Boeke et al. 1985; Curcio and Garfinkel 1992). Therefore, transposition into *RDN1* could play a role in the regulation of transposition by acting as a domain where Ty1 elements can insert without creating deleterious mutations or causing total Ty1 RNA to increase above a level that would overcome transpositional dormancy. Considering this, it is notable that Ty1 elements are underrepresented in the rDNA in

typical yeast strains, despite the fact that we have shown that Ty1 elements are targeted efficiently to the rDNA array and are mitotically stable in *RDN1* relative to Ty1 elements located elsewhere. Hence, it may be that the rDNA array is used more efficiently at times when Ty1 transcription is elevated, such as during induction by *GAL1*:Ty1 overexpression or during exposure to DNA-damaging agents. This hypothesis can be tested by quantitating transposition into the rDNA array during growth under different environmental conditions.

Materials and methods

Yeast strains

All yeast strains used in this study are congenic derivatives of GRF167 (*MAT α* , *his3 Δ 200*, *ura3-167*) (Boeke et al. 1985), except strains JC820 (*MAT α* , *his3 Δ 200*, *ura3*, ::Ty1*his3AI*-820), and JC821 (*MAT α* , *his3 Δ 200*, *ade2-101*, *leu2 Δ* , *ura3*, ::Ty1*his3AI*-821). Strains JC234 (::Ty1*his3AI*-234), JC236 (::Ty1*his3AI*-236), JC242 (::Ty1*his3AI*-242), JC272 (::Ty1*his3AI*-272), JC273 (::Ty1*his3AI*-273), JC815 (::Ty1*his3AI*-815), and JC816 (::Ty1*his3AI*-816) were isolated after galactose-induction of plasmid pGTy1-H3*mhis3AI* in strain GRF167, as described previously (Curcio and Garfinkel 1991b). The presence of a single genomic Ty1*his3AI* transposition event was demonstrated by Southern blotting with a *HIS3* probe. Strains JC820 and JC821 are ascospores of a cross between an *spt3-101* derivative of GRF167 containing Ty1*his3AI* transposition events. Strain JSS104-32A (*MAT α* , *ade2-101*, *his3 Δ 200*, *leu2- Δ 1*, *ura3-52*) was kindly provided by J.N. Strathern (NCI-FCRDC, Frederick, MD).

Isogenic derivatives of strains GRF167, JC234, JC236, JC242, JC272, JC815, JC816, JC820, and JC821 harboring the *ubc2 Δ ::hisG* allele were constructed by integrative transformation using plasmid pJJ313, as described previously (Curcio and Garfinkel 1994).

The *his3AI* gene was replaced with *HIS3* by transforming strains JC234, JC236, JC242, JC272, and JC820 with a 0.83-kb *Cla*I fragment of pGEM-HIS3 (Curcio and Garfinkel 1992), and selecting for His⁺ transformants. The presence of a Ty1*HIS3* allele was confirmed by Southern analysis.

The *SIR2* gene was disrupted in strains JC234, JC242, JC272, and JC273 by integrative transformation, using the 4.9-kb *Xba*I-*Xho*I fragment of pBST KS+ sir2::URA3gb (see below). The sir2::URA3 disruptions were verified by a nonmating phenotype and Southern analysis. The mating-type of each of the four strains was switched from α to *a*/ α ::URA3 by two-step gene disruption using plasmid pSC11, a URA3-based plasmid containing MAT α (Adams et al. 1995). Subsequently, the mating type was switched to *a* by selection for 5-fluoro-orotic acid (5-FOA)-resistant derivatives of the *a*/ α haploids.

The *HTA1-HTB1* locus encoding histones H2A and H2B was disrupted in strains JC236, JC242, JC272, JC273, and JC816 by integrative transformation of the *Eco*RI-*Msc*I fragment of pUC9 Δ trt1::URA3 (Norris and Osley 1987). The *hta1-htb1 Δ ::URA3* disruptions were verified by Southern analysis.

The *TOP1* gene was disrupted in *trp1 Δ* derivatives of JC236, JC242, JC272, and JC273 by integrative transformation of the *Nco*I-*Pst*I fragment of pTP1-4 *top1 Δ ::TRP1*. The *top1* disruptions were verified by Southern analysis. Plasmid pTP1-4 *top1 Δ ::TRP1* was generously provided by M. Bjornsti (Thomas Jefferson University, Philadelphia, PA).

Plasmid construction

Plasmid pBST KS+ sir2 was constructed by ligating a 1063-bp *Bam*HI-*Eco*RV fragment generated by PCR amplification of the

SIR2 gene from strain GRF167 using oligomers sir2 5.1 (CC-GAGTGATCAAGATAACG) and sir2 3' (CGTTCCATTCT-TATGCGG) into the *Bam*HI-*Eco*RV interval of the pBST KS+ (Stratagene). Plasmid pBST KS+ sir2::URA3gb was constructed by ligating the 3.8-kb *Bam*HI-*Bgl*III *hisG-URA3-hisG* fragment from plasmid pNKY51 (Alani et al. 1987) into the unique *Bgl*III site in *SIR2* sequences of pBST KS+ sir2.

Transposition assays

Transposition rates in strains containing Ty1*his3AI* elements were determined by the maximum likelihood method of Lea and Coulson (1949). Strains were grown to saturation in YPD medium at 30°C. For each strain, 9 or 11 tubes containing 2 ml of YPD medium were inoculated with ~500 cells and grown to saturation at 20°C. For *HTA1-HTB1* and *Δ hta1-htb1* strains, after dilution of cells into YPD, the cultures were grown at 24°C because the *Δ hta1-htb1* strains exhibit a severe growth defect at 20°C. Three cultures were titered on YPD plates, and all cultures were plated to synthetic complete medium lacking histidine (SC-His) and grown at 30°C to quantify His⁺ prototrophs. In all cases, *UBC2* and *ubc2 Δ ::hisG*, *SIR2*, and *sir2::URA3*, *HTA1-HTB1*, and *Δ hta1-htb1*, or *TOP1* and *top1 Δ* isogenic pairs were tested in the same experiment.

For complementation experiments, strains JC236*ubc2 Δ* and JC272*ubc2 Δ* were transformed with YCp50, a URA3-based CEN vector, or derivatives carrying *UBC2*, *ubc2-149*, *ubc2-153* (plasmid pR67, pR615, and pR616, respectively; Morrison et al. 1988) or *ubc2 Δ 1-9* (plasmid pR68; Watkins et al. 1993). Plasmids carrying *UBC2* alleles were generously provided by L. Prakash (University of Texas, Galveston). Plasmid-containing strains were grown to saturation in SC-Ura medium. For each strain, 11 tubes containing 2 ml of SC-Ura medium were inoculated with ~500 cells and grown to saturation at 20°C. The cultures were titered on SC-Ura and plated to SC-Ura-His to quantify His⁺ Ura⁺ prototroph formation.

CHEF gel analysis

Agarose-embedded genomic DNA was prepared from 1 ml of an overnight culture in YPD medium (Gerring et al. 1991). In the case where genomic DNA was subject to digestion with *Bam*HI, plugs were preincubated in PMSF, and then incubated with 40 units of enzyme for 15 hr at 37°C. Using a BioRad CHEF Mapper apparatus, samples were separated by electrophoresis on a 1% agarose gel (SeaKem Gold) in 0.5 $\times$ TBE buffer at 14°C using 200 V and 50- to 90-sec pulse times for 24 hr. The gel was stained with ethidium bromide and photographed. After UV irradiation for 2 min in a Spectrolinker (Spectronics), DNA was transferred onto a Hybond-N+ membrane (Amersham) and probed with a *HIS3* riboprobe synthesized as described below. The probe was stripped from the blot in 0.4 N NaOH at 37°C and rehybridized to an *RDN1* riboprobe synthesized from pGEM-18S, which contains an *Eco*RI fragment of the 18S rDNA gene cloned into pSP70 (Promega). The sizes of rDNA-hybridizing *Bam*HI fragments (Fig. 3) were estimated from Lambda Ladder standards (Bio-Rad) and added to determine the total size of *RDN1*. For strains JC234 and JC236, the *RDN1* locus was estimated to be 1900 kb. Variability in the sizes of the *RDN1* locus and its fragments was noted in repeats of these experiments. This is not unexpected as the size of *RDN1* can vary during cell growth (Olson 1991).

Isolation and sequencing of DNA flanking Ty1*his3AI* insertions

The 3' end of Ty1*his3AI* insertions and flanking genomic DNA was cloned by the integration and eviction method of Winston

et al. (1983). Plasmid pGEM-HIS3 contains an 890-bp fragment of the yeast *HIS3* gene (Garfinkel et al. 1988) subcloned into pSP70 (Promega). The plasmid was linearized within *HIS3* by *NheI* and transformed into yeast strains JC234, JC236, JC242, JC272, JC815, JC816, and JC820 by the lithium acetate method (Gietz et al. 1992). His⁺ plasmid integrants were single-colony purified. To evict pGEM-HIS3 and sequences flanking the Ty1*his3AI* element, 100 ng of genomic DNA prepared from independent His⁺ transformants was digested with *AatII*, which cleaves once in pGEM-HIS3 but not in *his3AI* or the Ty1-LTR. The resulting *AatII* fragment contains vector sequences, the 3' LTR, and flanking genomic DNA. *AatII*-digested DNA was phenol extracted, ethanol precipitated, resuspended in 1× TE buffer (pH 8), and 1 of 50 µl was ligated in a 20-µl reaction. Ligations were used to transform *Escherichia coli* strain DH5 to ampicillin resistance. The presence of a 0.45-kb *BamHI* fragment of *his3AI* in plasmid evictants from three independent His⁺ prototrophs was used to verify eviction of the Ty1*his3AI* junction fragment. DNA sequencing from the 3' LTR was performed using the oligomer AX009 (CTTTATCAACAATGGAATCCC) and Sequenase, version 2.0 (U.S. Biochemical).

Assay for mitotic stability of Ty1HIS3 elements

The *his3AI* marker in strains JC234, JC236, JC242, JC272, and JC820 was replaced with a *HIS3* marker by integrative transformation. A single colony of each His⁺ transformant was taken from SC-His medium and used to inoculate 10 ml of YPD medium. In addition, single colonies of the His⁻ auxotroph JC242 (pregrown on YPD) and the Ty1HIS3 derivative of JC242 were mixed in 10 ml of YPD medium. An aliquot of the JC242+JC242-Ty1HIS3 mixed culture was plated onto YPD medium, and then replicated to SC-His medium to determine the fraction of His⁻ colonies before prolonged growth in nonselective medium. All cultures were grown overnight at 30°C. One microliter of each culture was then diluted into 10 ml of fresh YPD and grown to stationary phase (32–40 hr) at 30°C. The 1:10,000 dilution was repeated a total of eight times. Cells from the ninth serial culture were plated onto YPD plates, and replicated to determine the fraction of His⁻ auxotrophs.

Northern blot analysis

Yeast strains were grown overnight in YPD medium at 30°C to saturation, and then diluted 1:25 in fresh YPD, except for *ubc2Δ::hisG* strains, which were diluted 1:12.5, and all were grown at 20°C for 4 hr to exponential phase. Total RNA was isolated from 5×10^8 to 7×10^8 cells as described by Schmitt et al. (1990). RNA samples were denatured by treatment with glyoxal as described previously (Curcio et al. 1990), separated by electrophoresis in 1% agarose (SeaKem Gold, FMC), and transferred to Hybond-N nylon membrane (Amersham). Radiolabeled RNA probes were synthesized in vitro using Riboprobe Gemini System II (Promega). Plasmids pGEM-HIS3, pGEM-PYK1, and pGEM-Ty1A (Curcio and Garfinkel 1992) were used as templates for in vitro transcription of sense-strand *HIS3* and antisense-strand *PYK1* and Ty1 probes, respectively. Northern blots were quantified by scanning densitometry using a Howtek Scanmaster 3+ scanner and Scanalytics software. Ty1*his3AI* RNA levels normalized to *PYK1* RNA were determined for each strain and the ratio of Ty1*his3AI*/*PYK1* RNA for each strain pair is reported in the legends to Figures 4 (*ubc2Δ/UBC2*) and 5 (*sir2/SIR2*). The ratio of *PYK1* RNA to rRNA (as determined by ethidium staining of RNA) was not significantly altered by *ubc2Δ*.

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