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The Role of RAP1 in the Regulation of the *MAT*α Locus

DONNAMARIE GIESMAN,[1,2] LEWIS BEST,[2] AND KELLY TATCHELL[2]*

Graduate Group of Molecular Biology and Genetics, University of Pennsylvania, Philadelphia, Pennsylvania 19104,[1] and Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615[2]

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The *RAP1* gene of *Saccharomyces cerevisiae* encodes an abundant DNA-binding protein, also known as GRF1, TBA, or TUF, that binds to many sites in the yeast genome in vitro. These sites define a consensus sequence, 5′-$^{AA}_{GC}$ACCCANNCA$^{TT}_{CC}$-3′, and deletion analyses of genes that contain this sequence have implicated the involvement of RAP1 in numerous cellular processes, including gene activation and repression. The *MAT*α locus, required for determination of the α cell type in yeast cells, contains a RAP1 binding site; this site coincides with the *MAT*α upstream activating sequence (UAS) and is necessary for expression of the two genes encoded by the *MAT*α locus, *MAT*α*1* and *MAT*α*2*. We show that the *MAT*α UAS is sufficient to activate transcription from a promoterless gene fusion of the yeast *CYC1* upstream region and the *lacZ* gene. Constructs containing only the *MAT*α UAS generated elevated levels of β-galactosidase activity which were indistinguishable from those of constructs containing the entire *MAT*α intergenic region. Further, the *MAT*α UAS has an intrinsic polarity of transcriptional activation; transcription of *CYC1-lacZ* was six- to sevenfold higher when the UAS was oriented in the direction normally associated with *MAT*α*2* transcription. Point mutations in the *MAT*α UAS that reduce *MAT*α expression three- to fivefold resulted in a bi-mating phenotype, while a mutation that reduced *MAT*α expression still further resulted in an a-mating phenotype. We isolated plasmids from a high-copy-number yeast library that suppressed the bi-mating defect of point mutations in the *MAT*α UAS, and the most effective dosage suppressor contained the gene encoding RAP1. A temperature-sensitive *rap1* mutant bi-mates at the semipermissive temperature. Double mutants at *rap1* and *mat*α mate exclusively as a cells, at all temperatures, and do not express detectable levels of *MAT*α RNA. These data provide evidence that the *RAP1* gene product functions at the *MAT*α UAS in vivo.

The yeast *Saccharomyces cerevisiae* can exist as one of three cell types, mating-competent haploid cell types, called **a** and α, and a diploid cell type, the product of productive mating between opposite haploid cell types. These cell types can be distinguished on the basis of cell morphology, cell size, and expression of specific pheromones, receptors, and cell agglutinins. The molecular basis for this regulation is complex, but the ultimate regulator of cell type is the *MAT* locus (1, 2, 33, 46, 51; for reviews, see references 10, 19, and 20). Cells expressing the *MAT*α allele have the α cell type, and those expressing the *MAT***a** allele have the **a** cell type. Cells expressing both *MAT*α and *MAT***a** have the diploid cell type. The products of the *MAT*α locus, α1 and α2, are DNA-binding proteins that function to activate and repress, respectively, genes required to determine the α or **a** cell type (α-specific and **a**-specific genes). Mutants in *mat*α*1* fail to acquire the α cell type because they fail to express α-specific genes; mutants in *mat*α*2* are defective because they fail to repress otherwise constitutively expressed **a**-specific genes. The *MAT***a** allele encodes the **a**1 protein that acts in conjunction with α2 to establish the diploid cell type.

*MAT*α*1* and *MAT*α*2* are transcribed divergently; only 200 bp separate the 5′ ends of the two RNAs. Four DNA elements, defined by deletion analysis, in the intergenic region modulate transcription at *MAT*α. The two TATA elements situated just upstream of the *MAT*α*1* and *MAT*α*2* genes do not affect the level of transcription but do determine the start site of mRNAs (44). Another regulatory element, the **a**1/α2 box, is responsible for repression of *MAT*α*1* in diploid cells (45). Finally, a single upstream activating sequence (UAS) is required for the transcription of both *MAT*α*1* and *MAT*α*2* (44). The *MAT*α UAS coincides with the consensus sequence for the DNA-binding protein RAP1 (also known as GRF1, TBA, and TUF) (5, 23, 30, 42). Purified RAP1 has been shown to bind to the *MAT*α UAS in vitro (5).

GRF1 (for general regulatory factor), TUF (translational upstream factor), TBA (telomere binding activity), and RAP1 (repressor-activator protein) probably represent a single protein. All of these proteins were shown to bind to the same consensus sequence, 5′-$^{AA}_{GC}$ACCCANNCA$^{TT}_{CC}$3′, in vitro (defined in reference 5). The reported molecular masses (estimated by electrophoretic analyses) of GRF1, TBA, and RAP1 are all between 116 and 120 kDa. TBA, TUF, and RAP1 manifest the same patterns of proteolytic digestion and can be immunoprecipitated by the same antibodies. Also, GRF, TUF, TBA, and RAP1 were shown to have similar binding affinities for known as well as synthetic, versions of the consensus sequence (5, 30, 53, 54). It is still a formal possibility that some or all of these factors represent different members of a family of proteins. However, for the purposes of this report, we will proceed with the assumption that they are a single protein.

Binding sites for RAP1 are found upstream of a diverse set of genes, including ribosomal protein genes, the genes for enzymes involved in glycolysis (8, 9, 36, 52), the gene for the regulatory subunit of protein kinase A (50), and the membrane ATPase gene (5, 7). It is also found in some ARS (autonomously replicating sequences) elements (3, 5), at telomeres (6, 30), and in the silencer elements of *HMR* and *HML* (42). RAP1 apparently participates in a diverse set of physiological processes. RAP1 binding sites are required for transcriptional activation (5, 7, 39, 44, 55) and transcriptional repression at the silent mating type loci *HML* and *HMR* (5, 27, 43), and they can confer plasmid stability (3). In most of

* Corresponding author.

TABLE 1. Strains

Strain	Genotype	Mating phenotype	Reference or source
EG123	*MAT***a** *his4 leu2 trp1 ura3*	**a**	44
23α67	*MAT*α *his4 leu2 trp1 ura3*	α	48
246.1.1	*MAT*α *his4 leu2 trp1 ura3*	α	44
80	*MAT***a** *leu2 lys1 trp1*	**a**	44
80α52	*MAT*α *leu2 lys1 trp1*	α	44
265.7.4	*MAT***a** *hom3 ilv1*	**a**	34
265.5.4	*MAT*α *hom3*	α	34
αX8	*MAT*αΔ *his4 leu2 trp1 ura3*	alf	48
G245-24a	*MAT***a** *bar1-1 his6 leu2 trp1 tyr6*	**a**	George Sprague
grc4	*MAT***a** *leu2 trp1 ura3 rap1*(Ts)	**a**	W. Spevak and M. Breitenbach
DG167	*mat*α^{1615} *his4 leu2 trp1 ura3*	α	This work
DG168	*mat*α^{1613} *his4 leu2 trp1 ura3*	bi	This work
DG169	*mat*α^{1617} *his4 leu2 trp1 ura3*	bi	This work
DG170	*mat*α^{1618} *his4 leu2 trp1 ura3*	alf	This work
DG101	*MAT*α *bar1-1 leu2 trp1 tyr6 ura3*	α	This work
DG110	*mat*α^{1615} *bar1-1 leu2 trp1 tyr6 ura3*	α	This work
DG115	*mat*α^{1613} *bar1-1 leu2 trp1 tyr6 ura3*	α	This work
DG116	*mat*α^{1617} *bar1-1 leu2 trp1 tyr6 ura3*	α	This work
DG145	*mat*α^{1618} *bar1*::*LEU2 his4 leu2 trp1 ura3*	alf	This work
DG174	*mat*α^{1613} *rap1*(Ts) *his4 leu2 trp1 ura3*	**a**	This work
DG175	*MAT*α *rap1*(Ts) *his4 leu2 trp1 ura3*	α	This work
DG184	*mat*α^{1617} *rap1*(Ts) *his4 leu2 trp1 ura3*	**a**	This work

these processes, the binding site for RAP1 is only one of a number of important *cis*-acting sites, suggesting that RAP1 may act in combination with a variety of other factors to elicit the required activity (4). The RAP1 protein has been purified to homogeneity, and the gene encoding RAP1 has been cloned and sequenced; disruption of the chromosomal copy of the gene is lethal (42). The coding sequence predicts a molecular mass of 93 kDa, and protein synthesized in vitro comigrates with the factor purified from yeast cells. Though RAP1 is known to bind DNA, the sequence does not reveal any of the canonical DNA-binding motifs (18, 42). However, RAP1 has been shown to bend DNA, a property that has been associated with the physiological processes attributed to RAP1 (54). In addition, RAP1 copurifies with scaffold proteins and is required for reconstitution of the nuclear scaffold in vitro. It is also involved in forming chromatin loops in vitro with DNA fragments containing the silent *MAT* locus, *HML*, and its flanking sequences (22).

In this report, we present evidence that RAP1 functions at the *MAT*α UAS in vivo to regulate gene expression and that the only sequence necessary for this regulation is the RAP1 binding site. We show that the *MAT*α UAS is not only necessary but also sufficient for transcriptional activation. Also, point mutations in the UAS have significant effects on mating behavior and cell-type-specific gene expression. We show that a multicopy suppressor containing the *RAP1* gene can suppress these effects and that strains carrying a temperature-sensitive allele of *rap1* mate as both **a** and α cells at the semipermissive temperature; strains carrying both the point mutations at *MAT*α and the temperature-sensitive *rap1* allele mate exclusively as **a** cells at all temperatures and do not express detectable levels of *MAT*α RNA.

MATERIALS AND METHODS

Yeast strains and media. Strains were grown at 30°C in YPD medium (2% glucose, 2% yeast extract, 2% Bacto-Peptone) or, if nutritional selection was imposed, yeast minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with the appropriate amino acids (41). Strains were induced to sporulate on plates containing 1% potassium acetate, 0.25% yeast extract, 0.1% glucose, and 2% Bacto-Agar. Mutant and wild-type strains characterized in this study are all congenic with strains EG123 and 246.1.1 (Table 1). For many of the studies, strain 23α67 was used as the wild-type *MAT*α strain. This strain contains an 8-bp *Xho*I linker inserted into the *MAT*α intergenic region at a site between the *MAT* UAS and the **a**1/α2 box at nucleotide 1642. 23α67 is indistinguishable from the congenic wild-type strain 246.1.1 in mating, barrier, and halo assays and expresses wild-type levels of *MAT*α*1* and *MAT*α*2* mRNAs (unpublished results). Quantitative and qualitative mating tests were conducted with either the congenic **a** and α derivatives of strain 80 or strains 265.9.4 and 265.7.4. Strains DG175 and DG176 were derived by six serial backcrosses of the temperature-sensitive *rap1* strain grc4 (generously provided by Walter Spevak and Michael Breitenbach) into the EG123 background. The inability to grow above 36°C segregated 2:2 through meiosis in all of the backcrosses. The diploid strain containing point mutation *mat*α^{1618} at *MAT*α and the wild-type *MAT***a** allele was constructed by first transforming strain *mat*α^{1618} with *MAT*α on a yeast replicating plasmid (YRp7) and mating the resulting transformant to strain 80. Diploids were selected on the appropriate yeast minimal media supplemented with amino acids and then cured of the plasmid.

Plasmids. Wild-type copies of the entire *MAT*α locus or of *MAT*α*1* and *MAT*α*2* alone (contained on *Hin*dIII fragments derived from DNAs αX38 and αX113, respectively; 48) were cloned into the *Hin*dIII site adjacent to the tetracycline resistance gene of YRp7. YRp7 is a multicopy plasmid in yeast cells, derived from pBR322, which contains the yeast selectable marker *TRP1* and the ARS1 sequences that permit it to replicate autonomously. Deletions of the *MAT*α intergenic region were made by *Xho*I-*Hin*dIII restriction of BAL 31 deletion mutants C21, C23, and C6 (44) and *Xho*I linker mutants αX52 and αX109 (43a). The two resulting fragments contain intact copies of either *MAT*α*1* or *MAT*α*2* and some

portion of the intergenic region. These deletions were cloned into the *Xho*I site of plasmid pLG670-Z in both possible orientations, formally replacing either *MATα1* or *MATα2* with *lacZ*. A synthetic copy of the *MATα* UAS was formed by phosphorylating oligonucleotides 896A (TCGACTAATG ATGTCTGGGTTTTGTG) and 897A (TCGACACAAAACC CAGACATCATTAG) with T4 polynucleotide kinase, annealing the two, and ligating them into the *Xho*I-*Sal*I fragment of pLG670-Z. pLG670-Z is a derivative of pLG669-Z (15) which lacks the *CYC1* UAS. Plasmids containing the synthetic UAS were sequenced to determine the number and orientation of UAS inserts. Plasmid GR1 (gift from Jeffrey Strathern) was used in the gap repair of *MATα* mutant and wild-type strains. GR1 is a derivative of YEp213 which contains a fusion of *MATα* *Xho*I linker mutants αX141 and αX154 (48) inserted into the unique *Xho*I site. A yeast genomic library in multicopy shuttle vector YEp13, used to isolate dosage suppressors, has been described previously (34).

Site-directed mutagenesis and sequencing. The *Eco*RI-*Hin*dIII fragment of *MATα* was subcloned into bacteriophage M13mp10, and site-directed mutagenesis was conducted as described by Kunkel et al. (28). *Escherichia coli* strains used were CJ236 [*dut-1 ung-1 thi-1 relA1* (*pCJ105*) (*Cm*^{r})] and DH5αF′ [*F′ ϕ80d lacZΔ(lacZYA-argF)U169 recA1 endA1 hsdR17* [r_K^- m_K^-) *supE44 thi-1 gyrA relA1*] (16, 28). Mutagenized cassettes of *MATα* were integrated into recipient strain 23α67 (44). This strain contains an *Xho*I linker insertion in the *MATα* intergenic region; loss of the *Xho*I restriction site was used to confirm gene replacement events. Single- and double-stranded sequencing was performed essentially as described previously (40), with the following modifications. Prior to the annealing step, double-stranded DNA isolated from a CsCl gradient and treated with RNase was denatured in 0.2 N NaOH for 10 min at room temperature; 0.4 volume of 5 M ammonium acetate (pH 4.5) and 4 volumes of 100% ethanol were added, and the DNA was precipitated for 10 min at −70°C. Sequencing reactions were conducted by using the Sequenase reaction mixes and enzyme purchased from United States Biochemical Corp.

Yeast transformations and genetic and biochemical assays. Yeast strains were transformed either by the spheroplast method (21) or by lithium acetate treatment (25). Gap repair and integrative transformations were done as described previously (38, 44). Quantitative mating experiments were conducted by the filter mating assay as described previously (17). Qualitative mating experiments involved patching strains of interest to a YEPD plate, applying a lawn of **a** or α tester strain, mating for 12 to 16 h at 30°C, and replica plating to selective media on which only diploids survive. Halo and barrier assays were done essentially as described previously (47) except that α strain 80α52 was used as a source of α-factor (in the barrier assay) and strain G245-24a was used as the **a** tester in the α-factor halo assay. For β-galactosidase assays, strains were grown in yeast minimal medium supplemented with the appropriate amino acids and quantitative β-galactosidase assays were performed by the chloroform lysis method (14). Cells used in the assay were grown to an optical density at 600 nm (OD_{600}) of 0.8 to 1.0 in yeast minimal medium lacking uracil at 30°C. Units of β-galactosidase activity were obtained from the following formula: $OD_{420} \times (1{,}000/OD_{600}) \times$ time (in minutes) (14).

Northern (RNA) analyses. A 50-μg sample of RNA prepared by method of Domdey et al. (11) was subjected to Northern analysis as follows. RNA was glyoxalated, electrophoresed (11), and transferred to GeneScreen nylon membrane (DuPont) (49). The membrane was treated with UV light (26) and baked under vacuum at 80°C for 2 h. DNA probes for the *MATα1* and *MATα2* transcripts were derived from the 630-bp *Xba*I-*Xho*I fragment and the 530-bp *Xho*I-*Bst*NI fragment of *MATα* linker mutant αX67 cloned into YRp7 (48). The DNA probe for the *URA3* transcript was isolated by *Pst*I and *Nco*I restriction from a pUC19 derivative containing the 1-kb *Xba*I fragment of *URA3*. Probes were labeled with ^{32}P by nick translation as described previously (32). Prehybridization was conducted in 10 to 25 ml of a solution containing 50% formamide, 0.2% each Ficoll, polyvinylpyrrolidone (PVP), and bovine serum albumin (BSA), 5× SSC, 0.2% sodium dodecyl sulfate (SDS), and 50 mM sodium phosphate (pH 6.5) for 1.5 h at 50°C and 1.5 h at 37°C. Hybridization was conducted overnight at 37°C in a solution containing 50% formamide, 0.02% each Ficoll, PVP, and BSA, 5× SSC, 10 mM EDTA, 5% dextran sulfate, and 50 mM sodium phosphate (pH 6.5). Blots were washed for 30 min in 2× SSC–0.1% SDS at 60°C and for 30 min in 0.1× SSC–0.1% SDS at room temperature, wrapped in plastic wrap, and exposed to film for 16 to 24 h at −70°C with an intensifying screen. Blots were stripped for reprobing by washing for 2 h at 80°C in 2.5 mM Tris hydrochloride (pH 8.0)–0.1 mM EDTA–0.25% sodium pyrophosphate–0.001% each BSA, Ficoll, and PVP. mRNA levels were quantitated by scanning the autoradiograms on a Shimadzu densitometer, cutting out and weighing the peaks obtained, and normalizing for the amount of RNA in a given sample.

Yeast plasmid DNA preparation. Cells were harvested from a 5-ml overnight culture, washed once with sterile water, and resuspended in 150 μl of 1 M sorbitol–0.1 M sodium citrate–6 mM EDTA (pH 7.0) to which 180 μg of zymolyase and 1.2 μl of β-mercaptoethanol had been freshly added. Cells were then incubated at 37°C for 30 min; 150 μl of 2% SDS–100 mM Tris hydrochloride (pH 9.7)–10 mM EDTA (pH 8.5) was added, and the cells were heated to 65°C for 5 min. After addition of 150 μl of 5 M potassium acetate, the cells were incubated on ice for 60 min. Debris was pelleted by centrifugation, and the supernatant was transferred to a new tube; 200 μl of 5 M ammonium acetate and 1 ml of isopropanol were added, and the mixture was incubated at −20°C for 15 min. DNA was pelleted by centrifugation and resuspended in 90 μl of TE. Then 10 μl of 5 M ammonium acetate and 200 μl of isopropanol were added, and the DNA was pelleted as before. DNA was washed in 70% ethanol and resuspended in 50 μl of TE. Before transformation into *E. coli* DH5αF′ (Bethesda Research Laboratories), DNA was centrifuged for 2 min to pellet cell debris.

RESULTS

The *MATα* UAS is necessary and sufficient for *MATα* expression. A deletion analysis of the intergenic region of the *MATα* locus defined a single sequence 15 bp in length (Fig. 1; bp 1608 to 1622) which was necessary for transcription of both *MATα1* and *MATα2* in vivo (44). To determine whether this sequence was sufficient to activate transcription, plasmids were constructed by cloning nested deletions of the *MATα* intergenic region, as well as a synthetic copy of the putative UAS element itself, into the *Xho*I site of the yeast shuttle vector, pLG670-Z (see Materials and Methods). β-Galactosidase activity was measured in **a**, α, and **a**/α diploid strains transformed with these plasmids (Fig. 2).

pLG670-Z contains the start sites of transcription and the TATA element of the *CYC1* gene fused to the *lacZ* gene but

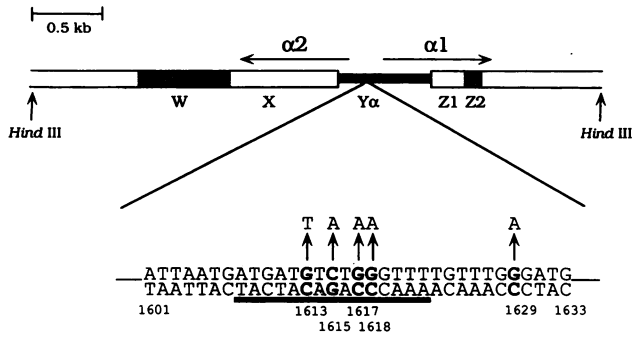

FIG. 1. The *MATα* intergenic region. The 4.3-kb *Hin*dIII fragment that contains the *MATα* locus is presented with the location of the intergenic region that contains the UAS. The bar under the sequence localizes the RAP1/GRF1/TBA/TUF binding site as defined by Buchman et al. (5). Base pairs in boldface type are targets of site-specific mutagenesis; base substitutions are above the wild-type sequence. The numbering system is that of Siliciano and Tatchell (44). The relative directions of transcription of *MATα1* and *MATα2* are also shown.

lacks the *CYC1* UAS. Yeast strains transformed with pLG670-Z synthesized low levels of β-galactosidase activity. Plasmids that contained deletions of the *MATα* UAS (C21-L and C21-S) also synthesized low levels of β-galactosidase, indicating that the UAS was necessary for gene expression. Plasmids that contained almost the entire *MATα* intergenic region (αX67-L and αX109-S) expressed elevated levels of β-galactosidase, the same levels as those that contained a synthetic 20-bp oligonucleotide encoding the UAS (pUAS-1 and pUAS-2). This result implies that no other sequences in the *MATα* locus are required for full expression. Furthermore, though the distance between the UAS and the *CYC1* TATA element did not significantly affect the level of transcriptional activation (compare C23-L with αX67-L and C6-S with αX109-S), transcriptional activation by the UAS was orientation dependent; transcription in the direction normally associated with *MATα2* (pUAS-2, C6-S, αX109-S, and pUAS-22) was approximately 20-fold higher than in the direction of MATα1 (pUAS-1, C23-L, αX67-L, and pUAS-11). Finally, transcriptional activation by this promoter was not cell type specific. The diploid-specific repression exhibited by constructs αX109-S and C6-S was due to the presence of the a1/α2 box (45); constructs C23-L and C6-S, which did not contain the a1/α2 box, displayed no diploid-specific repression.

Mutants in the UAS reduce expression and affect mating behavior. To further characterize the UAS of *MATα*, four site-specific point mutations within the UAS were synthesized in vitro by oligonucleotide-directed mutagenesis. These mutations (Fig. 1) were then integrated into the normal chromosomal locus of *MATα*, replacing the wild-type *MAT* promoter with each of the point mutations, using the selection procedure described previously (44). Each point mutant consistently produced the same phenotype in independent transformants, and this phenotype segregated 2:2 with the *MATα* locus in crosses. To confirm that each transformed strain contained the in vitro-generated point mutation and no other, we cloned the *MATα* locus from each transformant by gap repair (38) and sequenced the *MATα*

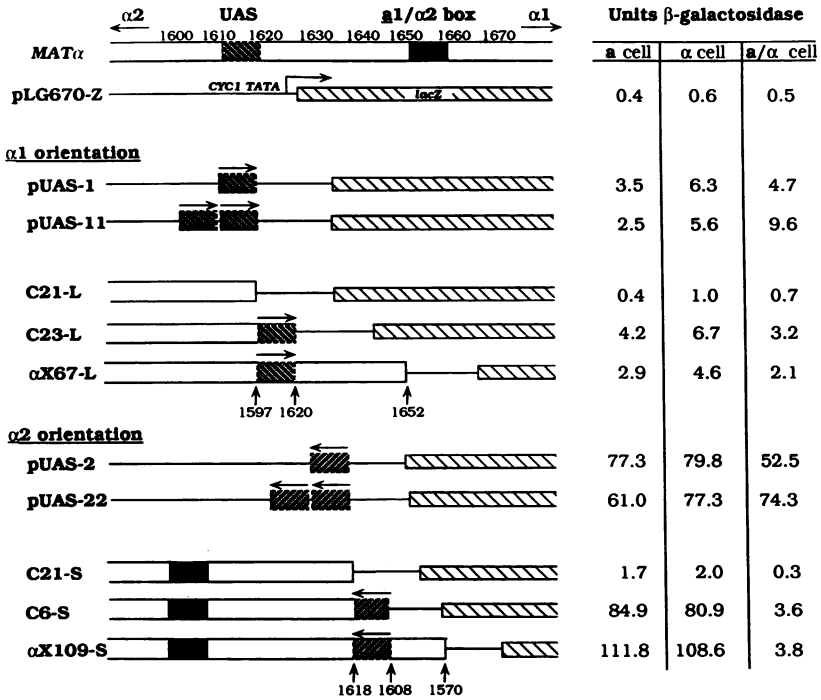

FIG. 2. Gene fusions between the *MATα* intergenic region and *CYC1-lacZ*. Structures of the *MATα* intergenic region and the promoterless *CYC1-lacZ* gene fusion are presented in the top two lines. β-Galactosidase assays are described in Materials and Methods. The hatched box represents the UAS, and the black box represents the a1/α2 binding site. In the α1 orientation, *lacZ* effectively replaces the *MATα1* gene with respect to the UAS. Likewise, in the α2 orientation, *lacZ* replaces *MATα2* with respect to the UAS. β-Galactosidase activities are representative of at least three experimental trials and were assayed in cells of all three types, a, α, and a/α. The higher level of β-galactosidase activity obtained in strains containing αX109-S are not significant since the increase was not found in other experiments.

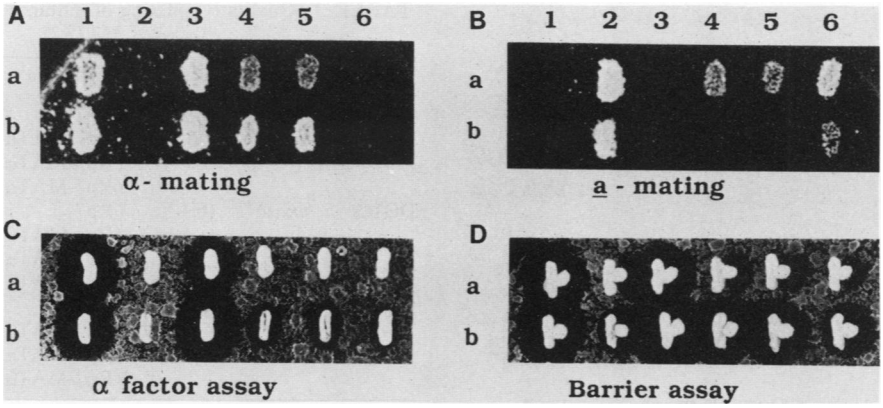

FIG. 3. Qualitative mating, α factor, and barrier assays of mutant strains. (A and B) Strains were overlayed with **a** and α tester strains 265.7.4 (**a**) and 265.5.4 (α) and were incubated at 30°C for 24 h; the plates replicated to minimal medium and allowed to grow overnight. The photographs illustrate the ability of strains to mate as α (A) and **a** (B). (C) Strains were patched onto an overlay of a culture sensitive to α-factor (strain G245-24a). Strains secreting α-factor produce a zone of growth inhibition (α-factor halo) around the culture. (D) Barrier activity was assayed by the ability of strains secreting barrier to inhibit the growth of an α-factor halo. A patch of α strain 80α52 was placed to the right of each strain to act as a source of α factor. Inhibition of the α-factor halo indicates the secretion of barrier. Strains in row a: 1, 23α67 (*MAT*α); 2, EG123 (*MAT***a**); 3, DG167 (*mat*α^{1615}); 4, DG168 (*mat*α^{1613}); 5, DG169 (*mat*α^{1617}); 6, DG170 (*mat*α^{1618}). Strains in row b: 1, DG101 (*MAT*α *bar1-1*); 2, G245-24a (*MAT***a** *bar1-1*); 3, DG110 (*mat*α^{1615} *bar1-1*); 4, DG115 (*mat*α^{1613} *bar1-1*); 5, DG116 (*mat*α^{1617} *bar1-1*); 6, DG145 (*mat*α^{1618} *bar1-1*).

intergenic region. Only the expected point mutation was observed.

The four mutations generated three phenotypic classes, as judged by mating behavior (Fig. 3 and Table 2). Strain DG167 (*mat*α^{1615}) mated as an α cell but with only 19% the efficiency of a congenic wild-type α strain. Strains DG168 (*mat*α^{1613}) and DG169 (*mat*α^{1617}) were bi-maters (bi); they mated both as **a** cells and as α cells with approximately 5 and 6% the efficiencies of the wild type, respectively. Strain DG170 (*mat*α^{1618}) mated exclusively as an **a** cell but with only 30% the efficiency of a congenic wild-type **a** strain.

*MAT***a**/*MAT*α diploid strains heterozygous for these mutations also had mutant phenotypes. A normal diploid is sterile but can undergo meiosis and sporulate. **a**/α diploid containing *mat*α^{1613} or *mat*α^{1617} continued to mate as **a** cells, albeit poorly, and were still able to sporulate. In contrast, α/α diploids heterozygous for *mat*α^{1613} or *mat*α^{1617} mated as wild-type α cells and could not sporulate because of the absence of the MAT**a**1 protein. Diploids resulting from conjugation between two bi-maters continued to mate as bi-maters and failed to sporulate.

DG170 (*mat*α^{1618}) mated only as an **a** cell (**a**-like faker [alf]). Two lines of evidence indicated that this mutant was not a null mutant. First, DG170 mated at only 30% the level of a wild-type *MAT***a** cell (Table 2) or a *MAT* deletion mutant (αX8) (data not shown). Second, a diploid constructed between DG170 and a wild-type **a** strain was still able to sporulate although it continued to mate as an **a** cell. Tetrad analysis of this cross yielded the expected four **a**-mating progeny.

A second RAP1 consensus sequence was found adjacent to the UAS at *MAT*α from bp 1619 to 1633 (Fig. 1). It diverged from the RAP1 consensus site at four base pairs and was not required for transcription (as shown by deletion analysis). To assess any possible function of this second site, point mutants were generated with a C-to-A transversion at bp 1629 (corresponding to the mutation causing the most severe phenotype in the UAS [*mat*α^{1618}]), coupled with the mutations in *mat*α^{1613}, *mat*α^{1615}, and *mat*α^{1617}. Since mating behavior was quite sensitive to changes in the level of *MAT*α expression (see below), we reasoned that any effects of these new mutations would be detectable in strains harboring the UAS point mutations. However, the double point mutants displayed the same decreases in mRNA levels, mating behavior, and mating efficiencies as the original point mutants (data not shown). Therefore, it is unlikely that this sequence plays a role in *MAT*α expression.

The bi-mating phenotypes of DG168 (*mat*α^{1613}) and DG169 (*mat*α^{1617}) suggested that both **a**- and α-specific gene products were being synthesized in these strains. This hypothesis was tested by assaying for an α-specific gene product, α-factor, and an **a**-specific protein, barrier (Fig. 3 and Table 2). Strains DG168, DG169, and DG170 (*mat*α^{1618}) did not secrete a detectable amount of α-factor but did produce barrier (Fig. 3 and Table 2). The α-mating strain DG167 (*mat*α^{1615}) produced an α-factor halo but at roughly half the size of a wild-type halo. Though this strain did not appear to produce barrier according to this assay, the introduction of a *bar1* (*sst1*) mutation, which eliminates the expression of barrier, allowed a strain containing the

TABLE 2. Mating efficiencies and phenotypes of *mat*α promoter mutants[a]

Strain	Relevant genotype	% **a**-Mating[b]	% α-Mating[b]	α-Factor halo	Barrier
EG123	*MAT***a** (**a**)	85	0	−	+
23α67	*MAT*α (α)	0	71	+	−
DG167	*mat*α^{1615} (α)	0	19	+	−
DG168	*mat*α^{1613} (bi)	6	5	−	+
DG169	*mat*α^{1617} (bi)	5	6	−	+
DG170	*mat*α^{1618} (alf)	31	0	−	+

[a] Cells were grown for 12 h at 30°C in YEPD medium; 2 × 10^6 cells of each experimental strain were mixed with an equal number of either an **a** or an α tester strain (80 or 80αX52) and placed onto a nitrocellulose filter. Filter matings were performed at 30°C in YEPD medium for 6 h before plating onto selective and rich media. α-Factor halo and barrier assays are shown in Fig. 3; see legend for experimental details.

[b] 100× (no. of diploids/total no. of cells in mating mix). Data represent averages of at least three experimental trials.

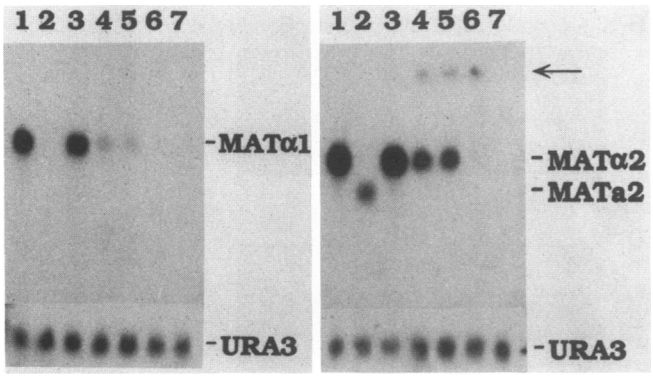

FIG. 4. Northern analysis of *MATα* UAS mutants. Total yeast RNA was subjected to Northern analysis as described in Materials and Methods. *MATα1* and *MATα2* mRNAs comigrate and were analyzed on separate blots. Blots were then stripped and reprobed for *URA3* mRNA to detect the relative amounts of RNA loaded in each lane. Lanes contain RNA from the following strains: 1, 23α67 (*MATα*); 2, EG123 (*MATa*); 3, DG167 (*matα*^{1615}); 4, DG168 (*matα*^{1613}); 5, DG169 (*matα*^{1617}); 6, DG170 (*matα*^{1618}); 7, αX8 (*matαΔ8*). RNA marked with arrow is detected in strains with a crippled or deleted *MATα* UAS and is probably the same as that characterized by Siliciano (43a) for other *matα* deletion strains. It is of a length consistent with transcription initiating in the *MATα1* gene and proceeding through the *MATα2* gene.

matα^{1615} mutation (DG110) to form an α-factor halo comparable to that of the wild type. Also, the mating efficiency of DG110 (*matα*^{1615} *bar1-1*) was equal to that of the wild type, a fourfold increase over the value for original mutant strain (data not shown). The *bar1* (*sst1*) mutation also had a dramatic effect on strains containing the *matα*^{1613} and *matα*^{1617} mutations. The *bar1* (*sst1*) mutation not only allowed these strains to produce an α-factor halo but also caused them to mate exclusively as α cells (Fig. 3 and Table 2). Nevertheless, they continued to express the **a**-specific gene *STE2* (data not shown). Strains containing the *matα*^{1618} mutation and the *bar1* mutation continued to mate as **a** cells and did not produce an α-factor halo.

Mutations in the UAS reduce the level of *MATα1* and *MATα2* mRNAs. *MATα1* and *MATα2* mRNA levels were assayed in the point mutants by Northern analysis as described in Materials and Methods. The level of *MATα* mRNA correlated well with the mating phenotype (Fig. 4). The nearly silent mutant DG167 (*matα*^{1615}) (Fig. 4, lane 3) contained levels of *MATα1* and *MATα2* mRNAs indistinguishable from the wild-type levels (Fig. 4, lane 1). Bi-mating mutants DG168 (*matα*^{1613}) and DG169 (*matα*^{1617}) showed a significant reduction of steady-state mRNA levels (Fig. 4, lanes 4 and 5); densitometric analysis of the autoradiograms indicated at least a three- to fivefold reduction in the *MATα* message levels. *MATα1* and *MATα2* mRNAs in the **a**-like faker strain, DG170 (*matα*^{1618}), were not detected (Fig. 4, lane 6). All point mutations in the *MATα* UAS affected the expression of both *MATα1* and *MATα2*, consistent with the hypothesis that a single protein or protein complex is responsible for the transcriptional activation of *MATα*.

Increased expression of either *MATα1* or *MATα2* is sufficient to eliminate a-mating of bi-maters and the a-like faker. The results of the Northern blot analysis suggested that relatively small perturbations in the expression of *MATα* had significant effects on mating behavior. However, it was unclear whether lowering the expression of either *MATα1* or *MATα2* alone was sufficient to cause these effects. To address this issue, plasmids containing only *MATα1* or *MATα2* were transformed into mutant strains DG168 (*matα*^{1613}), DG169 (*matα*^{1617}), and DG170 (*matα*^{1618}), and qualitative mating tests were conducted with the resulting transformants. The bi-mating phenotype of mutant strains DG168 and DG169 required reduced expression at both *MATα1* and *MATα2*; supplying either gene in *trans* suppressed the bi-mating phenotype and increased α-mating (Table 3). Surprisingly, *MATα1* or *MATα2* in high copy number conferred α-mating on mutant strain DG170 (*matα*^{1618}). In each case, the mating efficiency was low, but α-mating was clearly restored. Overexpression of *MATα2* abolished **a**-mating in DG170 as expected, but overexpression of *MATα1* did not. That this mutant required increased expression of only one of the two genes of *MATα* to mate as an α cell provides additional evidence that this mutation is not a null, despite our inability to detect mRNA by Northern blot analysis (Fig. 4).

TABLE 3. Qualitative mating of mutants overexpressing *MATα1* or *MATα2*[a]

Strain	Relevant genotype	Plasmid	a-Mating	α-Mating
DG168	*matα*^{1613} (bi)	YRp7	+	+
		YRp7-MATα	−	+++
		YRp7-MATα1	−	++
		YRp7-MATα2	−	++
DG169	*matα*^{1617} (bi)	YRp7	+	+
		YRp7-MATα	−	+++
		YRp7-MATα1	−	++
		YRp7-MATα2	−	++
DG170	*matα*^{1618} (alf)	YRp7	++	−
		YRp7-MATα	−	+++
		YRp7-MATα1	+/−	+
		YRp7-MATα2	−	+

[a] Experimental strains were mated to tester strains 80 and 80α52. Matings were conducted on YEPD medium for 12 to 16 h before replica plating to minimal medium lacking tryptophan, lysine, and uracil. Plates were incubated at 30°C for 24 h. Residual (+/−) mating was observed only after 48 h at 30°C.

Overproduction of RAP1 suppresses the bi-mating phenotype. As discussed above, the bi-mating phenotype of DG168 (*matα*^{1613}) and DG169 (*matα*^{1617}) appeared to be extremely sensitive to the level of *MATα* expression; an increased dosage of either *MATα1* or *MATα2* eliminated the bi-mating phenotype. We therefore asked whether increased dosages of any genes in addition to *MATα* could suppress the bi-mating phenotype. Strains DG168 and DG169 were transformed with a yeast genomic library in the multicopy shuttle vector YEp13. Transformants with an increased level of α-mating were observed at a frequency of 10^{-3} to 10^{-4}. The plasmids responsible for the α-mating phenotype were recovered from the yeast cells and retransformed into the bi-mating strains to confirm the ability of the plasmids to suppress the bi-mating phenotype. Plasmids containing the *MATα* locus were eliminated by Southern hybridization using a *MATα*-specific probe. Four plasmids were obtained that did not cross-hybridize with *MATα* but conferred an increased level of α-mating and a low level of **a**-mating. Our analysis of these plasmids is ongoing, but the plasmid that conferred the greatest α-mating ability, 13H3, was found to contain the structural gene for the RAP1 protein. 13H3 contained restriction endonuclease fragments that comigrated and cross-hybridized with fragments from the *RAP1* gene (generously provided by David Shore). Tn*5* transposon insertions that eliminated the ability of 13H3 to suppress

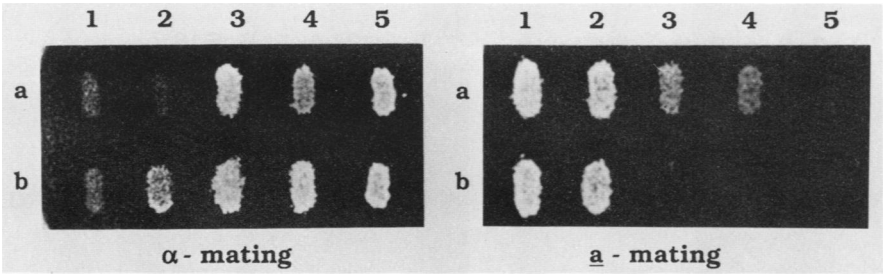

FIG. 5. Qualitative mating assays of strains overexpressing RAP1. Assays were conducted as described for Fig. 3 except that the selective medium was yeast minimal medium lacking leucine, uracil, and lysine, and tester strains were 80 (**a**) and 80α52 (α). Row a represents strains transformed with YEp13; row b represents strains transformed with 13H3. Strains are numbered as follows: 1, αX8 (*matαΔ8*); 2, DG170 (*matα*^{1618}); 3, DG169 (*matα*^{1617}); 4, DG168 (*matα*^{1613}); 5, 23α67 (*MATα*).

bi-mating mapped within the *RAP1* coding sequence. 13H3 also complemented the growth defect of a temperature-sensitive *rap1* strain, grc4. Furthermore, it increased α-mating and eliminated **a**-mating in the bi-mating strains DG168 and DG169 and conferred a weak bi-mating phenotype to the **a**-like faker, DG170 (*matα*^{1618}) (Fig. 5). However, it had no effect on the **a**-mating phenotype of a *MATα* deletion strain αX8. Northern analysis of strains DG168, DG169, and DG170 transformed with 13H3 showed that the presence of this plasmid increased both *MATα1* and *MATα2* mRNA levels (Fig. 6). Densitometric analysis revealed that plasmid 13H3 causes at least a twofold increase in mRNA levels in both bi-mating strains.

A temperature-sensitive *rap1* mutation reduces expression from *matα*^{1613} and *matα*^{1617}. If the *RAP1* gene product acts at the *MATα* locus in vivo, mutations in *RAP1* might be expected to affect mating. To test this possibility, a temperature-sensitive *rap1* mutant (DG175) isogenic to EG123 was mated to a series of strains containing different *MATα* alleles and analyzed by tetrad analysis. Strains containing this temperature-sensitive *rap1* allele fail to grow above 35°C and will be characterized in detail elsewhere (45a). The mating phenotypes of representative strains are presented in Fig. 7. We observed that all meiotic progeny that contained both the *rap1* mutation and either of the bi-mating *MAT* mutations (*matα*^{1613} and *matα*^{1617}) mated strictly as **a** cells (Fig. 7b). *rap1* mutants with a wild-type MATα allele acquired a bi-mating phenotype at the semipermissive temperature of 34°C (Fig. 7d). The mating defect of *rap1* strains was α cell specific; no mating defect has been observed for **a** cells. Interestingly, we observed that the bi-mating mutations

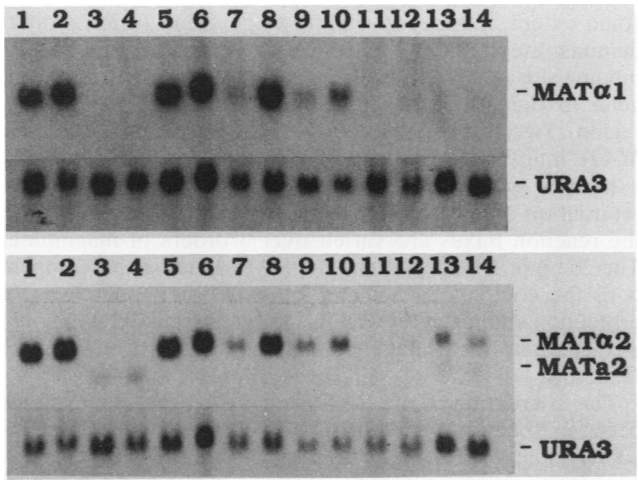

FIG. 6. Northern analysis of strains overexpressing RAP1. Analysis was conducted as described for Fig. 4. Odd-numbered lanes contain RNA from strains transformed with YEp13; even-numbered lanes contain RNA from strains transformed with 13H3. Lanes contain RNA from the following strains: 1 and 2, 23α67 (*MATα*); 3 and 4, EG123 (*MATa*); 5 and 6, DG167 (*matα*^{1615}); 7 and 8, DG168 (*matα*^{1613}); 9 and 10, DG169 (*matα*^{1617}); 11 and 12, DG170 (*matα*^{1618}); 13 and 14, EG123 × 23α67 diploid.

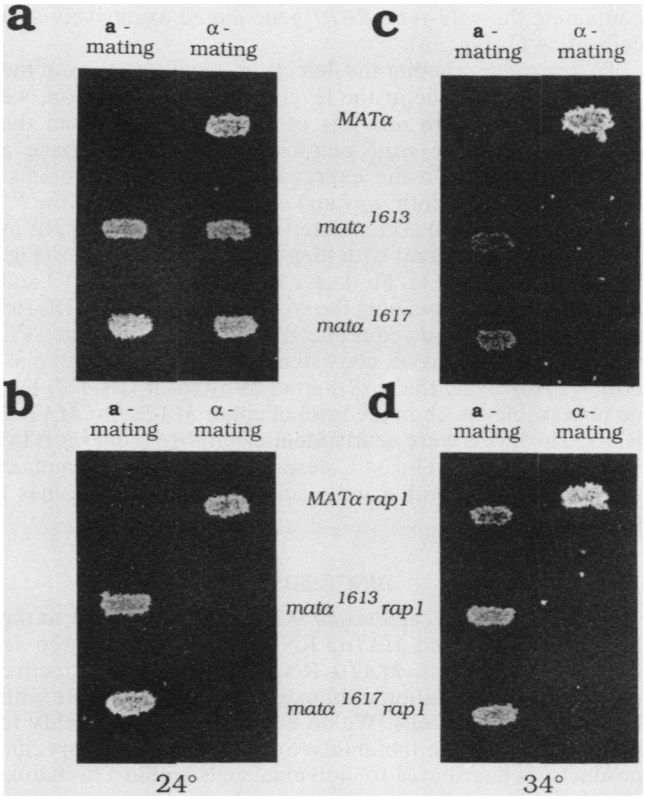

FIG. 7. Qualitative mating assays of strains temperature sensitive for RAP1. Assays were conducted as described for Fig. 3 except the mating events took place at either 24°C (a and b) or 34°C (c and d). (a and c) Parental strains 246.1.1 (*MATα*), DG168 (*matα*^{1613}), and DG169 (*matα*^{1617}); (b and d) the same strains in an isogenic *rap1* background, DG175 (*MATα rap1*), DG184 (*matα*^{1613} *rap1*), and DG174 (*matα*^{1617} *rap1*).

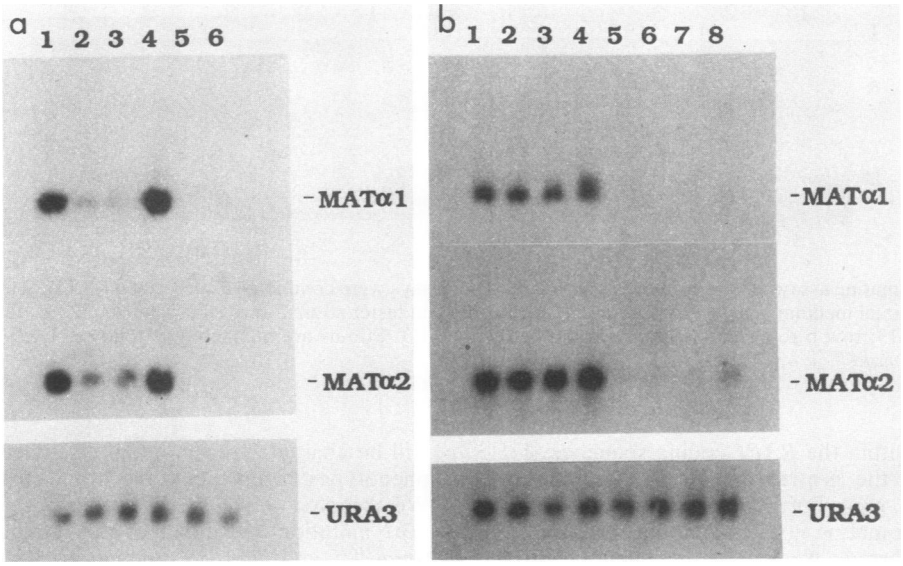

FIG. 8. Northern analysis of strains temperature sensitive for RAP1. Analysis was conducted as described for Fig. 4, but RNA was isolated at 24 or 34°C, and a single blot was probed with α1, stripped, and reprobed for α2 and then URA3. Lanes in panel a contain RNA from the following strains, all isolated at 24°C: 1, 246.1.1 (*MAT*α); 2, DG168 (*matα*^{1613}); 3, DG169 (*matα*^{1617}); 4, DG175 (*MAT*α *rap1*); 5, DG184 (*matα*^{1613} *rap1*); 6, DG174 (*matα*^{1617} *rap1*). In panel b, odd-numbered lanes represent RNAs isolated at 24°C and even-numbered lanes represent RNAs isolated at 34°C. Lanes contain RNA from following strains: 1 and 2, 246.1.1; 3 and 4, DG175; 5 and 6, DG184; 7 and 8, DG174.

containing the wild-type *RAP1* gene mated exclusively as **a** cells at 34°C (Fig. 7c).

To determine whether the defects in α-mating ability of the *rap1* (Ts) mutants lie at the level of *MAT*α expression, we conducted Northern analysis of RNA prepared from the above strains. The *rap1* mutation was found to have a synergistic effect on the expression of the *mat*α mutants. Strains containing both *rap1* and either *matα*^{1613} or *matα*^{1617} (DG184 and DG174) accumulated little if any *MAT*α RNA at 24 and 34°C, consistent with their **a**-mating phenotypes (Fig. 8a, lanes 5 and 6; Fig. 8b, lanes 5 through 8). *matα*^{1613} and *matα*^{1617} strains containing the wild-type *RAP1* gene (DG168 and DG169) also showed reduced *MAT*α expression at 34°C (data not shown), again consistent with their **a**-mating phenotype. However, the *rap1* mutation by itself (DG175) had no observable effect on the level of either *MAT*α*1* or *MAT*α*2* RNA. The levels were nearly identical in *RAP1* and *rap1*(Ts) strains grown at 24 and 34°C despite the fact that the mutant strain mated with both **a** and α cells at 34°C (Fig. 8b, lanes 1 through 4).

DISCUSSION

Mating type in *S. cerevisiae* is extremely sensitive to the levels of *MAT*α*1* and *MAT*α*2* RNAs. As little as a three- to fivefold reduction in *MAT*α RNA results in a distinctive phenotype: a population of bi-mating cells, which mate with both **a** cells and α cells. We do not know how the ability to mate, and therefore the ability to express **a**- and α-specific products, is distributed to individual cells within a bi-mating population. *matα2* mutants express both **a**- and α-specific genes but, in contrast to bi-mating strains, are sterile. It is likely that the higher levels of expression of **a**- and α-specific genes in the *matα2* mutants result in sterility. Since the total level of mating in the bi-mating strains is little more than 10% of the wild-type level, it is possible that most of the cells in these strains are also sterile. While reduced *MAT*α*2* expression results in partial activation of **a**-specific genes (barrier and α-factor receptor), a similar reduction of *MAT*α*2* expression in diploids (Fig. 6, lane 14) does not lead to the appearance of these **a**-cell-specific products. We do not have an explanation for this discrepancy.

*MAT*α gene expression is reduced or abolished by single base pair changes in the *MAT*α UAS. This UAS coincides with the consensus sequence for the DNA-binding protein RAP1 and is the only upstream sequence required for transcription at *MAT*α, although a sequence adjacent to the *MAT*α UAS is required for the repression of *MAT*α*1* (and to some extent *MAT*α*2*) in diploid cells. Gene fusion studies demonstrate that 20 bp of DNA surrounding the RAP1 binding site at *MAT*α is sufficient to activate a UAS-deficient gene to the same extent as the entire *MAT*α intergenic region. Two hundred base pairs of labeled DNA from the *MAT*α intergenic region, incubated with crude cell extracts, exhibit a single high-molecular-weight complex in a gel retardation assay even when the protein concentrations in the reaction mixes are varied over 3 orders of magnitude. The 20 bp of DNA encoding the RAP1 binding site compete with the complex in a molar ratio of 1:1 (13a). DNase I protection studies of the *MAT*α intergenic region reveal only a single footprint which encompasses the RAP1 binding site (5, 13a).

The orientation of the UAS element, but not the distance between the UAS and the *CYC1* TATA element, significantly affects the level of transcriptional activation; in the orientation associated with *MAT*α*2* expression, the UAS stimulates transcription six- to sevenfold more than in the opposite orientation. Two copies of the UAS, in either orientation, do not increase the level of transcription above that obtained for a single copy. This is in contrast to results obtained by Woudt et al. (55). A Northern analysis of the *galK* gene under the transcriptional control of the *L25* upstream region displayed the same polarity of transcrip-

tional activation as the *MATα* UAS. However, two copies of the RPG box placed head to tail in the less active orientation stimulated transcription as well as did a single copy in the more active orientation. Two important differences between the experiments of Woudt et al. and those presented in this report may explain the discrepancy. First, the synthetic UAS or RPG box used in the *L25-galK* fusion (5′-CCCAT ACATTT-3′) is an abbreviated form of the RAP1 consensus binding site and differs in both length and sequence from the synthetic *MATα* UAS. Also, the distance between adjacent RAP1 sites in the *L25-galK* fusions is 12 bp less than in the *MATα* fusions.

In other systems, RAP1 binding sites exist in the context of additional *cis*-acting sequences. Deletion analyses of these regulatory regions reveal that the RAP1 binding sites are often important, though not necessarily sufficient, for proper gene regulation. The upstream control regions of genes such as *PGK* (which encodes phosphoglycerate kinase), *RNR2* (which encodes ribonucleotide reductase), and ribosomal protein genes require a RAP1 binding site for full transcriptional activity (8, 12, 24, 29, 31), but the RAP1 site is not sufficient to regulate expression (8, 12, 24, 39). The silent *MAT* loci, *HMR* and *HML*, depend on the RAP1 consensus sequences to maintain full transcriptional repression but also require at least one additional DNA sequence element to remain transcriptionally silent (43).

Several lines of evidence support the hypothesis that RAP1 binds to and functions at the *MATα* upstream region in vivo. First, a good correlation exists between the steady-state levels of *MATα1* and *MATα2* mRNAs expressed from mutant *MATα* loci and the affinity of mutant sites for the binding factor in vitro. Vignais et al. (53) have isolated 30 point mutations in the optimal binding sequence for RAP1 (TUF) and determined the equilibrium dissociation constant for each of the mutant binding sites. Their sequence (1-ACACCCATACATTT-14) differs from the RAP1 binding site at MATα (1-AAACCCAGACATC-13) at three positions. Vignais et al. have directly tested two of these differences (positions 2 and 13) and find no effect on binding affinity. The third difference, which corresponds to *matα*^{1615}, has no mating defect. However, a mutant sequence in which an A replaces the central C of the conserved C triplet has a very low affinity for the binding factor. This change is analogous to the mutation in *matα*^{1618}, which almost completely abolishes expression of *MATα*. A similar mutation at the *HMR* E site (known to bind GRF1) also abolishes binding in vitro and transcriptional activation in vivo (5). A point mutation at bp 1615 has no effect on binding affinity, and strains containing this mutation have almost normal levels of *MATα* expression. The binding affinities of point mutations analogous to those which cause bi-mating (*matα*^{1613} and *matα*^{1617}) are reduced to 15% of the wild-type level. *MATα* expression is reduced to 20 to 30% of the wild-type level in bi-mating strains. It should be noted that Nieuwint et al. made point mutations in the optimal binding site for RAP1 (TUF), in the context of the ribosomal protein gene L25, and examined the effects on mRNA levels in vivo (35). Their assay suggests that a C-to-A transversion at the position equivalent to *matα*^{1613} has no effect on mRNA levels in vivo. However, there are several important differences between their assay and ours. They monitor mRNA levels of a plasmid-born reporter gene (*galK*) under the transcriptional control of a synthetic RPG box. There is also an additional *cis* element present in the upstream region of their gene fusion, the pyrimidine stretch or T-rich region, which is thought to be involved in the transcriptional activation of ribosomal protein genes (39, 55). It may be that interactions between the RPG box and the pyrimidine stretch mask the effects of changes at this particular base pair. Results obtained by Nieuwint et al. for changes at positions equivalent to *matα*^{1617} and *matα*^{1618} are in good agreement with both our results and those obtained by Vignais et al. (53). We therefore suggest that the strong correlations between the in vitro binding affinities (53) and our in vivo studies strengthen the hypothesis that RAP1 binds to the *MATα* UAS in vivo.

The second line of evidence supporting an in vivo role for RAP1 stems from the observation that the *RAP1* gene influences the level of expression of *MATα* in the bi-mating *matα* mutants. Increased gene dosage of *RAP1*, from a multicopy plasmid, restores α-mating to the bi-mating strains (*matα*^{1613} and *matα*^{1617}) and, to a lesser extent, the **a**-like faker strain (*matα*^{1618}). Steady-state levels of *MATα1* and *MATα2* mRNAs are increased in such strains. Although other explanations are possible, we favor the hypothesis that the restoration of α-mating to these strains is accomplished by increasing the concentration of RAP1 in the cell, thus restoring the binding of RAP1 to those sites with reduced affinity. In contrast, a temperature-sensitive mutation in *rap1*, which presumably alters or reduces the activity of RAP1, essentially eliminates *MATα* expression from *matα*^{1613} and *matα*^{1617}.

We and others (28a) have observed that temperature-sensitive *rap1* mutants have a bi-mating phenotype at elevated temperatures. Surprisingly, no reduction in *MATα* RNA has been observed under conditions in which the mating defect is observed. We have no explanation for this discrepancy, but it stands to warn against proposing too simple a model for the role of RAP1 in *MATα* regulation. It is possible that RAP1 plays additional roles in mating-type regulation, acting on genes downstream in the pathway. However to our knowledge, no genes in the mating pathway other than *MAT*, *HMR*, and *HML* are known to contain a RAP1 binding site.

Is RAP1 the only gene product that acts to direct the transcriptional machinery at *MATα*? Fassler and Winston (13) and Nishizawa et al. (37) have shown that the *MATα* mRNA is decreased in a *spt13* (*gal11*) mutant. The SPT13 (GAL11) protein could possibly bind to MATα, overlapping RAP1 or TATA binding sites, or it could, as proposed by Nishizawa et al. (37), act to facilitate the interaction between the upstream regulatory proteins, in this case RAP1, and the TATA binding factor.

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