RAP1 protein activates and silences transcription of mating-type genes in yeast

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RAP1 is a sequence-specific DNA-binding protein essential for cell growth. The occurrence of RAP1-binding sites in many promoter regions, the mating-type gene silencer elements, and telomeres suggests that RAP1 has multiple functions in the cell. To assess its role in transcription, temperature-sensitive mutations in *RAP1* were generated. Analysis of *rap1^{ts}* strains provides evidence that RAP1 functions in both transcriptional activation and silencing of mating-type genes. Several observations indicate that *rap1^{ts}* strains are defective in the expression of *MAT α* , whose upstream activation sequence (UAS) contains a RAP1-binding site. At nonpermissive temperatures, decreases in *MAT α* steady-state transcript levels can be detected in *MAT α rap1^{ts}* strains. Furthermore, these strains are deficient in α -pheromone production and simultaneously express at least two *a*-specific genes. These phenotypes can be reversed by replacing the RAP1-binding site at *MAT α* with a binding site for the GAL4 transcriptional activator. Certain *rap1^{ts}* alleles have an opposite effect on the silent mating-type locus *HMR*, which becomes partially derepressed at nonpermissive temperatures.

[Key Words: *Saccharomyces cerevisiae*; transcriptional activation; silencer; mating type; transcription factor]

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RAP1 (TUF/GRF1/TBA) has been independently identified as a sequence-specific DNA-binding protein in studies of ribosomal protein gene promoters (Huet et al. 1985), silencer elements at the nontranscribed mating-type loci *HML* and *HMR* (Shore et al. 1987; Buchman et al. 1988a), and the poly(C₁₋₃A) tracts at telomeres (Berman et al. 1986; Dunn 1989). The RAP1 protein has been purified, and its gene has been cloned and shown to be essential for growth (Shore and Nasmyth 1987). RAP1-binding sites have since been identified in the promoters of a large number of genes in addition to those involved in translation, including many glycolytic enzyme genes and the *MAT α* mating-type genes (for review, see Buchman et al. 1988b; Capieaux et al. 1989). The location of RAP1-binding sites at both upstream activation sequences (UASs) and silencers has led to the suggestion that RAP1 has a dual role in regulating transcription. Recent studies have demonstrated directly that RAP1 is also involved in controlling the length of poly(C₁₋₃A) tracts at telomeres (Lustig et al. 1990).

The idea that RAP1 functions in vivo as an activator of transcription is supported by a number of deletion analyses of promoter regions containing RAP1-binding sites. Deletion of these sites from promoters typically results in a moderate reduction in expression (Rotenberg and Woolford 1986; Chambers et al. 1988; Elledge and Davis 1989; Hurd and Roberts 1989), although in some cases the effect appears to be more severe (Siliciano and Tatchell 1984; Nishizawa et al. 1989). Similarly, analyses of silencer elements has suggested a repressor function for

RAP1: Deletion of the RAP1-binding site at the *HMR* silencer causes a partial derepression of this locus (Brand et al. 1987; Kimmerly et al. 1988). The function of RAP1 at a particular locus appears to depend on the context of its binding site within other regulatory sequences rather than the precise sequence of the site, and presumably results from interactions with other regulatory factors. So, for example, UAS-associated RAP1-binding-sites can substitute for the binding site normally found at the *HMR* silencer (Shore and Nasmyth 1987), and RAP1 binding sites taken from either UAS elements, silencers, or telomeres activate transcription when placed upstream of a TATA element (Buchman et al. 1988b).

The presence of RAP1-binding sites at both the *MAT α* promoter and the *HML* and *HMR* silencers suggests that RAP1 might play a complex role in cell type determination. The *MAT* locus, which controls cell type (either *a* or α), is situated near the centromere of chromosome III and can contain either the *a* or α allele. The *MAT α* allele encodes two regulatory proteins (α 1 and α 2) that control the expression of a number of cell type-specific genes in haploids (e.g., pheromone and pheromone receptor genes). The *MAT α 1* protein is an activator of α -specific genes, whereas *MAT α 2* is a repressor of the otherwise constitutive *a*-specific genes. Therefore, expression of both α 1 and α 2 represses *a*-specific genes and activates α -specific genes, leading to the α -mating phenotype. In the absence of *MAT α* expression cells display an *a*-mating phenotype. The *MAT α* locus has no function in haploids but is required in *a*/ α diploids, which are nonmat-

ing cells capable of sporulation. Two identical, yet non-transcribed, copies of mating-type genes are present near the telomeres of chromosome III, at loci called *HMR* and *HML*, which usually contain a and α alleles, respectively. Silencing of these loci is essential for mating. In haploid cells containing a wild-type *HO* gene, the silent loci are used as donors of information in a mating-type switching event that results in a conversion of the allele present at the *MAT* locus (for review, see Nasmyth and Shore 1987; Herskowitz 1989).

Several studies have defined DNA sequence elements and genes necessary for maintaining transcriptional silencing. At *HMR*, a flanking sequence called *HMRE* is required for repression of the locus (Abraham et al. 1984; Brand et al. 1985). The *HMRE* silencer is itself composed of three elements (called A, E, and B), one of which (element E) is a RAP1-binding site that is required for full repression (Brand et al. 1987; Shore and Nasmyth 1987; Kimmerly et al. 1988). Element A is an autonomous replicating sequence (ARS) consensus sequence, and element B is a binding site for ARS-binding factor 1 (ABF1) (Shore et al. 1987; Buchman et al. 1988a; Diffley and Stillman 1988). Deletions of either A or B alone have no effect on silencing, but either element, in combination with the RAP1-binding site, provides complete repression in the context of *HMRE*. A number of unlinked *trans*-acting genes have been identified that are required for silencing. Mutations in the nonessential genes *SIR2*, *SIR3*, or *SIR4* result in a complete loss of silencer function (Rine and Herskowitz 1987), and mutations in *SIR1* appear to affect the establishment, but not the maintenance, of the repressed state (Pillus and Rine 1989). In addition, deletions of the amino terminus of histone H4 lead to derepression of the silent loci (Kayne et al. 1988); and mutations in *NAT1* and *ARD1*, components of an amino-terminal protein acetylase, result in a partial loss of silencing (Whiteway et al. 1987; Mullen et al. 1989). No mutations in the genes encoding the known silencer binding proteins RAP1 and ABF1 have been isolated previously in screens for silencing mutants (Miller and Nasmyth 1984; Rine and Herskowitz 1987), perhaps because their functions are redundant at the silencers and both are essential genes.

To investigate the role of RAP1 in transcriptional regulation, we have isolated conditionally lethal mutations in the *RAP1* gene. Strains containing these mutations were then used to examine the requirement for RAP1 in gene expression at *MAT α* and repression at *HMR*. These experiments provide genetic evidence that RAP1 has a fundamental role in transcriptional activation and repression and demonstrate the importance of RAP1 in establishing and maintaining cell type in yeast.

Results

Isolation and initial characterization of rap1^{ts} mutants

Temperature-sensitive mutations in *RAP1* were generated by mutagenesis of the cloned gene. Because the *RAP1* gene is essential, we used a plasmid shuffle tech-

nique (Boeke et al. 1987; Mann et al. 1987) to introduce a mutagenized copy of the gene into cells deleted for the chromosomal copy. Four independent temperature-sensitive mutants were obtained using this procedure. Plasmids recovered from these strains conferred a temperature-sensitive phenotype upon retransformation of the original strain and subsequent plasmid shuffling, confirming that the temperature-sensitive mutations were plasmid encoded. The mutant alleles were isolated from these plasmids, subcloned into an integrating vector, and used to replace the *RAP1* locus. All of the mutations are recessive; they are complemented by plasmids containing the wild-type *RAP1* gene, and heterozygous diploids grow normally at 37°C. The integrated alleles segregate as expected (2+ : 2- for growth at 37°C) in crosses to a wild-type strain. Strains containing these *rap1^{ts}* alleles at the genomic *RAP1* locus were used in all experiments presented below.

The effect on cell growth of the four *rap1^{ts}* alleles was assessed at permissive (25°C) and restrictive (37°C) temperatures. As shown in Figure 1A, the mutants display a range of different growth phenotypes relative to the wild-type strain. At 25°C, strains containing the *rap1-2* or the *rap1-5* mutation grow at near wild-type rates (2 hr doubling time). At 37°C, these strains gradually arrest their growth after 3 to 4 doublings. Strains containing either the *rap1-1* or *rap1-4* mutation have a more dramatic growth phenotype. At 25°C, both of these strains have a pronounced increase in doubling time (4 hr for *rap1-1* and 7 hr for *rap1-4*) and arrest growth after 1 to 2 doublings when shifted to 37°C. The relative growth differences among the four mutant strains is reflected in their efficiency of plating after a shift to 37°C. The strains that grow poorly at 25°C lose viability at the restrictive temperature more rapidly than the strains that grow normally at 25°C. Strains carrying the *rap1-2* or *rap1-5* mutations show only a twofold decrease in viability for every 4 hr of incubation at 37°C, whereas the *rap1-1* and *rap1-4* strains show a more dramatic loss of viability (~10-fold reduction for every 4 hr at 37°C; Fig. 1B). In contrast, wild-type cells show no detectable loss of viability after similar periods of incubation at 37°C.

None of the *rap1^{ts}* mutants appear to arrest growth at a particular stage in the cell cycle. Strains containing *rap1^{ts}* mutations have a larger cell size than wild-type cells even at permissive growth conditions. Many of the cells in a population carrying a *rap^{ts}* mutation have pronounced vacuoles and grossly altered morphologies relative to wild-type cells after a temperature shift to 37°C (data not shown).

On the basis of relative differences in growth between the four temperature-sensitive mutations, it seemed likely that each mutant represented a unique *rap1* allele. We mapped the mutations to regions in the *RAP1* gene by a series of restriction fragment swap experiments (see Materials and methods). Appropriate mutant fragments were then subcloned and sequenced. The four mutants are unique missense mutations in the *RAP1*-coding sequence distributed over a region of ~250 amino acids in the middle of the coding sequence (Fig. 2).

Figure 1. Growth phenotypes of *rap1^{ts}* mutants at 25°C and 37°C. (A) Log-phase cultures of wild-type (YDS3) and *rap1^{ts}* strains (YDS406, 409, 410, 413) were split and maintained in YEPD media at 25°C or shifted to 37°C. Aliquots were removed at regular intervals, and the optical density at 600 nm was determined. OD₆₀₀ values are plotted vs. time of incubation. (B) Survival of *rap1^{ts}* mutants after incubation at the restrictive temperature. Log-phase cultures of wild-type (YDS3) and *rap1^{ts}* strains (YDS406, 409, 410, 413) were shifted from 25°C to 37°C. At various intervals, equal OD₆₀₀ units were diluted, spread on YEPD plates, and incubated at 25°C. After 5 days, the number of surviving colonies was determined and plotted vs. time of incubation at 37°C.

RAP1 is required for the proper regulation of genes controlled by the MAT α locus

In constructing strains for the purpose of isolating *rap1* mutants we noticed an unusual property of MAT α cells carrying both a deletion of the genomic *RAP1* locus and a plasmid containing the wild-type *RAP1* gene. In patch mating assays, a portion of these cells were capable of mating to MAT α testers, whereas otherwise isogenic strains containing the MAT α allele mated normally (Fig. 3). A quantitative mating analysis indicated that the MAT α , *rap1* deletion strains mate with the inappropriate cell type [MAT α] 200-fold more frequently than the parental wild-type MAT α strain (Table 1). We were able to show by a simple genetic test that this aberrant mating behavior occurred only in cells that had lost the *RAP1* plasmid; selection for the *URA3* gene on this plasmid

abolished the ability of this strain to form diploids with the MAT α tester strain. This result suggested that loss of the *RAP1*-containing plasmid was a prerequisite for a-like mating, an event that rescues these cells from death. Because RAP1 binds in vitro to the MAT α UAS (Shore and Nasmyth 1987; Buchman et al. 1988a), we suspected that a reduction in RAP1 activity in cells that have lost the *RAP1* plasmid might directly affect transcription of the MAT α locus. Previous studies have shown that the absence of functional MAT α gene products, either due to mutations in both $\alpha 1$ and $\alpha 2$ or promoter deletions that abolish MAT α transcription, results in an a-mating phenotype (Strathern et al. 1981; Tatchell et al. 1981; Siliaciano and Tatchell 1984).

To test whether RAP1 is required for the expression of the MAT α locus, we measured the activities of several gene products known to be directly regulated by MAT α .

RAP 1

ts allele	nucleotide change	amino acid change
rap 1-1	2451: G->C	ALA ⁵⁶³ -> PRO
rap 1-2	2136: G->A	ALA ⁴⁵⁸ -> THR
rap 1-4	1863: G->A	GLU ³⁶⁷ -> LYS
rap 1-5	2845: C->T	PRO ⁶⁹⁴ -> LEU

Figure 2. Allele map of *rap1^{ts}* mutations. *rap1^{ts}* mutations were mapped and sequenced as described in Materials and methods. The nucleotide and amino acid changes are listed for each allele. The line drawing, representing the *RAP1* gene, indicates the positions of restriction sites used in mapping, the *RAP1*-coding region (solid bar), and the region containing the temperature-sensitive mutations (stippled bar).

The $\alpha 1$ and $\alpha 2$ proteins regulate genes encoding both α - and *a*-specific functions (Nasmyth and Shore 1987; Herskowitz 1989): the $\alpha 1$ protein is an activator of α -specific genes, such as *MF α 1* and *MF α 2*, which encode α -factor, whereas $\alpha 2$ is a repressor of *a*-specific genes, including the genes that encode *a*-factor and barrier, a protease that inactivates α -factor. The presence or absence of the mating factors and the barrier protease, which are all readily detectable in a bioassay, provides a sensitive measure of $\alpha 1$ and $\alpha 2$ activities.

rap1^{ts} strains were tested for the synthesis of α -factor using a halo assay (Julius et al. 1983) in which the production of α -factor by a patch of *MAT α* cells is measured

as a zone of growth inhibition in a surrounding lawn of *MAT α* cells. All four *MAT α rap1^{ts}* strains produce halos typical of *MAT α* cells at the permissive temperature (25°C). However, at semipermissive temperatures (30–33°C) these strains fail to produce α -factor halos (Fig. 4; data presented is for the *rap1-1* allele). The severity of this phenotype correlates with the overall growth rates of the different *rap1^{ts}* alleles: *rap1-1* and *rap1-4* fail to produce halos when transferred directly from 25°C to 30°C tester plates, whereas the *rap1-2* or *rap1-5* alleles require a 6-hr preincubation at 33°C before the loss of α -factor activity is evident. Because the α -factor genes require $\alpha 1$ protein for expression, this observation is consistent with the notion that *rap1^{ts}* strains are defective in the expression of *MAT α 1*. In the experiments described below we show that the failure to produce α -factor halos in *rap1^{ts}* strains is not an indirect effect arising from diminished growth rate or cell viability at elevated temperatures but, rather, is due to a specific defect in transcriptional activation of *MAT α* .

Coincident with the decrease in α -factor production in the *MAT α rap1^{ts}* strains is the inappropriate expression of several *a*-specific genes. At semipermissive temperatures, *MAT α rap1^{ts}* strains express *a*-factor, a peptide normally secreted by *MAT α* strains and detectable in a halo assay (Fig. 5A; *rap1-1* allele is shown). Because *a*-factor gene expression is repressed in *MAT α* cells by the $\alpha 2$ protein (Brake et al. 1985; Johnson and Herskowitz 1985), this observation suggests that *rap1^{ts}* strains are also defective in the expression of $\alpha 2$. Consistent with

Figure 3. *rap1* deletion strains mate inappropriately. Patches of wild-type and *rap1* deletion strains were printed onto minimal plates spread with a lawn of *MAT α* tester strain YDS32 (A) or *MAT α* tester strain YDS31 (B). Plates were incubated at 30°C for 2 days. (a) *MAT α* (YDS3); (b) *MAT α* (YDS2); (c) *MAT α , rap1 :: leu2* (YDS203-2d); (d) *MAT α , rap1 :: leu2* (YDS203-6b). Strains 203-2d and 203-6b contain a *CEN* plasmid bearing a wild-type *RAP1* gene.

Table 1. Relative mating efficiencies of wild-type and *rap1* deletion strains

Strain	Mating type	Efficiency of mating	
		$\times$ MAT <i>a</i>	$\times$ MAT α
YDS 3	α	0.48	1.2×10^{-6}
YDS 203-2d	α	0.56	2.6×10^{-4}
YDS 2	<i>a</i>	1×10^{-6}	0.7
YDS 203-6b	<i>a</i>	1×10^{-6}	0.83

Figure 4. *rap1^{ts}* strains are defective in α -Factor production at elevated temperatures. α -Factor production was measured using a halo assay at 25°C (permissive) and 30°C (semirestrictive). Fresh colonies of each strain were toothpicked onto plates spread with a lawn of strain RC634 (*MATa sst1-3*) and incubated at the designated temperature for 2 days. (a) Wild-type *RAP1*, *MATa* (YDS3); (b) *rap1-1*, *MATa* (YDS406); (c) wild-type *RAP1*, *MATa* (YDS2); (d) *rap1-1*, *MATa* (YDS407).

this idea, we found that another *a*-specific gene, *BAR1/SST1*, which encodes barrier and is also repressed by $\alpha 2$ (Chan 1977; Sprague and Herskowitz 1981), is expressed in the *MATa rap1^{ts}* strains at semipermissive temperatures. Barrier production was measured by patching the *MATa rap1^{ts}* strains next to a wild-type *MATa* strain on a lawn of *MATa sst1* cells (Fig. 5B). The growth of the lawn is inhibited by α -factor secreted from the streak of *MATa* wild-type cells. At 25°C, the *MATa rap1^{ts}* strains secrete α -factor, and consequently the lawn does not overcome this growth inhibition. Yet at 30°C, the patch of *MATa rap1^{ts}* cells protected the lawn from growth inhibition, indicating that the *rap1^{ts}* strains produce barrier at the elevated temperature. A *MATa* strain, which produces barrier constitutively, protected the lawn at both temperatures. In addition to suggesting a defect in $\alpha 2$ activity, these experiments show that although *MATa rap1^{ts}* strains are unable to produce an α -factor halo, they are still capable of expressing and secreting other gene products (*a*-factor and barrier) at semipermissive temperatures. These phenotypes are observed in strains containing any of the four *rap1^{ts}* alleles after a shift to semipermissive temperatures (data not shown).

Because the aberrant production of barrier in the *MATa rap1^{ts}* strains could also account for the loss of α -factor activity at semipermissive temperatures, we examined the production of α -factor in these strains in the absence of the *BAR1* gene. *MATa, rap1^{ts}, bar::LEU2* strains produce near wild-type levels of α -factor at 25°C; however, at 30°C, these strains produce little or no detectable α -factor (Fig. 6A). Therefore, the aberrant production of barrier in the *MATa rap1^{ts}* strains cannot alone account for their reduced α -factor production. This result further supports the conclusion that expression of $\alpha 1$, the activator of the α -factor genes, is also reduced in the *rap1^{ts}* strains following a temperature shift. Finally, the *MATa*-specific phenotypes of the *rap1^{ts}* mutants do not result from a derepression of the silent *HMRa* locus, as they are observed in strains carrying a deletion of the *a1* and *a2* genes at *HMR* (data not shown).

Steady-state levels of transcripts from *MATa* and from the $\alpha 1$ -regulated gene encoding α -factor (*MFa1*) were measured in *rap1^{ts}* strains following a shift to nonpermissive conditions. These RNAs were analyzed on Northern blots. A probe specific for *URA3*, a gene that does not have a *RAP1*-binding site in its regulatory region, was included as an internal control. There is a temperature-dependent decrease in the amounts of *MATa1* and *MATa2* mRNAs in strains containing the *rap1-1* (Fig. 7A) or *rap1-4* allele (data not shown) over the course of an 18-hr incubation at 33°C. We note that the decrease in the levels of *MATa1* and *MATa2* messages is not always as apparent in these strains and is difficult to detect in strains containing either the *rap1-2* or *rap1-5* alleles (data not shown). The behavior of strains with the *rap1-2* and *rap1-5* alleles is consistent with properties described above, indicating that they are weak alleles; these strains are defective in α -factor production and aberrantly produce *a*-factor and barrier only after a preincubation at nonpermissive temperatures.

We also note that the amount of *MFa1* message drops significantly following temperature shift (Fig. 7A, *MFa1*), and this decrease is apparent before there is a detectable drop in *MATa1* transcript levels. This may indicate that *MFa1* transcription is extremely sensitive to small fluctuations in levels of *MATa1* mRNA, the

Figure 5. *rap1^{ts}* strains express *a*-specific genes at elevated temperatures. (A) α -Factor halo assay at 25°C and 30°C on plates spread with a lawn of strain XBH8-2c (*MATa sst2-4*). (a) *rap1-1*, *MATa* (YDS406); (b) wild-type *RAP1*, *MATa* (YDS3); (c) *rap1-1*, *MATa* (YDS407); (d) wild-type *RAP1*, *MATa* (YDS2). (B) Barrier assay at 25°C and 30°C on plates spread with a lawn of strain RC634 (*MATa, sst1-3*). Strains were patched alongside the streak of *MATa* cells that continues across the plate. (a) *rap1-1*, *MATa* (YDS406); (b) wild-type *RAP1*, *MATa* (YDS3); (c) *rap1-1*, *MATa* (YDS407); (d) wild-type *RAP1*, *MATa* (YDS2).

Figure 6. A heterologous promoter restores the activation defect in *rap1^{ts}* strains. (A) α -Factor halo assay at 25°C and 30°C on plates spread with a lawn of strain RC634 (*MAT α* , *sst1-3*). (a) Wild-type *RAP1*, *MAT α* (YDS3); (b) *rap1-1*, *MAT α* (YDS406); (c) *rap1-1*, *MAT α* (YDS407); (d) *rap1-1*, *MAT α* , *bar :: LEU2* (YDS540); (e) *rap1-1*, *UAS_{Gal}-MAT α* , *GAL80⁺* (YDS558); (f) *rap1-1*, *UAS_{Gal}-MAT α* , Δ *gal80* (YDS447). (B) Barrier assay at 25°C and 30°C on plates spread with a lawn of strain RC634. (a) *rap1-1*, *MAT α* (YDS406); (b) wild-type *RAP1*, *MAT α* (YDS2); (c) wild-type *RAP1*, *MAT α* (YDS3); (d) *rap1-1*, *UAS_{Gal}-MAT α* , *gal80 Δ* (YDS447); (e) *rap1-1*, *MAT α* (YDS407); (f) *rap1-1*, *MAT α* , *bar1 :: LEU2* (YDS540).

MAT α 1 mRNA produced after temperature shift is non-functional, or *RAP1* directly affects transcription of *MF α 1* (see Discussion). We therefore devised another experiment, described below, to assess the affect of the *rap1^{ts}* mutations on *MAT α* transcription.

A heterologous promoter at *MAT α* restores α -specific functions in *rap1^{ts}* strains

Because a known *RAP1*-binding site has been shown by deletion analysis to be an essential element in the *MAT α* UAS and because mutations in this binding site lead to mating-specific phenotypes very similar to those observed in the *rap1^{ts}* mutants (Giesman et al. 1991), a different experiment was designed to test whether *MAT α* transcription is defective in the *rap1^{ts}* strains. We reasoned that if the mating-specific phenotypes of the *rap1^{ts}* mutants were due to an inability to properly transcribe the *MAT α* genes, then replacing the *MAT α* UAS (*RAP1*-binding site) with a heterologous UAS element should abolish these phenotypes and restore the α -cell characteristics.

We therefore replaced the *RAP1*-binding site in the

MAT α promoter with a single binding site for the *GAL4* transcriptional activator (see Materials and methods). This *UAS_{Gal}-MAT α* construct was inserted at the *MAT* locus in a strain that contained the *rap1-1* mutation and a *gal80* gene deletion. The *gal80* mutation was used to facilitate the analysis of the *UAS_{Gal}-MAT α* fusion. Because the product of the *GAL80* gene is a negative regulator of *GAL4* activity in glucose-grown cells (Johnston et al. 1987; Lue et al. 1987; Ma and Ptashne 1987), deletion of this gene should result in constitutive (galactose-independent) expression from *UAS_{Gal}-MAT α* , allowing us to perform halo assays under optimal growth conditions.

rap1^{ts} strains containing the *UAS_{Gal}-MAT α* gene and the *gal80* mutation produce wild-type levels of α -factor at all temperatures (Fig. 6A, strain f) and no longer produce barrier (Fig. 6B, strain d) or α -factor (data not shown) at the elevated temperatures, indicating that the *MAT α* -regulated functions have been restored. The restoration of α -specific functions is not the consequence of overexpression from the heterologous *UAS_{Gal}-MAT α* promoter because Northern blot analysis indicates that transcript levels produced from the hybrid gene are similar to those produced from the wild-type promoter (Fig. 7B). These results suggest that the mating-specific phenotypes associated with the *rap1^{ts}* mutations are due to an inability to properly transcribe the *MAT α* genes, and we conclude from this that one function of the wild-type *RAP1* protein is to activate transcription at *MAT α* .

rap1^{ts} alleles affect transcriptional silencing at HMR

Deletion of the *RAP1*-binding site in the *HMR* silencer results in a partial derepression of the locus (Brand et al. 1987; Kimmerly et al. 1988). To determine whether the *rap1^{ts}* alleles affect silencing, we measured the transcrip-

Figure 7. *rap1-1* mutants are defective in *MAT α* transcription. (A) Northern blot analysis of *MAT α* , *rap1-1* strain YDS406. RNAs were isolated from a continuous culture at 0, 2, 6, 12, 15, and 18 hr following a temperature shift from 25°C to 33°C. The filter was hybridized successively with probes for the $\alpha 1$, $\alpha 2$, α -factor [*MF α 1*], and *URA3* transcripts. (B) Northern blot analysis of *MAT α* transcripts from the *UAS_{Gal}-MAT α* construct. RNAs were isolated from YDS439; *MAT α* , *rap1-1*, Δ *gal80* grown at 25°C (lane 1) and from YDS447; *MAT α* , *rap1-1*, Δ *gal80*, *UAS_{Gal}-MAT α* grown at 25°C (lane 2) and 8 hr after a shift to 33°C (lane 3). The filter was hybridized successively with probes for the $\alpha 1$ and $\alpha 2$ transcripts.

tion of *HMRa1* after a temperature shift. Because the *HMR* silencer is only partially dependent on the RAP1-binding site (element E), we also measured *HMRa1* transcription in strains carrying deletions of either of the two other silencer regulatory elements, A and B. Both the *hmrΔA* and *hmrΔB* silencers are totally functional, but unlike the wild-type silencer they are dependent on the RAP1-binding site for repression (Brand et al. 1987). Therefore, we expected that any effect on silencing in the *rap1^{ts}* strains would be more pronounced in the *hmrΔA* or *hmrΔB* silencer backgrounds.

RNAs were prepared from strains containing both the *rap1^{ts}* alleles and either a wild-type, *hmrΔA*, or *hmrΔB* silencer after a shift from 25°C to 37°C. The steady-state level of the *HMRa1* transcript was quantified by an S1 nuclease protection assay, using the *SIR3* transcript as an internal control (Miller 1984). In strains containing the *rap1-5* allele, this analysis detected *a1* transcripts 4 hr after the temperature shift in a strain containing the *hmrΔA* silencer and 8 hr after the shift in a strain containing the *hmrΔB* silencer (Fig. 8). Derepression of *HMR* was also detected in a strain containing the *rap1-4* allele and a *hmrΔA* silencer but not in strains containing the *rap1-1* or *rap1-2* alleles (data not shown). No derepression of *HMR* was detected in strains containing a *rap1^{ts}* allele and a wild-type silencer (A, E, and B present). Because a deletion of the RAP1-binding site (*hmrΔE*) only partially derepresses *HMR* (~10%), it may be the case that cell death precludes detection of expression at *HMR* in *rap1^{ts}* strains containing a wild-type silencer. Derepression of *HMR* in strains carrying the *rap1-5* allele is the

consequence of temperature elevation on this allele; no derepression of these silencer constructions occurs in wild-type *RAP1* strains at any temperature (see Fig. 8).

DNA-binding activity is heat labile in *rap1^{ts}* strains

All of our *rap1^{ts}* mutants map within or close to a large region in the middle of the protein (from approximately amino acid 360 to 600) that appears to be required for sequence-specific DNA binding (Henry et al. 1990; D. Balderes and D. Shore, unpubl.). To determine whether the various *rap1^{ts}* alleles affected the DNA-binding activity of the mutant proteins, cell extracts were prepared from each of the four temperature-sensitive strains before and after a shift to the restrictive temperature. Strains were maintained in culture at 37°C for 12 hr, and aliquots were removed every 2 hr for preparation of cell extracts. Gel mobility-shift assays were performed at 20°C on the extracts using a high-affinity RAP1-binding site (*TEF2*) and the binding site for ABF1 (another silencer-binding protein whose activity should be unaffected by these temperature-sensitive mutations). To reduce the effects of general proteolysis, these strains were made protease deficient by disruption of the *PEP4* gene (Ammerer et al. 1986). Extracts prepared from *rap1^{ts}* strains maintained at 25°C produced a protein-DNA complex with the *TEF2* probe that was indistinguishable from that produced in extracts containing wild-type RAP1 (Fig. 9A). In strains carrying the *rap1-1* or *rap1-4* alleles, RAP1-binding activity, although diminished relative to wild-type strains, remains after prolonged incubation at 37°C. Strains containing the *rap1-2* and *rap1-5* alleles have wild-type levels of RAP1 DNA-binding activity under these culture conditions. Although these extracts were prepared from cells maintained at 37°C, the observed levels of RAP1 DNA-binding activity may be the consequence of renaturation during the course of the binding assay, which was done at 20°C. To determine whether the temperature-sensitive extracts were capable of binding at nonpermissive temperatures, RAP1-binding activity was assessed at 37°C. The RAP1-binding activity in all of the *rap1^{ts}*, but not wild-type *RAP1* strains, is heat labile: Binding reactions performed at 37°C do not produce RAP1-DNA complexes, whereas the binding activity of ABF1 in the same extracts is unaffected (Fig. 9B). The loss of RAP1-binding activity in extracts from *rap1^{ts}* strains does not appear to be the consequence of proteolysis during the binding reaction, as a RAP1 antiserum detects equivalent amounts of full-length protein in Western blots of extracts incubated at 25°C or 37°C (data not shown).

Discussion

The importance of RAP1 to *MATα* expression was anticipated from previous studies in which the deletion of a small region of the *MATα* promoter (containing a RAP1-binding site) was shown to abolish transcription and cause α cells to mate inappropriately, becoming *a*-like "fakers" (Siliciano and Tatchell 1984). More recent stud-

Figure 8. *rap1-5* mutants are defective in silencing *HMR* at elevated temperatures. S1 nuclease mapping of transcripts produced from *HMRa1* in *MATα* strains containing *rap1-5* (YDS410), *rap1-5* and an *hmrΔA* silencer (YDS550), and *rap1-5* and an *hmrΔB* silencer (YDS551). RNAs were extracted from aliquots of continuous cultures at various times after a shift from 25°C to 37°C. RNAs were also prepared from wild-type *RAP1*, *MATα* strains with partial silencer deletions [*hmrΔA* (YDS36), *hmrΔB* (YDS37)] that do not produce *a1* at any temperature (lanes 0 hr and 10 hr, *hmrΔA*; lanes 0 hr and 10 hr, *hmrΔB*) and from strains that produce *a1* transcripts irrespective of temperature [*hmrΔE* (YDS39), *hmrΔEB* (YDS338), *MATα* (YDS2)]. Lane designations indicate hours after the shift to 37°C.

Figure 9. RAP1 DNA-binding activity is heat labile in *rap1^{ts}* strains. (A) Gel mobility-shift assay of RAP1 DNA-binding activity using a high-affinity RAP1-binding site (*TEF2*). Extracts from wild-type (YDS560) and *rap1-1* (YDS561) strains were prepared from continuous cultures following a temperature shift from 25°C to 37°C. Lane designations indicate 2-hr intervals after temperature shift; (–) a control reaction in which no extract was added. (B) Gel mobility-shift assay of RAP1 and ABF1 DNA-binding activities in extracts prepared from wild-type (YDS560) or *rap1-1* (YDS561) strains maintained at 37°C for 12 hr. Extracts were incubated in binding assays at 25°C or 37°C, as indicated; (–) a control reaction in which no extract was added.

ies in which point mutations were introduced into the RAP1-binding site at *MAT α* have revealed similar mating and transcriptional defects (Giesman et al. 1991). We have presented two types of experiments that, together, strongly suggest that the RAP1 protein mediates transcriptional activation of *MAT α* . First, *MAT α rap1^{ts}* strains fail to produce an α -factor halo and inappropriately express two α -specific gene products, α -factor and barrier, a phenotype characteristic of *mata1 mata2* double mutants. Additionally, in the strongest *rap1^{ts}* mutants, a significant reduction in the steady-state levels of *MAT α 1* and *MAT α 2* transcripts can be observed. Furthermore, the loss of RAP1 in strains carrying only a plasmid-borne copy of the gene leads to α -like mating in *MAT α* cells, again a property of cells unable to express the *MAT α* genes. In a second set of experiments we have shown that the α -specific defects resulting from the loss of RAP1 activity can be overcome by replacing the RAP1-binding site at *MAT α* with a heterologous UAS element, the GAL4 activator-binding site. This rescue by GAL4 is not an indirect effect owing to overexpression of the *MAT α* genes, as Northern analysis has shown that the steady-state mRNA levels of both α 1 and α 2 are similar to those produced from the wild-type *MAT α* promoter (Fig. 7B). These experiments indicate that the altered mating phenotypes resulting from diminished RAP1 activity are a consequence of reduced transcription at the *MAT α* locus and support the idea that RAP1 protein functions at the *MAT α* promoter to activate transcription.

A decrease in the steady-state levels of *MAT α 1* and *MAT α 2* mRNAs could account for the defect in α -specific functions observed in *rap1^{ts}* strains. However, we note that this decrease is not always detected in RNAs isolated from *rap1-1* or *rap1-4* strains and is difficult to observe in the *rap1-2* and *rap1-5* strains. Furthermore, the levels of *MF α 1* transcript decrease before there is a detectable drop in the transcript levels of *MAT α 1* (a known activator of *MF α 1*). One explanation for this observation is that a small decrease in *MAT α* mRNA levels can result in a dramatic reduction in *MF α 1* transcript

levels. Consistent with this explanation, Giesman et al. (1991) have shown that RAP1-binding site mutations at *MAT α* that result in only a partial decrease in *MAT α* steady-state mRNA levels can abolish α -factor production and lead to inappropriate expression of α -specific genes. It is also possible that alterations in transcription start sites or mRNA transport, which are not detected by Northern blot analysis, occur after temperature shift in *rap1^{ts}* strains and further diminish the pool of functional *MAT α 1* messages. We consider it unlikely that RAP1 is directly involved in *MF α 1* expression, as there are no known RAP1-binding sites in the *MF α 1* promoter or in the promoter of *MCM1*, another gene required for *MF α 1* expression. Moreover, the restoration of *MF α 1* function by altering only the *MAT α* promoter (UAS_{GAL}–*MAT α*) indicates that the phenotypes observed in the *rap1^{ts}* mutants result from a defect in transcription at *MAT α* . Finally, we cannot exclude the possibility that *MAT α* transcription is deficient in the *rap1^{ts}* strains, for example, during a critical period in the cell cycle required for α 1 and α 2 function. It is not difficult to imagine how a small drop in *MAT α 2* transcription in an individual cell could lead to a change in cell phenotype. The half-life of α 2 protein is <5 min (Hochstrasser and Varshavsky 1990), so this protein is likely to be rapidly depleted from the cell under conditions where *MAT α* expression is not optimal. It is worth noting that the normal process of mating-type switching in homothallic strains also leads to a change in cell type in the course of a single cell cycle.

The relationship between phenotype and mRNA levels that we observe at *MAT α* may have important implications for understanding the cause of death of the *rap1^{ts}* mutants. We have failed to detect significant temperature-dependent changes in the transcript levels of other genes (*RP73*, *RNR2*, *PYK1*, *LSR*, and *BCY1*; data not shown) that contain RAP1-binding sites in their promoter regions. Given that RAP1 is a transcriptional activator at *MAT α* , we think it is likely that it has a similar function at these other genes, particularly in cases where promoter deletion analyses have shown that a RAP1-binding site is a UAS element. As RAP1 becomes limit-

ing in the temperature-sensitive strains following a temperature shift, we imagine that a complex series of events ensues, considering the large number of genes that may be activated by RAP1. It is possible that the function of some essential genes becomes limiting shortly after the temperature shift, for example, due to a stronger dependence on RAP1 for activation, weaker upstream binding sites for the protein, a requirement of full transcription for activity, or a combination of these and other factors. In this case, death may occur before the steady-state mRNA levels of many RAP1-activated genes are affected. As is the case with *MAT α* , a sensitive biological assay may be the most effective way of assessing the effect of RAP1 on the transcription of a particular gene. Finally, we have observed that the poly(C₁₋₃A) tracts at telomeres slowly become shorter at semipermissive temperatures for all of the *rap1^{ts}* alleles (Lustig et al. 1990). Therefore, it is possible that some aspect of chromosome stability (perhaps related to telomere function) is significantly perturbed at the nonpermissive temperature in these mutants and ultimately results in lethality.

The contribution of RAP1 to repression of the silent mating-type loci was suggested by previous studies in which deletions of the RAP1-binding site at *HMR* were shown to result in partial derepression (Brand et al. 1987; Shore and Nasmyth 1987; Kimmerly et al. 1988). One of the *rap1^{ts}* alleles (*rap1-5*), in combination with a deletion of a silencer regulatory element (either A or B), resulted in a temperature-dependent derepression of the *HMR* locus. The level of derepression, although not as dramatic as that observed for a complete silencer deletion, may in part reflect the extent of derepression attainable in a population of cells facing impending death. The allele specificity of the derepression we observe suggests that silencing and activation may be two mechanistically different and separable functions of RAP1. For example, the *rap1-2* and *rap1-5* alleles have similar growth characteristics, yet differ in their effects at *HMR*. Likewise, the *rap1-4* and *rap1-1* alleles affect growth more severely than does the *rap1-5* allele, yet display little or no derepression of *HMR*. The separation of essential and silencing functions of RAP1 has been demonstrated directly by the isolation of *rap1* point mutants defective in silencing that grow at wild-type rates and display no defect in *MAT α* activation (L. Sussel and D. Shore, in prep.). All of these silencer-specific *rap1* mutants map to the carboxy-terminal 100 amino acids of RAP1. It may be noteworthy that the temperature-sensitive mutant that most severely affects silencing (*rap1-5*) maps closer to the carboxy-terminal end of the RAP1 protein than the other temperature-sensitive alleles.

We have not examined the effect of the *rap1^{ts}* mutants on silencing at the *HML* locus. The *cis* requirements for silencing at *HML* differ from those at *HMR* in that sequences flanking either side of the locus (*HMLE* and *HMLI*; left and right flanking, respectively) are by themselves sufficient for silencing (Mahoney and Broach 1989). In addition, the *HMLI* silencer, unlike either *HMLE* or the *HMR* silencer, appears to lack a strong

RAP1-binding site (Shore et al. 1987; Buchman et al. 1988a). Therefore, one might predict, that the *rap1^{ts}* mutants would only affect silencing at *HML* in the absence of the *HMLI* silencer.

There is a striking parallel between the role of RAP1 in mating-type gene expression and the role of the MCM1 (PRTF/GRM) protein in the regulation of downstream α - and α -specific genes (for review, see Herskowitz 1989; Sprague 1990). In α cells the MCM1 protein binds upstream of α -specific genes and activates their transcription, whereas in α cells MCM1 functions both as a corepressor (together with $\alpha 2$) to block the expression of α -specific genes and as a coactivator (with $\alpha 1$ protein) to stimulate the transcription of α -specific genes (Bender and Sprague 1987; Keleher et al. 1988; Tan et al. 1988; Passmore et al. 1989). The ability of sequence-specific DNA-binding proteins to function as both repressors and activators of transcription through combinatorial interactions with other regulatory proteins may be a general theme in eukaryotic transcriptional regulation (Diamond et al. 1990). In the case of MCM1, the factors that determine whether it acts positively or negatively appear to be clearly defined: the presence or absence of the interacting $\alpha 1$ and $\alpha 2$ proteins and sequences flanking the MCM1-binding sites that are recognized by these proteins. The factors that influence RAP1 function are less clearly defined. Although the *SIR* gene products are required in *trans* for silencing and the *GAL11/SPT13* gene product appears to be required for activation of *MAT α* (Fassler and Winston 1989; Nishizawa et al. 1990), it is still unclear whether any of these proteins interact directly with RAP1 and/or flanking DNA regulatory sequences.

Materials and methods

Yeast media and methods

Strains were maintained at 25°C (fully permissive conditions) in rich medium (YEED) or on minimal media supplemented with the appropriate amino acids, as described in Sherman et al. (1983) [see Table 2]. For the analysis of temperature-sensitive strains, log-phase cultures growing at 25°C were shifted to 37°C (fully restrictive conditions) or 30–33°C (semirestrictive conditions). The temperature-sensitive phenotype was routinely checked before and after time course experiments to ensure that revertants had not accumulated. Yeast strains were transformed by the spheroplast method (Beggs 1978) or by the LiOAc method (Ito et al. 1983), as indicated.

Quantitative mating analysis was performed according to Sprague and Herskowitz (1981). Values of relative mating efficiencies were normalized to the number of viable cells plated. Halo assays were done as described in Julius et al. (1983). All strains tested in halo assays were taken from freshly streaked YEED plates. Colonies were patched on YEED plates to which a dilute lawn of responder cells (either strain RC634 or XBH8-2c) had been spread. Plates were then incubated at 25°C or 30–33°C to examine temperature-dependent effects.

Generation of *rap1^{ts}* strains

Plasmid D130, which contains *CEN IV*, *TRP1*, and the wild-type *RAP1* gene, was mutagenized in vitro by incubating 30 μ g

Table 2. *Strains*

YDS 2: <i>HMLα MATα HMRα ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1</i>
YDS 3: <i>HMLα MATα HMRα ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura3-1</i>
YDS31: <i>MATα his1</i>
YDS32: <i>MATα his1</i>
YDS36: YDS3 <i>hmrΔA</i> (silencer deletion 352–358; Brand et al. 1987).
YDS37: YDS3 <i>hmrΔB</i> (silencer deletion 300–256; Brand et al. 1987)
YDS39: YDS3 <i>hmrΔE</i> (silencer deletion 331–324; Brand et al. 1987)
YDS203-2d: YDS3 <i>rap1 :: LEU2</i> , pD145[YCP50, <i>RAP1</i> , <i>URA3</i> , <i>sup4-o</i>]
YDS203-6b: YDS2 <i>rap1 :: LEU2</i> , pD145[YCP50, <i>RAP1</i> , <i>URA3</i> , <i>sup4-o</i>]
YDS338: YDS3 <i>hmrΔEB</i> (silencer deletions 331–324 and 274–256; Brand et al. 1987)
YDS406: YDS3 <i>rap1-1</i>
YDS407: YDS2 <i>rap1-1</i>
YDS408: YDS2 <i>rap1-2</i>
YDS409: YDS3 <i>rap1-2</i>
YDS410: YDS3 <i>rap1-5</i>
YDS411: YDS2 <i>rap1-5</i>
YDS412: YDS2 <i>rap1-4</i>
YDS413: YDS3 <i>rap1-4</i>
YDS438: YDS3 Δ <i>gal80</i>
YDS439: YDS406 Δ <i>gal80</i>
YDS444: YDS438 UAS _{Gal} - <i>MATα</i>
YDS447: YDS439 UAS _{Gal} - <i>MATα</i>
YDS539: YDS3 <i>bar1 :: LEU2</i>
YDS540: YDS406 <i>bar1 :: LEU2</i>
YDS544: YDS406 <i>hmrΔA</i>
YDS545: YDS406 <i>hmrΔB</i>
YDS546: YDS409 <i>hmrΔA</i>
YDS547: YDS409 <i>hmrΔB</i>
YDS548: YDS413 <i>hmrΔA</i>
YDS549: YDS413 <i>hmrΔB</i>
YDS550: YDS410 <i>hmrΔA</i>
YDS551: YDS410 <i>hmrΔB</i>
YDS558: YDS406 UAS _{Gal} - <i>MAT</i>
YDS560: YDS3 <i>pep4 :: URA3</i>
YDS561: YDS406 <i>pep4 :: URA3</i>
YDS562: YDS409 <i>pep4 :: URA3</i>
YDS563: YDS410 <i>pep4 :: URA3</i>
YDS564: YDS413 <i>pep4 :: URA3</i>
RC634: <i>MATα sst1-3 ade2 his6 met1 ura1 rml</i> (from R. Chan)
XBH8-2c: <i>MATα sst2-4 cryI^r his6 ura1 met1</i> (from L. Blair)

of plasmid DNA in a solution of 1 M hydroxylamine/0.45 N NaOH for 20 hr at 37°C (Rose and Fink 1987). The mutagenesis reaction was quenched by the addition of NaCl to a concentration of 0.1 M and bovine serum albumin to 0.1 μ g/ml. The solution was precipitated twice with ethanol, resuspended in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA, and used directly to transform strain YDS203, a yeast strain carrying a deletion of the chromosomal *RAP1* gene and a plasmid-borne copy of the wild-type gene, using the spheroplast transformation procedure. Transformants were then grown selectively for both plasmids at 37°C to eliminate cells that had acquired a chromosomal temperature-sensitive-lethal mutation during transformation. Col-

onies were then replica-plated to media containing canavanine, an arginine analog, resulting in loss of the plasmid carrying the wild-type *RAP1* gene. Transformants (now carrying only the mutagenized plasmid) were screened for conditional growth (lethality at 37°C). Using this screen, four colonies, identified from a population of 5500 independent transformants, contained plasmids to which the temperature-sensitive phenotype was linked. Confirmation that the mutations were plasmid linked was obtained by isolating the plasmids and using them to transform the original strain, YDS203. All of the transformants were temperature sensitive. Additionally, the temperature-sensitive phenotype always segregated with the plasmid in appropriate test crosses. Plasmids from the four temperature-sensitive strains were digested with *EcoRI* to recover a 4.2-kb fragment containing the mutant *rap1* alleles. These fragments were subcloned into a pUC-based vector that also contained the *URA3* selectable marker and used to replace the wild-type *RAP1* locus by transformation (Ito et al. 1983) and subsequent growth on minimal plates containing 5-fluoro-orotic acid (Boeke et al. 1984). Colonies that were temperature sensitive and Ura⁻ were analyzed by Southern blotting to confirm the expected structure of the locus. Temperature-sensitive colonies were analyzed further by genetic complementation to confirm the integration of the *rap1^{ts}* alleles; transformation with a plasmid containing the wild-type *RAP1* gene and crosses to an isogenic strain containing a wild-type *RAP1* gene complemented the growth defect at 37°C.

Mapping and sequencing of *rap1^{ts}* alleles

The location of each mutation was determined by complementation of the temperature-sensitive lethality with hybrid *RAP1* genes constructed by interchanging fragments of the mutant allele with homologous fragments from the wild-type *RAP1* gene. Fragments containing the entire coding region of the mutant *rap1* alleles (from the *PstI* site at nucleotide position 853 to the *XbaI* site at position 3670) were subcloned into pRS315, a *CEN* vector that contains the *LEU2* selectable marker (Sikorski and Hieter 1989). These plasmids were then digested with *PstI* and *SphI* or *PstI* and *BglIII*. Vector fragments isolated from these digests, and now lacking the respective 1507- or 2011-bp fragments from the *rap1^{ts}*-coding region, were then ligated with the corresponding fragments isolated from digestion of the wild-type *RAP1* gene. Plasmids containing the hybrid genes were then introduced into the temperature-sensitive strains (YDS406, 409, 410, 413) by transformation, and growth at 37°C was assessed. Complementation of the temperature-sensitive phenotype, indicated by growth at 37°C, correlated with a particular wild-type fragment for each *rap1* allele. Smaller fragments of the wild-type *RAP1* gene (generated from the *HindIII* sites at positions 1843 and 2837) were used to further define the region complementing the temperature-sensitive phenotype to ~500 bp. The corresponding fragments obtained from the mutant alleles were then subcloned into the M13 vectors mp18 and mp19 and sequenced using the Sanger dideoxynucleotide method (Bankier et al. 1987). Mutations were confirmed by sequencing both strands of the appropriate fragment.

Construction of *MAT α* UAS replacement

A 4-kb fragment containing the *MAT α* gene, deleted in the promoter from base pair 1602 to 1618, was removed by digestion with *HindIII* from a YRP7-based plasmid provided by K. Tatchell (an *XhoI* linker had been inserted at the site of the deletion) and inserted at the *HindIII* site in pIC20 to form plasmid D1446.

This plasmid was then digested with *Xho*I, treated with calf alkaline phosphatase, and ligated to a 22-bp oligonucleotide (5'-TCGACGGAAGACTCTCCTCCGC-3' and its complement 5'-TCGAGCGGAGGAGAGTCTTCCG-3') containing a GAL4-binding site to form UAS_{GAI}-MAT α in plasmid D1452. The 4-kb UAS_{GAI}-MAT α fragment from D1452 was isolated after digestion with *Hind*III and inserted into pRS306, a integrating vector that also contains the yeast *URA3* gene. This plasmid was used to transform the *rap^{ts}* strains. Stable Ura⁺ prototrophs were analyzed by Southern blotting to verify that the integration occurred at the *MAT* locus. Thus, these transformants contain a duplication at the *MAT* locus in which the UAS_{GAI}-MAT α construct is adjacent to the endogenous MAT α locus. Transcription of the UAS_{GAI}-MAT α construct is constitutive in strains that contain a deletion of the *GAL80* locus (YDS444, YDS447) and repressed in glucose-grown strains that contain a wild-type *GAL80* gene (YDS558; see Fig. 6). Strains that express the UAS_{GAI}-MAT α construct produce wild-type levels of MAT α 1 and MAT α 2 transcripts at 25°C and at 33°C as determined by Northern blots.

Isolation and analysis of RNA

RNAs were isolated from aliquots of log-phase cultures maintained at 25°C, 30°C, 33°C, or 37°C for various times by breaking cells with a vortex blender with glass beads and with phenol and chloroform (Kurtz and Lindquist 1984). RNAs were precipitated in ethanol and analyzed by Northern blotting or with S1 nuclease after hybridization with prime-cut probes for the MAT α 1 and *SIR3* transcripts, as described (Miller 1984). Northern blots were prepared after electrophoretic separation of 20 μ g of total RNA for each time point on 1.4% agarose-formaldehyde gels. Filters were reprobed after boiling twice in 0.1 $\times$ SSC for 5 min.

Gel mobility-shift assay

Extracts were prepared from strains grown in rich media at 25°C or at various times after a shift to 37°C. Cells were collected in Eppendorf tubes, washed in sterile water, and resuspended in breakage buffer [0.2 M Tris-HCl (pH 8), 10 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol, 1 mM PMSF, 20 μ g/ml pepstatin A] with ~0.2 g of glass beads (0.5 mm). Cells were broken by vortexing at maximum speed for four 1-min bursts interspersed with incubation on ice. After centrifugation in a microfuge for 15 min, supernatants were removed and the protein concentrations were determined by using the Bio-Rad assay. Gel mobility-shift assays were performed as described (Shore and Nasmyth 1987; Shore et al. 1987) by using 2 μ g of cell extract per reaction and probes (labeled with [α -³²P]dATP) containing RAP1- (*TEF2*) or ABF1-binding sites. Note that the RAP1-binding sites at *TEF2* and MAT α are nearly identical matches to the consensus-binding site (see Buchman et al. 1988a). These sites have binding affinities for RAP1 that are similar, and these sites compete similarly for RAP1 binding.

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