Changes in histone gene dosage alter transcription in yeast

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Chromatin structure is believed to be important for a number of cellular processes, including transcription. However, the role of nucleosomes in transcription is not well understood. We have identified the yeast histone locus *HTB1-HTB1*, encoding histones H2A and H2B, as a suppressor of solo δ insertion mutations that inhibit adjacent gene expression. The *HTA1-HTB1* locus causes suppression either when present on a high-copy-number plasmid or when mutant. These changes in *HTA1-HTB1* alter transcription of the genes adjacent to the δ insertions. On the basis of this result, we have examined the effects of increased and decreased histone gene dosage for all four yeast histone loci. From the types of histone gene dosage changes that cause suppression of insertion mutations, we conclude that altered stoichiometry of histone dimer sets can alter transcription in yeast.

Many studies have demonstrated the presence of altered chromatin structure in transcriptionally active regions of the genome (for reviews, see Mathis et al. 1980; Pederson et al. 1986). However, other work has shown that the level of transcription does not affect chromatin structure at certain loci (Nasmyth 1982; for review, see Mathis et al. 1980). Thus, the cause-and-effect relationship between chromatin structure and transcription remains unclear.

The major subunit of chromatin is the nucleosome, which consists of DNA wrapped around a histone octamer that is composed of two H2A-H2B dimers and an H3-H4 tetramer (for review, see Pederson et al. 1986). Since histones are the protein components of nucleosomes, it is reasonable to hypothesize that alteration of histone levels would affect chromatin structure. A genetic approach to study the effect of chromatin structure on transcription and other cellular processes would be to alter histone levels by mutation or by gene amplification and then to screen for resulting mutant phenotypes.

Changes in histone levels have been reported to cause various phenotypes. Meeks-Wagner and Hartwell (1986) showed that a high copy number of the genes encoding H2A and H2B or of the genes encoding H3 and H4 causes increased chromosome loss during mitosis in *Saccharomyces cerevisiae*. Yeast cells depleted for H2B arrest in

mitosis, with defects in chromatin structure and nuclear segregation (Han et al. 1987). Norris and Osley (1987) demonstrated that deletion of one of the two H2A-H2B loci affects many aspects of the yeast life cycle, including the mitotic cell cycle and the stress response. In *Drosophila melanogaster*, a reduction of histone gene copy number suppresses gene inactivation associated with position-effect variegation (Moore et al. 1983). These studies suggest that altering histone levels can cause pleiotropic phenotypes, perhaps due to perturbations in chromatin structure.

If a wild-type level of histones is required for normal transcription, a genetic screen for transcription mutants might identify mutations in histone genes. We have isolated a large number of transcription mutants in the yeast *Saccharomyces cerevisiae* as suppressors of Ty and solo δ insertion mutations that inhibit normal transcription of adjacent genes (Winston et al. 1984b, 1987; Fassler and Winston 1988). Mutations in *SPT* genes (SPT = suppressor of Ty) suppress Ty and δ insertion mutations by restoration of functional transcription to the adjacent genes (Silverman and Fink 1984; Winston et al. 1984a; Fassler and Winston 1988). Additionally, mutations in *SPT* genes confer various other mutant phenotypes, and some *SPT* genes are essential for growth, suggesting that they are required for more general aspects of cellular transcription (Winston et al. 1984a,b; Clark-Adams and Winston 1987; Fassler and Winston 1987).

Recently, we have shown that increased dosage of the wild-type *SPT6* gene causes suppression of δ insertion

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mutations at the transcriptional level (Clark-Adams and Winston 1987; unpubl.). On the basis of this result, we have screened for other high-copy-number clones that can suppress δ insertion mutations. One of the high-copy-number clones isolated by this screen contains the genes previously identified as *SPT11* and *SPT12* (Fassler and Winston 1988). Genetic and molecular evidence demonstrates that these are the same genes as the histone genes *HTA1* and *HTB1*. Analysis of altered gene dosage of this locus and of the other histone loci in yeast suggests that a change in the normal stoichiometry of histone proteins alters transcription in yeast.

Results

Isolation of high-copy-number suppressors of δ insertion mutations

Previous studies of the *SPT6* gene demonstrated that increased dosage of the wild-type *SPT6* gene, as well as mutations in *SPT6*, causes suppression of δ insertion mutations (Winston et al. 1984b; Clark-Adams and Winston 1987). Based on this result, we screened for other genes that in high copy number can suppress insertion mutations. We transformed a yeast library in the high-copy-number plasmid YEp24 (Carlson and Botstein 1982) into strain FW1238 (*MATα* his4-912δ lys2-128δ ura3-52). Ura⁺ transformants were screened for suppression of the solo δ insertion mutation *lys2-128δ* from Lys⁻ to Lys⁺. Thirty-one Lys⁺ candidates were isolated from approximately 50,000 Ura⁺ transformants. Thirty of the candidates also suppressed *his4-912δ*, resulting in a His⁺ phenotype; the single Lys⁺ His⁻ clone contained the *LYS2* gene as determined by restriction endonuclease mapping. The remaining clones fell into two classes by restriction endonuclease mapping and Southern hybridization analysis. Class 1 contained 17 members; class 2 contained 13 members. Our failure to reisolate the *SPT6* gene from this particular screen probably reflects the large size of *SPT6* (4.5 kb) (Clark-Adams and Winston 1987; Neigeborn et al. 1987), since the 4.2-kb *LYS2* gene was isolated only once.

To confirm that the suppression phenotype caused by the class 1 and class 2 clones was due to high copy number, we subcloned large restriction fragments from each class into the low-copy-number centromere-containing vector YCp50 (Johnston and Davis 1984) and into the high-copy-number vector YEp24. The low-copy-number subclones do not cause suppression of *his4-912δ* or *lys2-128δ*, whereas the high-copy-number subclones do cause suppression. Therefore, the DNA fragments carried on the class 1 and class 2 clones cause suppression only when present in high copy number. The experiments in this paper focus on results with the class 2 clone.

The class 2 clone contains the SPT11 and SPT12 genes

We isolated the class 2 clone as a high-copy-number suppressor of δ insertion mutations. To determine if null

mutations in the class 2 locus would cause suppression or some other mutant phenotype, we constructed disruptions at this locus by Tn10-LUK transposon insertion mutagenesis in *E. coli* (Huisman et al. 1987; see Experimental procedures). Eleven Tn10-LUK insertion mutations that eliminated the class 2 high-copy-number suppression phenotype were recombined into the genome of strain FW1237, replacing the wild-type locus (Rothstein 1983; see Experimental procedures). Haploid strains with these Tn10-LUK insertions grow more slowly than wild-type strains but are viable, demonstrating that the class 2 locus is not essential for growth. In addition, strains that contain Tn10-LUK disruptions of the class 2 locus exhibit the Spt⁻ phenotype of suppression of δ insertion mutations. Thus, either overproduction or disruption of the class 2 locus causes suppression of δ insertion mutations.

Since the class 2 disruption alleles exhibited an Spt⁻ phenotype, we tested whether the class 2 locus could complement mutations in any previously identified *SPT* gene. We transformed strains containing representative mutant alleles of the 14 different SPT genes with a low-copy-number plasmid carrying the class 2 locus. This plasmid complemented the Spt⁻ phenotype of either *spt11* or *spt12* mutant strains. Mutations in *SPT11* and *SPT12* are very tightly linked to each other and are suppressors of solo δ and complete Ty insertion mutations (Fassler and Winston 1988). To prove that the class 2 locus contains the *SPT11* and *SPT12* genes, we showed that certain class 2 Tn10-LUK disruption alleles fail to complement *spt11* and *spt12* mutations. Furthermore, all of these mutations segregate in crosses as tightly linked markers. These results demonstrate that the *SPT11* and *SPT12* genes cause the high-copy-number suppression phenotype of the class 2 clone.

SPT11 and SPT12 are the histone genes HTA1 and HTB1

We mapped the *SPT11* and *SPT12* locus to chromosome IV by the 2μ-mapping method (Falco et al. 1982; Falco and Botstein 1983). By tetrad analysis we showed that the *SPT11* and *SPT12* locus is 12 cM from *LYS4* and 16 cM from *PET14*. From the map distance and the relative frequencies of recombinants between markers in the same cross, we conclude that the map order is *CEN4-PET14-(SPT11* and *SPT12)-LYS4*. This is very close to the genetic map position for the histone genes *HTA1* and *HTB1* (Norris and Osley 1987), suggesting that *SPT11* and *SPT12* may be these two histone genes. This idea was supported by the observation that the restriction map of the *SPT11* and *SPT12* locus is identical to that of the *HTA1-HTB1* locus (Hereford et al. 1979) with respect to *Hind*III, *Pvu*II, and *Bgl*II restriction sites. Southern hybridization analysis between a plasmid that contains the *HTA1-HTB1* locus and one that contains the *SPT11* and *SPT12* locus demonstrated that these two loci contain homologous sequences (data not shown).

If *SPT11* and *SPT12* are *HTA1* and *HTB1*, we would expect that mutations in *HTA1* and *HTB1* would exhibit

suppression of δ insertion mutations. We observed that a deletion of the *HTA1-HTB1* locus does exhibit suppression of insertion mutations (strain CC232). To confirm that the *SPT11* and *SPT12* genes are *HTA1* and *HTB1* we showed that a deletion of the *HTA1-HTB1* locus fails to complement either *spt11* or *spt12* mutations for suppression.

To verify that the high-copy-number suppression phenotype of the class 2 clone was caused by the *HTA1* and *HTB1* genes, we examined the effects of 32 Tn10-LUK transposon insertion mutations (see Experimental procedures) in the class 2 clone on suppression of insertion mutations. Eleven of the Tn10-LUK insertions eliminated high-copy-number suppression; all of these insertions mapped within the *HTA1-HTB1* region by restriction endonuclease analysis (Fig. 1). The remaining 21 Tn10-LUK insertions that did not affect suppression mapped to regions on either side of the *HTA1-HTB1* locus, including the region containing the Protein 1 (*PRT1*) gene, whose function is unknown (Hereford et al. 1979).

To determine the exact position of five of the Tn10-LUK insertion mutations that eliminated high-copy-number suppression, we sequenced their sites of insertion. Two of these insertion mutations are in the coding regions for *HTA1* and *HTB1*: *hta1-3 : : Tn10-LUK* is inserted 27 bp 3′ of the *HTA1* translation initiation codon; *htb1-5 : : Tn10-LUK* is inserted 98 bp 3′ of the *HTB1* initiation codon. The remaining insertions appear to be located within sequences encoding untranslated portions of the *HTA1* and *HTB1* transcripts: *hta1-1 : : Tn10-LUK* and *hta1-2 : : Tn10-LUK* are both inserted 74 bp 3′ of the *HTA1* translation stop codon; *htb1-4 : : Tn10-LUK* is located 4 bp 5′ of the *HTB1* translation start codon. These results prove that high copy number of the *HTA1-HTB1* locus causes suppression of δ insertion mutations. Apparently, overproduction of both H2A and H2B is necessary to cause suppression, since Tn10-LUK insertions into either *HTA1* or *HTB1* destroy high-copy-number suppression. Thus, suppression is probably caused by an elevated level of the heterodimer H2A-H2B, since high copy number of either *HTA1* or *HTB1* alone does not cause suppression.

Increased dosage of other histone loci causes suppression of δ insertion mutations

The *HTA1-HTB1* locus was isolated as a high-copy-number suppressor of δ insertion mutations; therefore, we tested whether the other histone loci, when in high copy number, would also cause suppression. *Saccharomyces cerevisiae* has four unlinked histone loci organized as divergently transcribed gene pairs. There are two H2A-H2B gene pairs located at the *HTA1-HTB1* and *HTA2-HTB2* loci, and two H3-H4 gene pairs encoded by the *HHT1-HHF1* and *HHT2-HHF2* loci (Hereford et al. 1979; Smith and Murray 1983).

We first tested the high-copy-number *HTA2-HTB2* plasmid, pDN242, in strain FW1237 and found that it caused suppression of both *his4-912δ* and *lys2-128δ* from His⁻Lys⁻ to His⁺Lys⁺ (Table 1). Since the two histone loci *HTA1 -HTB1* and *HTA2 -HTB2* share some restriction sites and limited homology at the DNA level (Hereford et al. 1979), we did more detailed restriction mapping and Southern hybridization analysis of the 13 original class 2 clones. We discovered that four of the 13 are actually *HTA2 -HTB2* clones. Therefore, both of the H2A-H2B loci were isolated as high-copy-number suppressors of δ insertion mutations.

We also tested the high-copy-number plasmids pCC64 and pCC66 containing the H3-H4 loci *HHT1-HHF1* and *HHT2-HHF2* (Smith and Murray 1983; Smith and Andresson 1983). Both pCC64 and pCC66 suppress *his4-912δ* from His⁻ to His⁺ but only weakly suppress *lys2-128δ* from Lys⁻ to Lys⁻/⁺ (Table 1). The weak suppression of *lys2-128δ* explains why neither of the H3-H4 loci were isolated in the original screen for strong Lys⁺ transformants. These results demonstrate that increased dosage of any one of the four histone gene pairs causes suppression of δ insertion mutations (Fig. 2).
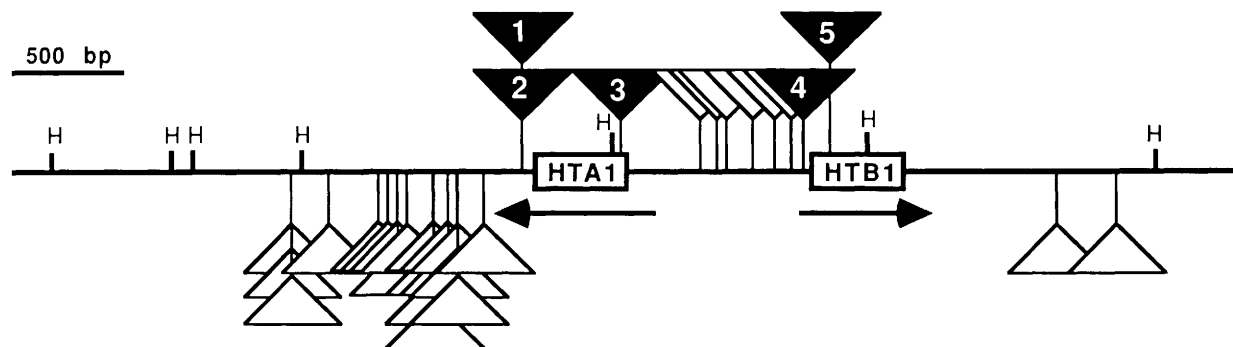
Figure 1. Tn10-LUK insertions at the *HTA1-HTB1* locus. Each triangle represents a single 6.09-kb Tn10-LUK element. Triangles below the line indicate Tn10-LUK insertions that did not affect the high-copy-number suppression phenotype of plasmid pCC61. Triangles above the line represent Tn10-LUK insertions that eliminated the high-copy-number suppression phenotype of pCC61. The 11 insertions shown above the line were transplaced into the genome as described in Experimental procedures. The black triangles represent Tn10-LUK elements whose sites of insertion were sequenced. The black arrows indicate the approximate positions of the *HTA1* and *HTB1* transcripts. (H) *Hind*III restriction sites.

Table 1. *Suppression of insertion mutations by histone genes in high copy number*

Plasmid	Histone locus in high copy number	*his4-912δ*	*lys2-128δ*
YEp24	none	−	−
pCC61	*HTA1-HTB1*	+	+
pDN242	*HTA2-HTB2*	+	+
pCC64	*HHT1-HHF1*	+	−/+
pCC66	*HHT2-HHF2*	+	−/+
pCC67	*HTA1-HTB1, HHT1-HHF1*	−	−

Symbols indicate growth on media lacking histidine for *his4-912δ* and on media lacking lysine for *lys2-128δ*. (+) Strong growth; (−/+) weak growth; (−) no growth. Suppression was determined by replica plating onto SD plates supplemented with the appropriate amino acids.

Overproducing all four histones eliminates suppression

We have demonstrated that increased dosage of histone gene pairs that encode either an H2A-H2B or an H3-H4 dimer set causes suppression of δ insertion mutations. To test whether suppression is due to the creation of an imbalance between dimer sets, we constructed a high-copy-number plasmid, pCC67, which contains all four histone genes from the *HTA1-HTB1* and *HHT1-HHF1* loci. This plasmid does not confer suppression when transformed into strain FW1237; the strain remains His⁻ and Lys⁻ (Table 1; Fig. 2). The *HTB1* and *HHT1* transcript levels in this transformant are overproduced to approximately the same degree (two- to fivefold) as in strains that contain high-copy-number clones of the individual *HTA1-HTB1* or *HHT1-HHF1* loci (approximately threefold overproduction; Fig. 3). We conclude that altering the ratio of H2A-H2B to H3-H4 dimers causes suppression of δ insertion mutations. This result is similar to that of Meeks-Wagner and Hartwell (1986), whose experiments suggested that the ratio of H2A and

H2B to H3 and H4 is important for the normal fidelity of mitotic chromosome transmission.

Reduced histone gene dosage causes suppression

We next examined the effect of reduced histone gene dosage on suppression of δ insertion mutations for all four histone loci. Since each histone locus is duplicated, any gene disruption will halve the gene dosage (the level of histone protein may not decrease twofold if the different loci are not expressed at the same level or if the remaining gene can compensate for the reduced gene dosage). We have demonstrated that deletion of the *HTA1-HTB1* locus causes suppression of δ insertion mutations and that Tn10-LUK insertion mutations in either *HTA1* or *HTB1* also cause suppression (Table 2).

While deletion of *HTA1-HTB1* causes suppression, deletion of *HTA2-HTB2* does not cause suppression (Table 2). One explanation for this result is the observation that the *HTA1-HTB1* locus can increase its expression to compensate for the loss of *HTA2-HTB2*, but that *HTA2-HTB2* is unable to compensate for the loss of *HTA1-HTB1* (Norris and Osley 1987). Alternatively, suppression of δ insertion mutations caused by Δ(hta1-htb1) could be due to the loss of specific H2A and H2B protein subtypes encoded at the *HTA1-HTB1* locus (Wallis et al. 1980; Choe et al. 1982). To distinguish between these possibilities, we duplicated the *HTA2-HTB2* locus in a Δ(hta1-htb1) strain. Duplication of *HTA2-HTB2* eliminates the suppression phenotype of Δ(hta1-htb1) (Table 2). This result demonstrates that suppression of δ insertion mutations caused by deletion of the *HTA1-HTB1* locus is due to a reduction in H2A and H2B levels. Most of the phenotypes Norris and Osley (1987) observed as a result of deleting *HTA1-HTB1* were also due to a reduction in H2A and H2B levels.

Deletion of either of the H3-H4 loci does not cause suppression of *his4-912δ* and *lys2-128δ* (Table 2). Perhaps each H3-H4 locus can compensate for loss of the other, so that deletion of one locus does not result in a reduction in the level of H3 and H4 proteins. Alternatively, a reduction in H3-H4 levels may not cause suppression of δ insertion mutations.
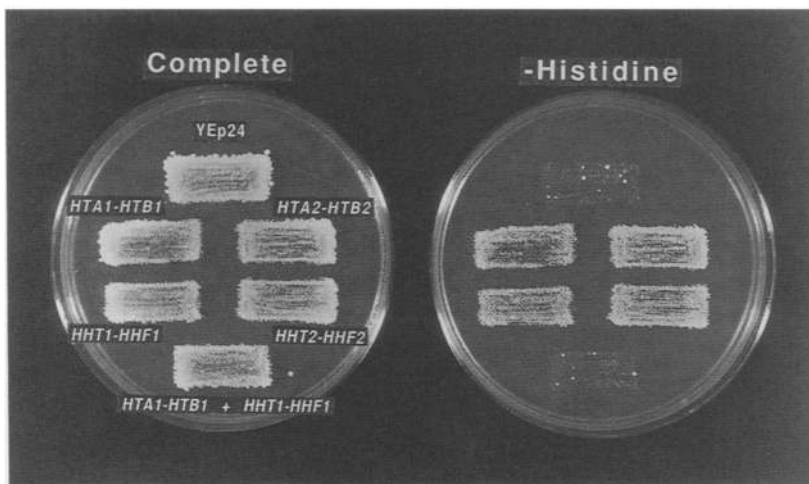
Figure 2. Suppression of *his4-912δ* by high-copy-number histone genes. Strain FW1237 was transformed with high-copy-number plasmids carrying the histone loci indicated in the figure: YEp24; pCC61(*HTA1-HTB1*); pDN242(*HTA2-HTB2*); pCC64(*HHT1-HHF1*); pCC66(*HHT2-HHF2*); pCC67(*HTA1-HTB1* and *HHT1-HHF1*). The transformants were grown in patches on an SD plate supplemented with all amino acid requirements and then replica-plated onto media lacking histidine (-Histidine) and to media containing all amino acid requirements (Complete).

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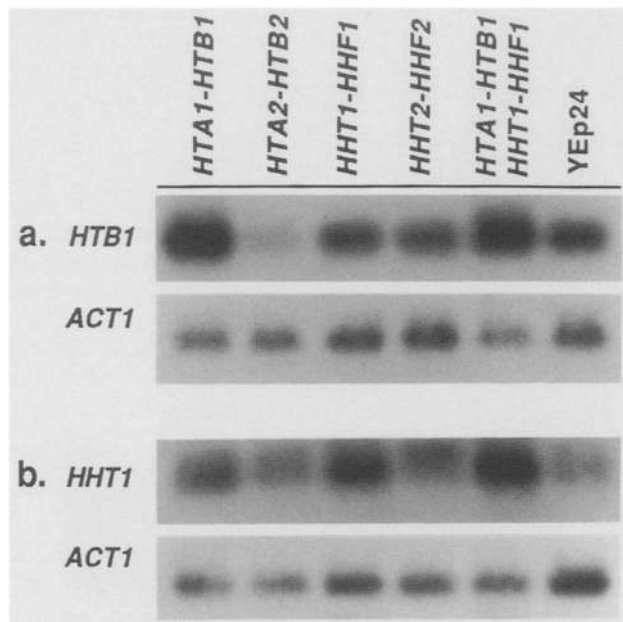
Figure 3. Northern analysis of histone RNA in strains with histone genes in high copy number. Strain FW1237 was transformed with high-copy-number plasmids carrying the histone loci indicated in the figure: pCC61(*HTA1-HTB1*); pDN242(*HTA2-HTB2*); pCC64(*HHT1-HHF1*); pCC66(*HHT2-HHF2*); pCC67(*HTA1-HTB1* and *HHT1-HHF1*); YEp24. Total RNA was isolated from the transformants. (*a*) Four micrograms of RNA was run per lane. The probes were an *HTB1*-specific fragment and an *ACT1*-specific fragment. *HTB1* mRNA is approximately 0.6 kb in length; *ACT1* mRNA is 1.4 kb. (The amount of *HTB1* transcript is reduced four- to fivefold in the strain containing pDN242 HTA2-HTB2) relative to the amount in the strain containing YEp24, probably reflecting dosage compensation by the *HTA1-HTB1* locus.) (*b*) One microgram of RNA was run per lane. The probes were an *HHT1*-specific fragment and an *ACT1*-specific fragment. *HHT1* mRNA is approximately 0.7 kb long.

Deletion of HTA1 *and* HTB1 *alters transcription of* his4-912δ *and* lys2-128δ

In all cases studied, *spt* mutations suppress insertion mutations at the transcriptional level (Silverman and Fink 1984; Winston et al. 1984a; Clark-Adams and Winston 1987; Fassler and Winston 1988). Northern hybridization analysis shows that suppression of *his4-912δ* and *lys2-128δ* by Δ(*hta1-htb1*) also occurs via specific transcriptional alterations (Fig. 4). Wild-type *HIS4* and *LYS2* transcription appear unaffected by Δ(*hta1-htb1*).

In *his4-912δ* strains, *HIS4* transcription occurs in both *HTA1-HTB1* and Δ(*hta1-htb1*) genetic backgrounds, but the predominant transcripts differ in size. In an *HTA1-HTB1 his4-912δ* strain, which is His⁻, the most abundant *HIS4* transcript initiates in the δ sequence and is nonfunctional for *HIS4* expression (Silverman and Fink 1984) (Fig. 4a, lane 3). In the Δ(*hta1-htb1*) *his4-912δ* strain, which is His⁺, two transcripts are apparent. A wild-type-size *HIS4* transcript is present in addition to a reduced amount of the δ-initiated transcript (Fig. 4a, lane 4). Thus, Δ(*hta1-htb1*) restores a functional *HIS4*

transcript to *his4-912δ*, apparently by altering the initiation site of the *HIS4* mRNA. We have observed similar transcriptional alterations at *his4-912δ* caused by high-copy-number clones containing any one of the four histone loci (data not shown).

For *lys2-128δ*, a different type of transcriptional alteration was observed. In contrast to *his4-912δ*, at which the δ sequence is inserted in the 5′ noncoding region of *HIS4*, the δ sequence at *lys2-128δ* is inserted within the 5′ end of the *LYS2* coding region (Clark-Adams and Winston 1987). We used two *LYS2* probes to study transcription of *lys2-128δ*: pFW47, an internal *LYS2* probe, and pFW112, a probe containing 5′ noncoding and coding sequences (see Experimental procedures). These probes were used individually in initial experiments and were mixed for later experiments. For an *HTA1-HTB1 lys2-128δ* strain, which is Lys⁻, only one *LYS2* transcript is present: a 580-bp transcript, detected with the 5′ probe pFW112 (Fig. 4b, lane 3; Clark-Adams and Winston 1987). The size of this transcript is consistent with that of a transcript that initiates at the normal *LYS2* transcription start site and terminates in the δ sequence. No major *LYS2* mRNA is detected with the internal *LYS2* probe. In a Δ(*hta1-htb1*) *lys2-128δ* double mutant (Fig. 4b, lane 4), which is Lys⁺, two transcripts are present. The 5′ *LYS2* probe detects a short transcript similar in size to the 5′ transcript seen in the *HTA1-HTB1 lys2-128δ* strain. In addition, the internal *LYS2* probe detects a new *LYS2* transcript that is slightly shorter than the wild-type *LYS2* mRNA. We believe this new transcript is functional since the strain is Lys⁺. Apparently, the Δ(*hta1-htb1*) mutation alters transcription at *lys2-128δ* to create a functional *LYS2* transcript. (In both *HTA1-HTB1* and Δ(*hta1-htb1*) backgrounds, the internal *LYS2* probe sometimes detects a minor *LYS2* transcript of approximately 3.8 kb).

Discussion

Our results demonstrate that changes in the dosage of the histone locus *HTA1-HTB1* can alter transcription in

Table 2. *Suppression of insertion mutations by mutations in histone loci*

Relevant genotype	his4-912δ	lys2-128δ
Wild type	−	−
hta1-3::Tn10-LUK	+	+
htb1-5::Tn10-LUK	+	+
Δ(hta1-htb1)	+	+
Δ(hta2-htb2)	−	−
Δ(hta1-htb1), HTA2-HTB2/HTA2-HTB2	−	−
Δ(hht1-hhf1)	−	−
Δ(hht2-hhf2)	−	−

Symbols indicate growth on media lacking histidine for *his4-912δ* and on media lacking lysine for *lys2-128δ*. (+) Strong growth; (−) no growth. Suppression was determined by replica plating onto SD plates supplemented with the appropriate amino acids. *HTA2-HTB2/HTA2-HTB2* indicates a duplication of this locus.
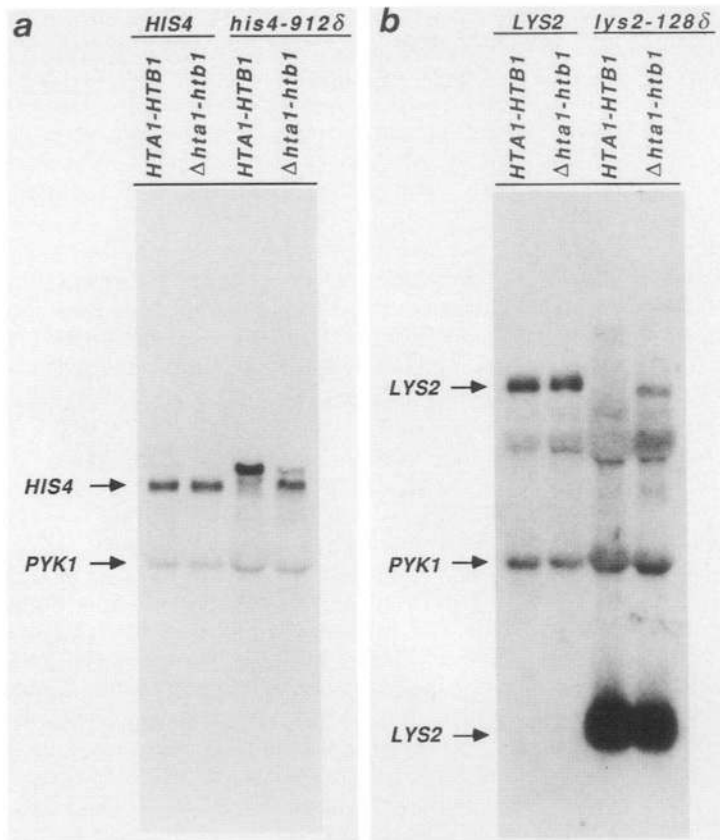
Figure 4. Northern analysis of *HIS4*- and *LYS2*-specific RNA in *HTA1-HTB1* and Δ(*hta1-htb1*) strains. Total RNA was isolated from the strains indicated. One microgram of RNA was run in lanes *1* and *2*; 5 μg was run in lanes *3* and *4*. The strains represented are (*left to right*): S288C, DN106, FW1237, and CC232. (*a*) The probes were pFW45(HIS4) and pFR2(PYK1). *HIS4* mRNA is 2.6 kb long. (*b*) The probes were pFW47(internal *LYS2*), pFW112(5' *LYS2*), and pFR2. *LYS2* mRNA is 4.2 kb. The faint band present in all four lanes is caused by the presence of 25S rRNA.

S. cerevisiae. The cases for which we have observed transcriptional changes are solo δ insertion mutations at the *HIS4* and *LYS2* genes. The transcriptional alterations result in suppression of these insertion mutations, changing the phenotypes they confer from His⁻ and Lys⁻ to His⁺ and Lys⁺. The suppression phenotype has allowed us to score the effects of both amplification and mutation of all four *S. cerevisiae* histone loci on transcription. Our analysis suggests that it is the alteration of the proper stoichiometry of histone dimer sets that causes the transcriptional changes.

Two results directly suggest that an imbalance of histone dimer sets, rather than an imbalance of individual histone proteins or a change in the absolute level of histones, is responsible for the effects we have observed. First, both histones H2A and H2B must be overproduced to cause suppression of insertion mutations; a mutation in either *HTA1* or *HTB1* on a high-copy-number plasmid abolishes the suppression phenotype. Second, high-copy-number plasmids that contain any one of the histone gene pairs cause suppression of solo δ insertion mutations, yet when two different loci encoding all four histones are on the same high-copy-number plasmid, no suppression is observed.

While high copy number of any of the four histone loci causes suppression of insertion mutations, deletion of only one particular histone locus, *HTA1-HTB1*, causes suppression. At this locus, either a deletion encompassing both genes or mutations within either gene alone cause suppression. Suppression is observed for the Tn10-LUK insertion mutations described in this work and for the spontaneous *HTA1* and *HTB1* mutations isolated as *spt11* and *spt12* suppressor mutations (Fassler and Winston 1988). This result is consistent with the model that the level of histone dimers is the critical parameter causing a transcriptional change; either a deletion of *HTA1-HTB1* or a single mutation in one of the two genes should reduce the level of H2A-H2B dimers.

A deletion of the other H2A-H2B locus, *HTA2-HTB2*, does not cause suppression of insertion mutations; this is probably due to dosage compensation by *HTA1-HTB1* in strains deleted for *HTA2-HTB2* (Norris and Osley 1987). In such strains, the level of expression of *HTA1-HTB1* is increased, resulting in near-wild-type levels for these two histones. By this hypothesis, it is the level of H2A-H2B dimers, rather than an alteration in the relative levels of a particular H2A or H2B subtype (Wallis et al. 1980; Choe et al. 1982), that is responsible for the suppression phenotype. This model is supported strongly by our observation that a strain containing an *HTA1-HTB1* deletion and a duplication of *HTA2-HTB2* does not suppress insertion mutations.

Deletion of either of the H3-H4 loci, *HHT1-HHF1* or *HHT2-HHF2*, also does not cause suppression. One explanation is that these loci may both dosage compensate for each other, as does *HTA1-HTB1* for *HTA2-HTB2*. If this were true, then neither deletion would lower the level of H3 and H4 enough to cause a suppression phenotype. Alternatively, it is possible that a reduction of H3-H4 dimers does not lead to the same alteration in

transcription as does a reduction in H2A-H2B dimers. The latter possibility is more difficult to reconcile with the result that a high-copy-number plasmid that contains either *HHT1-HHF1* or *HHT2-HHF2* causes suppression.

Deletion of the *HTA1-HTB1* locus causes a variety of mutant phenotypes (Norris and Osley 1987). Therefore, it is conceivable that the transcriptional changes we have observed are indirect, caused by some other effect of altered histone gene dosage. One possibility is that the transcriptional alterations are occuring via the heat shock response, which is expressed constitutively in strains deleted for *HTA1-HTB1* (Norris and Osley 1987). If transcription from δ sequences were reduced under heat shock conditions, then the δ insertion mutations could be suppressed in the Δ(*hta1-htb1*) strain because the normally strong δ promoters are reduced in efficiency, allowing weaker promoters at *HIS4* and *LYS2* to compete for transcription factors. However, we have observed no change in the level of Ty transcripts during heat shock as measured by Northern hybridization analysis (D. Norris and H. Iida, unpubl.; Clark-Adams and Winston, unpubl.).

A plausible explanation for the transcriptional alterations we have observed is that changing the level of histone dimer sets either reduces the number of nucleosomes or alters nucleosomal structure, thereby causing a perturbation in chromatin structure that allows different transcription initiation sites to be utilized. Lorch et al. (1987) demonstrated that, in vitro, transcription cannot initiate in a nucleosome. Perhaps the insertion mutations we have studied change nucleosome phasing such that nucleosomes block transcription initiation sites at *HIS4* and *LYS2*. When the number of nucleosomes is reduced or nucleosomal structure is altered, an initiation site for a functional transcript would now be accessible to the cell's transcription machinery. Micrococcal nuclease and DNase I digestion studies will help to identify changes in chromatin structure at *his4-912δ* and *lys2-128δ* caused by altering histone gene dosage.

Either overproduction or underproduction of histone dimer sets can cause transcriptional changes. There is evidence that an equimolar ratio of H2A-H2B to H3-H4 is necessary for proper chromatin formation in vitro (Camerini-Otero et al. 1976). An increase or decrease in the level of either dimer set would upset this ratio, and, as discussed by Meeks-Wagner and Hartwell (1986), might promote the assembly of aberrant nucleosomal structures and/or reduce the number of nucleosomes. The level of functional complexes involved in other processes, including bacteriophage morphogenesis (Floor 1970; Sternberg 1976) and bacterial chemotaxis (DeFranco and Koshland 1981) has been shown to be sensitive to altered stoichiometry.

The transcriptional alterations we have observed as the result of changing histone gene dosage occur at insertion mutations at the *HIS4* and *LYS2* genes. We did not see any alteration in transcription of the wild-type *HIS4*, *LYS2*, and *PYK1* genes. Likewise, Han et al. (1987) did not see any effect on transcription of the *S. cerevi-*

siae CUP1 gene under conditions in which no histone H2B is being synthesized. The level of expression of wild-type genes may be unaffected by the degree to which we have perturbed histone levels. Alternatively, transcriptional effects caused by altered histone levels may be detected only under certain conditions such as mutation of a promoter or absence of a *trans*-acting factor.

The original purpose of the screen for high-copy-number suppressors was to isolate new genes that shared phenotypes with the *SPT6* gene. Either too many or too few copies of the *SPT6* gene cause suppression of δ insertion mutations (Clark-Adams and Winston 1987). We isolated the *HTA1-HTB1* locus as a high-copy-number suppressor and subsequently demonstrated that mutations in the locus also cause suppression of δ insertion mutations. In addition, the transcriptional alterations at *his4-912δ* and *lys2-128δ* observed in the Δ(*hta1-htb1*) strains are similar to those seen in *spt6* mutant strains (Clark-Adams and Winston 1987). Whether the function of the *SPT6* gene product is related to histone function will be addressed in future experiments.

In conclusion, we have demonstrated that alterations in histone gene dosage can cause specific transcriptional changes in yeast. Suppression of insertion mutations, the system by which we have detected these changes, is sensitive to small variations in levels of gene expression that are easy to detect. We anticipate that this system will provide a powerful genetic tool to assay for changes in chromatin structure caused by mutations in histone genes as well as by mutations in other genes whose products are important for chromatin structure.

Experimental procedures

Yeast strains and genetic methods

The *S. cerevisiae* strains constructed for these studies are derived from strain S288C and are listed in Table 3. We have used standard genetic nomenclature for the histone genes. Uppercase letters denote the dominant (wild-type) allele and lowercase letters denote the recessive (mutant) allele. The Δ(*hta1-htb1*) and Δ(*hta2-htb2*) alleles are described in Norris and Osley (1987). The Δ(*hht1-hhf1*) and Δ(*hht2-hhf2*) alleles were provided by M. Mitchell Smith. Standard methods for mating, sporulation, and tetrad analysis were used (Mortimer and Hawthrone 1969; Sherman et al. 1978). All media were made as described by Sherman et al. (1978).

Transformations

Yeast cells were transformed by the lithium acetate method (Ito et al. 1983). *Escherichia coli* HB101 (Boyer and Roulland-Dussoix 1969) was transformed to ampicillin resistance as decribed by Maniatis et al. (1982).

Enzymes

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc., and used as suggested by the supplier.

Table 3. *Yeast strains*

Strain	Genotype
S288C	*MATα ga12 hap1*
FW1237	*MATa his4-912δ lys2-128δ ura3-52*
FW1238	*MATα his4-912δ lys2-128δ ura3-52*
CC232	*MATa Δ(hta1-htb1) his4-912δ lys2-128δ ura3*
CC217	*MATα Δ(hta2-htb2) his4-912δ lys2-128δ ura3-52*
CC249	*MATa Δ(hht1-hhf1) his4-912δ lys2 ura3-52*
CC251	*MATa Δ(hht2-hhf2) his4-912δ lys2 ura3-52*
CC204	*MATα htb1-5::Tn10-LUK his4-912δ lys2-128δ ura3-52*
CC240	*MATa hta1-3::Tn10-LUK his4-912δ lys2-128δ ura3-52*
CC260	*MATa Δ(hta1-htb1) HTA2-HTB2/HTA2-HTB2 his3 lys2-128δ ura3*
DN106	*MATα Δ(hta1-htb1) his3 ura3*
CC131	*MATa/MATα his4-912δ/his4-912δ lys2-128δ/lys2-128δ ura3-52/ura3-52*

DNA preparation and analysis

Yeast DNA minipreps for recovery of plasmids from 10-ml cultures were done as described by Winston et al. (1983). *E. coli* plasmid mini-preps were prepared by the alkaline lysis method (Birnboim and Doly 1979). DNA restriction fragments were analyzed by electrophoresis through 0.6% Seakem ME agarose gels that were run in 0.5 × E buffer (Roeder and Fink 1980). We purified restriction fragments from agarose gels by electroelution. Southern blot hybridization analysis of yeast genomic DNA was performed as described (Roeder and Fink 1980). ^{32}P-Labeled DNA probes were prepared by nick-translation (Rigby et al. 1977).

Plasmids

Plasmid vectors used for subcloning were YCp50 (Johnston and Davis 1984), YEp24 (Botstein et al. 1979), YIp5 (Parent et al. 1985), and pCGS42 (provided by Collaborative Research). High-copy-number plasmids of histone genes were constructed as described below and are diagrammed in Figure 5. A 6-kb *Sal*I restriction fragment containing the *HTA1-HTB1* locus was cloned into the *Sal*I site of YEp24 to create pCC61. In addition to *HTA1* and *HTB1*, the *Sal*I fragment in pCC61 contains a

gene of unknown function, *PRT1* (Protein 1) (Hereford et al. 1979). Plasmid pDN242 contains a 3.55-kb *Eco*RI–*Hind*III fragment from the *HTA2-HTB2* locus cloned into the *Eco*RI and *Bam*HI sites of YIp5 and a 2.24-kb *Eco*RI fragment from 2μ circle cloned into the *Eco*RI site of YIp5. Plasmid pCC64 contains a 6.7-kb *Hind*III fragment containing the *HHT1-HHF1* locus (provided by M. Mitchell Smith) cloned into the *Hind*III site of YEp24. In addition to *HHT1* and *HHF1*, the insert in pCC64 contains *SMT1*, a gene of unknown function (Smith and Murray 1983). Plasmid pCC66 contains two *Hind*III fragments, 2.6 kb and 1.48 kb, from the *HHT2-HHF2* locus (provided by M. Mitchell Smith) cloned into the *Hind*III site of pCGS42. Plasmid pCC67 contains four histone genes (*HTA1*, *HTB1*, *HHT1*, and *HHF1*) and was constructed by cloning a 6.4-kb *HTA1-HTB1 Bam*HI fragment and a 5.4-kb *HHT1-HHF1 Bam*HI-*Hind*III fragment into the *Hind*III and *Bam*HI sites of pCGS42.

Hybridization probes

Plasmids and DNA fragments used as probes were as follows: for *HIS4*, pFW45, a *HIS4* internal *Bgl*II–*Sal*I restriction fragment in pBR322 (Winston et al. 1984a); for *LYS2*, pFW47, a *LYS2* internal *Bgl*II–*Xho*I restriction fragment in pBR322, and pFW112, a *LYS2 Eco*RI–*Bgl*II fragment spanning the 5' end of *LYS2*, cloned into pBR322 (Clark-Adams and Winston 1987); for *PYK1*, pFR2, a clone containing the *PYK1* gene (provided by Pratima Sinha); for *ACT1*, a 3.2-kb *Bam*HI–*Eco*RI restriction fragment containing the *ACT1* gene (derived from plasmid pRB149 provided by David Botstein); for *HTB1*, a 1.3-kb *Hind*III restriction fragment containing the 3' of the *HTB1* gene; for *HHT1*, a 3.5-kb *Hind*III–*Sma*I restriction fragment containing the *HHT1* gene and the *SMT1* gene.

RNA blot hybridization analysis

Cells for RNA isolation were grown in supplemented SD medium to 1 × 10⁷ cells/ml, and yeast RNA isolated as described (Carlson and Botstein 1982). For RNA blots and hybridization analysis, we followed the instructions for GeneScreen (New England Nuclear) using the dextran sulfate method. RNA was cross-linked onto GeneScreen by exposure to UV radiation (1200 μW/cm² for 2 min) (Church and Gilbert 1984). The amount of RNA per lane was standardized by hybridization to pFR2 or to a restriction fragment containing the *ACT1* gene. Bands on autoradiograms were quantitated by densitometry on an LKB Bromma Ultroscan XL Laser Densitometer.
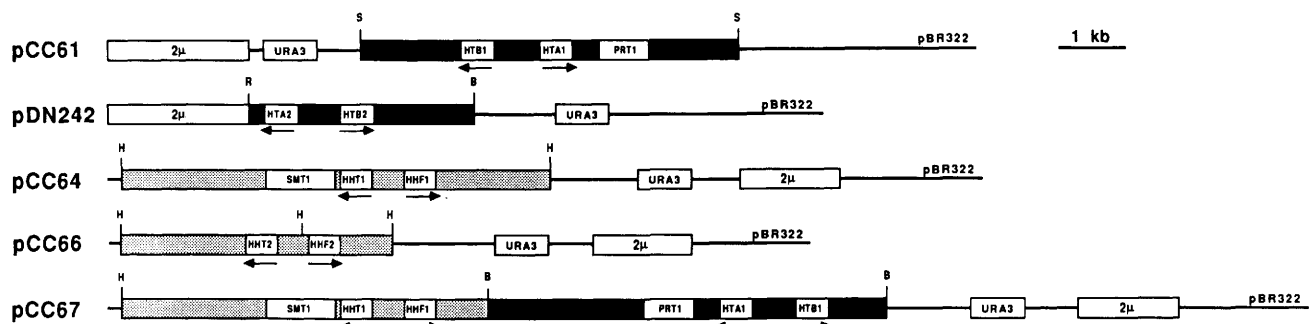
Figure 5. High-copy-number plasmids containing the histone loci. The autonomously replicating plasmids diagrammed all contain the 2μ origin of replication and the *URA3* gene as a selectable marker in yeast. Each plasmid is shown with the *Eco*RI site of pBR322 located at the far left end. The plasmids were constructed as described in Experimental procedures. Only relevant restriction sites are listed. (S) *Sal*I; (R) *Eco*RI; (B) *Bam*HI; (H) *Hind*III.

Construction and analysis of HTA1-HTB1 *null alleles*

A *Sal*I restriction fragment containing the *HTA1-HTB1* locus was subcloned into YEp24. The resulting subclone, pCC61, was subjected to Tn10-LUK transposon insertion mutagenesis (Huisman et al. 1987). The Tn10-LUK element carries the *URA3* gene as a selectable marker in yeast. Derivatives of pCC61 that contain individual Tn10-LUK insertions were transformed into strain FW1237 and Ura⁺ transformants were screened for the ability to suppress δ insertion mutations. The positions of the Tn10-LUK insertions were determined by restriction mapping. Eleven Tn10-LUK insertions within the *HTA1-HTB1* locus which eliminate the suppression phenotype of pCC61 were transplaced into the yeast genome as follows: *Sal*I restriction fragments containing the *HTA1-HTB1* locus disrupted by a given Tn10-LUK insertion were purified and used to transform yeast by selection for Ura⁺. A double crossover event will replace one of the genomic *HTA1-HTB1* locus alleles with the Tn10-LUK-disrupted *HTA1-HTB1* allele (Orr-Weaver et al. 1981; Rothstein 1983). Two of the Tn10-LUK insertions were transformed into the diploid strain CC131. To analyze the phenotype of the disruption in haploid cells, we sporulated the Ura⁺ transformants and dissected tetrads. The remaining nine Tn10-LUK insertions in the *HTA1-HTB1* locus were transplaced directly into haploid strain FW1237. Southern blot hybrdization analysis of the Ura⁺ haploid transformants confirmed that all eleven Tn10-LUK insertions were integrated at the *HTA1-HTB1* locus.

DNA sequence analysis

The positions of the Tn10-LUK transposon insertion mutations at the *HTA1-HTB1* locus were determined by DNA sequence analysis, using a primer complementary to the end of the Tn10-LUK element: 5′d(CAAGATGTGTATCCACC)-3′ (made by Paul Tempst with an Applied Biosystems 380B DNA Synthesizer). Plasmids containing the Tn10-LUK insertions were digested with restriction enzymes to produce a restriction fragment with one end inside the Tn10-LUK element and the other end within the *HTA1-HTB1* locus. These restriction fragments were isolated by electroelution, denatured, annealed to primer, and sequenced using a modification of the Sanger technique as described by Bartlett et al. (1986). [α-³⁵S] dATP was purchased from New England Nuclear.

Acknowledgments

References

Bartlett, J.A., R.K. Gaillard, Jr., and W.K. Joklik. 1986. Sequencing of supercoiled plasmid DNA. *Biotechniques* **4:** 208–210.

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7:** 1513–1523.

Botstein, D., S.C. Falco, S.E. Stewart, M. Brennan, S. Scherer, D.T. Stinchcomb, K. Struhl, and R.W. Davis. 1979. Sterile host yeasts (SHY): A eukaryotic system of biological containment for recombinant DNA experiments. *Gene* **8:** 17–24.

Boyer, H.W. and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *E. coli. J. Mol. Biol.* **41:** 458–472.

Camerini-Otero, R.D., B. Sollner-Webb, and G. Felsenfeld. 1976. The organization of histones and DNA in chromatin: Evidence for an arginine-rich histone kernel. *Cell* **8:** 333–347.

Carlson, M. and D. Botstein. 1982. Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. *Cell* **28:** 145–154.

Choe, J., D. Kolodrubetz, and M. Grunstein. 1982. The two yeast histone H2A genes encode similar protein subtypes. *Proc. Natl. Acad. Sci.* **79:** 1484–1487.

Church, G.M. and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci.* **81:** 1991–1995.

Clark-Adams, C.D. and F. Winston. 1987. The *SPT6* gene is essential for growth and is required for δ-mediated transcription in *Saccharomyces cerevisiae Mol. Cell. Biol.* **39:** 679–686.

DeFranco, A.L. and D.E. Koshland, Jr. 1981. Molecular cloning of chemotaxis genes and overproduction of gene products in the bacterial sensing system. *J. Bacteriol.* **147:** 390–400.

Falco, S.C. and D. Botstein. 1983. A rapid chromosome-mapping method for cloned fragments of yeast DNA. *Genetics* **105:** 857–872.

Falco, S.C., Y. Li, J.R. Broach, and D. Botstein. 1982. Genetic properties of chromosomally integrated 2μ plasmid DNA in yeast. *Cell* **29:** 573–584.

Fassler, J.S. and F. Winston. 1988. Isolation and analysis of a novel class of suppressor of Ty insertion mutations in *Saccharomyces cerevisiae. Genetics* **118:** 203–212.

Floor, E. 1970. Interaction of morphogenetic genes of bacteriophage T4. *J. Mol. Biol.* **47:** 293–306.

Han, M., M. Chang, U. Kim, and M. Grunstein. 1987. Histone H2B repression causes cell-cycle-specific arrest in yeast: Effects on chromosomal segregation, replication, and transcription. *Cell* **48:** 589–597.

Hereford, L., K. Fahrner, J. Woolford, Jr., M. Rosbash, and D.B. Kaback. 1979. Isolation of yeast histone genes H2A and H2B. *Cell* **18:** 1261–1271.

Huisman, O., W. Raymond, K. Froehlich, P. Errada, N. Kleckner, D. Botstein, and M.A. Hoyt. 1987. A Tn*10-lacZ-kanR-URA3* gene fusion transposon for insertion mutagenesis and fusion analysis of yeast and bacterial genes. *Genetics* **116:** 191–199.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153:** 163–168.

Johnston, M. and R.W. Davis. 1984. Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **4:** 1440–1448.

Lorch, Y., J.W. LaPointe, and R.D. Kornberg. 1987. Nucleosomes inhibit the initiation of transcription but allow chain elongation with the displacement of histones. *Cell* **49:** 203–210.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mathis, D., P. Oudet, and P. Chambon. 1980. Structure of transcribing chromatin. *Prog. Nucl. Acad. Res. Mol. Biol.* **24:** 1–55.

Meeks-Wagner, D. and L.H. Hartwell. 1986. Normal stoichiometry of histone dimer sets is necessary for high fidelity of

mitotic chromosome transmission. *Cell* **44:** 53–63.

Moore, G.D., D.A. Sinclair, and T.A. Grigliatti. 1983. Histone gene multiplicity and position effect variegation in *Drosophila melanogaster Genetics* **105:** 327–344.

Mortimer, R.K. and D.C. Hawthorne. 1969. Yeast genetics. In *The yeasts*, (ed. A.H. Rose and J.S. Harrison), vol. 1, pp. 385–460. New York. Academic Press.

Nasmyth, K.A. 1982. The regulation of yeast mating-type chromatin structure by *SIR*: An action at a distance affecting both transcription and transposition. *Cell* **30:** 567–578.

Neigeborn, L., J.L. Celenza, and M. Carlson. 1987. *SSN20* is an essential gene with mutant alleles that suppress defects in *SUC2* transcription in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **7:** 672–678.

Norris, D. and M.A. Osley. 1987. The two gene pairs encoding H2A and H2B play different roles in the *Saccharomyces cerevisiae* life cycle. *Mol. Cell. Biol.* **7:**3473–3481.

Orr-Weaver, T.L., J.W. Szostak, and R.J. Rothstein. 1981. Yeast transformation: a model system for the study of recombination. *Proc. Natl. Acad. Sci.* **78:** 6354–6358.

Parent, S.A., C.M. Fenimore, and K.A. Bostian. 1985. Vector systems for the expression, analysis and cloning of DNA sequences in *S. cerevisiae. Yeast* **1:** 83–138.

Pederson, D.S., F. Thoma, and R.T. Simpson. 1986. Core particle, fiber, and transcriptionally active chromatin structure. *Annu. Rev. Cell. Biol.* **2:** 117–147.

Rigby, P.W.J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specfiic activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* **113:** 237–251.

Roeder, G.S. and G.R. Fink. 1980. DNA rearrangements associated with a transposable element in yeast. *Cell* **21:** 239–249.

Rothstein, R.J. 1983. One-step gene disruption in yeast. *Methods Enzymol.* **101:** 202–211.

Sherman, F., G.R. Fink, and C.W. Lawrence. 1978. *Methods in yeast genetics*, rev. ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Silverman, S.J. and G.R. Fink. 1984. Effects of Ty insertions on *HIS4* transcription in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **4:** 1246–1251.

Smith, M.M. and O.S. Andresson. 1983. DNA sequences of yeast H3 and H4 histone genes from two non-allelic gene sets encode identical H3 and H4 proteins. *J. Mol. Biol.* **169:** 663–690.

Smith, M.M. and K. Murray. 1983. Yeast H3 and H4 histone messenger RNAs are transcribed from two non-allelic gene sets. *J. Mol. Biol.* **169:** 641–661.

Sternberg, N. 1976. A genetic analysis of bacteriophage λ head assembly. *Virology* **71:** 568–582.

Wallis, J.W., L. Hereford, and M. Grunstein. 1980. Histone H2B genes of yeast encode two different proteins. *Cell* **22:** 799–805.

Winston, F., F. Chumley, and G.R. Fink. 1983. Eviction and transplacement of mutant genes in yeast. *Methods Enzymol.* **101:** 211–228.

Winston, F., K.J. Durbin, and G.R. Fink. 1984a. The *SPT3* gene is required for normal transcription of Ty elements in *S. cerevisiae. Cell* **39:** 675–682.

Winston, F., D.T. Chaleff, B. Valent, and G.R. Fink. 1984b. Mutations affecting Ty-mediated expression of the *HIS4* gene of *Saccharomyces cerevisiae. Genetics* **107:** 179–197.

Winston, F., C. Dollard, E.A. Malone, J. Clare, J.G. Kapakos, P. Farabaugh, and P.L. Minehart. 1987. Three genes are required for trans-activation of Ty transcription in yeast. *Genetics* **115:** 649–656.

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