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Role of α2 Protein in Donor Locus Selection during Mating Type Interconversion

LISA SZETO AND JAMES R. BROACH*

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544-1014

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The homeodomain protein α2p plays a role both in transcriptional repression in the process of cell type determination and in donor selection during mating interconversion. We have explored the mechanism of α2p-directed donor selection by examining the effects on donor preference of mutants deficient in α2p-mediated transcriptional repression. As a transcriptional regulator, α2p interacts with Mcm1p, Tup1p, and Ssn6p to repress a-specific genes and with a1p, Tup1p, and Ssn6p to repress haploid-specific genes. We have found that mutant alleles of *MATα2* that specifically diminish the interaction of α2p with Mcm1p or Tup1p behave as null alleles with regard to donor preference, while mutations of *MATα2* that specifically diminish interaction of α2p with a1p behave as wild-type *MATα2* in this capacity. Tup1p plays an essential role in α2p-mediated transcriptional repression, while Ssn6p has only a modest effect in repression. In a similar vein, we find that *TUP1*, but not *SSN6*, is required for proper donor selection. These results suggest that, in addition to regulating a-specific gene expression to establish the mating type of the cell, α2p-Mcm1p-Tup1p complex may indirectly regulate donor preference through transcriptional control of an a-specific gene. Alternatively, this complex may play a direct role in establishing donor preference via its DNA binding and chromatin organization capacity.

Mating type interconversion in *Saccharomyces cerevisiae* provides a mechanism to allow single haploid cells to become diploid (reviewed in reference 7). In strains carrying the *HO*, or homothallism, allele, a single haploid cell of one mating type gives rise to four granddaughter cells, two of the same mating type as the initial cell and two of the opposite mating type. The pairs of opposite mating type cells can then mate to yield two diploid progeny of the initial haploid cell. Since diploids are more resistant to stress and have access to survival strategies unavailable to the haploid—pseudohyphal growth to facilitate foraging or sporulation as a response to starvation or desiccation—mating type switching is critical to the persistence of the organism.

The mating type of a haploid yeast cell is determined by the allele present at the mating type locus, *MAT*, on chromosome III (7). Cells carrying the *MAT***a** allele have an **a** mating type while cells carrying the *MAT*α allele have an α mating type. Mating type interconversion results from replacement of the allele resident at *MAT* with a copy of the opposite mating type information located in storage loci, *HMR* and *HML*, located near the right and left ends of chromosome III, respectively. The mating type genes resident at these loci do not contribute to the phenotype of the cell, since they are maintained in a transcriptionally inactive state (16). Mating type switching is initiated when the product of the *HO* gene, a site-specific endonuclease, induces a double-strand DNA cleavage within the *MAT* locus (14, 15, 27). The double-strand break at *MAT* is then repaired by gene conversion, using either *HML* or *HMR* as the template, resulting in the unidirectional transposition to *MAT* of the mating type information resident at the selected donor locus.

Mating type interconversion is highly regulated. Switching is initiated only in cells that have budded at least once and only during the G_1 phase of the cell cycle. These constraints on switching arise from transcriptional regulation of *HO*; the gene is expressed only in G_1 and in mother but not daughter cells (1, 19, 23). In addition, donor locus selection is controlled by the mating type of the cell: **a** cells select *HML* as donor in greater than 80% of switching opportunities while α cells select *HMR* as donor in greater than 95% of the possible events (11, 33). Since *HML* usually carries an α allele and *HMR* usually carries an **a** allele, this bias in donor preference dictates that most *HO*-initiated events result in a change in mating type. In total, these constraints on switching ensure that the second-generation progeny of any haploid homothallic cells will almost invariably consist of two pairs of cells of opposite mating type (26).

The fact that yeast cells exhibit cell-specific donor preference in the process of mating type interconversion indicates that the organism can promote selective interaction between *MAT* and one or the other donor locus as a function of the mating type of the cell. This implies that the two donor loci possess some distinguishable feature that is recognized in a cell-specific manner. Neither the critical distinguishing feature of the donor loci nor the apparatus responsible for making the distinction has been defined (32, 33). However, of the three transcriptional regulators—**a**1p, α1p, and α2p—encoded by the two mating type alleles, only α2p plays a role in donor selection (8, 29, 34). That is, strains that lack α2p, e.g., *MAT***a** cells or *matα2*$^-$ cells, preferentially select *HML* as donor. In contrast, cells expressing α2p select *HMR* preferentially as donor, even in cells in which other mating type regulators are concurrently expressed.

In its capacity as a regulator of mating type in yeast, α2p functions as a repressor to prevent transcription of two distinct sets of cell type genes (Fig. 1). In **a** cells, Mcm1p, the constitutively expressed MADS box protein, acts as a transcriptional activator of **a**-specific genes by binding as a dimer to a dyad Mcm1p binding site upstream of the promoters for these genes (20). In α cells, α2p represses transcription of these **a**-specific

* Corresponding author. Phone: (609) 258-5981. Fax: (609) 258-6175. E-mail: jbroach@watson.princeton.edu.

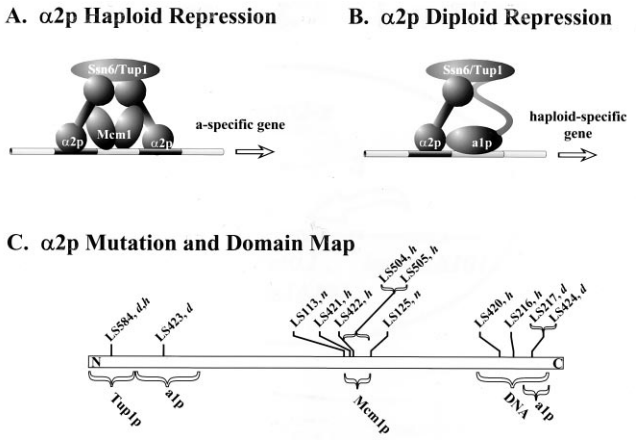

FIG. 1. Domains of the α2 protein. (A and B) Schematic diagram of the interactions of α2p with Mcm1p, Tup1p, and Ssn6p in repression of **a**-specific genes (A) and with **a**1p, Tup1p, and Ssn6p in repression of haploid-specific genes (B). In the former case, α2p and Mcm1p bind as a heterotetramer to a partially symmetrical 31-bp sequence, composed of a central core Mcm1p binding site [encompassing a consensus $CC(A/T)_6GG$ sequence] bracketed by symmetrical 9-bp α2p binding sites (containing a consensus TGTA sequence in each site). Binding is stabilized by α2p-Mcm1p interaction through a flexible linker domain separating the DNA binding domain from the Tup1p-Ssn6p interaction domain. In the latter case, α2p and **a**1p bind to an asymmetric sequence consisting of a single **a**1p binding site and an adjacent α2p binding site. Binding is stabilized by interactions between **a**1p and α2p through both the amino- and carboxyl-terminal domains of α2p. In both cases, the bound complex recruits the Tup1p-Ssn6 general repression complex through interaction between the WD40 repeats of Tup1p and a domain within the amino-terminal region of α2p. (C) Linear map of α2p indicating the positions of mutations in alleles of *α2* carried in the designated strains used in this study (above the map) and the functional domains of the protein (below the map). *h*, mutation that abolishes **a**-specific gene repression but retains haploid-specific gene repression; *d*, mutation that abolishes haploid-specific gene repression but retains **a**-specific gene repression; *n*, silent mutation.

genes by binding to two inversely oriented sites immediately flanking the Mcm1p binding site (9, 22). Binding of α2p to these sites, and resultant repression, is stabilized by direct interaction between Mcm1p and an unstructured, hydrophobic central domain of the α2p protein (9, 18). In diploid **a**/α cells, α2p protein also combines with **a**1p to bind as a heterodimer to sites upstream of haploid-specific genes (*hsg*), such as *HO*, and represses expression of these genes (4, 5). Interaction with **a**1p required for *hsg* repression occurs through the C-terminal region of α2p, distinct from the domain involved in Mcm1p association. Both Mcm1p/α2p-mediated repression of **a**-specific genes and **a**1p/α2p-mediated repression of haploid-specific genes result from recruitment of the general repression complex, Tup1p-Ssn6p (10). Recruitment is accomplished by direct binding of the WD40 repeat domain of Tup1p to the amino-terminal region of α2p (13). Thus, point mutations in the Tup1p-interacting domain of α2p alleviate repression of **a**-specific genes in α cells and haploid-specific genes in **a**/α diploids. Similarly, α2p-mediated repression is fully alleviated in *tup1* strains and partially alleviated in *ssn6* strains (3, 10).

In this work, we have dissected the role of α2p in the mechanism of donor selection in α cells. By determining the donor preference of strains carrying defined *matα2* point mutations, we have been able to establish a correlation between mutations in α2p that are unable to repress **a**-specific genes and those that cause α cells inappropriately to use *HML* as a donor during switching. That is, mutations of *MATα2* that disrupt the interaction of α2p with its DNA target site, with Mcm1p, or with Tup1p abolish normal donor preference. In contrast, alleles of *MATα2* that disrupt the interaction of α2p with **a**1p, needed for repression of haploid-specific genes, do not affect donor preference. We have concluded from these observations that α2p functions in donor locus selection in a manner similar to the way it functions in repression of **a**-specific genes. Two possible models are presented to explain this result. In one, α2p represses an **a**-specific gene that is responsible for the selection of *HML* (or inhibition of *HMR* selection). In the other, α2p acts directly, in conjunction with Mcm1p and Tup1p, to block selection of *HML*.

MATERIALS AND METHODS

Strains and plasmids. Table 1 provides a list of the yeast strains used in this work, which were obtained as follows. We constructed strain Y2200 to test the effect on donor preference of different *matα2* mutant alleles. The strain was derived from strain Y1014 (*hmlα1*$^+$ *α2*$^-$ *HMR***a** *HO*), which carries a frameshift mutation at codon 26 in the *hmlα2* coding region, by a two-step replacement of *HMR***a** with an allele consisting of both the wild-type *HO* cutting site and a deletion of the **a***1* coding region. First, *HMR***a** was deleted from strain Y1014 by transforming the strain to Leu$^+$ with a 5.3-kb *Hin*dIII fragment from plasmid pLS16. This fragment spans the *HMR* locus in which the X, Ya, and Z1 regions are precisely replaced by the yeast *LEU2* gene, derived by insertion of a 2.2-kb *Sal*I fragment from plasmid pLK9 spanning the *LEU2* gene into the *Xho*I site of plasmid pKSW5 (32). One such Leu$^+$ transformant, designated strain LS67, was transformed to Ura$^+$ with plasmid pLS17 digested with *Mlu*I to yield strain LS77. Plasmid pLS17 consists of plasmid YIp5 containing a *Hin*dIII-*Eco*RI fragment spanning the *hmr***a***1Δ101* allele. The **a***1Δ101* allele was created by the Promega Altered Sites Mutagenesis Kit, according to the instructions provided, with the oligonucleotide 5′-GTTCTTTCGGGGAATTCAAGTAAGAGTTTGGG-3′ to introduce within the **a***1* gene a 462-bp deletion marked with an *Eco*RI recognition site. The deletion is restricted to the **a***1* coding region and does not impinge on the *HO* recognition site. Excision of the integrated pLS17 plasmid to leave the **a***1Δ101* allele in place of *hmr*::*LEU2* was accomplished by identifying 5-fluoro-orotic acid-resistant derivatives of strain LS77 that were also Leu$^-$ Ura$^-$. One such isolate was confirmed by PCR to carry the *hmr***a***1Δ101* allele and was designated strain Y2200.

The cloned *α2* alleles were kindly supplied by J. Strathern, J. Mead, A. Vershon, and A. Johnson. Except for the $\alpha2^{L10S}$ allele, all the alleles were cloned as *Eco*RV-*Eag*I fragments into the *URA3-CEN4* plasmid YCp50 digested with *Eag*I and *Nru*I. The *α1* gene does not reside on this fragment. The $\alpha2^{L10S}$ allele was obtained from plasmid pKK63, which carries the *MAT*α locus with the $\alpha2^{L10S}$ allele and a deletion allele of the *α1* gene, created by excision of a 0.7-kb *Nde*I fragment internal to the α1 gene. A *Hin*dIII fragment from plasmid pKK63 spanning this *mat*α1Δ$\alpha2^{L10S}$ was ligated into the *Hin*dIII site of plasmid YCp50 to create plasmid pLS34. All α2 alleles were sequenced after final recloning to confirm the presence of the single, expected mutation. As control strains for these experiments, we introduced plasmid YCp50 into strain Y2200 to create strain LS133 (*MATα2* null allele) and the YCp50-*MATα2* plasmids pLS20 and pLS21 into strain Y2200 to create strains LS419 and LS425 (*MATα2*$^+$ allele), respectively. Plasmids pLS20 and pLS21 both contain an *Eco*RV-*Eag*I fragment spanning *MATα2* cloned into YCp50 as above, with pLS20 containing the wild-

TABLE 1. Strains used in this study

Strain	Genotype	Reference
Y1014	(*matα2 MAT***a**) *hmlα2 HMR***a***-stk ura3-52 leu2-3,112 his3Δ1 trp1-289 HO mfα1-lacZ*	32
LS67	Y1014 *hmr*Δ::*LEU2*	This study
LS77	Y1014 *hmr*Δ::*LEU2*-pUC-*URA3-hmr***a***1Δ101*	This study
Y2200	(*matα2 mat***a***1Δ101*) *hmlα2 hmr***a***1Δ101 ura3-52 leu2-3,112 his3Δ1 trp1-289 HO mfα1-lacZ*	This study
Y2201	*MAT***a**/*MATα hmlα1*$\alpha2_{inc}$/*hmlα1*$\alpha2_{inc}$*hmr***a***1Δ101*$_{inc}$/*hmr***a***1Δ101*$_{inc}$ *ura3-52*/*ura3-52 leu2-3,112*/*leu2-3,112 his3Δ1*/*his3Δ1 trp1-289*/*trp1-289 HO*/*HO*	33
LS616	Y2201 *tup1*::*URA3*/*TUP1*	This study
Y2202	LS616 *MAT***a**::*HIS3*/*MATα*	This study
Y2203	LS616 *MAT***a**/*MATα*::*HIS3*	This study
LS617	Y2201 *ssn6*::*URA3*/*SSN6*	This study
Y2204	LS617 *MAT***a**::*HIS3*/*MATα*	This study
Y2205	LS617 *MAT***a**/*MATα*::*HIS3*	This study
Y2206	Y2201 *mat***a***2Δ1*/*MATα*	This study
Y2207	Y2201 *mat***a***2-2*/*MATα*	This study

type *MAT*α*2* sequence and pLS21 containing a version carrying a number of silent mutations introduced for cloning purposes (18).

The construction of strain Y2201, used as a platform for analyzing the effects of mutations in various chromosomal genes on donor preference, has been described elsewhere (33). This strain carries the **a***1*Δ*101* allele at *HMR* and an α*1*α*2* deletion allele at *HML* (distinct from the α*2* frameshift allele carried in strain Y2200). Both donor loci also carry an *inc* mutation, which prevents cleavage of the allele by *HO*. The *inc* allele at *HML* was obtained by gap repair of strain K673 and provided by Amar Klar (32). The *inc* allele at *HMR* was introduced during mutagenesis creating the **a***1*Δ*101* allele and consists of C-to-T mutation at position 6 and an A-to-T mutation at position 7 of the Z1 domain. To disrupt *TUP1*, strain Y2201 was transformed to Ura$^+$ with a *Pvu*II fragment of plasmid pKK365, which spans a deletion allele of *TUP1* marked with *URA3*. One such transformant was retained as strain LS616. To disrupt *SSN6*, strain Y2201 was transformed to Ura$^+$ with a *Sal*I-*Sph*I fragment of plasmid pKK436, which spans a deletion allele of *SSN6* marked with *URA3*. One such transformant was retained as strain LS617. To facilitate mating type identification in pedigree analysis, we inserted the *HIS3* gene immediately adjacent to the *MAT* locus in strains LS616 and LS617. This was accomplished by circular integration of plasmid pLS33 into chromosome III, obtained by transforming the strains to His$^+$ with *Bsr*GI-digested plasmid DNA. Plasmid pLS33 consists of pUC18 in which the *Hin*dIII-*Ava*I region has been replaced by the *Bam*HI fragment spanning *HIS3* and a 0.9-kb *Bgl*II-*Hin*dIII fragment immediately centromere distal to *MAT* and spanning a unique *Bsr*GI site has been inserted at the *Eco*RI site. His$^+$ transformants of strains LS616 and LS617 were analyzed by Southern hybridization to determine at which allele of *MAT* the plasmid had inserted. Strains Y2202 and Y2203 are derivatives of strain LS616 in which the *HIS3* gene resides adjacent to *MAT***a** or *MAT*α, respectively. Strains Y2204 and Y2205 are derivatives of strain LS617 in which the *HIS3* gene resides adjacent to *MAT***a** or *MAT*α, respectively.

To create *mat***a***2* derivatives of strain Y2201, we introduced in vitro-generated deletion and frameshift alleles of *MAT***a***2* into the strain. The *mat***a***2*Δ*1* allele, consisting of a 431-bp *Bsr*BI-to-*Bsa*BI deletion in the X region of **a***2*, was constructed in plasmid pLS7, which consists of the *Eag*I-*Hin*dIII portion of *MAT***a** in plasmid YIp5, and inserted into the genome of strain Y2201 by the two-step replacement technique (2). One such strain, in which the presence of the deletion was confirmed by PCR and Southern hybridization, was retained as strain Y2206. To create the *mat***a***2-2* frameshift allele, we performed oligonucleotide-directed mutagenesis of plasmid pLS35, by using the Promega Altered Sites Mutagenesis protocol and the oligonucleotide 5′-CTTCTATCGTTTTCTCGAGCTGCGCA TTTC-3′ to generate a *Xho*I site and a 1-bp insertion at codon 95 of the *MAT***a***2* coding region. A *Hin*dIII fragment spanning the resultant *mat***a***2-2* allele was cloned into plasmid YIp5, which in turn was used to introduce the allele into strain Y2201 by the two-step gene replacement procedure. One such strain was confirmed by Southern hybridization analysis to carry the allele and was retained as strain Y2207.

Assay of donor locus preference dictated by different α2 alleles. As described in Results, assays for donor locus preference as a function of different α2 alleles were conducted by determining the predominant mating type cassette present at the *MAT* locus in cultures of homothallic strains undergoing mating type interconversion in successive generations. We determined the proportion of *mat*α*2* (derived from *hml*α*2*) to *mat***a***1*Δ*101* (derived from *hmr***a***1*Δ*101*) in such strains by either PCR or Southern hybridization analysis. In the PCR analysis, two oligonucleotides, 5′-CTTGAGACGATTTGGCCCTG-3′ and 5′-CCGTCCCGTATA GCCAATTCG-3′, homologous to a site within the W region of *MAT* and to a site immediately centromere distal to *MAT*, respectively, were used to amplify both *mat* alleles in a population of cells. In Southern hybridization analysis, both *mat* alleles as well as the *hml*α*2* donor locus were visualized by digestion of genomic DNA with *Hin*dIII and *Eco*RI, fractionation by gel electrophoresis, transfer to Hybond-N, and hybridization with a 603-bp fragment extending from the first *Eco*RV site centromere proximal to *MAT* to the *Eag*I site within the W region of homology shared by *MAT* and *HML*. Quantitation of the ratio of *mat***a***1*Δ*101* to *mat*α*2* was achieved by PhosphorImager analysis using ImageQuant software.

Donor preference in *tup1*, *ssn6*, and a*2* mutants. In order to establish the donor preference in strains carrying null alleles of *tup1*, *ssn6*, or *mat***a***2*, we used a PCR-based assay similar to one we previously described (33). Both donor loci of the strains used in these assays carry an *inc* mutation in their *HO* endonuclease recognition sites and deletion mutations that allow them to be distinguished by PCR from each other and from either of the alleles initially resident at *MAT*. Transfer of the nonfunctional *HO* recognition site from the donor locus to *MAT* at the initial switch prevents further switching events in the progeny of the switched cell. Thus, the donor preference in the initial switching event in each spore clone can be inferred from the predominant mating type cassette at the *MAT* locus in cells of the colony originating from that spore.

Diploid strains homozygous for *HO* and heterozygous for either *tup1*, *ssn6*, or *mat***a***2* were sporulated, and the mating type of each of the resulting spores was determined as described below. Colonies derived from each of the spores were tested by replica plating for the auxotrophic marker disrupting the gene of interest. Only colonies from spores disrupted for the gene of interest were analyzed for donor preference. The mating type of the spores carrying the *tup1* or *ssn6* mutations was established ex post facto by determining the histidine auxotrophy or prototrophy of spore clones, which specified the mating type allele since *HIS3* was inserted immediately adjacent to either *MAT***a** or *MAT*α in these strains. The mating type of spores carrying **a***2* mutations was determined by confrontation with an α-factor-producing strain as follows. Diploid strains were induced to sporulate by patching onto solid YEPD media (media are as described in the work of Rose et al. [21]), incubating for 24 h, replica plating to sporulation media, and then incubating for an additional 3 to 5 days. Cells were scraped off the sporulation plate and resuspended in 100 μl of water containing 5 μl of 10-mg/ml Zymolyase. Cells were incubated for 10 min at room temperature and then diluted with 900 μl of water. Approximately 100 μl of this treated cell suspension was applied to a YEPD plate on which a thick bisecting strip of strain 17α cells had been previously grown. Tetrads were dissected, and individual spores were placed adjacent to the swath of 17α cells. The plates were incubated for 6 to 8 h, which time the morphology of the cells was examined microscopically. Those spores that had undergone cell division were scored as α cells, while those that failed to undergo cell division but rather assumed an elongated shape were scored as **a** cells. Following determination of mating type, all spore progeny and shmoos were removed from the vicinity of the mating type tester strip and allowed to form spore colonies.

PCR analysis to determine the allele resident at *MAT* in individual spore clones was performed directly on cells from each colony of interest. Cells from the colonies were boiled for 3 min in a 50-μl cocktail containing 1.5 mM $MgCl_2$; 200 μM each deoxynucleoside triphosphate; 500 nM each oligonucleotides 5′-C TTGAGACGATTTGGCCCTG-3′ and 5′-CCGTCCCGTATAGCCAATTCG-3′, corresponding to sites within W and centromere distal to *MAT*, respectively; and the buffer recommended by the manufacturer (Promega). The mixture was cooled to room temperature, and Promega *Taq* DNA polymerase was added. The reaction mix was overlaid with 50 μl of light mineral oil and subjected to 35 cycles of incubations of 92°C for 1.5 min, 53°C for 2 min, and 72°C for 3 min with a 14-s ramping speed. The products of the reactions were visualized on a 1% agarose gel. This analysis readily distinguished among all the possible *MAT* alleles: **a**, α, **a***1*Δ*101*-inc and α*1*α*2*-inc. Colonies predominantly exhibiting the *mat***a***1*Δ*101* allele were scored as selecting *HMR* as donor, colonies predominantly exhibiting *mat*α*1*α*2* were scored as selecting *HML* as donor, and colonies that failed to exhibit one predominant allele were scored as ambiguous. These latter colonies resulted either from diminished switching efficiency, in which a significant number of cells carrying the original wild-type allele resident at *MAT* are present in the colony, or from random donor preference, in which both donor loci are represented at *MAT* in cells in the colony.

RESULTS

The effects of α2 mutations on donor preference. The homeodomain protein α2p plays a role both in transcriptional repression in the process of cell type determination and in donor selection during mating interconversion. As a transcriptional repressor, α2p can associate with Mcm1p to repress the dispersed collection of **a**-specific genes and, independently, with **a**1p to repress a group of haploid-specific genes. To examine the mechanism of α2p-mediated donor locus selection, we have tested which, if any, of the various activities that α2p exhibits in transcriptional regulation are responsible for its ability to promote discrimination between the two donor loci. Accordingly, we have examined the consequences on donor selection of different mutations in *MAT*α*2* with well-defined effects on the interaction of α2p with its various partners in transcriptional regulation.

The haploid tester strain Y2200 used to assess donor preference in response to different α*2* alleles is represented in Fig. 2. The strain carries the wild-type *HO* allele as well as an α*2* frameshift mutation in *HML* and the **a***1*Δ*101* null mutation in *HMR*. These mutant donor loci are unaffected in their ability to donate their resident alleles during mating type interconversion. As a result of this configuration of donor loci alleles, the strain is capable of mating type switching, although such switching does not yield mating-competent cells of both mating types. As a consequence, the strain remains haploid, undergoing *HO*-mediated mating type interconversion essentially every generation to yield a steady-state mixture of *mat***a***1*Δ*101* and *mat*α*2* cells in the culture. To assay donor preference dictated by different α*2* alleles, we introduced each specific allele into the strain on a centromeric plasmid. Since **a**1p is never expressed in this strain, none of the α*2* alleles imposes repression of *HO* expression nor do any of the alleles result in production of mating-competent cells of both mating types. In addition,

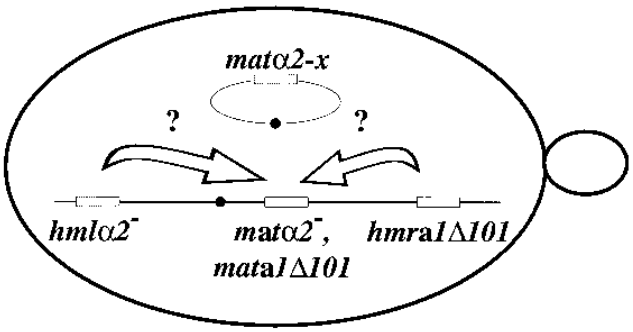

FIG. 2. Assay for examining the effects of α2 mutations on donor preference. Strain Y2200, depicted in the diagram, carries the wild-type *HO* locus as well as an *α2* frameshift allele in *HML* and an **a***1Δ101* null mutation in *HMR*. Since the strain cannot yield mating-competent cells of both mating types, it undergoes continual mating type interconversion during growth of the culture. If the allele of *α2* carried on the tester plasmid in the strain is wild type for donor preference, then cells will most often select *HMR* as the donor locus during mating type switching and the predominant *MAT* allele in the culture will be **a***1Δ101*. In contrast, if the *α2* allele is defective for donor preference, then cells will most often select *HML* as the donor locus and the predominant *MAT* allele will be *α2*$^-$. Thus, the ratio of *mat***a***1Δ101* to *matα2*$^-$ in a culture of strain Y2200 carrying a particular *α2* allele reveals the donor preference phenotype of that allele.

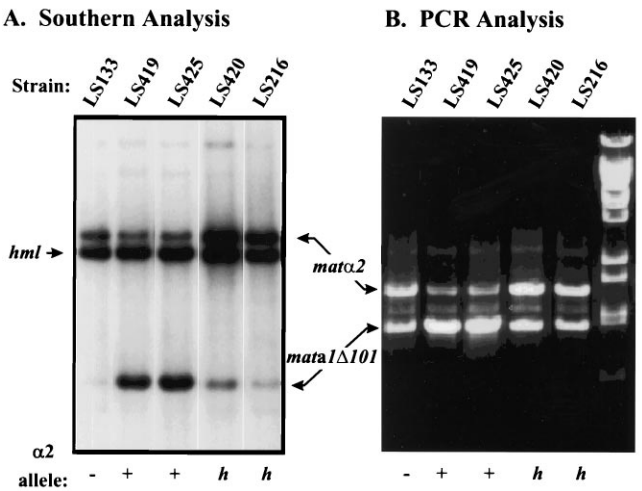

FIG. 3. Mutations in the homeodomain of α2p affect donor preference. Strains listed across the top of the gels are derived from strain Y2200 by introduction of the *α2* allele (identified in Table 2), which elicits the repression phenotype listed at the bottom of the gels (−, null allele; +, wild type; *h*, deficient in **a**-specific gene repression but not in haploid-specific gene repression). DNA was isolated from cultures of each of the strains, and the relative representations of the **a***1Δ101* and *α2*$^-$ alleles at *MAT* were determined either by Southern analysis (A) or by PCR analysis (B) as described in Materials and Methods. The positions of migrations of fragments corresponding to the two *mat* alleles are indicated, as is that of the cross-reacting *hml* locus. The right-hand lane in panel B is *Bst*EII fragments of phage λ DNA. Quantitation of the Southern results is presented in Table 2.

the α2 expression plasmid does not include sequences to allow it to serve as donor during mating type interconversion, so the introduced α2 gene participates in switching only as a *trans*-acting regulator of donor preference. Thus, donor preference dictated by an *α2* allele can be readily determined by measuring the ratio of *mat***a***1Δ101*, derived from *HMR*, to *matα2*, derived from *HML*, in cultures of strain Y2200 carrying the *α2* allele of interest on the plasmid vector.

The homeodomain region of α2p is required for proper selection of *HMR*. Previous work has shown that homothallic strains expressing α2p select *HMR* preferentially and those lacking α2p select *HML* preferentially (8, 29, 34). We confirmed this observation in our assay system. As evident in Fig. 3 and Table 2, the predominant mating type cassette at the *MAT* locus in a population of cells carrying a null allele of *α2* (strain LS133) is *matα2*, demonstrating that the preferred donor locus for a strain lacking the *α2* gene is *HML*. Reciprocally, the predominant mating type cassette at the *MAT* locus in a population of cells expressing wild-type *α2* (strains LS425 and LS419) is *mat***a***1Δ101*, indicating that the preferred donor locus for a strain containing a wild-type *α2* gene is *HMR*. The results are the same whether the analysis of the *MAT* locus is done by PCR analysis or the Southern hybridization technique.

Vershon et al. (30) created a number of mutations in the homeodomain of *α2*, corresponding to the DNA binding domain of the protein, and showed that many of these mutations abolished the ability of the protein to promote repression in vivo of **a**-specific genes but did not perturb the protein's ability to repress haploid-specific genes. That is, the mutant proteins retained their diploid-specific function, exerted in concert with **a**1p, but had lost their haploid function, accomplished in concert with Mcm1p. Mutations that yielded this phenotype are designated *h* in Fig. 1. We examined two such mutant proteins, N182A and K188I, present in strains LS420 and LS216, respectively, for their ability to promote proper donor preference. As evident in Fig. 3 and Table 1, both strains had *matα2* as the predominant allele, indicating that both proteins elicited a preference for *HML* as donor. Thus, a mutation in the DNA binding domain of *α2* that abolished the protein's ability to repress **a**-specific, but not haploid-specific, genes yielded the equivalent of a null phenotype with regard to donor preference.

Interaction between α2p and Mcm1p is required for proper donor preference. α2p binds cooperatively with Mcm1p to defined DNA sites lying upstream of various **a**-specific genes and through which repression of these genes is effected. Coopera-

TABLE 2. Summary of effects of α2 mutations on donor preference

Mutation	Reference	Strain	asg repression[a]	hsg repression[b]	Donor preference *HMR*/*HML*[c]
Δ	This study	LS133	−	−	0.35
+	This study	LS419	++	++	2.33
+	18	LS425	++	++	2.36
+	6	LS588	++	++	3.25
L10S	6	LS584	−	−	0.38
C33Y	30	LS423	++	−	2.04
G113A	18	LS502	++	++	2.16
F116A	18	LS421	−	++	0.16
N117A	18	LS422	+	++	3.14
114-120r11	18	LS504	−	++	0.06
114-120r12	18	LS505	−	++	0.07
N125A	18	LS503	++	++	2.44
N182A	30	LS420	−	++	0.12
K188I	30	LS216	−	++	0.06
L196S	30	LS217	++	−	3.84
L196S	25	LS424	++	−	3.20

[a] Derived from in vivo analysis of repression of an **a**-specific reporter gene in a strain carrying the designated α2 allele as described in the indicated reference. ++, repression activity essentially equivalent to that of the wild-type protein; −, essentially no repression activity; +, repression yielding 10 to 30% of the fully derepressed level of the reporter gene.

[b] Derived from in vivo analysis of repression of an **a**-specific reporter gene in a strain carrying the designated α2 allele as described in the indicated reference. ++, repression activity essentially equivalent to that of the wild-type protein; −, essentially no repression activity.

[c] Calculated from the phosphorimager analysis of the data presented in Fig. 3, 4, and 5, by dividing the intensity of the hybridization to the band corresponding to *mat***a***1Δ101* by that of the band corresponding to *matα2*.

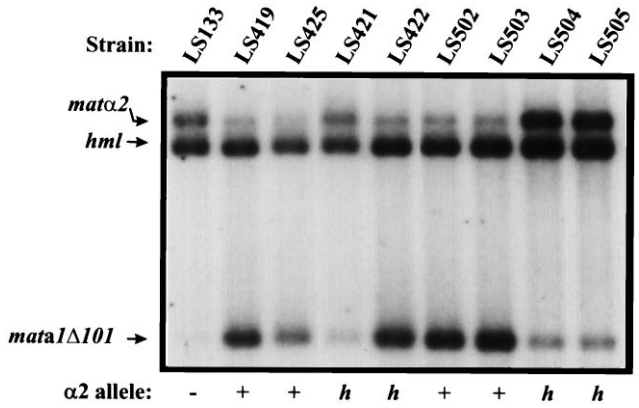

FIG. 4. Mutations in the linker region of α2p affect donor preference. See the legend to Fig. 3A.

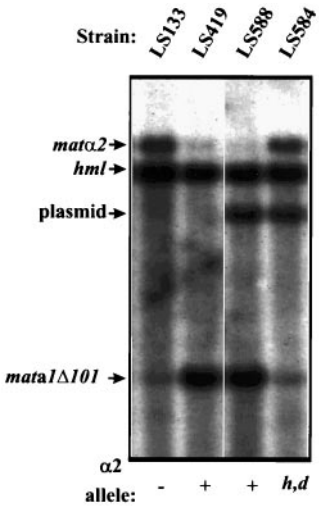

FIG. 5. A mutation interfering with Tup1p-α2p association affects donor preference. See the legend to Fig. 3A. Strains LS588 and LS584 carry the α2 allele on a plasmid that gives a cross-reacting fragment whose position of migration is indicated. The α2 allele in LS584 is defective in repression of both **a**-specific and haploid-specific genes.

tive binding with Mcm1p and attendant repression of **a**-specific genes depend on a short hydrophobic region of α2p, within an unstructured linker region, which makes direct contact with Mcm1p when both proteins are bound to DNA (31). Mead et al. (18) performed extensive mutagenesis of this linker domain and characterized the effects on Mcm1p binding, on in vivo repression of **a**-specific genes, and on repression of haploid-specific genes of a variety of amino acid changes within this linker region. These results confirmed the importance of and refined the nature of the linker region in the interaction between α2p and Mcm1p with regard to **a**-specific gene repression.

We examined the effects on donor selection of various mutations within the linker region of α2p. Two of the alleles, F116A and N117A, carried in strains LS421 and LS422, respectively, are single point mutations, while two other alleles, r11 and r12, present in strains L504 and L505, respectively, were created by randomizing the residues between 114 and 120. All four alleles exhibited an *h*, or haploid repression-defective, phenotype. As evident in Fig. 4, three of the four alleles promoted selection of *HML* as donor; that is, they exhibited a null phenotype for donor preference, while the fourth allele, N117A, promoted selection of *HMR* donor, at a level equivalent to that obtained with the wild-type allele. Although the nature of the discrepancy among the four alleles is unknown, the three mutants that displayed a null phenotype exhibited a more severe defect in **a**-specific gene repression than did the allele that elicited a wild-type donor preference. That is, in vivo expression of an **a**-specific reporter gene construct was at 80% of the fully derepressed level in cells carrying the F116A, r11, or r12 allele, while expression of the reporter gene was at only 25% of the fully derepressed levels in cells carrying the N117A allele (18). Finally, two control alleles were examined in this assay. Mutations G113A and N125A, which bracket the hydrophobic region, had no effect on **a**-specific gene repression and, as seen in Fig. 4, elicited a wild-type phenotype with regard to donor preference. We conclude from these results that interaction with Mcm1p is important for α2p to promote selection of *HMR* as donor.

Interaction between α2p and Tup1p is required for proper donor preference. The L10S allele of α2p prevents binding to the global repressor protein Tup1p and therefore interdicts repression of both **a**-specific genes and haploid-specific genes (13). We examined the effects of this allele, carried in strain LS584, on donor preference. As evident from the data in Fig. 5 and Table 2, this allele promoted selection of *HML* as donor, exhibiting the identical behavior of a control strain containing no α2p. This result suggested that recruitment of Tup1p by α2p is critical for α2p to perform its role in donor preference. This also suggested that Tup1p should play a role in donor preference, a hypothesis confirmed by results presented below.

Mutations that render α2p unable to repress only haploid-specific genes do not interfere with donor selection. Two mutations that diminished the interaction of α2p with **a**1p and thus abrogated the ability of α2p to effect repression of haploid-specific genes but not of **a**-specific genes were assayed for donor preference. Strain LS423 contains a mutation, C33Y, in the N-terminal region required for **a**1p binding, and strain LS434 contains a mutation, L196S, in a region of the C-terminal tail that makes contacts in the homeodomain region of **a**1p to stabilize DNA binding by the heterodimer (17, 25). As evident from the results in Fig. 6, these mutations causing a *d*, or diploid-defective, phenotype did not alter donor preference. That is, these mutations promoted selection of *HMR* as the

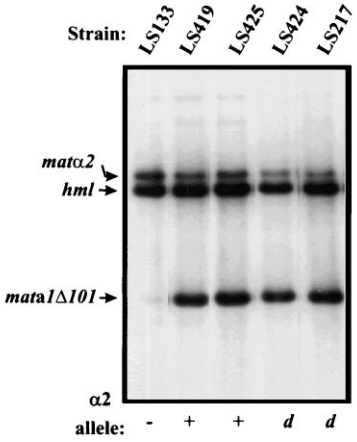

FIG. 6. Mutations in the **a**1p interaction domains of α2p do not affect donor preference. See the legend to Fig. 3A. *d*, defective in haploid-specific gene repression but not in **a**-specific gene repression.

TABLE 3. Effect of **a**2 mutations on donor preference

Strain	Genotype	Cell type	% Donor selected[a]	
			HML	*HMR*
Y2201	*MAT***a**/*MAT*α	**a**	81 (152)	13 (24)
Y2206	*mat***a**2Δ*1*/*MAT*α	**a**	75 (36)	2 (1)
Y2207	*mat***a**2*-2*/*MAT*α	**a**	93 (55)	3 (2)
Y2206	*mat***a**2Δ*1*/*MAT*α	α	1 (1)	99 (77)

[a] Donor preference was determined as described in Materials and Methods by examining the predominant allele at *MAT* in colonies derived from individual spores of the indicated cell type obtained following dissection of the indicated strain. Numbers in parentheses are the absolute number of spore clones that exhibited the indicated donor preference. Only those colonies that gave a single predominant *MAT* allele are listed. The remainder for each strain represents colonies in which both donor alleles are represented essentially equally in the spore clone.

TABLE 4. Effect of *TUP1* or *SSN6* inactivation on donor preference

Strain	Genotype	% Donor selection in cells[a]:			
		α		**a**	
		HML	*HMR*	*HML*	*HMR*
Y2201	*TUP1 SSN6*	2 (6)	95 (239)	81 (152)	13 (24)
Y2202, Y2203	*tup1*::*URA3*	38 (15)	33 (13)	79 (63)	5 (4)
Y2205, Y2204	*ssn6*::*URA3*	14 (5)	81 (30)	86 (31)	3 (1)

[a] Donor preference was determined as described in Materials and Methods by examining the predominant allele at *MAT* in colonies derived from individual spores of the indicated cell type with the designated marker obtained following dissection of the indicated strain. Numbers in parentheses are the absolute number of spore clones that exhibited the indicated donor preference. Only those colonies that gave a single predominant *MAT* allele are listed. The remainder for each strain represents colonies in which both donor alleles were represented essentially equally in the spore clone or, for the case of *tup1* cells, in which the parental *MAT* allele remained present in the clone.

donor with the same efficiency as that of the control strain carrying the wild-type α*2* allele. Thus, we concluded that the region of α2p required for its interaction with **a**1p is not required for α2p to execute its role in donor preference.

The a*2* gene is not required for proper donor selection during mating type switching. The *MAT***a***2* gene encodes and expresses a protein of 119 amino acids identical to the C-terminal half of α2p (7). However, no role for this protein in mating type regulation or in any other cellular process has been identified. We were interested in exploring whether this protein might play a role in donor preference in **a** cells, especially since **a**2p contains the homeodomain and Mcm1p binding linker regions of α2p but not the N-terminal half of α2p that makes contacts with the Tup1p-Ssn6p complex required for transcriptional repression. To test the effect on donor preference of eliminating **a**2p, we used an assay for donor preference previously described (33). Basically, the diploid strains used for this analysis had alleles at *HML* and *HMR* that could be distinguished by size from each other and from either allele initially present at *MAT*. In addition, each donor locus allele carried an *inc* mutation to prevent further conversions of *MAT* following an initial switching event. Accordingly, the particular donor locus selected during the first switching event within a spore clone could be determined simply by performing PCR analysis on a sample of DNA from the colony. Accordingly, we created a deletion within the **a***2* gene at *MAT***a** in strain Y2201. The resulting strain, Y2206, was sporulated, and colonies from **a** spores were examined to assess the efficiency of selecting *HML* as the donor locus during mating type interconversion. As evident from the results presented in Table 3, *mat***a**2Δ*1* cells selected *HML* preferentially as donor at the same frequency as did *MAT***a** cells from the parent strain Y2201. This was not a consequence of any defect in the function of *HMR* as donor, since α spore progeny of strain Y2206 exhibited the expected bias for *HMR* as donor (Table 3). One caveat of the analysis of donor selection using the **a***2*Δ*1* allele is that the deletion removed a significant portion of the homology between the *MAT* locus and *HMR*. Accordingly, we also created a frameshift mutation in *MAT***a***2* (*mat***a***2-2*, strain Y2207) and examined the consequences of this mutation on donor preference. As noted in Table 3, this mutation also did not diminish the ability of **a** spore progeny to select *HML* as the preferred donor. Thus, we conclude that **a**2p is not required for proper donor selection in **a** cells.

Tup1p is required for efficient switching and proper donor preference in α cells. As noted above, the L10S α2 mutant protein, which fails to bind to and recruit Tup1p, was defective in promoting selection of *HMR* as donor during mating type interconversion. This suggested that Tup1p might play a role in appropriate donor selection. We tested this possibility by examining donor preference in *tup1* spore clones of strains Y2202 and Y2203. As shown in Table 4, **a** *tup1* spore clones selected *HML* as donor at a frequency equivalent to that of *TUP1* cells. In contrast, α *tup1* spore clones exhibited a random pattern of donor selection during mating type interconversion. Thus, we conclude that Tup1p plays no role in donor selection in **a** cells but is essential for proper donor selection in α cells.

The distribution of *MAT* alleles present in the progeny of α *tup1* spores from the above strains suggested that, in addition to a role in determining which donor locus will participate in the conversion event, Tup1p was also required for ensuring efficient *HO*-mediated gene conversion in α cells. In contrast to *TUP1* spore clones, in which most often the predominant allele at *MAT* corresponded to that initially resident at one or the other of the donor loci, α *tup1* spore clones retained a significant proportion of the *MAT*α allele in the population (Fig. 7). This suggested that the efficiency of HO-mediated conversion was reduced in α *tup1* cells, possible reasons for which are addressed in the Discussion.

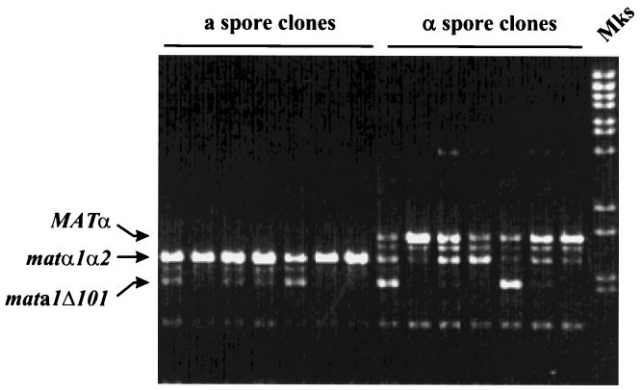

FIG. 7. α *tup1* strains are inefficient in mating type interconversion. Individual **a** and α spore clones from strains Y2202 and Y2203 (*MAT***a**/*MAT*α *HO*/*HO tup1*/*TUP1*) were analyzed by PCR for the allele present at *MAT*, as described in Materials and Methods. The positions of migrations of fragments corresponding to *MAT*α (initially resident at *MAT*), *mat*α*1*α*2* (derived from *HML*), and *mat***a***1*Δ*101* (derived from *HMR*) are indicated. The fragment corresponding to *MAT***a** migrates at a position readily distinguishable from that of the above three alleles. Mks, *Bst*EII-digested phage λ DNA.

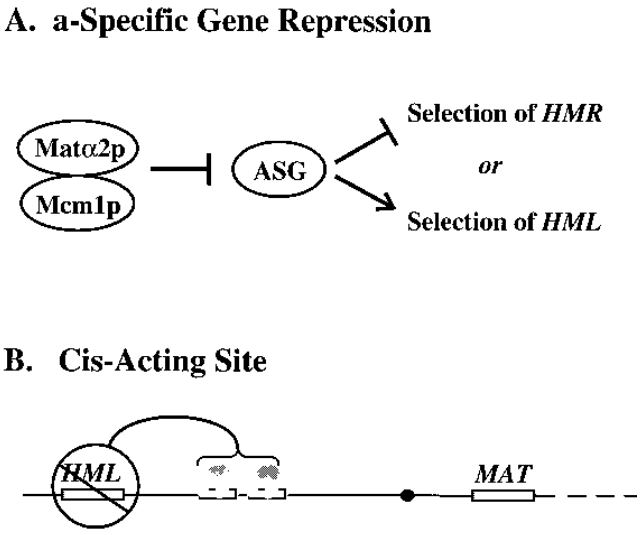

FIG. 8. Two models for the role of **a**2p in donor preference. (A) Donor preference gene model. α2p affects donor preference through regulation of an as yet undefined **a**-specific gene (ASG) that is responsible for promoting access to *HML* (or blocking access to *HMR*). In the absence of α2p, this gene is active and *HML* is selected; in the presence of α2p, this donor selection gene is repressed and *HMR* becomes the preferred donor. (B) Direct effect model. In the second model, α2p-Mcm1p would bind to a site on chromosome III either to prevent the selection of *HML* or to promote directly the selection of *HMR* in α cells. This model predicts the presence of an α2p-Mcm1p binding site(s) (*cis*-acting site) on chromosome III that is required for proper donor preference. The recent identification of such sites and their involvement in donor preference enhance the likelihood of this model (28).

***SSN6* is not required for proper donor selection.** We anticipated that, since α2, Mcm1p, and Tup1p were all required for proper donor selection in α cells, Ssn6p, which is required for efficient repression of **a**-specific genes by the α2p-Mcm1p complex, would also be required. However, this was not quite the case. We measured donor selection in spore colonies of strains Y2205 and Y2204, which carry disruptions of *SSN6*. As shown in Table 4, **a** spore clones exhibited appropriate donor preference at a level equivalent to the wild-type parent control strains and α *ssn6* exhibited appropriate donor preference at a level near, but somewhat lower than, that of wild type. The significance of these results in distinguishing the role of α2p in specifying proper donor selection from its role in **a**-specific gene repression is discussed below.

DISCUSSION

Models for the role of α2p in donor selection. The results presented in this report establish a strong correlation between the role of α2p in repression of **a**-specific genes and its role in promoting selection of *HMR* during mating type interconversion. As shown in this report, donor preference depends on the ability of α2p to bind DNA specifically, to associate with Mcm1p, and to recruit Tup1p but is not dependent on the ability of α2p to associate with **a**1p. Donor preference also requires the activity of Tup1p and, most likely, Mcm1p, although, due to the absence of viable mutations in Mcm1p that abolish α2p-mediated repression, we have not been able to demonstrate directly the requirement for Mcm1p. In sum, these observations prompt two distinct models for the role of α2p in establishing donor preference, which are outlined in Fig. 8 and described below.

(i) Donor selection through regulation of an a-specific gene. One model consistent with the above observations is that α2p affects donor preference through regulation of an as yet undefined **a**-specific gene that is responsible for promoting access to *HML* (or blocking access to *HMR*). Thus, in the absence of α2p, this gene is active and *HML* is selected; in the presence of α2p, this donor selection gene is repressed and *HMR* becomes the preferred donor. This would account for the correlation between mutations defective in donor preference and those defective in **a**-specific gene repression. The two discrepancies in this correlation—the α2 N117A allele and the *ssn6* null allele—can readily be accommodated on quantitative grounds. While both mutations are defective in α2p-mediated repression of **a**-specific genes but promote normal donor preference during mating type interconversion, both of these mutations cause only a limited defect in repression. For instance, while an **a**-specific reporter gene was essentially fully derepressed in an α *tup1* strain, the same gene exhibited only 10% of the fully derepressed level in an α *ssn6* strain (3). Perhaps noteworthy, the limited data suggest that α *ssn6* cells exhibit a slightly increased error frequency in donor selection, at a level remarkably similar to that of the defect that these cells exhibit in repression. Similar considerations apply to the α*2* N117A allele, as described in Results. Thus, if we assume that selection of *HML* promoted by the unknown **a**-specific gene requires relatively robust expression of this gene, then we would expect that the *ssn6* mutation or the N117A allele would behave essentially like wild type in donor preference, rather than like the mutant. Accordingly, the results in this report are consistent with the function of α2p in donor selection simply being an outcome of its role in **a**-specific gene repression.

(ii) A direct effect of α2p on donor preference. In the second model, α2p would bind to a site on chromosome III either to prevent the selection of *HML* or to promote directly the selection of *HMR* in α cells. Our results suggest that such a site should be similar to α2p binding sites upstream of **a**-specific genes in that it would be comprised of both the α2p binding domain and the Mcm1p binding domain. Our results would further suggest that the activity of this site would depend on Tup1p. Simpson (24) has provided a precedent for α2p affecting the activity of a *cis*-acting site by showing that positioning an α2p-Mcm1p binding site at an appropriate distance from an origin of replication (autonomously replicating sequence element) could yield α-specific repression of the replication potential of the autonomously replicating sequence element. This effect, as well as the ability of α2p-Mcm1p to repress the activity of a contiguous promoter, results at least in part from the ability of α2p to elicit nucleosome phasing outward from its binding site, an effect that is dependent on Tup1p and, to a lesser extent, on Ssn6p (3). This would account for the overlap in the genetic requirements for donor selection versus **a**-specific gene repression. That is, although the target sites for regulation in the two situations would be distinct—a *cis*-acting site affecting the structure or accessibility of the surrounding DNA, in the case of donor selection, versus a promoter for a cell-type-specific gene, in the case of **a**-specific gene repression—the machinery responsible for imposing both forms of regulation would be the same. For reasons discussed below, we favor this model.

Tup1p and conversion efficiency. We have found that Tup1p is required both for proper donor selection and for efficient completion of the switching process. This reduced efficiency could have resulted either from a reduced frequency of initiation of the conversion event—due to reduced expression of *HO*, for example—or from a reduced efficiency of completing the conversion event. The fact that **a** *tup1* spore clones did not exhibit a decrease in switching efficiency suggested that the frequency of initiation was not affected in *tup1* cells. Thus, we

favored the interpretation that the majority of the mother cells in an α *tup1* colony initiated a switching event but were unable to find a donor to repair the double-strand break at *MAT*. As demonstrated by Klar et al. (12), cells that fail to complete a switching event do not progress through the cell cycle and eventually die. The remainder of the colony would be composed of daughter cells that had not initiated a switch and therefore retained the wild-type *MAT*α allele. Occasionally, a mother cell in the colony would complete a switching event, and because Tup1p was not present to promote proper donor selection, a donor would be chosen at random. Thus, we conclude that Tup1p is required both for proper donor selection and for efficient completion of switching in α cells.

Recombination potential and α2p-Mcm1p binding sites on chromosome III. In a separate study, we have explored what regions of chromosome III are required for the cell to distinguish *HML* from *HMR* during mating type interconversion. Our previous studies demonstrated that neither the allele at either donor locus, the structure of either locus, nor any feature near either donor locus or the *MAT* locus provides a marker used by the cell to select one donor locus versus the other during interconversion (32). Nonetheless, since we found that donor preference is maintained even when *MAT* is moved to a different chromosome, we concluded that the two donor loci reside in intrinsically distinct chromosomal domains that define accessibility of the loci in a cell-type-specific manner.

More recently, we have used chromosomal inversions to identify the region on chromosome III that acts in *cis* to specify donor selection. From these studies, we found that the region between 24 and 60 kb from the left arm of chromosome III plays a major role in defining the accessibility of the contiguous donor locus in donor selection (28). This region spans two α2p-Mcm1p binding sites separated by a distance of approximately 1.6 kb (at 29 and 30.6 kb from the left end of the chromosome), which are significant in three aspects. First, besides the α2p-Mcm1p binding sites associated with known **a**-specific genes, these two sites conform to the consensus binding sequence for this repression complex better than any other site within the yeast genome. Second, neither of these sites is associated with an open reading frame. Third, deletion specifically of these sites has a profound effect on donor preference, as described recently by Wu and Haber (35) and by Szeto et al. (28). The fact that these sites play a role in donor preference even though they are not associated with an open coding region is consistent with the second model presented above, in which α2p affects donor preference through direct interaction with a *cis*-acting donor preference control locus. On the other hand, α2p-Mcm1p binding at this site might regulate expression of an **a**-specific RNA that could directly affect the recombination potential of this arm of the chromosome. Specific RNA transcripts have been implicated in chromosome function in X-chromosome inactivation and imprinting in mammalian cells.

These results can be appreciated in the context of recent work by Wu and Haber (34, 35). These investigators have recently suggested that the mechanism underlying cell-specific donor preference involves activation of a large segment of the left arm of chromosome III for recombination in a cell-type-specific manner. In particular, they note that the leftmost 60 kb of chromosome III, comprising roughly 20% of the chromosome, exhibits an approximately 20-fold higher frequency of mitotic recombination in an **a** cell than in an α cell (*HML* is located ca. 12 kb from the left end of the chromosome). This is true not only for recombination involving mating type loci but also for other nonmating type genes inserted at various sites within this region. Thus, they suggest that this enhanced mitotic recombination potential of the left arm of chromosome III in **a** cells renders *HML* the preferred donor, while this enhanced recombination potential is lost in α cells and *HMR* becomes the default donor.

Our results provide some of the molecular context for this model. We suggest that suppression of the recombination potential of the left arm of chromosome III in α cells is achieved by binding of α2p-Mcm1p to the sites at 29 and 30 kb and subsequent recruitment of Tup1p (and perhaps Ssn6p) to this region. The potential for this complex to modify dramatically the local chromatin structure provides obvious directions for further exploration. Nonetheless, how the recombination potential is established in **a** cells and how binding of α2p-Mcm1p to sites located ca. 20 kb away from HML suppresses this recombination potential over such monumental distances await further study.

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