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# Tup1p represses Mcm1p transcriptional activation and chromatin remodeling of an a-cell-specific gene

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**In yeast, a number of regulatory proteins expressed only in specific cell types interact with general transcription factors in a combinatorial manner to control expression of cell-type-specific genes. We report a detailed analysis of activation and repression events that occur at the promoter of the a-cell-specific *STE6* gene fused to a β-galactosidase gene in a yeast minichromosome, as well as factors that control the chromatin structure of this promoter both in the minichromosome and in the genomic *STE6* locus. Mcm1p results in chromatin remodeling and is responsible for all transcriptional activity from the *STE6* promoter in both wild-type a and α cells. Matα2p cooperates with Tup1p to block both chromatin remodeling and Mcm1p-associated activation. While Matα2p represses only Mcm1p, the Tup1p-mediated repression involves both Mcm1p-dependent and -independent mechanisms. Swi/Snf and Gcn5p, required for full induction of the *STE6* gene, do not contribute to chromatin remodeling. We suggest that Tup1p can contribute to repression by blocking transcriptional activators, in addition to interacting with transcription machinery and stabilizing chromatin.**

*Keywords*: **a**-cell-type-specific gene/chromatin/Mcm1p/Ssn6p–Tup1p/transcriptional regulation

## Introduction

Transcriptional regulation in eukaryotes involves a complex interplay of non-histone *trans*-acting factors functioning in a chromatin environment. Promoter regions of a number of genes are organized in nucleosomal arrays, implicated in transcriptional regulation. Positioned nucleosomes often flank activator or repressor binding sites and occlude other *cis*-acting elements. Eukaryotic cells have a number of mechanisms to overcome repressive chromatin, including remodeling by ATP-dependent complexes and modification of histones (Pollard and Peterson, 1998; Kingston and Narlikar, 1999). Binding of activators to nucleosome-free sites may alter nearby chromatin, enhancing transcription (Verdone *et al*., 1996; Beato and Eisfeld, 1997; Lohr, 1997). On the other hand, repressors such as Matα2p may organize or stabilize chromatin (Simpson *et al*., 1993; Roth, 1995; Bortvin and Winston, 1996).

Seven genes are specifically expressed only in yeast cells of the **a** mating type. In α cells, Matα2p cooperates with Mcm1p to repress these **a**-cell-type-specific genes (Johnson, 1992, 1995). Promoters of **a**-specific genes contain a 32 bp sequence, the α2 operator, which includes two recognition sequences for Matα2p flanking a central Mcm1p binding site. Matα2p binds to DNA cooperatively with a homodimer of Mcm1p (Johnson, 1992) and recruits the Ssn6p–Tup1p general transcriptional repressor complex (Keleher *et al*., 1992; Komachi *et al*., 1994). Mcm1p and Ste12p have been implicated in transcriptional activation of **a**-specific genes in **a** cells (Dolan and Fields, 1991). Ste12p binds cooperatively with Mcm1p to pheromone response elements (PREs), located downstream of the α2 operator (Errede and Ammerer, 1989; Hwang-Shum *et al*., 1991; Mueller and Nordheim, 1991). The relative contribution of Mcm1p to transcriptional activation of **a**-specific genes is unsettled, since *MCM1* is an essential gene. In **a** cells, Mcm1p contributes significantly to *STE2* expression (Ammerer, 1990; Elble and Tye, 1991; Hwang-Shum *et al*., 1991) and deletion of the α2 operator reduces transcription of the *BAR1* gene (Kronstad *et al*., 1987). In addition, α2 operator-dependent activation in **a** cells markedly decreases as the distance between the operator and the promoter is increased (Patterton and Simpson, 1994). On the other hand, the *STE6* α2 operator has only weak upstream activating sequence (UAS) activity when placed upstream of a heterologous reporter gene in **a** cells (Johnson and Herskowitz, 1985; Keleher *et al*., 1988; Acton *et al*., 1997).

Chromatin around all endogenous α2 operators examined to date is organized as a well-defined nucleosomal array in α cells that appears to be disrupted in **a** cells (Ganter *et al*., 1993; Simpson *et al*., 1993; Weiss and Simpson, 1997). It was proposed that the organized chromatin was associated with Matα2p binding and implicated in repression of **a**-specific genes (Simpson *et al*., 1993; Roth, 1995). The well-defined nucleosomal organization at the *STE6* promoter (Cooper *et al*., 1994) and the recombination enhancer (Weiss and Simpson, 1997) require Tup1p, which has been suggested to repress transcription through stabilization of chromatin structure by direct interaction with the core histones (Edmondson *et al*., 1996). Tup1p also associates with *STE6* nucleosomes *in vivo* (Ducker and Simpson, 2000). However, the Matα2p–Mat**a**1p–Tup1p-mediated repression of haploid-specific genes does not require positioned nucleosomes, although histones are involved in repression (Huang *et al*.,

1997). Nor is organized chromatin required for a low level of repression of promoters with an isolated sequence of the α2 operator *in vitro* (Herschbach *et al*., 1994) or *in vivo* (Redd *et al*., 1996). In this study, we investigated the role of chromatin and other factors involved in transcriptional regulation of **a**-specific genes by studying both activation and repression events taking place at the *STE6* promoter in **a** and α cells.

## Results

### *Chromatin remodeling and transcriptional activation require Mcm1p binding in a cells*

Correspondence between an α2 operator in the native context of a transcriptionally repressed **a**-cell-specific gene in α cells and an organized nucleosomal array *in vivo* is well established (Shimizu *et al*., 1991). Although positioned nucleosomes have been observed abutting the α2 operator in repressed, heterologous genes (Roth *et al*., 1990; Patterton and Simpson, 1994), recent evidence (Redd *et al*., 1996; Wu *et al*., 1998) suggests that the sequence context of the α2 operator may have a significant effect on chromatin structure. Disruption of nucleosome positioning abutting the α2 operator at the promoters of **a**-specific genes in **a** cells correlates with but does not require transcription (Ganter *et al*., 1993; Cooper *et al*., 1994). The change in *STE6* chromatin structure in **a** cells may be caused by binding of *trans*-acting factors, e.g. Mcm1p, in the absence of Matα2p.

Mcm1p occupies the central P element of the α2 operator in **a** as well as in α cells *in vivo* (Keleher *et al*., 1992; Ganter *et al*., 1993; Murphy *et al*., 1993). Mutations in the P element reduce Mcm1p binding and the ability to affect transcription from heterologous promoters

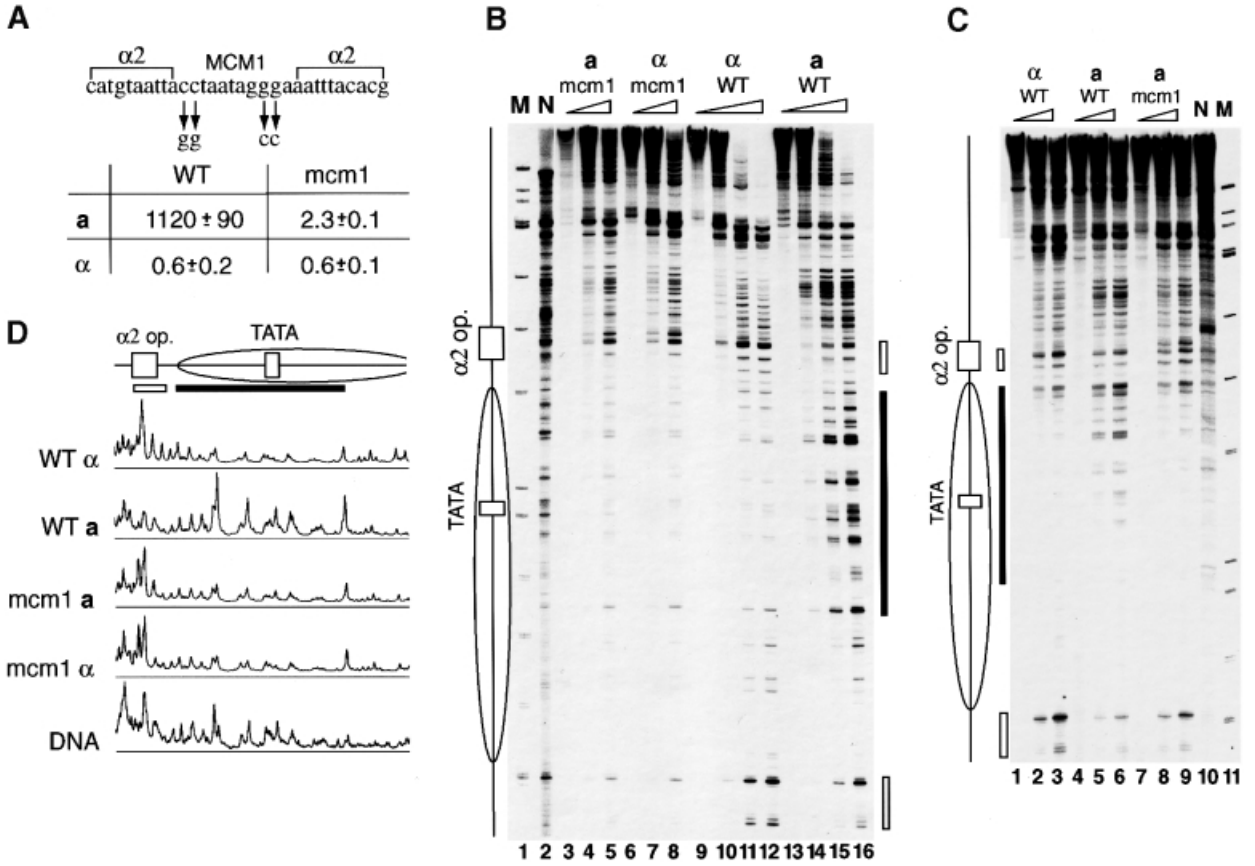


**Fig. 1.** Mcm1p binding is a prerequisite to chromatin remodeling and transcriptional activation at the *STE6* promoter in **a** cells. (**A**) Mcm1p binding is responsible for all transcriptional activation from the *STE6* promoter in **a** cells. The β-galactosidase activity (in M.u.) per 10 plasmids per cell is shown for the *STE6–lacZ* fusion plasmid carrying either the WT α2 operator or the operator bearing four single point mutations in the Mcm1p binding site (mcm1) in **a** as well as in α cells. Sequence of the α2 operator, positions of Matα2p and Mcm1p binding sites (Smith and Johnson, 1994) as well as mutations introduced into the Mcm1p recognition sequence are shown on the top. (**B**) Mcm1p remodels chromatin structure at the *STE6* promoter in minichromosomes in **a** cells. Nuclei isolated from cells from experiments shown in (A) were digested with increasing amounts of micrococcal nuclease. Primer corresponding to positions +40 to +17 of the *lacZ* sequence was extended using DNA purified from digested nuclei as a template. The first chromatin lane from each type of cell corresponds to DNA from undigested nuclei showing *Taq* polymerase pauses. Purified plasmid DNA digested with MNase (N) is shown as a control for MNase sequence specificity. M is DNA molecular weight standards from *Hin*fI digest of ϕX174 RF DNA corresponding to 726, 713, 553, 500, 427, 417, 413, 311, 249, 200, 151, 140, 118 and 100 nucleotides. Positions of the α2 operator, TATA element (Patterton and Simpson, 1994) (rectangles) and inferred position of a nucleosome downstream of the α2 operator (an ellipse) are shown on the left. The regions of more (filled bar) and less (open bars) cleavage in **a** cells compared with α cells are shown on the right. (**C**) Mcm1p remodels chromatin around the genomic *STE6* α2 operator. Primer extension analysis of the *STE6* promoter at the chromosomal location using the primer corresponding to +76 to +47 of the coding region is shown. Bars show the same sequences as in (B). (**D**) Loss of nucleosome positioning downstream of the α2 operator depends on Mcm1p binding. Scanning profiles of the last lanes in the sets of chromatin probes from the gel in (B) are shown to compare the chromatin structure around the TATA box for different cells.

(Ammerer, 1990; Acton *et al*., 1997). For example, the DNA substitutions shown in Figure 1, at sites that make predominant contacts with the protein (Tan and Richmond, 1998), reduce Mcmlp affinity by at least 100-fold (Smith and Johnson, 1994). We mutated the Mcm1p recognition sequence at the *STE6* promoter fused to a *lacZ* gene in pSTEZL, a plasmid that has chromatin structure similar to the native *STE6* locus in both α and **a** cells (Patterton and Simpson, 1994). Following transformation, β-galactosidase activity and chromatin structure around the α2 operator were determined (Figure 1). For pSTEZL the β-galactosidase activity was 1120 Miller units (M.u.) in **a** cells and 0.6 M.u. in α cells (Figure 1A). When pSTEZL mutated in the P element was introduced into **a** cells, the β-galactosidase activity was 2.3 M.u., only 3.6-fold higher than that in α cells. This 500-fold decrease in transcriptional activity from the mutated *STE6* promoter suggests that Mcmlp binding is a prerequisite for transcriptional activation of **a**-cell-specific genes. Slightly higher transcription in **a** relative to α cells likely results from weak residual Mcm1p binding to the mutated P element (Smith and Johnson, 1994), as transcription is decreased when Matα2p is present. In contrast, in α cells, Mcm1p binding has no effect on repression because no other transcriptional activator is present at the *STE6* promoter. We conclude that Mcm1p binding contributes to repression of the *STE6* promoter only by enhancing Matα2p binding.

Chromatin structure around the α2 operator was mapped by micrococcal nuclease (MNase) digestion and extension mapping from a primer corresponding to positions +40 to +17 of the *lacZ* sequence. The 168 bp region downstream of the hypersensitive site at the wild-type (WT) α2 operator is less accessible in α cells compared with naked DNA (Figure 1B and D). In contrast to the genomic locus, where a nuclease-sensitive site 16 bp from the edge of the α2 operator marks the edge of a positioned nucleosome (Figure 1C), this protected region is larger than expected for a nucleosome core. Nuclease cutting sites are present at ~10 bp intervals in this protected region adjacent to the operator, suggesting several translational positions for rotationally positioned nucleosomes. The differences between this structure for the promoter–*lacZ* fusion and the genomic copy of *STE6* may arise from contributions of prokaryotic sequences, since positioned nucleosomes span the length of the genomic gene but are limited to two for the promoter–*lacZ* fusion construct. Irrespective of these differences, the minichromosome does provide an example of organized chromatin at the *STE6* promoter for study of the relationship between chromatin and repression in an Mcm1p- and Tup1p-dependent system.

In **a** cells, strong cleavage appears at multiple sites within this region (filled bars in Figure 1B), resembling the digestion pattern of naked DNA. At the same time, the linker regions (open bars) are less accessible to the enzyme, indicating disruption of nucleosome positioning. When the P element was mutated, protection of this region was restored in **a** cells; chromatin structure is similar to that around the WT α2 operator in α cells (Figure 1B). In α cells, the same mutation had no effect on chromatin structure (Figure 1B). Thus, inability of Mcm1p to bind to the mutated α2 operator restores protection by a nucleosome in **a** cells, suggesting a role for Mcm1p in chromatin remodeling at the promoter regions of **a**-specific genes.

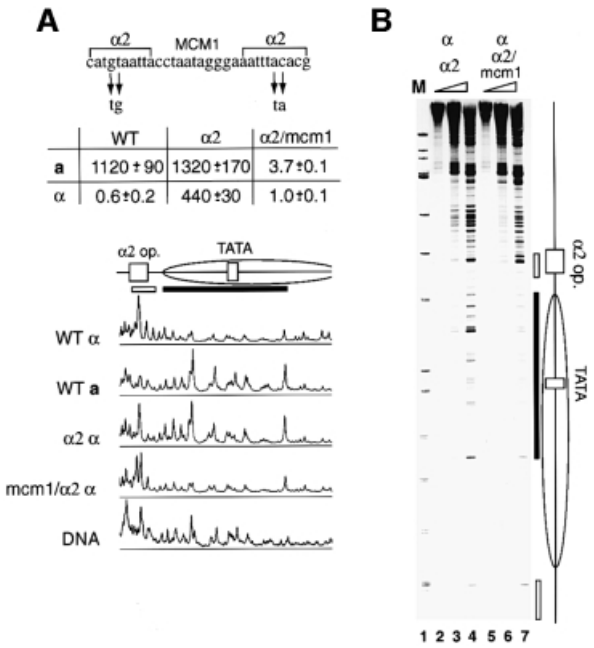


**Fig. 2.** Matα2p blocks Mcm1p-dependent transcriptional activation and chromatin remodeling at the *STE6* promoter in α cells. (**A**) Transcriptional activation in α cells in the absence of Matα2p binding is Mcm1p dependent. β-galactosidase assays of transcription from the *STE6* promoter bearing point mutations in both α2 half-sites either alone (α2) or in combination with the mutations in Mcm1p recognition sequence (α2/mcm1). Point mutations introduced into the Matα2p sites are shown on the top. The data for the WT **a** and α cells from Figure 1 are shown for comparison. The remaining β-galactosidase values were obtained from the same experiment. (**B**) Matα2p binding blocks Mcm1p chromatin remodeling activity in α cells. Primer extension analysis of the *STE6* chromatin structure in α cells from experiments shown in (A). (**C**) Disruption of a nucleosome downstream of the α2 operator in α cells in the absence of Matα2p binding requires Mcm1p. For other details see legend to Figure 1.

To examine whether Mcm1p is responsible for chromatin remodeling of the *STE6* genomic promoter, the same mutations were introduced into the α2 operator at the *STE6* locus, and chromatin structure was determined using the primer corresponding to +76 to +47 of the *STE6* coding region. The positioned nucleosome noted previously is present in α cells (Figure 1C; Shimizu *et al*., 1991; Cooper *et al*., 1994). The region downstream of the α2 operator indicated by the filled bar is accessible to MNase in **a** cells and protected in α cells. The linker region marked by open bars, hypersensitive in α cells, is less accessible in **a** cells. Point mutations introduced into the genomic P element restored protection due to a positioned nucleosome downstream of the α2 operator and the hypersensitivity of the linker region in **a** cells (Figure 1C) so that the chromatin structure is similar to that in α cells. Thus, Mcm1p binding to the genomic *STE6* promoter disrupts chromatin in **a** cells as well as in minichromosomes. Presence of the nucleosome downstream of the mutated α2 operator in **a** cell signals organized chromatin at the *STE6* promoter and supports the suggestion that sequences surrounding the α2 operator or other factors may contribute to chromatin organization in α cells.

### *Matα2p blocks Mcm1p-dependent transcription and chromatin remodeling*

Matα2p may block the Mcm1p-dependent transcriptional activation and chromatin remodeling seen in **a** cells. To explore this possibility, we mutated Matα2p binding sites in pSTEZL and examined both chromatin structure and *lacZ* expression in both cell types. The four substitutions shown in Figure 2A relieve repression and dramatically reduce Matα2p affinity for the *STE6* α2 operator with no apparent decrease in Mcm1p binding to the P element (Smith and Johnson, 1994). When all four mutations were introduced into pSTEZL and the resulting construct transformed into **a** cells, β-galactosidase activity from the mutated promoter was slightly higher than observed for the WT promoter (Figure 2A), suggesting that these mutations do not affect Mcm1p binding to the α2 operator. In α cells, *lacZ* expression from the mutated promoter was increased nearly 800-fold and was only 3-fold less than that in **a** cells. Consistent with the loss of transcriptional repression, the nucleosome downstream of the α2 operator was no longer positioned (Figure 2B and C). Intense cleavage in this previously protected region produced a pattern similar to that observed for the WT α2 operator in **a** cells (Figures 2B and C, and 1B). Although these results suggest that a role of Matα2p is to block Mcm1p activity, it is formally possible that transcription and chromatin remodeling in the absence of Matα2p in α cells may be activated by an Mcm1p-independent mechanism. Therefore, we made a double Matα2p/Mcm1p binding site mutant and examined both *lacZ* expression and chromatin structure in α and **a** cells. Mutation of the Mcm1p binding site reduced *lacZ* expression in the absence of Matα2p binding in α cells (Figure 2A). Consistent with the loss of transcriptional activation, no chromatin remodeling was observed in this construct (Figures 2B and C, and 1B). Thus, one role of Matα2p in repression of **a**-specific genes is to block Mcm1p transcriptional and chromatin remodeling activity in α cells.

### *Tup1p cooperates with Matα2p to block Mcm1p transcriptional and chromatin remodeling activity*

Ssn6p–Tup1p is a pleiotropic corepressor complex, thought to be targeted to promoters by sequence-specific DNA-binding proteins (Balasubramanian *et al*., 1993; Komachi *et al*., 1994; Treitel and Carlson, 1995; Huang *et al*., 1998; Park *et al*., 1999). The Ssn6p–Tup1p complex is required for Matα2p-mediated repression both *in vitro* (Redd *et al*., 1997) and *in vivo* (Keleher *et al*., 1992; Cooper *et al*., 1994). Matα2p interacts with Tup1p *in vitro* (Komachi *et al*., 1994; Komachi and Johnson, 1997). Three mechanisms for Ssn6p–Tup1p-mediated repression have been proposed. One suggests that Tup1p organizes a repressive chromatin structure through interaction with H4 and H3 (Cooper *et al*., 1994; Edmondson *et al*., 1996; Ducker and Simpson, 2000). A second model invokes direct interaction of Ssn6p–Tup1p with transcriptional machinery (Herschbach *et al*., 1994; Redd *et al*., 1996; Kuchin and Carlson, 1998). Finally, we and others have proposed that the Ssn6p–Tup1p complex blocks transcriptional activators (Gavin and Simpson, 1997; Geisberg and Struhl, 2000). Our finding that the Mcm1p-dependent transcriptional activation and chromatin remodeling at the *STE6* promoter are blocked by Matα2p implicates Ssn6–Tup1p in blocking Mcm1p activity.

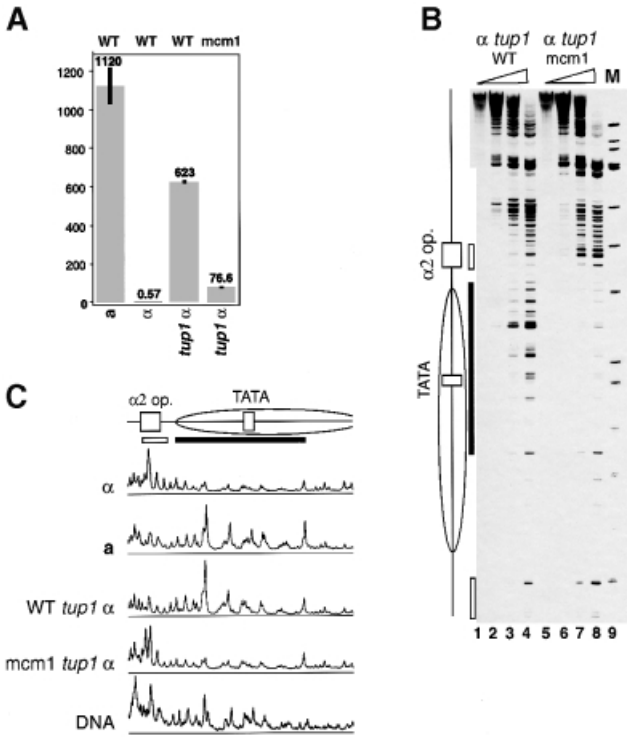


**Fig. 3.** Tup1p represses Mcm1p transcriptional and chromatin remodeling activity. (**A**) Transcriptional activation in α *tup1* cells is Mcm1p dependent. β-galactosidase assay of transcription from the *STE6* promoter bearing either the WT or mutated α2 operator, as indicated on the top, either in *tup1* mutants or in WT cells. Standard deviation is shown by the black bars. For comparison, the data for the WT **a** and α cells from Figure 1 are shown. The remaining β-galactosidase values were obtained from the same experiment. (**B**) Tup1p blocks chromatin remodeling by Mcm1p in α cells. Primer extension analysis of the chromatin structure around either the WT or mutated α2 operator in α *tup1* cells from (A). (**C**) Disruption of a nucleosome downstream of the α2 operator in α *tup1* mutants requires Mcm1p binding. For other details see legend to Figure 1.

We transformed pSTEZL-based constructs containing the WT or mutant Mcm1p binding site α2 operator into *tup1* α cells and examined *lacZ* expression and chromatin remodeling (Figure 3). The *tup1* mutation increases β-galactosidase expression from the WT *STE6* promoter in α cells ~1100-fold, only 2-fold less than in WT **a** cells. Chromatin structure around the α2 operator in the mutant background is similar to the WT **a** cells, with disruption of nucleosomes downstream of the α2 operator (Figures 3B and C, and 1B). These data correlate well with previous observations (Cooper *et al*., 1994) and with the change in expression and chromatin structure of the genomic *STE6* locus in *tup1* α cells (see below). Mutations in the P element reduced β-galactosidase activity ~8-fold in *tup1* cells, but the expression level was still two orders of magnitude higher than in WT α cells (Figure 3A). The near lack of Mcm1p binding to the mutant P element suggests that transcriptional activation in *tup1* mutants also involves an Mcm1p-independent mechanism. In spite of a high level of Mcm1p-independent transcriptional activity in *tup1* α cells, no disruption of chromatin structure was observed downstream of the mutated α2 operator (Figures 3B and 1B). Chromatin remodeling

downstream of the α2 operator in the absence of Tup1p in α cells requires Mcm1p binding. We suggest that recruitment of Tup1p by Matα2p to the close proximity of Mcm1p blocks its chromatin remodeling activity.

High transcriptional activity in *tup1* α cells in the absence of Mcm1p binding suggests that Tup1p represses transcription of **a**-specific genes by an alternative, Mcm1p-independent mechanism. Ste12p seems unlikely to contribute to this since Mcm1p recruits Ste12p to the promoters of **a**-specific genes (Errede and Ammerer, 1989; Hwang-Shum *et al*., 1991; Mueller and Nordheim, 1991). Mcm1p-independent Tup1p-mediated repression is difficult to explain in the context of either a model suggesting interaction of the Ssn6p–Tup1p complex with the general transcription machinery or Ssn6p–Tup1p-dependent formation of a repressive chromatin structure. Since we find no Tup1p associated with the *STE6* promoter in isolated minichromosomes in the absence of either Mcm1p or Matα2p (Ducker and Simpson, 2000), we think it is likely that the Mcm1p-independent effect of *tup1* mutation is indirect. The Ssn6p–Tup1p complex is required for expression of a large number of yeast genes and some of them may be involved in downstream regulation of other genes.

### *Chromatin disruption at the STE6 locus in a cells is independent of Swi/Snf and Gcn5p*

While the chromatin remodeling complex SWI/SNF and Gcn5p-containing histone acetyltransferase complexes are necessary for full activity of the *STE6* gene in **a** cells, they are dispensable for alterations of nucleosome positioning at the promoter of the genomic gene. Data supporting these conclusions are to be found in the Supplementary data, available at *The EMBO Journal* Online.

### *Matα2p binds the α2 operator in the absence of Tup1p*

The lack of Matα2p expression in **a** cells or its inability to bind the α2 operator in α cells has the same effect on *STE6* transcription and chromatin structure as a *tup1* mutation in α cells. Although it has previously been shown that Matα2p can bind the α2 operator in the absence of Ssn6p in α cells (Keleher *et al*., 1992), Ssn6p and Tup1p have distinct roles in *STE6* repression (Cooper *et al*., 1994). Northern blot analysis shows that *ssn6* mutations have a very small effect on *STE6* transcription in α cells compared with the deletion of *TUP1*. Thus, the *tup1* mutation might impair Matα2p binding to the *STE6* promoter. Therefore, we examined Matα2p binding to the α2 operator in α and **a** cells that were WT and *tup1*. These strains also harbored a single, integrated copy of *Sss* I DNA methyltransferase (M. *Sss* I), which enables sensitive detection of DNA–protein interactions *in vivo* (Kladde *et al*., 1996; Xu *et al*., 1998). MNase digestion of α cell nuclei (Roth *et al*., 1992; Cooper *et al*., 1994) indicated the presence of a positioned nucleosome downstream of the α2 operator at the genomic *STE6* locus, and protection against methylation of three CpG M. *Sss* I target sites confirmed this structural assignment (Figure 4). In contrast, this region was accessible to the methyltransferase in **a** WT and *tup1* strains of both mating types. These data rule out the possibility that the isolation of nuclei for the MNase analyses contributed to disruption of the

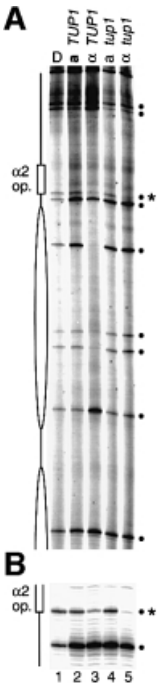


**Fig. 4.** Matα2p occupies the *STE6* α2 operator in the absence of Tup1p. (**A**) The Matα2p–operator interaction in haploid cells of each mating type (**a** or α) was footprinted *in vivo* in WT (*TUP1*) or mutant (*tup1*) by M. *Sss* I. Expression of the single-copy methyltransferase under control of the *GAL1* promoter was achieved by growth in galactose-containing media. Following rapid isolation of DNA, cytosines methylated in CpG target sites (indicated by filled circles) were identified by genomic bisulfite sequencing as described previously (Kladde *et al*., 1996). Band intensity is directly proportional to the degree of cytosine methylation at each CpG sequence. *In vitro* methylation of protein-free DNA (D) is shown in lane 1. The α2 operator is demarcated by the open bar and a CpG site within the α2 operator is marked by the asterisk. Note protection of three methylation sites adjacent to the α2 operator in α *TUP1* cells (lane 3) due to the presence of a positioned nucleosome (marked by open ellipse). Increased methylation at these three CpG sites in **a** cells (lanes 2 and 4) or when *TUP1* was deleted (lanes 4 and 5) indicates disruption of the nucleosome. (**B**) Operator binding by Matα2p in the same samples as in (A) was analyzed with a primer that anneals closer to the operator. Note protection of the operator CpG site (asterisk) in α cells (lanes 3 and 5) in the presence (lane 3) and absence (lane 5) of *TUP1* as compared with **a** cells (lanes 2 and 4).

positioned nucleosome in α *tup1* cells. Importantly, a CpG sequence located within the downstream α2 half-site of the operator (marked with an asterisk) was protected against methylation by M. *Sss* I in both WT and *tup1* α cells as compared with **a** cells (Figure 4). This Matα2p footprint was also present in α *tup1* cells at the α2 operator #1 of the recombination enhancer (Wu and Haber, 1996; data not shown). These data demonstrate that a null mutation of *TUP1* leads to loss of nucleosome positioning downstream of the α2 operator without affecting Matα2p binding. Therefore, the derepression of *STE6* transcription observed in α *tup1* cells (Figure 3A) is not due to a lack of binding of Matα2p at the operator.

Matα2p has a short half-life and is rapidly degraded by the ubiquitin–proteasome pathway (Chen *et al*., 1993). Proteins are often degraded when their partners in macromolecular complexes are absent; this type of observation suggests that the specific proteins may be components of a common complex (Peterson *et al*., 1994).

For example, association of Matα2p with Mat**a**1p significantly increases the stability of the former protein in diploid cells (Johnson *et al*., 1998). In this vein, Ssn6p–Tup1p might stabilize Matα2p, with its rapid degradation in the absence of Tup1p resulting in the loss of **a**-specific gene repression. Occupancy of the α2 operator by repressor in *tup1* mutants rules out this possibility.

## Discussion

### *Mcm1p binding is required for the Ste12p-dependent activation of a-specific genes*

Mcm1p is a member of the MADS-box family of transcription factors that is thought to cooperate with other proteins in the regulation of target genes (Shore and Sharrocks, 1995). In the present paper, we show that Mcm1p binding to the *STE6* promoter results in chromatin remodeling and transcriptional activation at the promoter of the *STE6* gene. Our findings are consistent with previous observations that Mcm1p is responsible for the majority of *STE2* expression in **a** cells (Ammerer, 1990; Elble and Tye, 1991; Hwang-Shum *et al*., 1991). However, expression of **a**-specific genes also requires Ste12p. Even in the absence of pheromone, the *STE12* deletion reduces mRNA levels from 5-fold for the *STE6* and *STE2* genes to >50-fold for the *MF***a***1* gene (Fields and Herskowitz, 1985; Fields *et al*., 1988), suggesting that Mcm1p can not significantly activate transcription on its own and requires Ste12p for full activity. This suggestion is consistent with the findings that an isolated P element or α2 operator displayed weak transcriptional activity in **a** cells (Johnson and Herskowitz, 1985; Keleher *et al*., 1988; Acton *et al*., 1997), while the α2 operator containing flanking sequences was able to activate a reporter gene to a significant level (Errede and Ammerer, 1989; Ammerer, 1990). It has been shown that Ste12p affinity for its DNA site *in vitro* is dramatically reduced in the absence of Mcm1p (Errede and Ammerer, 1989; Hwang-Shum *et al*., 1991; Mueller and Nordheim, 1991). This suggests a mechanism for transcriptional activation of **a**-specific genes in **a** cells where Mcm1p binds to the P element and recruits Ste12p to activate the gene. This scheme may also involve the Swi/Snf complex and Gcn5-containing acetyltransferase coactivator complexes that are required for maximal transcriptional activity. A similar model was suggested for the activation of α-specific genes (Dolan and Fields, 1991; Johnson, 1995), where cooperative binding of Mcm1p and Matα1p to the PQ elements at promoters of α-specific genes recruits Ste12p through direct protein–-protein interaction with Matα1p (Yuan *et al*., 1993).

### *Ssn6p–Tup1p represses transcription by interaction with transcriptional activators*

Matα2p expression in α cells abolishes both Mcm1p-dependent chromatin remodeling and activation of the *STE6* gene. However, Matα2p binding to the α2 operator alone is not sufficient to repress Mcm1p, and requires Tup1p, a component of the Ssn6p–Tup1p general repressor complex. It has been shown that when targeted, Tup1p does not require Ssn6p for repression (Tzamarias and Struhl, 1994), while Ssn6p represses transcription by recruiting Tup1p (Keleher *et al*., 1992; Tzamarias and Struhl, 1995). These observations are consistent with a small effect of *ssn6* mutations on expression and chromatin structure of **a**-specific genes (Cooper *et al*., 1994; Tzamarias and Struhl, 1995; this study). When neither Matα2p nor Mcm1p can bind to the α2 operator, no transcriptional activity was observed. These data argue against the model that Mcm1p contributes to the Matα2p-mediated repression of **a**-specific genes (Johnson, 1992). However, repression of activated transcription from heterologous promoters requires Mcm1p (Keleher *et al*., 1988; Smith and Johnson, 1994; Acton *et al*., 1997). This finding may be explained by the fact that Mcm1p binding to the α2 operator increases Matα2p affinity for its recognition sequences at least 500-fold (Keleher *et al*., 1989). Mcm1p has a high affinity for DNA *in vitro* (Acton *et al*., 1997) and binds to the α2 operator in the absence of Matα2p *in vivo* (Keleher *et al*., 1992; Ganter *et al*., 1993; Murphy *et al*., 1993; this study). In α cells, Mcm1p interacts with Matα2p (Vershon and Johnson, 1993; Mead *et al*., 1996; Tan and Richmond, 1998) and recruits Matα2p to the promoter. We have shown that Matα2p can bind to the operator in the absence of Tup1p *in vivo* and therefore ruled out a potential requirement for the Ssn6p–Tup1p complex in stabilization of operator binding. Upon DNA binding, Matα2p may recruit Tup1p through direct protein–protein interactions (Komachi *et al*., 1994; Komachi and Johnson, 1997). Once targeted, the Ssn6p–Tup1p complex represses activated transcription.

A number of studies have suggested that the Ssn6p–Tup1p complex represses transcription either through interaction with the general transcriptional machinery (Herschbach *et al*., 1994; Redd *et al*., 1996; Kuchin and Carlson, 1998) or by organizing a repressive chromatin structure (Simpson *et al*., 1993; Cooper *et al*., 1994; Edmondson *et al*., 1996). Based on the analysis of transcriptional regulation and chromatin structure of *SUC2*, a glucose repressible gene, we have proposed an alternative model for the Ssn6p–Tup1p-mediated repression that implies an inhibitory effect on pathway-specific chromatin remodeling transcription factors (Gavin and Simpson, 1997). Although the sequence-specific activators that are involved in *SUC2* expression and chromatin remodeling are yet to be identified, our finding that Tup1p blocks Mcm1p activation suggests that this mechanism may be realized in transcriptional regulation of other Ssn6p–Tup1p-dependent genes. Geisberg and Struhl (2000) have recently reached a similar conclusion regarding the mechanism of action of corepressor Tup1 based on an analysis of distinctive TATA binding protein mutants.

### *Role of chromatin structure in regulation of a-specific genes*

A current model for chromatin organization at the promoter regions of **a**-type-specific genes in α cells suggests that Matα2p repressor binding establishes positioned nucleosomes around the α2 operator by recruiting Tup1p, which interacts with the N-termini of histones H3 and H4 (Cooper *et al*., 1994; Roth, 1995; Edmondson *et al*., 1996). Alternatively, the positioned nucleosomes observed adjacent to naturally located α2 operators of the *STE6* and *BAR1* genes and a recombination enhancer region may be positioned by the underlying sequence. In the absence of Mcm1p binding, the chromatin structure around the

mutated α2 operator at the *STE6* promoter and at the recombination enhancer (Wu *et al*., 1998) in **a** cells is organized as arrays of positioned nucleosomes similarly, but not identically, to the array in α cells. This suggests that sequence elements flanking the 32 bp α2 operator may be required for the organization of adjacent chromatin. Although transcriptional repression was observed, organized chromatin was not found in minichromosomes bearing the α2 operator sequence at the promoter regions of a heterologous gene (Redd *et al*., 1996). On the other hand, the fact that positioned nucleosome arrays were found around all naturally occurring α2 operators in α cells suggests that these regions may have some unidentified elements that organize the chromatin structure in a similar way.

The observation that repression of cell-type-specific genes requires histone H3 and H4 N-terminal regions (Roth *et al*., 1992; Huang *et al*., 1997) suggests that chromatin plays an active role in transcriptional regulation of **a**-specific genes. Mcm1p binding appears to be the key element in *STE6* chromatin remodeling and transcriptional initiation. One of the possible scenarios for the events taking place at the *STE6* promoter during transcriptional initiation is as follows: when Mcm1p is inactive or not present at the *STE6* promoter, the PREs and TATA box are occluded by a positioned nucleosome and the gene is repressed. Following binding to the α2 operator, Mcm1p disorganizes chromatin structure, rendering accessibility to the PREs and the TATA box, and subsequent recruitment of Ste12p assists in preinitiation complex formation. In α cells, Matα2p cooperates with Tup1p to block Mcm1p transcriptional and chromatin remodeling activity. In addition, Tup1p likely stabilizes chromatin structure through interaction with histone N-terminal regions.

## Materials and methods

### Yeast strains, plasmids and media

Cell cultures were grown either in YPD or in selective media as described by Sherman (1991). Yeast strains FY23(*MAT***a**, *ura3-52*, *trp1-Δ63*, *leu2-Δ1*), FY24(*MAT*α, *ura3-52*, *trp1-Δ63*, *leu2-Δ1*), and their derivatives FY24 *ssn6*, FY24 *tup1* and FY24 *tup1 swi1*, were described earlier (Gavin and Simpson, 1997). Strains FY23 *swi1* and FY23 *swi1 tup1* were obtained as segregants from the same FY23/FY24 cross followed by *SWI1* replacement with *LEU2 (*Gavin and Simpson, 1997). The YPH499 *gcn5* strain is a segregant from a cross of YPH499 (Sikorski and Hieter, 1989) with CY569 (Pollard and Peterson, 1997). Strains for footprinting of the α2 operator are M. *Sss* I$^+$ segregants from a cross of YPH500ΔL.19-2 (Kladde *et al*., 1996) with MKY47, which is YPH499ΔL (Kladde *et al*., 1996) containing a *tup1*::*URA3* allele. All mutations were verified by PCR. Plasmid pSTEZL was described previously (Patterton and Simpson, 1994).

### Plasmid construction

All molecular biological manipulations were performed as described (Ausubel *et al*., 1997). The point mutations were introduced into the α2 operator as follows: the pSTEZL PCR fragments bearing mutations in the α2 operator were obtained using the primer 5′-CAGCTATGACCATGATTACGCCAAGC and either 5′-CAATTGCCAAGGTGCGAAGCAGCGTTAAAATTTCCCTATTAGGTAATTCAATGGCA for mutation of Matα2p binding sites, 5′-CAATTGCCAAGGTGCGAAGCAGCGTGTAAATTTGGCTATTACCTAATTAC for mutation of the Mcm1p binding site or 5′-CAATTGCCAAGGTGCGAAGCAGCGTTAAAATTTGGCTATTACCTAATTCAATGGCA for both α2 and MCM1 mutations. The fragments were cut with *Not*I and *Sty*I and were ligated into the pSTEZL replacing the WT α2 operator with the mutated one. The resulting plasmids were used to transform either FY23, FY24 or FY24 *tup1* to tryptophan prototrophy.

Mutations in the Mcm1p binding site at the endogenous *STE6* α2 operator were introduced by transforming FY23 with the pRS406 yeast integration plasmid bearing the pSTEZL MCM1 fragment cloned into the *Eco*RI and *Bam*HI sites. The plasmid was linearized with *Mfe*I prior to transformation. The integration event was confirmed by PCR using primers to the mutated α2 operator and *STE6* coding region.

### Micrococcal nuclease digestion and primer extension

Nuclei isolation, micrococcal nuclease digestion and primer extension were carried out according to Gavin and Simpson (1997).

### β-galactosidase assay and northern blot analysis

β-galactosidase assays were performed as described by Rose *et al*. (1990). Activities as reported are normalized to a plasmid copy number of 10 per cell. RNA isolation, northern blotting and hybridization to the *STE6* probe corresponding to position +97 to +668, or to the *ACT1* probe were carried out as described in Gavin and Simpson (1997) except that RNA samples were electrophoresed in a 1% agarose gel.

### In vivo footprinting with M. Sss I

Yeast cells were grown in YP galactose (2%) medium for 16 h to induce expression of the integrated copy of M. *Sss* I from the *GAL1* promoter. DNA was isolated and deaminated by sodium metabisulfite as described previously (Kladde *et al*., 1996). A 773 bp product encompassing –648 to +124 of the *STE6* genomic locus was amplified from the purified, deaminated DNA by PCR with primers *STE6*a1a (CCaaCACTAaaCCTaTTaCCACaaTAC; corresponding to position +98 to +124 at *STE6*) and *STE6*a2a (TAtTAtTTTTttAAGtTATAGGtAAATGGtAttTG; *STE6* –614 to –648), respectively, containing G→a or C→t transitions. The 32 bp *STE6* α2 operator is located at –183 to –214. The PCR product was purified and directly subjected to thermal cycle sequencing with [γ-$^{32}$P]-end-labeled primers to identify 5-methylcytosine residues (Kladde *et al*., 1996). The extension products were analyzed by electrophoresis on a 6% (w/v; 19:1 bisacrylamide:acrylamide), 50% (w/v) urea sequencing gel. For Figure 4A and B the oligonucleotides *STE6*a1a and *STE6*a1b (aaAaATAaTTCAaCCATATCCAa; *STE6* –75 to –97) were end-labeled and used as extension primers, respectively.

### Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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