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Amino Termini of Histones H3 and H4 Are Required for **a**1-α2 Repression in Yeast

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The *Saccharomyces cerevisiae* α2 repressor controls two classes of cell-type-specific genes in yeast through association with different partners. α2-Mcm1 complexes repress a cell-specific gene expression in haploid α cells and diploid a/α cells, while a1-α2 complexes repress haploid-specific genes in diploid cells. In both cases, repression is mediated through Ssn6-Tup1 corepressor complexes that are recruited via direct interactions with α2. We have previously shown that nucleosomes are positioned adjacent to the α2-Mcm1 operator under conditions of repression and that Tup1 interacts directly with histones H3 and H4. Here, we examine the role of chromatin in a1-α2 repression to determine if chromatin is a general feature of repression by Ssn6-Tup1. We find that mutations in the amino terminus of histone H4 cause a 4- to 11-fold derepression of a reporter gene under a1-α2 control, while truncation of the H3 amino terminus has a more modest (3-fold or less) effect. Strikingly, combination of the H3 truncation with an H4 mutation causes a 40-fold decrease in repression, clearly indicating a central role for these histones in a1-α2-mediated repression. However, in contrast to the ordered positioning of nucleosomes adjacent to the α2-Mcm1 operator, nucleosomes are not positioned adjacent to the a1-α2 operator in diploid cells. Our data indicate that chromatin is important to Ssn6-Tup1-mediated repression but that the degrees of chromatin organization directed by these proteins differ at different promoters.

Saccharomyces cerevisiae can exist as two haploid cell types, **a** or α, which can mate to form a third cell type, the **a**/α diploid cell (11). Cell type is largely controlled by the α2 repressor protein encoded by the *MAT*α locus. In α cells and **a**/α cells, dimers of α2 interact with dimers of the Mcm1 protein and these complexes bind a unique sequence upstream of the **a** cell-specific genes, thereby repressing their transcription (13, 16). In diploid cells, α2 forms heterodimers with the **a**1 protein (4) and this complex binds to a distinct sequence upstream of the haploid-specific genes to confer repression (7). In haploid **a** cells, α2 is not expressed, and so neither the **a** cell-specific nor the haploid-specific genes are repressed.

Although α2 is directed to different DNA sequences in different stoichiometries through association with either Mcm1 or **a**1, in both cases it recruits the Ssn6-Tup1 corepressor complex (17). Neither Ssn6 nor Tup1 binds directly to DNA, and these proteins are drawn to specific promoters through interactions with DNA binding factors such as α2 (18, 33). The need for the DNA binding factor can be bypassed by fusion of either *SSN6* or *TUP1* to a heterologous, LexA DNA binding domain, provided the target of repression contains a LexA binding site (17, 36). Interestingly, Ssn6-LexA repression requires the presence of *TUP1* (17), but Tup1-LexA can confer repression in the absence of *SSN6* (36). These findings suggest that Tup1 is the dominant repressor moiety in the Ssn6-Tup1 complex.

In addition to the **a** cell-specific and haploid-specific genes, Ssn6-Tup1 is required for repression of glucose-repressible genes such as *SUC2* (30, 35), DNA damage-inducible genes such as *RNR2* (40), and genes regulated by oxygen such as *ANB1* (41). The ability to regulate such diverse genes suggests that Ssn6-Tup1 inhibits transcription via some factor or element common to multiple promoters. Candidates include components of the basal transcription machinery or components of chromatin. Repression by α2 can be reconstituted to a limited extent in vitro (10, 22) in the absence of chromatin, and components of the RNA polymerase II holoenzyme are required for repression by α2 in vivo (38). However, the repression achieved in vitro is minor compared to that which occurs in vivo, and it is not clear whether components of the holoenzyme are directly or indirectly involved in repression (10, 38).

Nucleosomes are positioned adjacent to the α2-Mcm1 operators in the promoter regions of the **a** cell-specific genes, and both positioning and repression require *SSN6*, *TUP1*, and α2 (3, 25). Recently, we demonstrated that Tup1 directly interacts with the amino termini of histones H3 and H4 through its repression domain (6). In addition, mutations in the amino termini of these histones synergistically decrease repression of a *CYC1-lacZ* reporter gene carrying an α2-Mcm1 operator. These mutations also derepress a DNA damage-inducible reporter gene, indicating that repression of at least two classes of genes by Tup1 requires intact H3 and H4 (6).

Here, we report that repression of genes by **a**1-α2 also requires these histones, confirming that chromatin is an important element in Tup1-mediated repression. However, the chromatin structures of promoter regions repressed by **a**1-α2 are significantly less organized than those repressed by α2-Mcm1. These data suggest that different structures may be established by Tup1 at different promoters depending upon the type and stoichiometry of factors present to recruit the Ssn6-Tup1 complex.

MATERIALS AND METHODS

Yeast strains. *S. cerevisiae* strains were propagated according to standard procedures (24) in either rich (yeast extract-peptone-dextrose [YEPD]) or selective (synthetic complete [SC] medium lacking the appropriate amino acids or uracil) media. Strains used are presented in Table 1. LY1 was generated by directly mating AWY999 (derived from PKY999 [15], except with pAW499

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TABLE 1. Strain list

Strain[a]	Genotype or description[b]	Source (reference)
LY999	*MAT*α *ade2-101 his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 tyr thr hhf1::HIS3 hhf2::LEU2* pAW499 (*HHF2 TRP1*) $pCT_{\Delta G}$**a**1/α2-1	Derived from PKY999 (15)
LY1	Diploid cells generated from LY999 by mating with isogenic **a** cells (PKY899 [15])	This study
LY918*	Isogenic to LY999 except pPK618 [*hhf2* (del 4–19) *URA3*] instead of pAW499 and $pCT_{\Delta G}$**a**1/α2-2 instead of $pCT_{\Delta G}$**a**1/α2-1	Derived from PKY918 (15)
LY913*	Isogenic to LY918 except pPK613 [*hhf2* (del 4–23) *URA3*] instead of pPK618	Derived from PKY913 (15)
LY505*	Isogenic to LY918 except pPK305 (*hhf2 GLY-16 URA3*) instead of pPK618	Derived from PKY505 (14)
LY590	*MAT*α *ura3-52 lys2-Δ201 leu2-3 Δhht2-hhf2* $pCT_{\Delta G}$**a**1/α2-1	Derived from MSY590 (19)
LY591	Diploid cells generated from LY590 using an *HO* expression vector	This study
LY612*	Isogenic to LY590 except *MAT***a**, *hhf1-32* (H4 K12Q, K16Q)	Derived from MSY612 (19)
LY577*	Isogenic to LY612 except *hht1-2* (del 1–28) and *hhf1-327* (H4 K12Q, K16Q)	Derived from MSY577 (P. Megee and M. Smith)
LY890	*MAT***a**/α *ura3-52 leu2-3,112 lys2-801* Δ(*hht1 hhf1*) Δ(*hht2 hhf2*) pMS337 (*CEN ARS LEU2 hht1-1 HHF1*) $pCT_{\Delta G}$**a**1/α2-1	Derived from MSY890 (20)
LY892	Isogenic to LY890 except pMS358 [*CEN ARS LEU2 hht1-2* (del 1–28) *HHF1*] instead of pMS337	Derived from MSY892 (20)
LY893	Isogenic to LY892 except $pCT_{\Delta G}$UASc instead of $pCT_{\Delta G}$**a**1/α2-1	This study
LY2	*MAT***a**/α *ade2-101oc his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52* Δ(*hht1 hhf1*) Δ(*hht2 hhf2*) pRS414-F5 (*HHT2 HHF2 TRP1*), $pCT_{\Delta G}$**a**1/α2-1	This study
LY3	Isogenic to LY2 except pRS414-F8 [*hht2* (del 3–29) *HHF2 TRP1*] instead of pRS414-F5	This study
LY690	Isogenic to LY590 except $pCT_{\Delta G}$UASc instead of $pCT_{\Delta G}$**a**1/α2-1	This study
LY691	Same as LY591 except $pCT_{\Delta G}$UASc instead of $pCT_{\Delta G}$**a**1/α2-1	This study
LY677	Isogenic to LY577 except $pCT_{\Delta G}$UASc instead of $pCT_{\Delta G}$**a**1/α2-1	This study
LY590ΔTUP1	Isogenic to LY590 except ΔTUP1	This study
LY577ΔTUP1	Isogenic to LY577 except ΔTUP1	This study

[a] For strains marked with an asterisk, the silent mating loci are derepressed by the H4 mutation, providing α2 or **a**1 expression.
[b] Abbreviations: (del 4–19), (del 4–23), (del 1–28), and (del 3–29), deletion of codons for amino acids 4 to 19, 4 to 23, 1 to 28, and 3 to 29, respectively; (H4 K12Q, K16Q), mutation of H4 codons such that K is replaced by Q at positions 12 and 16.

[*HHF2 TRP1*] instead of pUK499 [*HHF2 URA3*]) and PKY899 (14) on YEPD plates overnight. Diploids were selected on SC medium lacking uracil and tryptophan. Diploid cells were then cultured on 5-FOA (5-fluoro-orotic acid)-containing media (24) to select for the loss of pUK499. Plasmid $pCT_{\Delta G}$**a**1/α2-1 was then transformed into 5-FOA-resistant cells as described previously (12). The same strategy was used to generate LY2 (with WZY05 and WZY10 [39a]) and LY3 (with WZY8 and WZY11 [39a]). LY591 was derived from MSY590 (19) by HO-induced mating type switching (after transformation with pAK32 bearing the HO gene; generous gift from A. Klar, National Cancer Institute, Frederick, Md.) and spontaneous mating of the resulting **a** cells with MSY590. The diploid cells were identified by mating and halo assays as described by Kayne et al. (15). LY890, LY892, and LY893 diploid strains were generated by this same strategy from MSY890 or MSY892 (20). LY690, LY691, and LY677 are the same as LY590, LY591, and LY577 except that the reporter plasmid does not carry the **a**1-α2 operator. The *TUP1* gene was disrupted in LY590Δ *TUP1* and LY577Δ *TUP1* as described previously (3).

Plasmids. Two primers corresponding to the 5′ and 3′ ends of the *CYC1* UASc region containing an *Eco*RI site (5′ primer) or an *Xho*I site (3′ primer) were used to amplify UASc from yeast genomic DNA by PCR. The PCR product was then ligated into *Eco*RI and *Xho*I sites of $pCT_{\Delta G}$136 vector, a CEN plasmid that contains a *lacZ* reporter gene controlled by the *CYC1* promoter (a gift from Steven Elledge, Baylor College of Medicine) to produce $pCT_{\Delta G}$UASc. The oligonucleotide duplex (LH24: 5′-TCG ACG CTT CCC AAT GTA GAA AAG TAC ATC ATA GC-3′; LH25: 5′-TCG AGC TAT GAT GTA CTT TTC TAC ATT GGG AAG CG-3′) representing the **a**1-α2 operator site was synthesized and inserted into the unique *Xho*I site of $pCT_{\Delta G}$UASc at position −244 to generate the $pCT_{\Delta G}$**a**1/α2-1 chimeric reporter gene (the *Xho*I site proximal to the UASc region was destroyed). $pCT_{\Delta G}$**a**1/α2-2 was produced by inserting the *TRP1* gene into the middle of *URA3* at the unique *Apa*I site of $pCT_{\Delta G}$**a**1/α2-1. Recombinant plasmids were transformed into *Escherichia coli*, and bacterial clones were identified by restriction analysis or colony hybridization (28). The sequence of UASc and the orientation and copy number of the **a**1-α2 operator were confirmed by DNA sequencing. DNA was prepared with a Qiagen Maxi kit by following the manufacturer's instructions.

β-Galactosidase assays. β-Galactosidase activities of reporter genes in wild-type cells or in strains carrying histone mutations were quantitated by standard *o*-nitrophenyl-β-D-galactopyranoside assays following the preparation of whole-cell extracts by a glass bead procedure (24).

Western blotting. Total cell yeast extracts were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting as described previously (5) by using 1:1,000 dilutions of anti-Tup1 or anti-Ssn6 polyclonal antibodies.

Nuclei preparation, MNase digestion, and primer extension footprinting of *RME1*. Transformed yeast cells were grown at 30°C in SC media without uracil, and nuclei were isolated from a 1-liter culture (optical density at 600 nm of 0.7 to 1.0) and digested with micrococcal nuclease (MNase) as described previously (32). MNase-cut sites in *RME1* chromatin and DNA were mapped by primer extension as described by Shimizu et al. (32) with primer SYR30 (5′-GTG CTT TCC CTC TTT AGT TTG GAC AGG GAT AGT GGG-3′).

Preparation and analysis of TALa1-α2 chromatin. Plasmid chromatin was prepared and digested with MNase, and DNA was purified and then cut with *Eco*RV, subjected to Southern blotting, and probed with an *Eco*RV-*Hin*dIII fragment from TAL**a**1-α2 as described previously (26).

In vivo UV and DMS footprinting. In vivo UV and dimethyl sulfate (DMS) footprinting were performed as described previously (21, 27). Transformed yeast cells were grown in 400 ml of SC medium to an A_{600} of 0.7 to 0.8. For UV photofootprinting, cultures were pelleted at 4,000 × *g* for 10 min, washed with 30 ml of phosphate-buffered saline (PBS; 0.2 M NaCl, 2.7 mM KCl, 15.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$), and then collected and resuspended in 12.5 ml of PBS. Portions (1.25 ml) of the suspension were irradiated with doses of 480 or 960 mJ/cm^2 with a Stratalinker UV Crosslinker (Stratagene), and DNA was isolated as described previously (21). Control, purified DNA was irradiated with doses of 120 mJ/cm^2. For DMS footprinting, cultures were divided into 50-ml portions, pelleted at 1,400 ×*g* for 5 min, resuspended in 1.5 ml of fresh SC medium, and treated with 0.13% or 0.065% DMS for 2 min at room temperature with vortexing and then DNA was isolated. Control, purified DNA was methylated by 0.125% DMS for 3 min at room temperature.

Primer extension assays. The MNase cleavage sites, sites of UV photoproducts, and DMS-induced methylation sites were analyzed by primer extension assay using *Taq* polymerase as described previously (1, 32). Twice as much DNA from diploid strains as from haploid strains was used as the template in the primer extension reaction to correct for the decreased plasmid-to-genomic-DNA ratio in diploid cells. Oligonucleotide LH30 (5′-GCA TGC CTG CAG GTC GAC TCT AGA ATT CGT CGA CCC C-3′; corresponding to nucleotides −418 to −455 of plasmid $pCT_{\Delta G}$**a**1/α2-1 [polylinker region]) was used as the primer.

RNA isolation and analysis. Yeast total RNA was isolated from wild-type and mutant cells as described previously (5) except that the final pellet was dissolved in 40 μl of sterile H_2O containing 0.05% diethyl pyrocarbonate. About 20 μg of total RNA from each strain was resolved on a 1.5% formaldehyde agarose gel, blotted to Gene Screen Plus (NEN), and then probed with an **a**1- or an α2-specific probe generated by PCR. Blots were reprobed with a PCR-generated DNA fragment from the actin gene.

RESULTS

Amino-terminal regions in histones H3 and H4 are required for efficient repression by a1-α2. To monitor the effects of

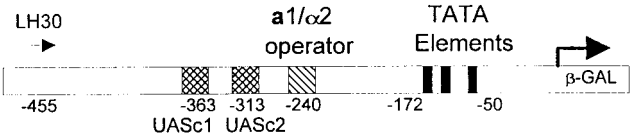

FIG. 1. **a**1-α2 reporter construct. The position of the **a**1-α2 operator relative to UASc1, UASc2, and TATA elements in the *CYC1* promoter is indicated, as is the position of the primer (LH30) used in chromatin mapping experiments. β-GAL, β-galactosidase.

various histone mutations on **a**1-α2-mediated repression, we inserted the **a**1-α2 operator sequence downstream of the UAS sequences in a *CYC1-lacZ* reporter gene (Fig. 1) and introduced this plasmid into strains carrying either wild-type or mutated histone genes. β-Galactosidase expression from this reporter is significantly repressed (90- to 200-fold) in wild-type diploid cells compared to isogenic haploid cells (compare LY1 to LY999 in Table 2 and LY591 to LY590 in Table 4). The level of **a**1-α2 repression we observe for our reporter is comparable to that observed by others for very similar constructs (17).

Mutations in the amino terminus of histone H4 cause a loss of silencing at the HML and HMR silent mating loci (8). Haploid cells carrying these mutations express both **a**1 and α2 (8) (see Fig. 2A) and therefore should repress **a**1-α2-sensitive promoters. Introduction of our **a**1-α2 reporter into cells containing three different H4 amino-terminal mutations (and no wild-type H4) indicates repression in these cells is incomplete relative to that observed in isogenic wild-type diploid cells. Deletion of amino acids 4 to 19 or 4 to 23 results in an 11-fold derepression (Table 2), comparable to the level of derepression observed by others in the absence of *SSN6* (17). Substitution of glycine (G) for lysine 16 (K16) also causes derepression, although to a lesser extent. Interestingly, simultaneous substitution of glutamine (Q) for residues K12 and K16 in H4 had no effect on repression (Table 4; compare LY591 to LY612). These data suggest that specific positions in the H4 amino terminus are important for **a**1-α2 repression and that the structure of residues at these positions dictates the level of repression achieved.

To test the importance of the H3 amino-terminal region for repression of our reporter gene, we constructed two sets of isogenic diploid cells carrying either wild-type H3 or a truncated version of H3. Deletion of amino acids 3 to 29 or 1 to 28 in H3 had very similar effects on repression. In both cases, we observed a modest (approximately threefold) derepression relative to cells carrying the wild-type allele (Table 3).

Even though truncation of amino acids 1 to 28 in H3 or substitution of Q for K12 and K16 in H4 individually had little or no effect, respectively, on expression of the **a**1-α2 reporter, the combination of these mutations in the same cell reduced repression more than 40-fold (Table 4; compare LY577 to LY591). Importantly, these mutations have no adverse effects on the expression of **a**1 or α2 (Fig. 2A) or the copy number of the reporter plasmid (data not shown). Steady-state levels of **a**1 or α2 RNA in all of the strains containing the histone mutations are, in fact, higher than in wild-type diploid cells where complete repression is observed. The derepression we observe in the strains carrying the histone mutations, then, is not due to insufficient levels of **a**1 or α2. We also examined levels of Ssn6 and Tup1 proteins in a subset of these cells (Fig. 2C). Multiple bands were detected for each protein, consistent with observations by ourselves and others that both Ssn6 and Tup1 are phosphoproteins (5a, 22, 29). Although the relative levels of phosphorylation observed in extracts from the different strains varied slightly from extract to extract, no consistent difference in phosphorylation or overall level of Ssn6 or Tup1 was observed in the presence of the histone mutations (Fig. 2C). If anything, the level of Tup1 was lowest in the wild-type diploid strain, LY591, which exhibited full repression.

TABLE 2. H4 mutations compromise **a**1-α2 repression

Strain	Cell type (*MAT*)	H4 type[a]	Presence of[b]:			β-Gal[d] level (U)	*n*[e]	Derepression (fold)
			a1	α2	**a**1-α2 op.[c]			
LY1	**a**/α	wt	+	+	+	1.0 ± 0.6	6	1.0
LY999	α	wt	−	+	+	94.3 ± 29	8	94.3
LY918[f]	α	del (4–19)	+	+	+	11.0 ± 5.4	6	11.0
LY913[f]	α	del (4–23)	+	+	+	11.1 ± 5.2	10	11.1
LY505[f]	**a**	K16G	+	+	+	3.7 ± 1.1	6	3.7

[a] wt, wild type; del (4–19), amino acids 4 to 19 deleted; del 4–23, amino acids 4 to 23 deleted; K16G, substitution of G for K at position 16.
[b] +, present; −, absent.
[c] Presence of **a**1-α2 operator in the reporter plasmid.
[d] β-Gal, β-galactosidase.
[e] *n*, number of determinations.
[f] The silent mating loci are derepressed, providing **a**1 or α2 expression.

Importantly, expression of a *CYC1-lacZ* construct that does not carry the **a**1-α2 operator sequence in wild-type diploid cells (LY691) is very similar to that in haploid cells bearing the combined H3 and H4 mutations (LY677), indicating that the diminished repression we observe (see above) in the presence of these mutations does not reflect a generalized increase in transcription of the *CYC1* promoter (Table 4). Similarly, comparable levels of expression of the *CYC1*–**a**1-α2 reporter are observed in haploid cells lacking *TUP1* in the presence of wild-type or mutant histones (LY590Δ*TUP1* and LY577Δ*TUP1*), although the overall level of expression of the *CYC1*–**a**1-α2 reporter is increased in the Δ*TUP1* cells. *TUP1*, then, may have indirect effects on *CYC1* expression (39). Our data indicate, however, that the histone mutations examined here do not influence these indirect effects, in contrast to their striking effects on **a**1-α2 repression.

The synergistic effects of the combined H3 and H4 mutations on expression of the *CYC1*–**a**1-α2 reporter not only demonstrate the importance of these histones to **a**1-α2 repression but are also consistent with our previous findings that Tup1 directly interacts with the amino termini of H3 and H4. In fact, the mutations examined here weaken (for H4 K12Q and K16Q) or abolish (for the H3 truncation) interaction of these histones with Tup1 (6).

Nucleosomes are not positioned adjacent to the a1-α2 operator. We next examined nucleosome location in the promoter region of our reporter in wild-type haploid and diploid cells to determine whether nucleosomes might be positioned

TABLE 3. H3 mutations slightly affect **a**1-α2 repression[a]

Strain	Cell type (*MAT*)	H3 type	Presence of:			β-Gal level (U)	*n*	Derepression (fold)
			a1	α2	**a**1-α2 op.			
LY2	**a**/α	wt	+	+	+	2.3 ± 0.4	4	1.0
LY3	**a**/α	del (3–29)	+	+	+	6.7 ± 1.1	4	2.9
LY890	**a**/α	wt	+	+	+	3.3 ± 0.5	10	1.0
LY892	**a**/α	del (1–28)	+	+	+	9.7 ± 1.0	6	2.9
LY893	**a**/α	del (1–28)	+	+	−	112 ± 9.0	6	33.9

[a] Symbols and abbreviations are as defined in the footnotes for Table 2.

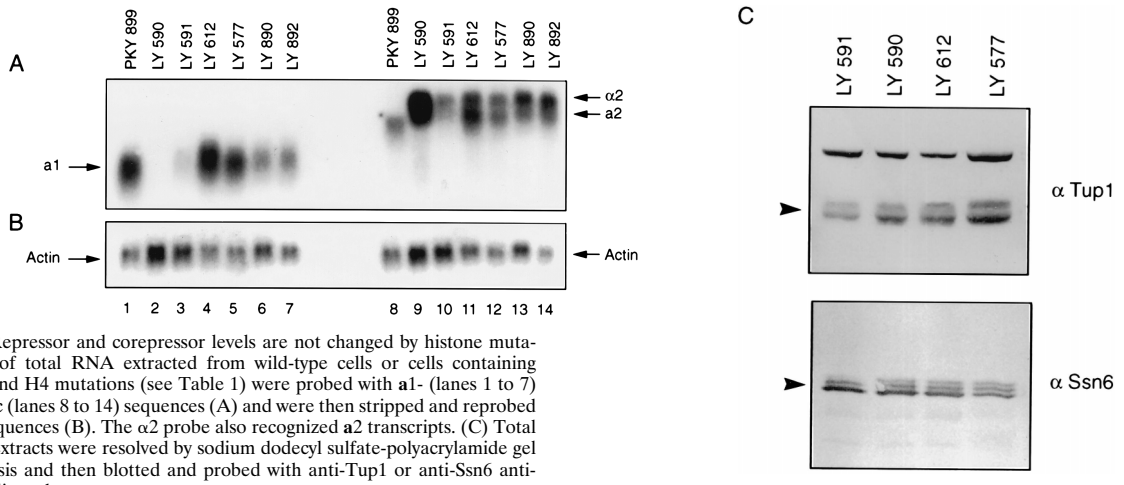

FIG. 2. Repressor and corepressor levels are not changed by histone mutations. Blots of total RNA extracted from wild-type cells or cells containing various H3 and H4 mutations (see Table 1) were probed with **a**1- (lanes 1 to 7) or α2-specific (lanes 8 to 14) sequences (A) and were then stripped and reprobed with actin sequences (B). The α2 probe also recognized **a**2 transcripts. (C) Total cell protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then blotted and probed with anti-Tup1 or anti-Ssn6 antibodies as indicated.

adjacent to the **a**1-α2 operator as they are positioned adjacent to the α2-Mcm1 operator under conditions of repression.

Nuclei were prepared from wild-type haploid or diploid cells and digested with MNase, which preferentially cleaves DNA between nucleosomes. Southern blot analysis indicated that the plasmid was packaged into chromatin in both cell types as evidenced by ladders of oligonucleosome-sized MNase fragments (data not shown). The locations of the micrococcal cut sites were then mapped by primer extension (Fig. 3A).

In all cases examined, the patterns of MNase cutting in haploid and diploid plasmid chromatin were identical (Fig. 3A). A subset of cut sites recognized in naked DNA was apparent in chromatin from both cell types. These cut sites are highly reproducible and are observed at several different levels of nuclease digestion. The sites of digestion in chromatin are not spaced at nucleosomal lengths, and only limited protection of cutting is observed relative to DNA, providing no evidence of nucleosome positioning.

We next asked whether nucleosome positioning is observed at an endogenous gene regulated by **a**1-α2. For this analysis, we chose to examine the chromatin structure of the *RME1* gene, since a single **a**1-α2 operator is found upstream of this gene in contrast to the multiple sites found upstream of several other genes regulated by **a**1-α2. Chromatin was prepared from haploid and diploid cells, digested with MNase, and analyzed as described above (Fig. 3B). Again, the patterns of digestion in haploid and diploid cells were identical, providing no evidence for nucleosome positioning.

Finally, we asked whether **a**1-α2 could direct nucleosome positioning in a context where we have previously observed positioning by α2-Mcm1. A TRP1/ARS1 derivative plasmid that carried the **a**1-α2 operator at a site analogous to that of the α2-Mcm1 operator in plasmid TALS was created (26). Positioned nucleosomes were readily detected in TALS chromatin from α and **a**/α cells in previous studies (26).

TAL**a**1-α2 was introduced into isogenic haploid or diploid cells, and chromatin isolated from these cells was digested with MNase. Cut sites were then examined by indirect end labeling (Fig. 3C). Once again, the patterns of digestion in diploid and haploid cells were identical. Some regions of protection were observed in chromatin relative to naked DNA, corresponding to the previously defined positions of nucleosomes I, II, and III in TRP1/ARS1 and TALS (26). However, since these protected regions were present in both cell types, their locations cannot be attributed to the presence or absence of **a**1-α2.

Activator binding to UAS sequences is decreased in diploid cells. The effects of the histone mutations together with the above mapping results indicate that some aspect of chromatin structure other than nucleosome positioning is involved in **a**1-α2 repression. A repressive chromatin structure might limit occupancy of promoter elements by transactivating factors in diploid cells. Activation of the *CYC1* promoter is dependent upon binding of Hap1 to UASc1 and Hap2-Hap3-Hap5 binding to UASc2. UASc1 directs the majority of regulation of this promoter when cells are grown in glucose (9). We examined the binding of these activators in the presence or absence of

TABLE 4. Combined H3 and H4 mutations synergistically derepress the **a**1-α2 operator[a]

Strain	Cell type (*MAT*)	H4 type	H3 type	Presence of:			β-Gal level (U)	*n*	Derepression (fold)
				a1	α2	**a**1-α2 op.			
LY591	**a**/α	wt	wt	+	+	+	1.3 ± 0.5	6	1
LY590	α	wt	wt	−	+	+	299.0 ± 71	8	230
LY612[b]	**a**	K12QK16Q	wt	+	+	+	1.6 ± 0.5	6	1
LY577[b]	**a**	K12QK16Q	del (1–28)	+	+	+	54.2 ± 11.3	8	42
LY690	α	wt	wt	−	+	−	279.0 ± 59	6	214
LY691	**a**/α	wt	wt	+	+	−	142.1 ± 40	6	109
LY677	**a**	K12QK16Q	del (1–28)	+	+	−	124.2 ± 14	4	95
LY590 ΔTUP1	α	wt	wt	−	+	+	744 ± 40	4	572
LY577 ΔTUP1	**a**	K12QK16Q	del (1–28)	+	+	+	782 ± 54	4	601

[a] Symbols and abbreviations are as defined in the footnotes for Table 2.
[b] The silent mating loci are derepressed in these strains, providing α2 expression.

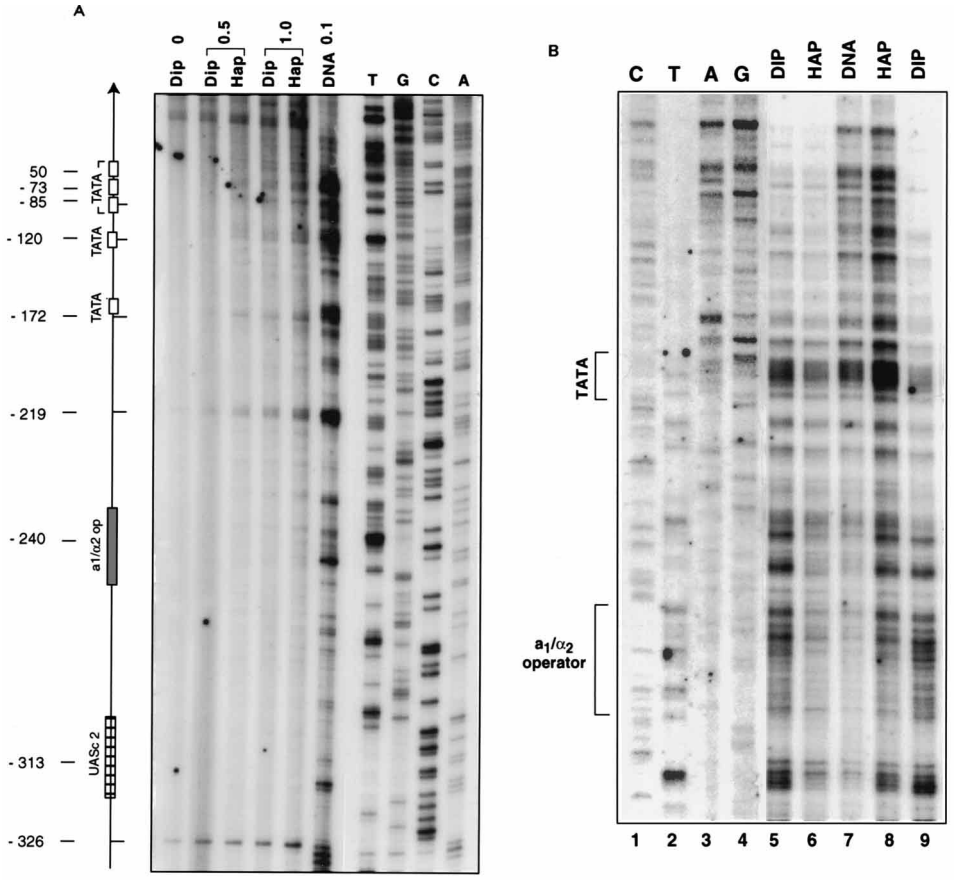

FIG. 3. Nucleosomes are not positioned adjacent to the **a**1-α2 operator. (A) MNase cut sites in the *CYC1*–**a**1-α2–*lacZ* reporter. Nucleosome positions were probed by primer extension analysis of MNase-digested chromatin (0, 0.5, or 1.0 U/ml) or naked DNA (0.1 U/ml) isolated from wild-type haploid (Hap) or diploid (Dip) cells. No positioned nucleosomes are observed in haploid or diploid chromatin. The locations of the **a**1-α2 operator and UASc2 and TATA elements are indicated to the left; the locations were determined by the sequence analysis shown in lanes marked T (ddA reaction), G (ddC reaction), C (ddG reaction), and A (ddT reaction). (B) MNase cut sites in *RME1* chromatin. Primer extension analysis of MNase cut sites in the *RME1* promoter were also mapped by primer extension analysis of chromatin from haploid or diploid cells. Again no protected regions indicative of positioned nucleosomes were observed. The concentrations of MNase used were 0.3 U/ml (lanes 5 and 8), 0.6 U/ml (lane 9), and 1.3 U/ml (lanes 6 and 7). (C) Nucleosome positions in plasmid TAL**a**1-α2. Plasmid chromatin (C) or naked DNA (D) was isolated from haploid (HAP) or diploid (DIP) cells as indicated and digested with micrococcal nuclease. After purification, samples were digested with *Eco*RV and subjected to indirect end label analysis by using an *Eco*RV-*Hin*dIII fragment of the plasmid as probe. The positions of ARS1 elements and the **a**1-α2 operator are indicated, as are the inferred positions of nucleosomes I, II, and III (following the nomenclature for nucleosomes in analogous positions in other TRP1/ARS1 plasmids).

a1-α2 in our reporter by in vivo UV or DMS footprinting of haploid and diploid cells.

Hap1 binding to UASc1 has been determined previously by others (31) to induce a site hypersensitive to UV-induced formation of pyrimidine dimers in vivo (Fig. 4). We also observe this hypersensitive site in our reporter in the presence of Hap1 (Fig. 4A) but not in its absence (in *hap1*Δ cells; data not shown). Interestingly, this site is reproducibly reduced in intensity by ~50% in constructs bearing the **a**1-α2 operator (quantitated by phosphorimage analysis) in diploid cells relative to isogenic haploid cells, suggesting a reduced or altered binding of Hap1. The accessibility of this site is increased in the presence of the combined H3 and H4 mutations (in LY577), reaching 86% of that seen in the wild-type haploid cells (Fig. 4B), consistent with the compromised repression observed above in the presence of these mutations. No difference in the intensity of this site is seen in constructs which lack the **a**1-α2 operator (data not shown, but see below), indicating that the

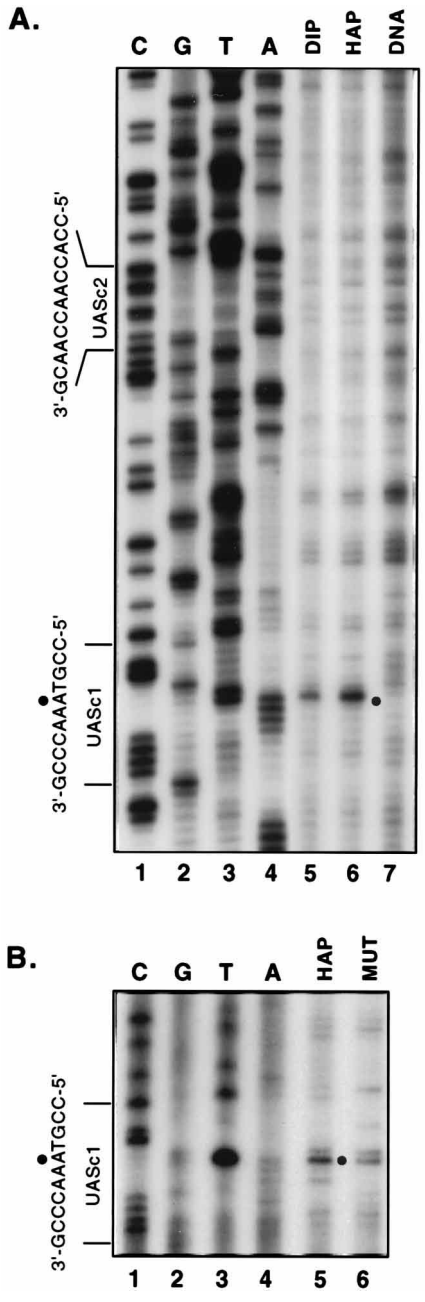

FIG. 4. UV photofootprinting of UASc1 and UASc2. (A) Primer extension analysis of UV-induced photoproducts in the UAS region upstream of the **a**1-α2 operator in the *CYC1-lacZ* reporter construct. The dot indicates a position in UASc1 where adduct formation is inhibited ~50% in wild-type diploid cells (LY591; lane 5) relative to wild-type haploid cells (LY590; lane 6). (B) Less inhibition of adduct formation (~24%) was observed at the UASc1 site in cells harboring H3 and H4 mutations (LY577; lane 6) relative to wild-type haploid cells (LY590; lane 5). Band intensities were quantified and compared by phosphorimage analysis. Sequencing reactions (lanes 1 to 4) are labeled as in Fig. 3A.

levels of Hap1 are not decreased in diploid cells and that the effects we see in operator-containing constructs are due to the presence of **a**1-α2. Together, these data indicate that Hap1 binding is inhibited when **a**1-α2 is bound to its operator ~80 bp downstream of UASc1.

The altered interaction of Hap1 with UASc1 is confirmed by in vivo DMS footprinting experiments. Again, specific residues are enhanced in their reactivity with DMS upon Hap1 binding while others are protected (Fig. 5). We find that the enhanced sites are less intense in diploid samples (compare bands marked by dots in lanes 6 and 7 of Fig. 5) and that protected sites are less protected (compare bands marked by arrowheads in lanes 5, 6, and 7). The pattern of methylation in the diploid cell, then, is more like that of naked DNA than that of haploid cells, consistent with decreased binding of Hap1. These changes are not seen in constructs lacking the **a**1-α2 operator (Fig. 5, lane 8), again indicating that Hap1 binding is inhibited by the presence of **a**1-α2.

Enhancement of certain sites for and protection of others against DMS reactivity upon activator binding to UASc2 has also been reported previously (31). We observe similar enhancements and protections (Fig. 5) in our reporter construct in haploid cells, and as for UASc1, these enhancements and protections are diminished in diploid cells. Again, these changes are not seen in constructs that lack the **a**1-α2 operator (Fig. 5, lane 8). Taken together with the effects of the histone mutations on repression described above, our data suggest that **a**1-α2 limits the binding of activators to UASc1 and UASc2 at a distance through formation of a repressive chromatin environment.

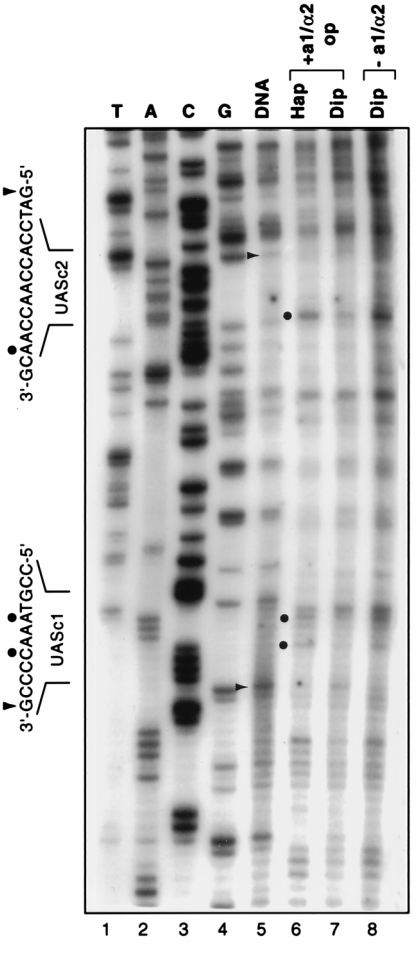

FIG. 5. DMS footprinting of UASc1 and UASc2. Primer extension analysis of methylation products. Dots indicate bands that are enhanced in haploid cells (lane 6) or in the construct lacking the **a**1-α2 operator (lane 8) relative to diploid cells (lane 7) or naked DNA (lane 5). Arrowheads indicate bands enhanced in diploid cells and naked DNA. Sequencing reactions (lanes 1 to 4) are as in Fig. 3A.

DISCUSSION

Our previous work suggested that Tup1 organizes chromatin through direct interactions with the amino termini of histones H3 and H4 in order to repress transcription (3, 6). Our finding that amino-terminal mutations in these histones synergistically derepress an **a**1-α2 reporter provides further evidence for this hypothesis. We have now demonstrated that three separate classes of genes (**a** cell specific, haploid specific, and DNA damage inducible) repressed by Tup1 are derepressed upon the combination of H3 and H4 mutations.

In contrast to previous studies of α2-Mcm1-mediated repression, however, repression by Tup1 via **a**1-α2 does not result in the positioning of nucleosomes. These data suggest that a different, perhaps more general, repressive aspect of chromatin is utilized for repression of haploid-specific genes. The decreased occupancy of UASc1 and UASc2 observed in diploid cells may directly reflect this repressive chromatin state. The interaction of Tup1 with histones could also contribute to repression by disrupting communication between factors bound to the UAS sequences and downstream factors bound to the *CYC1* promoter.

Interestingly, the *CYC1* promoter used here is the only RNA polymerase II-transcribed promoter described to date that escapes the positioning of nucleosomes adjacent to the α2-Mcm1 operator (23). These findings and our current data indicate that nucleosome positioning may play a secondary role in repression by α2-Mcm1. Nucleosome positioning could be precluded in the *CYC1* promoter by the presence of several TATA or TATA-like sequences that have been reported to be constitutively occupied by TFIID (2). Our inability to observe positioning adjacent to the **a**1-α2 operator in our *CYC1* reporter construct cannot be explained simply by the unusual nature of this promoter since we were also unable to detect positioning in a context in which α2-Mcm1 positioning of nucleosomes is readily detected (TAL-based plasmids [26]; Fig. 3C). Importantly, however, some aspect of chromatin structure is clearly required for the regulation of gene expression by both α2-Mcm1 and **a**1-α2, as evidenced by the synergistic derepression observed in the face of mutations in H3 and H4 (this work; 6).

Why might interaction of Tup1 with α2 in the context of α2-Mcm1 complexes lead to nucleosome positioning (at least in the majority of cases), but not in the context of **a**1-α2 complexes? One obvious possibility includes the differences in the arrangements and stoichiometries of α2 in these different contexts. Two molecules of α2 associate with Mcm1 at the α2-Mcm1 operator (34), whereas only one molecule of α2 associates with **a**1 (4). Since α2 interacts with both Tup1 and Ssn6 (17, 37), Ssn6-Tup1 complexes might be stabilized by interaction with multiple α2s at the α2-Mcm1 operator as compared to the single α2 at the **a**1-α2 operator. In addition, each α2 in the α2-Mcm1 complex could recruit a separate Ssn6-Tup1 complex, leading to an increased density of H3-H4 binding surfaces at these sites. Finally, Mcm1 dictates a particular spacing and orientation for α2 binding to DNA (34), which could in turn lead to a specialized arrangement of Ssn6-Tup1. Any of these possibilities could create a "nucleation point" for nucleosome positioning that would be absent at the **a**1-α2 operator.

Ssn6-Tup1 is likely recruited to multiple promoters through interactions with disparate DNA binding factors. Little is known about the molecular nature of these interactions at present, but both Ssn6 and Tup1 contain repeated motifs that may provide flexible protein-protein interaction surfaces. Different TPR domains in Ssn6, for example, are required for repression of different classes of genes (37), and the WD repeats in Tup1 provide redundant sites for interactions with proteins such as α2 (18). It has been suggested that this flexibility allows different conformations of Ssn6-Tup1 complexes at different promoters to accommodate different promoter architectures (18). Such flexibility could also create different faces for interactions with histones to organize or stabilize repressive chromatin structures. Understanding how these repressors are recruited and arranged at specific promoters should help us to understand how appropriate chromatin domains are established for the regulation of diverse genes.

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REFERENCES

1. **Axelrod, J. D., and J. Majors.** 1989. An improved method for photofootprinting yeast genes in vivo using Taq polymerase. Nucleic Acids Res. **17:** 171–183.
2. **Chen, J., M. Ding, and D. S. Pederson.** 1994. Binding of TFIID to the CYC1 TATA boxes in yeast occurs independently of upstream activating sequences. Proc. Natl. Acad. Sci. USA **91:**11909–11913.
3. **Cooper, J. P., S. Y. Roth, and R. T. Simpson.** 1994. The global transcriptional regulators, SSN6 and TUP1, play distinct roles in the establishment of a repressive chromatin structure. Genes Dev. **10:**1400–1410.
4. **Dranginis, A. M.** 1990. Binding of yeast a1 and alpha2 as a heterodimer to the operator of a haploid specific gene. Nature **347:**682–685.
5. **Dranginis, A. M.** 1989. Regulation of *STA1* gene expression by *MAT* during the life cycle of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**3992–3998.

5a. **Edmondson, D. G., and S. Y. Roth.** Unpublished data.

6. **Edmondson, D. G., M. M. Smith, and S. Y. Roth.** 1996. Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. Genes Dev. **10:**1247–1259.
7. **Goutte, C., and A. D. Johnson.** 1988. a1 protein alters the DNA binding specificity of alpha 2 repressor. Cell **52:**875–882.
8. **Grunstein, M.** 1990. Nucleosomes: regulators of transcription. Trends Genet. **6:**395–400.
9. **Guarente, L., B. Lalonde, P. Gifford, and E. Alani.** 1984. Distinctly regulated tandem upstream activation sites mediate catabolite repression of the CYC1 gene of S. cerevisiae. Cell **36:**503–511.
10. **Herschbach, B. M., M. B. Arnaud, and A. D. Johnson.** 1994. Transcriptional repression directed by the yeast alpha 2 protein in vitro. Nature **370:**309–311.
11. **Herskowitz, I.** 1989. A regulatory hierarchy for cell specialization in yeast. Nature **342:**749–757.
12. **Hill, J., I. G. Donald, and D. E. Griffiths.** 1991. DMSO enhanced yeast transformation. Nucleic Acids Res. **19:**5791.
13. **Johnson, A. D., and I. Herskowitz.** 1985. A repressor (MAT alpha 2 product) and its operator control expression of a set of cell type specific genes in yeast. Cell **42:**237–247.
14. **Johnson, L. M., P. S. Kayne, E. S. Kahn, and M. Grunstein.** 1990. Genetic evidence for an interaction between Sir3 and histone H4 in the repression of silent mating loci in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **87:**6286–6290.
15. **Kayne, P. S., U. Kim, M. Han, J. R. Mullen, F. Yoshizaki, and M. Grunstein.** 1988. Extremely conserved histone H4 amino terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell **55:**27–39.
16. **Keleher, C. A., C. Goutte, and A. D. Johnson.** 1988. The yeast cell-type-specific repressor alpha 2 acts cooperatively with a non-cell-type-specific protein. Cell **53:**927–936.
17. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell. **68:**709–719.
18. **Komachi, K., M. J. Redd, and A. D. Johnson.** 1994. The WD repeats of Tup1 interact with the homeo domain protein alpha 2. Genes Dev. **8:**2857–2867.
19. **Megee, P. C., B. A. Morgan, and M. M. Smith.** 1995. Histone H4 and the maintenance of genome integrity. Genes Dev. **9:**1716–1727.

20. **Morgan, B. A., B. A. Mittman, and M. M. Smith.** 1991. The highly conserved N-terminal domains of histones H3 and H4 are required for normal cell cycle progression. Mol. Cell. Biol. **11:**4111–4120.
21. **Murphy, M. R., M. Shimizu, S. Y. Roth, A. M. Dranginis, and R. T. Simpson.** 1993. DNA-protein interactions at the S. cerevisiae alpha 2 operator in vivo. Nucleic Acids Res. **21:**3295–3300.
22. **Redd, M. J., M. B. Arnaud, and A. D. Johnson.** 1997. A complex composed of Tup1 and Ssn6 represses transcription in vitro. J. Biol. Chem. **272:**11193–11197.
23. **Redd, M. J., M. R. Stark, and A. D. Johnson.** 1996. Accessibility of α2-repressed promoters to the activator Gal4. Mol. Cell. Biol. **16:**2865–2869.
24. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Roth, S. Y.** 1995. Chromatin-mediated transcriptional repression in yeast. Curr. Opin. Genet. Dev. **5:**168–173.
26. **Roth, S. Y., A. Dean, and R. T. Simpson.** 1990. Yeast α2 repressor positions nucleosomes in TRP1/ARS1 chromatin. Mol. Cell. Biol. **10:**2247–2260.
27. **Roth, S. Y., M. Shimizu, L. Johnson, M. Grunstein, and R. T. Simpson.** 1992. Stable nucleosome positioning and complete repression by the yeast alpha 2 repressor are disrupted by amino-terminal mutations in histone H4. Genes Dev. **6:**411–425.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Schultz, J., L. Marshall-Carlson, and M. Carlson.** 1990. The N-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**4744–4756.
30. **Schultz, J., and M. Carlson.** 1987. Molecular analysis of SSN6, a gene functionally related to the SNF1 kinase of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**3637–3645.
31. **Shimizu, M., W. Li, H. Shindo, and A. P. Mitchell.** 1997. Transcriptional repression at a distance through exclusion of activator binding in vivo. Proc. Natl. Acad. Sci. USA **94:**790–795.
32. **Shimizu, M., S. Y. Roth, C. Szent-Gyorgyi, and R. T. Simpson.** 1991. Nucleosomes are positioned with base pair precision adjacent to the alpha 2 operator in Saccharomyces cerevisiae. EMBO J. **10:**3033–3041.
33. **Smith, D. L., A. B. Desai, and A. D. Johnson.** 1995. DNA bending by the a1 and alpha 2 homeodomain proteins from yeast. Nucleic Acids Res. **23:**1239–1243.
34. **Smith, D. L., and A. D. Johnson.** 1992. A molecular mechanism for combinatorial control in yeast: MCM1 protein sets the spacing and orientation of the homeodomains of an alpha2 dimer. Cell **68:**133–142.
35. **Trumbly, R. J.** 1986. Isolation of *Saccharomyces cerevisiae* mutants constitutive for invertase synthesis. J. Bacteriol. **166:**1123–1127.
36. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cyc8-Tup1 corepressor complex. Nature **369:**758–761.
37. **Tzamarias, D., and K. Struhl.** 1995. Distinct TPR motifs of CYC8 are involved in recruiting the CYC8-Tup1 corepressor complex to differentially regulated promoters. Genes Dev. **9:**821–831.
38. **Wahi, M., and A. D. Johnson.** 1995. Identification of genes required for alpha2 repression in Saccharomyces cerevisiae. Genetics **140:**79–90.
39. **Zhang, L., and L. Guarente.** 1994. Evidence that TUP1/SSN6 has a positive effect on the activity of the yeast activator HAP1. Genetics **136:**813–817.
39a. **Zhang, W.** Unpublished data.
40. **Zhou, Z., and S. J. Elledge.** 1992. Isolation of crt mutants constitutive for transcription of the DNA damage inducible gene RNR3 in Saccharomyces cerevisiae. Genetics **131:**851–866.
41. **Zitomer, R. S., and C. V. Lowry.** 1992. Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. Microbiol. Rev. **56:**1–11.