Stable nucleosome positioning and complete repression by the yeast $\alpha 2$ repressor are disrupted by amino-terminal mutations in histone H4

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Nucleosomes are positioned in the presence of the yeast repressor $\alpha 2$ in minichromosomes containing the $\alpha 2$ operator and on the promoters of a-cell-specific genes regulated by $\alpha 2$. To investigate the possibility that $\alpha 2$ directs nucleosome position through an interaction with a component of the core particle, we analyzed chromatin structures adjacent to the operator in α cells containing mutations in the amino-terminal region of histone H4. Deletion or point mutation of specific amino acids in histone H4 altered the location and/or stability of nucleosomes adjacent to the $\alpha 2$ operator. These changes in chromatin structure were accompanied by partial derepression of a β -galactosidase reporter construct under $\alpha 2$ control, even though $\alpha 2$ remained bound to its operator sequence. Our data suggest that complete repression by $\alpha 2$ requires stable positioning of nucleosomes in promoter regions and this positioning involves the conserved amino-terminal region of histone H4.

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Activation or repression of cell-type-specific genes by products of the MAT locus determines mating type in the yeast *Saccharomyces cerevisiae* (for review, see Dranginis 1986; Nasmyth and Shore 1987; Herskowitz 1989; Dolan and Fields 1991). Two of these proteins, $\alpha 2$ and $\alpha 1$, share homology with homeo proteins important in the determination of cell type in other systems (Laughton and Scott 1984; Hall and Johnson 1987). $\alpha 2$ functions to repress a-cell-type-specific gene expression in haploid α cells and diploid a/α cells through cooperative interactions with a non-cell-type-specific protein, MCM1 (Wilson and Herskowitz 1986; Keleher et al. 1988, 1989). In addition, $\alpha 2$ forms a heterodimer with the MATa1 protein in diploid cells to repress haploid-specific gene expression (Jensen et al. 1983; Miller et al. 1985; Goutte and Johnson 1988; Dranginis 1990). The mechanisms by which the $\alpha 2$ /MCM1 or $\alpha 1$ / $\alpha 2$ complexes repress transcription are not known.

The $\alpha 2$ /MCM1 complex binds to a 32-bp sequence located ~200 bp upstream of the mRNA start sites of five a-cell-type-specific genes (Wilson and Herskowitz 1986;

Keleher et al. 1988, 1989; Passmore et al. 1988, 1989). This operator sequence can confer repression in the presence of $\alpha 2$ when inserted either upstream or downstream of heterologous promoter-activating elements (Johnson and Herskowitz 1985). The ability of the operator to act at a distance and in different contexts suggests that changes in chromatin structure could be involved in the repression of a-cell-type-specific gene expression by $\alpha 2$ /MCM1.

We reported previously a precise and stable positioning of nucleosomes in minichromosomes containing the $\alpha 2$ operator in haploid α and diploid a/α cells (Roth et al. 1990; Simpson 1990). A precise localization of nucleosomes in the minichromosomes was not observed in the absence of $\alpha 2$, in haploid a cells (Roth et al. 1990). Positioned nucleosomes adjacent to the $\alpha 2$ operator in α cells, but not in a cells, have also been observed upstream of *STE6* and *BAR1*, two chromosomal genes regulated by $\alpha 2$ (Shimizu et al. 1991). The occurrence of nucleosomes over different sequences in very similar positions relative to the operator in the minichromosomes and upstream of these genes suggests that their positions might be directly determined by $\alpha 2$, or the $\alpha 2$ /MCM1 complex. The ability of the operator to serve as a dominant nucleosome-positioning signal relative to an endog-

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Figure 1. Structure of TALS minichromosome, *STE6*, and H4 mutants. (A) The chromatin structure of the TRP1/ARS1 derivative plasmid TALS (Roth et al. 1990) in α and α/α cells is depicted. The positions of nucleosomes I–X and major MNase cut sites present in chromatin are indicated (in map units; arrowheads). The ARS1 elements are located in a hypersensitive region between nucleosomes X and I. The $\alpha 2$ operator is located between nucleosomes IV and V. The TRP1 transcription unit is depicted by the curved arrow. In α cells, nucleosomes are not stably positioned in TALS chromatin. (B) The chromatin structure of the 5' end of the *STE6* gene in α cells is depicted (Shimizu et al. 1991; also see Fig. 5). Major MNase cut sites in chromatin are shown (arrowheads), and inferred positions of nucleosomes are depicted. The locations of the $\alpha 2$ operator, a putative TATA element, and the transcription start site (bent arrow) are also indicated (Wilson and Herskowitz 1986). (C) The first 30 amino acids of histone H4 are shown, and sites of post-translational acetylation (+) or phosphorylation (–) are indicated (van Holde 1989). Deletion mutants used in this study are depicted below the sequence (Kayne et al. 1988). Point mutations (*) were also used in which amino acids 16, 17, or 18 were changed to glycyl residues (Johnson et al. 1990).

enous-positioning signal in a minichromosome in α cells supports this hypothesis further (Simpson 1990). Positioning of a nucleosome in promoter regions might inhibit transcription through occlusion of *cis*-acting elements (for review, see Grunstein 1990a,b; Wolffe 1990; Simpson 1991). Location of a *cis*-acting element involved in replication near the center of a nucleosome has been demonstrated to inhibit its function in vivo (Simpson 1990).

If nucleosomes are positioned adjacent to the $\alpha 2$ operator through an interaction between the $\alpha 2$ /MCM1 complex and a component of the core particle, then mutations, in particular histone molecules, might disrupt this interaction. To test this idea, we examined the structure of a minichromosome and *STE6* in α cells engineered to express amino-terminal mutations in histone H4. Deletions in H4 that extend into the globular core region necessary for nucleosome assembly are lethal (Kayne et

al. 1988). Removal of amino acids 4–19, 4–23, or 4–28 in histone H4 does not lead to cell death but results in a considerable extension of the cell cycle and sterility, as a result of aberrant expression of the silent mating loci (Kayne et al. 1988). Derepression of these loci in the presence of these H4 mutations is specific; other genes such as *PHO5* and *CUP1* are not activated. Point mutation (individually) of a repressor domain (Durrin et al. 1991), including amino acids 16–19, has effects similar to the above deletions in H4 (Johnson et al. 1990; Megee et al. 1990; Park and Szostak 1990) and can be suppressed by mutations in a known regulator of the silent mating loci, *SIR3* (Johnson et al. 1990). In addition, H4 amino-terminal sequences are required for efficient activation of a number of inducible promoters (Durrin et al. 1991) and are involved in position effects regulating gene expression near telomeres (Aparico et al. 1991).

Here, we report that mutations in the H4 amino-ter-

Figure 2. Effects of deletion of histone H4 or H2B amino-terminal regions on TALS chromatin structure. (A) MNase cut sites in TALS chromatin (C) or DNA (D) were mapped in a clockwise direction from an *EcoRV* restriction site at 385 map units (Fig. 1A) in α cells containing wild-type (WT) H4 or an H4 deletion mutant as indicated. Chromatin isolated from an unrelated α cell (SC3) used in previous studies (Roth et al. 1990) was also analyzed for comparative purposes and is shown in the right-most WT lane. The inferred positions of nucleosomes I–VIII are shown at the side, as is the location of the $\alpha 2$ operator (large arrowhead) and the nuclease-hypersensitive region HSR A. The naked DNA lanes (D) are presented twice to aid comparison between samples. Black dots indicate cut sites in chromatin that demarcate boundaries between nucleosomes in α cells with wild-type H4. Symbols next to the second lane indicate sites exposed in naked DNA that are protected in wild-type α -cell chromatin. Note the increased cutting (indicated by asterisks) in the regions normally protected by nucleosomes IV and V in the presence of del (4–19) and del (4–23). The dash indicates a cut site in the region of nucleosome II, which may also be somewhat more exposed in the presence of these deletions. Symbols between lanes 7 and 8 indicate sites in naked DNA that are still protected in the presence of del (4–19) and del (4–23) in the regions of nucleosomes I, II, III, V, VII, and VIII. (B) MNase cut sites in chromatin (C) and DNA (D) in cells containing wild-type (WT) H2B or an H2B amino-terminal deletion mutant ($\Delta H2B$) were mapped as above.

minimal region destabilize nucleosome position adjacent to the $\alpha 2$ operator in a minichromosome and upstream of *STE6*. The functional consequences of these alterations of chromatin structure were evaluated using a β -galactosidase reporter gene inserted into the α -cell-specific

gene *MFa2*. Although β -galactosidase expression is not detected in α cells containing wild-type H4, it is clearly evident in α cells containing H4 amino-terminal mutations, even though *in vivo* dimethylsulfate (DMS) footprints indicate that $\alpha 2$ is bound to its operator in these

cells. Our data support a direct role for nucleosome position in the regulation of α -cell-specific gene expression.

Results

Amino-terminal mutations in H4 alter the structure of the TALS minichromosome

In haploid α cells and diploid α/α cells, the TALS minichromosome is packaged into an array of stably positioned nucleosomes, excluding only those sequences 3' to the *TRP1* gene, which include the *ARS1* elements (Fig. 1A) (Roth et al. 1990). Two nucleosomes, IV and V, directly flank the $\alpha 2$ operator. High-resolution primer-extension mapping of this region indicates that these nucleosomes are positioned very precisely (Shimizu et al. 1991; Fig. 3, below) and are located within a few base pairs of the edge of the operator sequence as defined in vitro (Keleher et al. 1988). In α cells, in the absence of $\alpha 2$, these nucleosomes are not positioned stably, and nucleosomes on the plasmid are located nearly randomly.

To assess the effects of amino-terminal deletions in histone H4 on the structure of TALS, the plasmid was introduced into α cells bearing either a single-copy, plasmid-borne, wild-type H4 gene (PKY999) or an H4 gene with deletion of amino acids 4–14 [del (4–14); PKY917], 4–19 [del (4–19); PKY918], or 4–23 [del (4–23); PKY913] (Fig. 1C). Both chromosomal copies of H4 (H4-1, H4-2) are disrupted in these strains, and the plasmid-borne gene is the sole source of H4 (Kayne et al. 1988). Because strains bearing del (4–19) and del (4–23) express both α - and α -mating-type genes owing to derepression of the silent mating loci (Kayne et al. 1988), they are phenotypically diploid for mating-type functions. However, $\alpha 2$ represses α -cell-type-specific gene expression (Herskowitz 1989) and induces ordered nucleosome positions on the TALS minichromosome (Roth et al. 1990) in diploid α/α cells as well as in haploid α cells. Any changes observed in TALS structure in the presence of del (4–19) or del (4–23), then, should be related to changes in the structure of H4 rather than in the expression of $\alpha 2$ or other mating-type genes.

The structure of the TALS plasmid was first examined in the various strains by micrococcal nuclease (MNase) digestion and indirect end-labeling (Fig. 2A). Nucleosome position is inferred in these studies by the separation of MNase cut sites in chromatin by ~140 bp with protection of intervening sites present in naked DNA. Comparison of structures inferred from such data to those observed in electron micrographs of *TRP1/ARS1* chromatin indicate a close correspondence between the inferred and the actual structure of *TRP1/ARS1* (Pederson et al. 1986).

Indirect end-labeling of TALS chromatin isolated from the strains expressing a single copy of wild-type H4 or del (4–14) indicates that nucleosomes are positioned on the plasmid into an array identical to that observed previously in other α cells. A series of discrete MNase cut sites separated by 140 bp (dots in Fig. 2A), identical to those characterized previously in α cells (Roth et al. 1990), is observed in these strains (cf. lanes 3, 4, and 5,

Fig. 2A). Also, intervening sites exposed in naked DNA are protected from digestion in these chromatin samples (see symbols adjacent to lane 2, Fig. 2A). These data indicate that neither reduction in H4 copy number nor deletion of amino acids 4–14 alters the position or stability of nucleosomes on the TALS minichromosome.

In striking contrast, exposure of particular MNase cut sites is altered in the presence of del (4–19) and del (4–23). Nuclease-sensitive sites in chromatin that normally demarcate the borders between nucleosomes III and IV (fifth dot from top), IV and V (fourth dot from top), and V and VI (third dot from top) are diminished in these strains. Also, cut sites in these regions normally protected from digestion in chromatin relative to naked DNA are exposed (asterisks, Fig. 2A). These differences are most pronounced in the region adjacent to the operator normally protected by nucleosome IV. One cut site in the region protected by nucleosome II may also be somewhat more exposed relative to flanking sites in the presence of del (4–19) and del (4–23) (dash, adjacent to lane 6; Fig. 2A), but the surrounding hypersensitive sites and protection of other bands in this region are consistent with continued presence of nucleosome II in these strains. Positions of nucleosomes elsewhere on the plasmid are not affected by these deletions, as evidenced by the persistence of hypersensitive sites in chromatin and protection of intervening sites in naked DNA (see symbols adjacent to lane 7, Fig. 2A). The positions of nucleosomes I, VII, and VIII, for example (Fig. 2A), appear unchanged in the presence of these deletions. Other mapping experiments confirm that the positions of nucleosomes elsewhere on the plasmid (VI–X, Fig. 1) are not affected (data not shown). Thus, del (4–19) and del (4–23) largely affect the locations of nucleosomes near the $\alpha 2$ operator without causing general alterations in nucleosome positions elsewhere on the minichromosome.

The structure of TALS was also examined in an α strain bearing a 30-amino-acid deletion in the amino-terminal region of histone H2B [amino acids 3–32 (TSY155); Wallis et al. 1983]. No changes were observed in the positions of any nucleosomes on the plasmid (Fig. 2B), although cleavage at the edges of nucleosomes in the presence of the H2B deletion may be slightly more pronounced than in the presence of wild-type H2B (evidenced by a broadening of some bands in Fig. 2B). Thus, the changes we observe in the presence of the histone H4 mutations are not observed upon similar mutation of another histone.

High-resolution mapping of nucleosome positions in the TALS minichromosome

Changes in the positions of nucleosomes I–V in the presence of the H4 mutation del (4–23) were examined more closely by primer extension mapping (Fig. 3), which allows precise definition of the locations of nuclease-hypersensitive sites and protected regions in chromatin by comparison to cut sites in naked DNA and to sequencing ladders on the same gel.

In other studies, nucleosomes flanking the $\alpha 2$ operator in the TALS minichromosome, IV and V, have been shown to be both translationally and rotationally positioned in α cells (Shimizu et al. 1991; WT, Fig. 3). In particular, cutting by MNase is essentially blocked between 1387 and 1522 map units by the presence of nucleosome IV and between 1592 and 1737 map units by nucleosome V. Reappearance of cutting in these regions signals loss of stable translational positioning of these nucleosomes.

Primer extension analysis revealed alterations in sensitivity to MNase in the presence of del (4–23) in the region normally protected by nucleosome IV, as shown in Fig. 3A (lanes 2,4). Several sites protected in the presence of wild-type H4 (cf. lanes 1 and 3) are exposed in the presence of this deletion (arrowheads, Fig. 3A) producing a pattern very similar to that of naked DNA, consistent with results obtained in indirect end-labeling experiments above. Interestingly, a cut site near the $\alpha 2$ operator at 1522 map units, which includes sequences from the *STE6* promoter region, becomes hypersensitive to MNase in the presence of this deletion. These same sequences are hypersensitive in the presence of del (4–19) [and del (4–23)] at the *STE6* chromosomal locus (see site at –166, Fig. 5, below).

Some changes in the susceptibility of a few sites in the linker region between nucleosomes III and IV are evident in this analysis. By comparison with the above changes in the region protected by nucleosome IV, however, only minimal differences are observed in the regions of nucleosomes I, II, and III when examined at this level. Nuclease-sensitive sites are located in the same places in chromatin from the wild-type and del (4–23) strains in these regions, and sites exposed in naked DNA are protected (see dots in Fig. 3A) in chromatin from both cell types.

High-resolution mapping of the position of nucleosome V using MNase also revealed specific differences in the exposure of sequences adjacent to the $\alpha 2$ operator in the presence of del (4–23) (data not shown), indicating a loss of translational positioning of this nucleosome as well. Primer extension mapping of MNase cut sites provides additional evidence that del (4–23) affects the stability of nucleosome position near the $\alpha 2$ operator, in the regions of nucleosomes IV and V.

Rotational positioning of nucleosomes in the presence of H4 amino-terminal deletions

Rotational positioning refers to the orientation of DNA on the surface of a histone octamer, whereas translational positioning refers to the location of the nucleosome with respect to given sequences as defined above. DNase I is useful in evaluating the rotational positions of nucleosomes because it cleaves DNA within the core particle. If a nucleosome maintains a stable rotational position, a 10-bp periodicity of DNase I cuts will be observed that reflects the pitch of the double helix on the surface of the histone octamer.

Primer extension analysis of DNase I digests was per-

formed to determine the rotational positioning of nucleosomes IV and V in the presence of del (4–23). A periodic pattern of cutting is observed for the nucleosome V region in α cells expressing wild-type H4 (Fig. 3B), indicating that this nucleosome is rotationally positioned in these cells, in agreement with previous results (Shimizu et al. 1991). Disruptions of this pattern are observed in the presence of del (4–23) (Fig. 3B). A densitometric scan of the gel in Figure 3B is presented to aid comparison between samples. Cutting near the operator (to the right, as shown in the scan) in the presence of del (4–23) resembles more closely that in naked DNA than in wild-type chromatin. Cut sites normally protected in chromatin are exposed in the presence of this deletion (arrowheads, Fig. 3B). Other sites that should be cut to preserve the ~10-bp periodicity observed in wild-type chromatin are not susceptible to nuclease (open circles, Fig. 3B). This disruption of periodic cutting indicates that nucleosome V is not rotationally positioned adjacent to the $\alpha 2$ operator in the presence of del (4–23). Some sites in the distal region of nucleosome V are cut similarly in wild-type chromatin and in chromatin containing del (4–23) (dashes, Fig. 3B), indicating that a complete disruption of chromatin structure in this region has not occurred. DNase I digests of the nucleosome IV region also indicate that this nucleosome is no longer rotationally positioned in the presence of del (4–23) (data not shown). Primer extension analysis of MNase and DNase I cut sites indicates that the amino-terminal region of H4 is necessary for both stable translational and rotational positioning of nucleosomes IV and V normally observed in wild-type α cells adjacent to the $\alpha 2$ operator.

Changes in TALS topoisomer distribution in the presence of del (4–19) and del (4–23)

In previous studies we observed that the TALS plasmid isolated from α cells contained an extra negative supercoil relative to the plasmid isolated from a cells (Roth et al. 1990). Because each nucleosome imparts one negative supercoil to chromatin, these data are consistent with the presence of an additional nucleosome on the plasmid in α cells (see Discussion, below). When we examined the distribution of TALS topoisomers isolated from α strains containing either the wild-type H4 gene or del (4–14), we observed a supercoil density similar to that observed in other α cells (Fig. 4). A slight shift in supercoil density (less than one supercoil) may occur in the presence of del (4–14), but in general, the topoisomer distribution in the del (4–14) strain is equivalent to that of wild-type α cells. In α cells expressing either del (4–19) or del (4–23), the distribution of supercoil density is obviously shifted, to an extent normally observed in a cells, corresponding to the loss of one negative supercoil. This shift is not observed for plasmids lacking the $\alpha 2$ operator, including another TRP1/ARS1 derivative [TAL (Roth et al. 1990); data not shown] and the 2 μ circle (Kayne et al. 1988), indicating that it is a specific effect for plasmids that bind $\alpha 2$. These changes in TALS topoisomer distribution are consistent with the presence of

Figure 4. Distribution of TALS topoisomers in the presence of H4 amino-terminal deletions. Supercoiled DNA isolated from strains containing wild-type H4 (wt) or an H4 deletion mutant (del 4–14, del 4–19, or del 4–23 as indicated) was analyzed on agarose gels containing 40 μ g/ml of chloroquine diphosphate, transferred to nitrocellulose, and hybridized to a probe specific for the TALS plasmid. Under these conditions, more negatively supercoiled molecules migrate slower than less negatively supercoiled molecules. The center of distribution of TALS topoisomers in each lane is indicated (arrowheads). For comparison, the distribution of topoisomers in unrelated α (SC3) and a (E290) cells used in previous studies is also shown. All samples were analyzed on the same gel.

one less nucleosome on the plasmid in the presence of del (4–19) and del (4–23), reflecting the changes in stable nucleosome location indicated in the above mapping studies.

Structure of chromosomal a -cell-specific genes in the presence of amino-terminal deletions in H4

Changes in nucleosome position proximal to the $\alpha 2$ operator, like those observed in the minichromosome in the presence of del (4–19) and del (4–23), might also be expected in chromosomal copies of a -cell-specific genes regulated by $\alpha 2$. At least two of these genes, *STE6* and *BAR1*, are packaged into a positioned nucleosomal array in α cells but not in a cells, analogous to the packaging of the TALS plasmid in the two cell types (Shimizu et al. 1991; Fig. 1B).

Primer extension analysis of MNase cut sites in chromatin and naked DNA indicates that this organized structure is also observed for *STE6* in α cells containing a single copy of a wild-type H4 gene on a plasmid (Fig. 5, lanes 6,7,11). Nuclease-hypersensitive sites are observed at –166, –24, and +184 in chromatin isolated from these cells while many intervening sites present in naked DNA (lanes 5,16) are protected. In a cells, these hypersensitive sites are diminished (cf. lane 12 with lanes 6, 7, and 11). In addition, a subset of sites exposed in naked DNA are exposed in a -cell chromatin, and new hypersensitive sites are observed (cf. lane 12 with lanes 16 and 11). These data are consistent with a disruption of the chromatin structure observed in α cells. One hyper-

sensitive site in a -cell chromatin corresponds to the location of the TATA sequence in the *STE6* promoter (arrowhead, Fig. 5), consistent with an increased accessibility of this sequence in a cells. Surprisingly, the $\alpha 2$ operator sequence appears less sensitive to MNase digestion in a cells (lane 12) than in α cells (lane 11). We are investigating in detail in vivo protein–DNA interactions at the operator in separate studies.

We investigated the effects of del (4–19) on the chromatin structure of *STE6* both in an α strain isogenic to the wild-type strain above (Fig. 5, lane 8) and in strains bearing an integrated copy of this H4 deletion in which both the *HML* and *HMR* loci are converted to the same mating type as present at the *MAT* locus (Fig. 5, lanes 9,10). In effect, these strains possess either three functional copies of *MAT α* ($\alpha\alpha\alpha$; JTY222H; lane 10) or *MAT a* (aaa ; LJY438I; lane 9). Because only a single mating type is expressed in these cells, possible complications owing to secondary effects of derepression of *HML* and *HMR* are avoided.

In α strains carrying del (4–19), a *STE6* chromatin structure intermediate to that in wild-type α or a cells is observed (Fig. 5, lanes 8,10). Nuclease-hypersensitive sites at the ends of the regions protected by nucleosomes in normal α cells (e.g., at –166 and –24) are maintained in the presence of the deletion and, in some cases (at –166), appear to be even more sensitive to digestion. The maintenance of these sites indicates that the structure of this region is not disrupted completely by this amino-terminal deletion in H4. However, accessibility of numerous cut sites protected in the presence of wild-type H4 is clearly increased in the presence of del (4–19) (cf. bracketed region in Fig. 5, lanes 7,8,10,11), including the hypersensitive site that maps to the TATA sequence (arrowhead, Fig. 5) (Wilson and Herskowitz 1986) and two sites that map to sequences near pheromone response elements (dots, Fig. 5; Kronstad et al. 1987). The pattern of exposure of these sites in the *STE6* promoter region [bracket, lanes 8,10] in this strain strongly resembles that observed in chromatin isolated from a cells (lane 12). We interpret this increased accessibility as an indication of decreased nucleosome stability, which allows re-exposure of these sites. Cleavage sites farther downstream, in the *STE6*-coding region, which are normally protected by nucleosomes in α cells, are also exposed in the presence of del (4–19).

In the *aaa* del (4–19) strain, the pattern of MNase cutting in the *STE6* promoter region is similar to that observed in other a strains, as expected (cf. lanes 9 and 12). Many cut sites protected in α -cell chromatin are no longer protected in this strain, and hypersensitive sites are observed within exposed regions that are similar to those in wild-type a cells.

Alterations in *STE6* chromatin structure observed in α cells in the presence of del (4–19) were also observed in the presence of del (4–23). Surprisingly, del (4–14) also affected *STE6* chromatin structure, although to a lesser extent than the longer deletions (data not shown). Analogous experiments indicate that the structure of the *BAR1* promoter region is altered in a very similar man-

Figure 5. Chromatin structure of *STE6* in the presence of H4 amino-terminal deletions and point mutations. (A) The chromatin structure of sequences upstream of the α -cell-specific gene *STE6* was analyzed by primer extension mapping of MNase cut sites in chromatin (C) or DNA (D) isolated from unrelated α cells (SC3) (lanes 6,11), α cells containing a single copy of wild-type H4 [PKY999 (isogenic to H4 mutations); lane 7], unrelated α cells (E290; lane 12), or isogenic α cells containing H4 mutations (lanes 8–10,13–17) as indicated. The del (4–19) was analyzed in strains PKY918 (α), LJY438I (aaa), and JTY222H ($\alpha\alpha\alpha$). The H4 point mutations were in strains PKY505 (Gly-16), LJY921 (Gly-17), LJY933 (Gly-18), and LJY612 (Gln-16). The position of the mRNA start site is indicated by the bent arrow in the diagram at left, and the locations of the $\alpha 2$ operator, putative TATA sequences, and the 5' portion of the *STE6*-coding region are shown (Wilson and Herskowitz 1986). The ellipse indicates the inferred position of a nucleosome in the promoter region. (X) A band corresponding to an artifactual primer extension stop, not a MNase cut site; it is present in undigested samples (data not shown; Shimizu et al. 1991). The arrowhead marks cut sites that map to the TATA sequence. The dots correspond to cuts near pheromone response elements (Kronstad et al. 1987). Chromatin was digested with 5 U/ml (lanes 6–8,13–16), or 20 U/ml (lanes 9–12,17) of MNase. DNA was digested with 0.5 U/ml of MNase (lanes 5,15). Cleavage of chromatin from a given strain always produced the same pattern of bands, regardless of the concentration of nuclease used, except for a progressive loss of higher-molecular-weight bands with increasing concentrations of nuclease. Concentrations were chosen for display to optimize comparison between various samples.

ner in the presence of *del* (4–19), with exposure of sites normally protected by a nucleosome in α cells, but with maintenance of hypersensitive sites at the ends of this region (data not shown).

Point mutations in the H4 amino-terminal region affect STE6 chromatin structure

Point mutation of amino acids 15–19 in H4 elicits a phenotype similar to that of *del* (4–19), including an alteration of growth rate and derepression of the *HML* silent mating-type locus (Johnson et al. 1990). We investigated whether such mutations would also lead to alterations in the chromatin structure of the *STE6* promoter region. Examination of *STE6* structure in the presence of individual point mutations converting Lys-16, Arg-17, or His-18 (Fig. 1C) to glycyl residues revealed alterations in nucleosome stability extremely similar to those described above for *del* (4–19) (Fig. 5, cf. lanes 13, 14, and 15 with lane 10). Sites normally protected by nucleosomes in the promoter region and the first 184 bp of coding sequence are exposed in these strains, whereas hypersensitive sites at either end of these regions were maintained. Surprisingly, although substitution of glycine for Lys-16 (Gly-16) resulted in a changed *STE6* chromatin structure (lane 12), substitution of glutamine at this position (Gln-16), either on a plasmid (data not shown) or as an integrated allele, did not (lane 17; note similarity to lanes 6 and 7). The ability of the individual glycyl substitutions at residues 16, 17, or 18 to alter the structure of the *STE6* promoter region highlights the importance of this region of H4 to the stable positioning of nucleosomes normally observed adjacent to the $\alpha 2$ operator in α cells.

*Occupancy of the $\alpha 2$ operator is not changed in the presence of *del* (4–19)*

Changes in nucleosome position and stability might affect the association of *trans*-acting factors with chromatin. To rule out the possibility that the binding of $\alpha 2$ to its operator sequence might be altered in the presence of the H4 amino-terminal mutations, we performed *in vivo* footprints of the operator region by treating intact cells with DMS.

In vitro analysis of $\alpha 2$ binding to its operator by DMS footprinting indicates that symmetrical G residues at each end of the operator are protected by $\alpha 2$ (Sauer et al. 1988). These same residues are protected *in vivo* in wild-type α cells (M. Shimizu, unpubl.; Fig. 6) but not in α cells, in the absence of $\alpha 2$. In addition, other, more centrally located G residues are at least partially protected in both cell types (Fig. 6), consistent with the binding of MCM1 in this region. This pattern of protection is not changed in the presence of the *del* (4–19) H4 deletion, indicating that occupancy of the operator in these cells is equivalent to that in wild-type α cells (Fig. 6). A more detailed analysis of the binding of $\alpha 2$ and MCM1 to chromatin *in vivo* is the subject of a separate study (M. Shimizu, S.Y. Roth, M. Murphy, and R.T. Simpson, unpubl.).

Figure 6. *In vivo* DMS footprinting of the $\alpha 2$ operator. DNA purified from intact cells treated with DMS was subjected to multicycle primer extension. Sites of methylation in both strands of the $\alpha 2$ operator were mapped in wild-type α cells, α cells containing *del* (4–19), or wild-type α cells and compared to a ddC sequencing reaction (ddC) or DMS-treated naked DNA (DNA). Protected sites are indicated by dots next to the corresponding band in naked DNA and above the corresponding G residue in the sequences shown at left.

Incomplete repression by $\alpha 2$ in the presence of H4 amino-terminal mutations

If nucleosomes positioned adjacent to the $\alpha 2$ operator in the promoter regions of α -cell-specific genes contribute to the repression of these genes in α cells, increased expression should accompany alterations of nucleosome stability or position. To test this hypothesis, we replaced the α -cell-specific gene *Mfa2* (mating factor $\alpha 2$) in iso-

genic strains harboring the H4 amino-terminal mutations with a *MFa2*- β -galactosidase fusion gene.

Expression of the β -galactosidase reporter gene was monitored by development of blue color in colonies plated on media containing X-gal and by measurement of β -galactosidase activity (Fig. 7). Although no blue color developed in wild-type α cells (Fig. 7A), a faint blue color was observed in the del (4–14) strain, and more intense expression was observed in colonies bearing del (4–19), del (4–23), Gly-16, and Gly-18 (no transformants of the Gly-17 strain bearing the integrated reporter gene were recovered; see Materials and methods). Strong expression of the β -galactosidase fusion protein was observed in wild-type **a** cells, as expected.

β -Galactosidase activity measured in liquid assay indicated no expression above background in the wild-type α strain and minimal expression in the del (4–14) strain. A level of expression intermediate to that in wild-type α and **a** cells was observed in the presence of the other H4 mutations, reaching 4–15% of the maximal expression observed in **a** cells (Fig. 7B). Increased steady-state levels of *MFa2*- β -galactosidase mRNA were also observed in these strains (data not shown).

Importantly, partial derepression was observed in the presence of del (4–19) in two isogenic strains differing only at the *MAT* locus (PKY918 and PKY818) and in the nonisogenic $\alpha\alpha\alpha$ strain (JTY222H) (Fig. 7), indicating that derepression occurs in the presence of one, two, or three expressed copies of *MAT* α and is independent of the presence of **a**-mating-type information. We have also observed a partial derepression of a *STE6*- β -galactosidase reporter plasmid and of *BAR1* activity (Sprague and Herskowitz 1981) (data not shown). The partial derepression of these genes seems particularly significant in view of the DMS footprint data (Fig. 6), which indicate that the $\alpha 2$ operator is fully occupied in the presence of del (4–19). The $\alpha 2$ repressor is not capable of complete repression in the presence of this H4 deletion, even though it is bound to its operator in these cells.

The partial derepression observed is consistent with the partial change in *STE6* chromatin structure that occurs in the presence of the H4 mutations. Indeed, the lack of a more extensive derepression (relative to **a** cells) may reflect the residual organization of chromatin in the H4 mutant strains. Significantly, in every strain examined so far, mutations that alter nucleosome position/stability adjacent to the $\alpha 2$ operator also result in incomplete repression by $\alpha 2$. Conversely, mutations such as

the H2B amino-terminal deletion and the H4 Gln-16 mutation that do not affect *STE6* chromatin structure also do not affect repression by $\alpha 2$ (data not shown). Our finding that the H4 amino-terminal region is required both for stable positioning of nucleosomes adjacent to the $\alpha 2$ operator and for efficient repression by $\alpha 2$ indicates a direct role for chromatin structure in the regulation of **a**-cell-specific gene expression.

Discussion

Nucleosomes are precisely positioned adjacent to the $\alpha 2$ operator in the presence of $\alpha 2$ in several different sequence contexts (Roth et al. 1990; Simpson 1990; Shimizu et al. 1991). Here, we show that the amino-terminal region of H4 is required for stable translational and rotational positioning of these nucleosomes as well as for efficient repression by $\alpha 2$, indicating that chromatin structure directly contributes to the regulation of cell-type-specific gene expression in yeast.

Mechanism of nucleosome positioning by $\alpha 2$

Positioning of nucleosomes adjacent to the $\alpha 2$ operator could occur indirectly upon binding of $\alpha 2$ /MCM1 through formation of a boundary to nucleosome placement. However, statistical positioning around the operator (Kornberg 1981; Kornberg and Stryer 1988) cannot explain the ability of the operator to induce a shift in nucleosome position (Simpson 1990) or the precision of positioning observed adjacent to the operator (Shimizu et al. 1991; this paper). Also, because MCM1 binds the operator in both **a** and α cells (Keleher et al. 1988; Passmore et al. 1988), statistical positioning might be expected to occur in both cell types. Moreover, the positions of nucleosomes IV and V are destabilized in the presence of the H4 del (4–19) mutation, even though in vivo footprinting of the $\alpha 2$ operator indicates that it is occupied in these cells. Our data support a more direct mechanism for positioning of nucleosomes adjacent to the operator, involving the $\alpha 2$ /MCM1 complex and the H4 amino-terminal region.

It is surprising that nucleosome position is not randomized upon destabilization of nucleosomes IV and V in TALS, given the lack of organization observed in **a** cells (Roth et al. 1990). Although it is possible that $\alpha 2$

Figure 7. Expression of a reporter construct regulated by $\alpha 2$ in the presence of H4 mutations. (A) Cells containing the *MFa2*- β -galactosidase reporter construct grown on X-gal media. Wild-type α cells shown are PKY999; wild-type **a** cells are PKY899. Mutant H4 strains are as listed in B. The del (4–19) [PKY918 and JTY222H ($\alpha\alpha\alpha$)] and del (4–23) (PKY913) strains exhibit a characteristic slow-growth phenotype (Kayne et al. 1988). (B) β -Galactosidase expression in permeabilized cells was determined as described by Rose et al. (1990) and is expressed in Miller units. Values normalized by subtraction of activity present in cells without the reporter gene are shown (normalized), as are average values of activity in each strain (LacZ; $\pm$ S.D.). The number of determinations [n] is indicated for each. The % Max values represent activities divided by that present in isogenic, wild-type **a** cells. For all strains except PKY505 and LJY933, the isogenic **a** strain is PKY899; for these two strains, it is PKY501. PKY899 and PKY501 are isogenic except for the vector carrying the wild-type H4 allele. No % Max values are given for the $\alpha\alpha\alpha$ and **aaa** strains because they are not isogenic to the other strains. Strain names refer to the strains into which the reporter gene was introduced. The resultant transformants have the same names followed by “MFZ” to designate the integration.

Figure 7. (See facing page for legend.)

might act at a distance to maintain the positions of distal nucleosomes, it seems more plausible that nucleosomes IV and V may form transiently in the presence of the H4 mutations and thereby establish the ordered array normally observed in α cells. Other studies have indicated that preferred positions exist for nucleosomes in the region of *TRP1* and in the regions of nucleosomes I, II, and III (Thoma 1986), whereas *TRP1*/*ARS1* sequences incorporated into nucleosomes IV and V are hypersensitive to nuclease digestion in constructs lacking the $\alpha 2$ operator, indicating that they are not normally packaged into stable nucleosomes. In the absence of an intact H4 amino-terminal region to anchor nucleosomes IV and V adjacent to the operator in TALS, these nucleosomes appear to be selectively destabilized while more distal nucleosomes maintain their preferred positions. Because nucleosomes IV and V are never positioned in the absence of $\alpha 2$, the positioned nucleosomal array does not form in α cells.

Changes in the stability of nucleosome position adjacent to the operator in the presence of H4 mutations

The shift in the supercoil density of TALS observed in the presence of $\Delta(4-19)$ and $\Delta(4-23)$ is also consistent with the destabilization of a nucleosome adjacent to the operator, although other explanations are possible. Each nucleosome imparts one negative supercoil to chromatin (for review, see Morse and Simpson 1988), and the presence of an additional negative supercoil in strains expressing wild-type H4 or $\Delta(4-14)$ could indicate the presence of one more stable nucleosome on the plasmid in these strains. Increased acetylation of histones, which neutralizes positively charged lysyl residues, has been observed to decrease the negative linking number change per nucleosome (Norton et al. 1989). Deletion of amino-terminal lysines in H4 might mimic this effect, leading to an overall decrease in the negative superhelicity of chromatin as is observed for TALS in the presence of $\Delta(4-19)$ or $\Delta(4-23)$. However, our observation that such shifts in supercoil density do not occur for plasmids that do not contain the $\alpha 2$ operator indicates that a decreased negative superhelicity is not a general feature of chromatin in these cells. Changes in supercoil density might also be associated with changes in transcriptional activity. However, no induction of *TRP1* RNA accumulation was observed in the presence of $\Delta(4-19)$ or $\Delta(4-23)$ (data not shown), so the change in supercoil density is not easily explained by an increase in *TRP1* transcription, unless such an increase is accompanied by an increased turnover of *TRP1* RNA. It is also unlikely, therefore, that the changes in the positions of nucleosomes IV and V observed in the presence of these mutations stem from increased transcription of *TRP1*. The lack of an increase in *TRP1* expression is consistent with a continued repression of this gene by nucleosomes (VI, VII) positioned upstream of the *TRP1* RNA start site (at 105 map units, Fig. 1A).

Promoter accessibility and regulation of α -cell-specific gene expression

The involvement of chromatin structure in the repression of α -cell-specific gene expression could explain how repression can occur when the $\alpha 2$ operator is placed either upstream or downstream of UAS elements in heterologous promoters and the ability of the operator to act over varying distances (Keleher et al. 1988; Herskowitz 1989). Increased accessibility of promoter regions upon destabilization of a nucleosome adjacent to the $\alpha 2$ operator most easily explains the partial derepression of the β -galactosidase reporter gene that occurs in the presence of the H4 amino-terminal deletions. The lack of full induction of this gene correlates with the partial disruption of chromatin structure relative to α cells, which indicates that accessibility of promoter regions is still partially obscured in these strains.

Importantly, derepression of the reporter construct is not the result of some secondary effect resulting from derepression of the silent mating loci, and subsequent expression of both α and α mating information, as it also occurs in the $\alpha\alpha\alpha$ $\Delta(4-19)$ strain. It is also not the result of an altered association of $\alpha 2$ with its operator sequence in the presence of this mutation as indicated by *in vivo* footprinting experiments. Nor is it a reflection of a general increase in transcription in these cells because other repressed genes and some inducible promoters are not activated (Kayne et al. 1988; Durrin et al. 1991). Although we cannot rule out the possibility that the H4 amino-terminal region is involved in the regulation of some other gene required for repression of α -cell-specific genes, the alterations in chromatin structure adjacent to the $\alpha 2$ operator in the presence of the H4 mutations suggest a direct involvement of the H4 amino-terminal tail in the maintenance of this structure and, thereby, efficient repression by $\alpha 2$.

Other factors involved in regulation of α -cell-specific gene expression and chromatin structure

The incomplete disruption of chromatin structure in the presence of the H4 mutations indicates that other factors, or other regions of H4, may be involved in the maintenance of this structure. At least two other proteins are involved in the control of α -cell-type-specific gene expression, SSN6 and TUP1 (Carlson et al. 1984; Trumbly 1986; Schultz and Carlson 1987; Schultz et al. 1990; Williams and Trumbly 1990). Interestingly, glucose-repressible genes that are also regulated by SSN6 and TUP1 undergo changes in chromatin structure upon activation (Lohr 1984; Perez-Ortin et al. 1987). Other histones could be important in the maintenance of nucleosome position/stability adjacent to the $\alpha 2$ operator as well, although an amino-terminal deletion in H2B had minimal effects on the structure of the TALS minichromosome or *STE6*, and strains bearing amino-terminal deletions in H2A have no obvious phenotype or effect on mating (for review, see Grunstein 1990a). However, an amino-terminal deletion in H3 causes a constitutive ex-

pression of *GAL1* (Durrin et al. 1991), and mutations in H2A and H2B can restore transcriptional initiation downstream of solo δ and TY insertions, rescuing expression of downstream genes (Clark-Adams et al. 1988). These histones, then, are an important part of some regulatory chromatin structures.

Modulation of transcription by H4

The involvement of the H4 amino terminus in the activation of some genes (Durrin et al. 1991) and repression of others (Kayne et al. 1988; Johnson et al. 1990; Aparico et al. 1991; this paper) indicates that this histone is important in the organization of chromatin structures that either facilitate or inhibit transcription. Direct or indirect interactions between H4 amino-terminal domains and *trans*-acting factors such as GAL4 (Durrin et al. 1991), SIR3 (Johnson et al. 1990), and $\alpha 2$ may determine the type of structure formed. These interactions could easily be influenced by post-translational modification of the H4 amino-terminal tail, providing a mechanism whereby changes in promoter accessibility and transcriptional activity could be readily accomplished. The extreme conservation of the H4 amino-terminal region suggests that it may be involved in the regulation of gene expression in other organisms as well. A role in modulation of transcriptional activity might explain why this region of the H4 molecule is so highly conserved and yet is not essential for yeast cell viability.

Materials and methods

Yeast strains

S. cerevisiae strains were propagated according to standard procedures (Rose et al. 1990) in either rich media (YEED) or selec-

tive media (SDM containing appropriate amino acids, adenine, and uracil if needed) as necessary. Strains used are presented in Table 1.

Construction of plasmids and yeast strains containing the *MFa2*- β -galactosidase fusion gene

Construction of the yeast plasmid TALS has been described previously (Roth et al. 1990). One-step gene replacement of the *MFa2* gene in the various strains listed in Figure 7 was performed using standard techniques (Rose et al. 1990). Because no selection for the integration was available, a linear *HindIII* fragment from the plasmid pSM38 (generously provided by Susan Michaelis, Johns Hopkins University, Baltimore, MD) containing the *MFa2*- β -galactosidase fusion gene was cotransformed with a *TRP1*/*ARS1* plasmid at a ratio of $\sim 10:1$ (linear/plasmid) by mass. *TRP*⁺ transformants were replica plated to media containing X-gal. Cotransformants (*TRP*⁺; β -galactosidase⁺) arose at frequencies ranging between 0.5% and 3% in the H4 mutant strains with the exception of the Gly-17 mutant. For reasons not understood, this mutant reproducibly gave insufficient numbers of *TRP*⁺ transformants to facilitate screening for cotransformants. Blue colonies in the other strains were picked, and integration of the fusion gene at the *MFa2* locus was confirmed by Southern blot analysis using standard techniques (Sambrook et al. 1989). In the case of the wild-type α strain (PKY999), the reporter gene was repressed, as expected, so no blue color selection was available. In this case, colonies were screened directly by Southern analysis for the presence of the *MFa2*- β -galactosidase integration.

A *MFa2*- β -galactosidase reporter plasmid was constructed by ligating a 4.5-kb *HindIII* fragment from the plasmid pSM38 into the *HindIII* site of YCp50 (Rose et al. 1987). Bacterial clones were identified by restriction analysis; DNA was prepared and introduced into yeast using standard techniques (Rose et al. 1990). This plasmid was used to transform *ura3*⁻ strains including JTY222H ($\alpha\alpha\alpha$), LJY438I (*aaa*), LJY656, and LJY612 (Gln-16); *URA3*⁺ transformants were selected and the presence of the plasmid was confirmed by Southern analysis.

Table 1. Strain list

Strain	Genotype	Reference
PKY999	<i>MATα</i> , <i>ade2-101</i> , <i>arg4-1</i> , <i>his3</i> $\Delta 200$, <i>leu2-3</i> , <i>leu2,112</i> , <i>lys2-801</i> , <i>trp1</i> $\Delta 901$, <i>ura3-52</i> , <i>thr</i> , <i>tyr</i> , Δ <i>hhf1::HIS3</i> , Δ <i>hhf2::LEU2</i> , pUK499 (<i>HHF2</i>)	Kayne et al. (1988)
PKY917	isogenic to PKY999 except pPK917 [<i>hhf2 del(4-14)</i>] instead of pUK499	Kayne et al. (1988)
PKY918	isogenic to PKY999 except pPK618 [<i>hhf2 del(4-19)</i>] instead of pUK499	Kayne et al. (1988)
PKY818	isogenic to PKY918 except <i>MATα</i>	Kayne et al. (1988)
PKY913	isogenic to PKY999 except pPK613 [<i>hhf2 del(4-23)</i>] instead of pUK499	Kayne et al. (1988)
PKY899	isogenic to PKY999 except <i>MATα</i>	Kayne et al. (1988)
PKY505	isogenic to PKY899 except pPK305 (<i>hhf2</i> Gly-16 <i>URA</i> ⁺) instead of pUK499	Johnson et al. (1990)
LJY921	isogenic to PKY505 except pLJ921 (<i>hhf2</i> Gly-17 <i>URA</i> ⁺) instead of pPK305	Johnson et al. (1990)
LJY933	isogenic to PKY505 except pLJ933 (<i>hhf2</i> Gly-18 <i>URA</i> ⁺) instead of pPK305	Johnson et al. (1990)
PKY501	isogenic to PKY505 except pPK301(<i>HHF2</i>) instead of pPK305	Kayne et al. (1988)
LJY656I	<i>MATα</i> , <i>hhf1::HIS3</i> , <i>hht2::TRP1</i> , <i>trp1</i> , <i>lys2</i> , <i>ade2</i> , <i>his3</i> , <i>leu2</i> , <i>ura3</i>	L. Johnson
LJY612I	isogenic to LJY656I except <i>hhf2</i> Gln-16	L. Johnson
JTY222H	<i>HMLα</i> , <i>MATα</i> , <i>HMRα</i> , <i>leu2,3,112</i> , <i>ura3-52</i> , <i>trp1</i> , <i>ade2</i> , <i>lys2</i> , <i>hhf1::HIS3</i> , <i>hhf2 del(4-19)</i> , <i>hht2::TRP1</i>	M. Grunstein and J. Thompson
LJY438I	<i>HMLα</i> , <i>MATα</i> , <i>HMRα</i> , <i>ade2-101</i> , <i>his3-200</i> , <i>leu2-3,112</i> , <i>lys2-801</i> , <i>trp1-901</i> , <i>ura3-52</i> , <i>hhf1::HIS3</i> , <i>hhf2::del(4-19)</i> , <i>hht2::TRP1</i>	Johnson et al. (1990)
SC3	<i>MATα</i> , <i>ura3-53</i> , <i>trp1</i> , <i>his3</i> , <i>gal2</i> , <i>gal10</i> , <i>cir</i> ^o	Roth et al. (1990)
E290	<i>MATα</i> , <i>his4-290</i> , <i>trp1</i>	Roth et al. (1990)
TSY155	<i>MATα</i> , <i>ura3-52</i> , <i>htb1-1</i> , <i>htb2-1</i> , <i>ade2-101</i> , <i>trp1</i> , <i>met13</i> , pTS7 [<i>htb2 del(3-32)</i>]	Wallis et al. (1983)
YP47	<i>MATα</i> , <i>ura3-52</i> , <i>lys2-801</i> , <i>ade2-101</i> , <i>trp1</i>	Wallis et al. (1983)

Isolation and analysis of chromatin by indirect end-labeling

Chromatin was partially purified and digested with MNase as described previously (Thoma et al. 1986; Roth et al. 1990). Chromatin samples were digested with 2 U/ml (Fig. 2A), 5 U/ml, or 10 U/ml (Fig. 2B) of MNase, and DNA was digested with 0.5 U/ml (Fig. 2A) or 5 U/ml (Fig. 2B) of MNase at 37°C for 5 min. DNA was purified, subjected to restriction endonuclease digestion, fractionated on agarose gels, transferred to nitrocellulose, and probed with specific fragments of TALS labeled with ³²P as described previously (Roth et al. 1990). The blots in Figure 2 were probed with an *EcoRV*–*HindIII* fragment (230 bp) of TALS.

Analysis of TALS supercoil density

DNA was isolated and topoisomers were resolved as described previously (Roth et al. 1990) and fractionated on 1.5% agarose gels containing 40 µg/ml of chloroquine diphosphate (Sigma Chemical Co.) in both the gel and electrophoresis buffer. Under these conditions, more negatively supercoiled molecules migrate more slowly than less negatively supercoiled molecules. After electrophoresis, samples were transferred to nitrocellulose and analyzed as described previously (Roth et al. 1990).

Isolation of nuclei and primer extension analysis of nuclease cleavage sites

Nuclei were isolated as described by Szent-Gyorgyi and Isenberg (1983), except that dithiothreitol was used instead of 2-mercaptoethanol, and Zymolyase 100T (ICN) was used instead of lyticase (Roth and Simpson 1991). Nuclei were digested in digestion buffer [10 mM HEPES (pH 7.5), 0.5 mM MgCl₂, 0.05 mM CaCl₂] for 10 min at 37°C using concentrations of MNase or DNase I listed in the legends to Figures 3 and 4. DNA was purified (Szent-Gyorgyi and Isenberg 1983), and fragments were analyzed by either single cycle (Fig. 3) or multicycle (Fig. 4) primer extension using *Taq* polymerase, as described previously (Axelrod and Majors 1989; Shimizu et al. 1991), with the addition of a 5-min preincubation of samples at 95°C followed by quick chilling of samples in ice water before the addition of polymerase. Sequencing gel electrophoresis was performed by standard procedures (Sambrook et al. 1989). Single-cycle primer extension conditions were 95°C, 5 min; 48°C, 20 min; and 70°C, 5 min. Multicycle conditions were 94°C, 1 min; 55°C, 2 min; and 73°C, 3 min. Typically, 15 cycles were performed. Primers used included MS-2 (1735–1702 map units in TALS, for the analysis of nucleosome IV), 5'-TTA GGC ACC CCA GGC TT-T ACA CTT TAT GCT TCC GG-3'; MS-7 [–338 to –304 bp in *STE6* upstream sequences (Wilson and Herskowitz 1986; McGrath and Varshavsky 1989)], 5'-TGA TGG TCC TTG CAC TGA CAA ATA TGT TCC TTT CC-3'.

In vivo footprinting of the α2 operator

Intact, living cells were treated with DMS, and DNA was purified as described by Giniger et al. (1985) and Axelrod and Majors (1989), except that treatment with piperidine was omitted. Repeated heating to 94°C during the subsequent multicycle primer extension analysis (as above) was found to be sufficient for breakage at methylated G residues. Primers included MS-7 (above) and MS-8 (–55 to –89 in *STE6* upstream sequences, 5'-TCT GTG TGA ACG TAA CAA CCG GAG ATA GTT CAG-CC-3').

Measurement of β-galactosidase expression

Transformants were patched onto X-gal containing selective media prepared as described previously (Rose et al. 1990) and

incubated at 30°C or at room temperature for several days for development of blue color. β-Galactosidase activity was measured in permeabilized cells as described previously (Rose et al. 1990).

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