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Mating-Type Control in *Saccharomyces cerevisiae*: Isolation and Characterization of Mutants Defective in Repression by a1-α2

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The α2 protein, the product of the *MATα2* cistron, represses various genes specific to the a mating type (α2 repression), and when combined with the *MATa1* gene product, it represses *MATα1* and various haploid-specific genes (a1-α2 repression). One target of a1-α2 repression is *RME1*, which is a negative regulator of a/α-specific genes. We have isolated 13 recessive mutants whose a1-α2 repression is defective but which retain α2 repression in a genetic background of *ho MATa HMLα HMRa sir3* or *ho MATα HMRa HMRa sir3*. These mutations can be divided into three different classes. One class contains a missense mutation, designated *hmlα2-102*, in the α2 cistron of *HML*, and another class contains two missense mutations, *matα2-201* and *matα2-202*, in the *MATα* locus. These three mutants each have an amino acid substitution of tyrosine or phenylalanine for cysteine at the 33rd codon from the translation initiation codon in the α2 cistron of *HMLα* or *MATα*. The remaining 10 mutants make up the third class and form a single complementation group, having mutations designated *aar1* (a1-α2 repression), at a gene other than *MAT*, *HML*, *HMR*, *RME1*, or the four *SIR* genes. Although a diploid cell homozygous for the *aar1* and *sir3* mutations and for the *MATa*, *HMLα*, and *HMRa* alleles showed α mating type, it could sporulate and gave rise to asci containing four α mating-type spores. These facts indicate that the domain for α2 repression is separable from that for a1-α2 protein interaction or complex formation in the α2 protein and that an additional regulatory gene, *AAR1*, is associated with the a1-α2 repression of the α1 cistron and haploid-specific genes.

Cell types of *Saccharomyces cerevisiae* are determined by the codominant *MATa* and *MATα* alleles at the mating-type locus, *MAT*, on the right arm of chromosome III (for reviews, see references 16 and 37). A haploid *MATa* cell has the **a** mating type and can mate with a cell having the *MATα* genotype. Conjugation of **a** and α cells produces an **a**/α diploid cell which has the third cell type, nonmater. The **a**/α diploid cells undergo meiosis and form asci, and each ascus contains four ascospores bearing a haploid tetrad nucleus. Besides cell-type control, the *MAT* locus regulates various other genes such as *HO* for homothallic switching of the *MAT* locus (19) and the transcription of Ty elements (8, 9).

In addition to the *MAT* locus, complete but unexpressed copies of *MAT* information are encoded at two other loci, *HML* and *HMR*, on the same chromosome (16, 37). Homothallic switching of information at the *MATα* locus to **a** or *MATa* to α is achieved by transposition of the **a** or α cassette from the *HMR* or *HML* locus to the *MAT* locus. In general, *HMR* encodes the **a** information and *HML* has α, but their expression is normally repressed by the function of the *SIR* genes.

A hypothesis concerning the regulatory function of the *MAT* genes, known as the α1-α2 hypothesis (39), proposes that *MATα* consists of two cistrons, α1 and α2. The α1 cistron encodes a positive regulator for α-specific genes, and the α2 cistron encodes a negative regulator for **a**-specific genes (α2 repression). The *MATa* products do not function in haploid cells, but **a**1, in conjunction with the α2 product, represses the α1 cistron, the haploid-specific genes and the *RME1* gene (21, 33), and also expression from Ty promoters (a1-α2 repression). The *RME1* product is a negative regulator of the **a**/α-specific genes that are active in a cell expressing both the **a** and α information (27). Thus, the α2 polypeptide plays a role in two different repressor activities. In fact, the α2 protein recognizes a 32-base-pair (bp) DNA sequence located upstream of an **a**-specific gene, *STE6* (20), while the a1-α2 complex may recognize another 20-bp or 28-bp sequence in the nontranscribed region of the α1 cistron (26, 36) and various haploid-specific genes (7, 26). In addition, each of the α2 and **a**1 proteins has an apparent homeo domain which is believed to be a DNA-binding domain (35).

To analyze the dual function of the α2 protein, mutants defective in a1-α2 repression but functional for α2 repression were investigated. Three kinds of such mutants are reported here. Three of the mutations were amino acid substitutions of tyrosine or phenylalanine for cysteine at the 33rd codon of the open reading frame of the α2 cistron of *HMLα* or of *MATα*. Ten other mutants were found to have mutations at a locus, designated *AAR1*, unlinked to the *MAT*, *HML*, *HMR*, *RME1*, or four *SIR* loci.

MATERIALS AND METHODS

Microorganisms and plasmids. The *S. cerevisiae* strains used are listed in Table 1. All the strains have the *ho* genotype. Two *Escherichia coli* strains, JA221 (6) and JM83 (48), were used as the hosts for propagation and manipulation of plasmids. M13mp10 bacteriophage grown in another *E. coli* strain, JM101 (25), was used to determine nucleotide sequences by the dideoxy-termination method (34). Struc-

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TABLE 1. Characteristics of *S. cerevisiae* strains used

Strain	Mating type	Genotype[a]	Source or reference
ATG3-H	Non[b]	*MAT***a** *HMLα HMR***a** *sir3-4 ade2-1 leu2-3,112 trp5*	Our stock
KEN2-8A	Non	*MAT***a** *HMLα HMR***a** *sir3-4 pho3-1 PHO83 trp5*	Nonmater segregant from PS3-8A + PS3-8C fusant
KEN8-5B	**a**	*MAT***a** *HMLα HMR***a** *pho3-1 PHO83 his3 leu2-3,112 trp1*	Our stock
KO7-2A	**a**	*MAT***a** *HML***a** *HMR***a** *sir3-4 arg4 his4 his5 leu2-3,112 ura3*	Our stock
KO16-3C	α	*MAT*α *HMLα HMR***a** *pho3-1 PHO83 trp1*	Our stock
KYC53	**a**	*MAT***a** *lys1 trp3 ura1 ura2*	42
KYC54	α	*MAT*α *lys1 trp3 ura1 ura2*	42
PS3-8A	Non	*MAT***a** *HMLα HMR***a** *sir3-4 pho3-1 PHO83 his5-2 his6 leu1*	Our stock
PS3-8C	α	*MAT*α *HMLα HMR***a** *pho3-1 PHO83 trp5*	Our stock
SAT140-10B	α	*MAT***a** *hmlα2-102 HMR***a** *sir3-4 his3 his5 his6 leu2-3,112 trp1*	α Mating type segregant from PS3-8A-M1 + ATG3-H fusant
SH1240	**a**	*MAT***a** *HML***a** *HMR***a** *sir3-4 aar1-6 arg4 his4 leu2 ura3*	**a** Mating type segregant from KO7-2A × KEN2-8A-M6
SH1242	**a**	*MAT***a** *HML***a** *HMR***a** *sir3-4 aar1-6 his4 leu2 trp5*	**a** Mating type segregant from KO7-2A × KEN2-8A-M6
714	α	*mat***a**1 *HMLα HMR*α *sir1-351*^{ochre} *rme1 ade2 can1 cyh2 leu1 ura3*	I. Herskowitz
540	α	*mat***a**1 *HMLα HMR*α *sir2-540 rme1 ade1 can1 cyh2 leu1 ura3*	I. Herskowitz
529	α	*mat***a**1 *HMLα HMR*α *sir3-347 rme1 ade1 can1 cyh2 leu1 ura3*	I. Herskowitz
528	α	*mat***a**1 *HMLα HMR*α *sir4-167 rme1 ade1 can1 cyh2 leu1 ura3*	I. Herskowitz
M28	**a**[c]	*mat*::*LEU2*[d] *HML***a** *HMR***a** *sir3*(Ts) *ade2 his4 leu2 trp1-1 ura1*	Our stock
M30	α/Non[e]	*MAT*α *HML***a** *HMR***a** *sir3*(Ts) *ho*::*TRP1*[f] *trp1-1 ade2-1 can1-100 his3 leu2 ura3*	Our stock
K123	**a**/Non[e]	*MAT***a** *HMLα HMR***a** *sir3*(Ts) *ade2 leu2 trp1*	Our stock
K163	**a**	*MAT***a** *HMLα HMR***a** *SIR*$^{+}$ *ade1 gal1 his3 leu1 met14 trp1 ura3*	Our stock
DC16	**a**	*MAT***a** *HMLα HMR***a** *his1*	Our stock
DC17	α	*MAT*α *HMLα HMR***a** *his1*	Our stock

[a] All the strains are heterothallic as they have the *ho* genotype. The genetic symbols are those described by Mortimer and Schild (30).
[b] Non represents the nonmater phenotype.
[c] The mating type of this strain is **a** at both 25 and 34°C irrespective of the *sir3*(Ts) mutation.
[d] *mat*::*LEU2* denotes an insertion of the *LEU2* gene into the *Xho*I-linker mutation *MAT*αX8, which is α1^{-} and α2^{-} (43).
[e] Mating types of these strains are **a** or α at 25°C but nonmater at 34°C because of the genotype of *MAT*, *HML*, and *HMR* loci and the *sir3*(Ts) mutation.
[f] The *ho*::*TRP1* allele of M30 was constructed in vitro by replacing the *HO* DNA from nucleotide position −718 to +1,096 (26) (the A residue at the translation initiation codon ATG is taken as nucleotide position +1 according to the nucleotide sequence determined by Jensen et al. [19]) with the 850-bp *Eco*RV-*Bgl*II fragment of *TRP1* of which the nucleotide sequence has been published by Tschumper and Carbon (47). The ligated DNA fragment was inserted into the *HO* locus of strain M30.

tures and derivations of the plasmids used are illustrated in Fig. 1.

Media. The nutrient (YPAD), minimal, and sporulation media for yeast and the nutrient medium for *E. coli* were as described (46). The minimal media were supplemented with appropriate nutrients if necessary. YPAD contains sufficient inorganic phosphate to repress the *PHO5* gene encoding repressible acid phosphatase (45). To select and test yeast transformants with the *Rdhfr* marker conferring resistance against methotrexate and sulfanilamide (28), nutrient medium YPD-MTX, consisting of Bacto-Peptone (Sigma Chemical Co., St. Louis, Mo.; 0.25%), yeast extract (1%), and glucose (2%), and minimal medium MM-MTX, consisting of minimal medium with appropriate nutrients added, were prepared and supplemented with 40 μg of methotrexate and 5 mg of sulfanilamide per ml.

Genetic methods. Genetic methods for yeast cells were as described previously (42). To construct a hybrid clone between two yeast clones of the same mating type or a combination involving a nonmater clone(s), the cell fusion technique with protoplasted cells was employed. Yeast protoplasts were prepared by treatment with Zymolyase-100T (Seikagakukogyo, Co., Ltd., Tokyo, Japan), and cell fusants were selected by the complementation of the auxotrophic markers. Yeast was transformed by the method of Ito et al. (18), and *E. coli* was transformed by the method of Morrison (29). Yeast mutants were selected after ethyl methanesulfonate mutagenesis (22) or spontaneous mutation. Mating types of *S. cerevisiae* strains with auxotrophic markers were determined by cross-streaking them with standard haploid strains having the **a** (KYC53 and DC16) or α (KYC54 and DC17) mating type on a plate and checking for prototroph formation. For prototrophic diploid strains of *S. cerevisiae*, mating types were determined with their respiratory-deficient derivatives induced by streaking the cells onto YPAD medium containing ethidium bromide (50 μg/ml). In this case, the mating patches were replicated onto a minimal medium plate containing glycerol instead of glucose. For determination of mating types of transformant with the plasmids marked with the *Rdhfr* gene, the mating patches were replicated onto a plate containing MM-MTX.

Biochemical methods. Acid phosphatase activity of yeast colonies was detected by the staining method based upon a diazo-coupling reaction (45). Methods for preparation and manipulation of DNAs and RNAs were as described (32, 42).

RESULTS

Isolation of mutants defective for a1-α2 repression. (i) Mutant isolation with the aid of the *PHO83* gene mutation. To isolate mutants defective in a1-α2 repression but still capable of α2-repression, the *PHO83* mutation was employed. The *PHO83* mutation, most probably caused by insertion of a Ty element into the 5′ nontranscribed region of *PHO5*, is dominant over the wild-type allele and results in expression of the *PHO5* gene, even in high-phosphate medium, in a cell

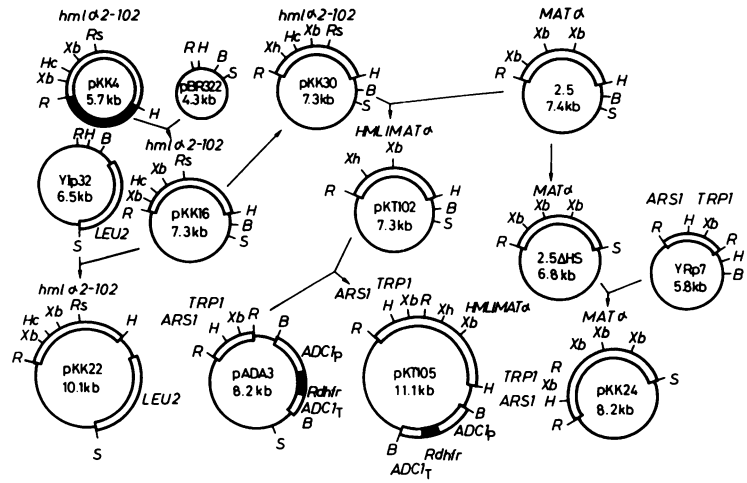

FIG. 1. Structure and construction of plasmids. Plasmid pKK4 was constructed by ligating the 3.0-kilobase-pair (kb) *Eco*RI-*Hin*dIII fragment bearing the *HML* locus prepared from the *hmlα2-102* mutant (PS3-8A-M1) at the *Eco*RI-*Hin*dIII gap of pUC9 (details are given in the text). The same 3.0-kb *Eco*RI-*Hin*dIII fragment bearing the *hmlα2-102* allele was transferred to the *Eco*RI-*Hin*dIII gap of pBR322 to construct pKK16. pKK22 was constructed by ligating the larger *Hin*dIII-*Sal*I fragment of pKK16 with the 3.2-kb *Hin*dIII-*Sal*I fragment containing the *LEU2* gene from plasmid YIp32 (4). Plasmid 2.5 is composed of the larger (4.4-kb) *Eco*RI-*Hin*dIII fragment of pBR322 and the 3.3-kb *Eco*RI-*Hin*dIII fragment of *S. cerevisiae* DNA bearing the *MATα* gene and was kindly provided by J. B. Hicks of the Scripps Research Institute and Clinic, La Jolla, Calif. Plasmid 2.5ΔHS was constructed by deleting a 622-bp *Sal*I-*Hin*dIII region of plasmid 2.5 and connecting the remainder with an 8-bp *Sal*I linker (Takara Shuzo Co. Ltd., Kyoto, Japan) at the *Hin*dIII end after filling in with Klenow fragment. pKK24 was constructed by inserting a 1.4-kb *Eco*RI fragment of YRp7 (38), which bears the yeast *TRP1* and *ARS1* sequences, into the *Eco*RI site of plasmid 2.5ΔHS. Plasmid pADA3 carrying the dehydrofolate reductase gene, *Rdhfr*, transcribed by the promoter, $ADC1_P$, and terminated by a 3′ downstream sequence, $ADC1_T$, of a gene encoding alcohol dehydrogenase, *ADC1*, was a gift from A. Miyajima of the DNAX Research Institute of Molecular and Cellular Biology, Inc., Palo Alto, Calif. Plasmid pKT105 was constructed by subcloning a 2.5-kb *Bam*HI fragment containing the $ADC1_P$-*Rdhfr*-$ADC1_T$ fused gene and a 1.4-kb *Eco*RI fragment containing the *TRP1-ARS1* sequence from pADA3 into the *Bam*HI and *Eco*RI sites, respectively, of plasmid pKT102, a chimeric plasmid constructed by ligating the smaller *Eco*RI-*Xba*I fragment of plasmid pKK30, a derivative of plasmid pKK16, whose *Xba*I site in the W region of the *hmlα2-102* allele was changed to an *Xho*I site by insertion of an 8-bp *Xho*I linker (Takara Shuzo), with the larger *Eco*RI-*Xba*I fragment of plasmid 2.5. The thin lines indicate the DNA fragments originated from pBR322. The thick lines on pADA3 and pKT105 represent *Rdhfr*, and the thick lines on pKK4 represent the pUC9 DNA. The open boxes represent DNA fragments derived from the *S. cerevisiae* chromosome. The symbols, B, H, Hc, R, Rs, S, Xb, and Xh indicate the restriction sites for *Bam*HI, *Hin*dIII, *Hin*cII, *Eco*RI, *Rsa*I, *Sal*I, *Xba*I, and *Xho*I, respectively.

with mating potency, but not in an **a**/α cell (44). Both the strains used for mutant isolation, PS3-8A and KEN2-8A, have the *ho MAT***a** *HML*α, *HMR***a** *sir3 pho3 PHO83* genotype. Expression of the *PHO5* gene is normally repressed in these strains because the strains are *sir3* mutant and therefore express all three of the *MAT***a**, *HML*α, and *HMR***a** loci; this leads to the expression of both **a** and α information, which results in **a**1-α2 repression of expression of a Ty promoter. Therefore, colonies of these strains were white to pale pink when stained for acid phosphatase activity. However, if they mutate to have a defect in **a**1-α2 repression while retaining α2 repression, the colonies should have the α mating type and stain red.

Strains PS3-8A and KEN2-8A were mutagenized with ethyl methanesulfonate and plated, and the plates were incubated at 30°C for 2 to 3 days. Approximately 20,000 of the colonies that developed were stained for acid phosphatase activity, and 735 pink or red colonies were isolated. One of them, PS3-8A-M1 from strain PS3-8A, developed a deep red color and showed a strong α mating type. Two other clones from PS3-8A (PS3-8A-M2 and PS3-8A-M3) and eight clones from KEN2-8A (KEN2-8A-M1 to KEN2-8A-M8) were pink and had weak α mating potency. In subsequent mass-mating experiments, however, we found that some of these 10 mutants and their tetrad progeny from hybrids with wild-type strains had extremely weak **a** mating potency as well as α mating type (bimating reaction). Another 13 clones from KEN2-8A were pink and had the **a** mating type. The remaining 711 pink isolates still had the nonmater phenotype. The 11 mutants showing substantial α mating type were further analyzed.

(ii) Isolation of mutants with the aid of an *ho*::*TRP1* fused gene. In another experiment, two mutants defective in **a**1-α2 repression were isolated from the haploid strain M30 having the *ho MAT*α *HML***a** *HMR***a** *sir3*(Ts) *ho*::*TRP1 trp1-1* genotype. Since *sir3*(Ts) is a temperature-sensitive mutant allele, strain M30 had α mating type at 25°C but was a nonmater when it was cultivated at 34°C because of the expression of both **a** and α information at the *HML***a**, *HMR***a**, and *MAT*α loci. The *TRP1* expression in the *ho*::*TRP1* allele (a detailed structure is given in Table 1) of strain M30 is subject to **a**1-α2–dependent repression. Therefore, the *TRP1* gene in *ho*::*TRP1* is transcribed at 25°C, but at 34°C the *HML***a** and *HMR***a** loci become derepressed and then the *TRP1* transcription is switched off by the **a**1-α2 repression. As a result, strain M30 grows on plates lacking tryptophan at 25°C but not at 34°C. A mutant cell defective in the **a**1-α2 repression but retaining the α2 repression should have α mating potency and the Trp^+ (tryptophan-prototrophic) phenotype at 34°C.

Approximately 2×10^8 cells of strain M30 were plated on Trp test plates, and 477 spontaneous Trp^+ colonies were isolated. Of these, 110 showed some α mating type. To select *mat*α mutants, these 110 mutants were crossed with strain M28 having the *ho matΔ HML***a** *HMR***a** *sir3*(Ts) *trp1-1* genotype. Four Trp^+ diploid clones showing the α mating type at 34°C were obtained, while diploids of the other mutants had Trp^- and nonmater phenotypes. Of the four mutants, two were discarded as they showed ambiguous

TABLE 2. Rationale for distinguishing four possible kinds of mutations showing the α mating type from the *ho MAT***a** *HML*α *HMR***a** *sir3* cell

Mutation[a]	Diploid phenotype[b] with tester:		
	1	2	3
*HML*α	α	α	Non
*hml*α*2*	α	Non	Non
AAR	α	α	α
aar	Non	Non	Non

[a] Gene symbols described by uppercase and lowercase letters indicate, respectively, dominant and recessive mutations with respect to the corresponding wild-type allele.

[b] Phenotypes of mating potency were determined for the diploid clones constructed by crossing a mutant with one of three mating type testers: tester 1 (strain KO7-2A) has the *ho MAT***a** *HML***a** *HMR***a** *sir3* genotype, tester 2 (KEN2-8A or PS3-8A) has the *ho MAT***a** *HML*α *HMR***a** *sir3* genotype, and tester 3 (KO16-3C) has the *ho MAT*α *HML*α *HMR***a** *SIR*$^+$ genotype (Table 1).

phenotypes. The two remaining clones, M30-5-9 and M30-8-2, were studied further.

Genetic classification of the mutants. The α mating-type clones from PS3-8A and KEN2-8A might have a mutation in the *HML*α*2* cistron, and the phenotype of such mutants was designated Aar$^-$ (**a**1-α2 repression defective). To discriminate among possible mutations in a gene other than *MAT*, *HML*, and *HMR*, the mutants were crossed by sexual conjugation or protoplast fusion with three tester strains (Table 2): tester 1 (KO7-2A: *ho MAT***a** *HML***a** *HMR***a** *sir3*); tester 2 (KEN2-8A or PS3-8A: *ho MAT***a** *HML*α *HMR***a** *sir3*); and tester 3 (KO16-3C: *ho MAT*α *HML*α *HMR***a** *SIR*$^+$). Each diploid clone should show the phenotype indicated in Table 2 depending on the mechanism and dominance-recessiveness of the mutation. According to this rationale, the mutants from PS3-8A and KEN2-8A were divided into at least two classes of recessive mutations. One of them, PS3-8A-M1, showed the α mating type in the hybrid cells with tester 1 and the nonmater phenotype with testers 2 and 3. These results strongly suggest that PS3-8A-M1 has a recessive mutation in the α2 cistron of *HML*α. This was confirmed by determination of the nucleotide sequence of the *HML*α locus of PS3-8A-M1 (described later). Thus, we designated the mutation *hml*α*2-102*. A diploid homozygous for *MAT***a**, *hml*α*2-201*, *HMR***a**, and *sir3* showed the α mating type and could not sporulate (Spo$^-$ phenotype; data not shown).

Similar diploids constructed with the remaining 10 Aar$^-$ mutants from PS3-8A and KEN2-8A and the three testers showed nonmater phenotype. This indicates that each of these Aar$^-$ mutations was recessive to the corresponding wild-type allele, and consequently occurred in a gene other than the *MAT*, *HML*, and *HMR* loci. Four of these 10 Aar$^-$ mutants, PS3-8A-M2, KEN2-8A-M4, KEN2-8A-M5, and KEN2-8A-M6, were examined by tetrad analysis after diploids had been constructed by cell fusion with strain KEN2-8A or PS3-8A. The diploids sporulated well and showed a 2non:2α segregation (non indicates a nonmater phenotype) in 5 to 10 asci tested for each cross. Thus, these four mutants each have a single recessive mutation, *aar*. By analogy, each of the other six Aar$^-$ mutants may have a single *aar* mutation but have not yet been tested. Diploids constructed by cell fusion between the *hml*α*2-102* mutation (PS3-8A-M1) and any one of the eight *aar* mutations (KEN2-8A-M1 to KEN2-8A-M8) had a nonmater phenotype. On the other hand, all the cell fusants between one of the eight *aar* mutations from KEN2-8A (KEN2-8A-M1 to KEN2-8A-M8) with one of the similar *aar* mutations from PS3-8A (PS3-8A-M2 and PS3-8A-M3) showed α mating type. These facts clearly indicate that these 10 *aar* mutations are allelic with each other, and the locus was designated *aar1*. Each of the *aar1* mutant alleles isolated from KEN2-8A was designated by its isolation number: e.g., the *aar1* mutation of KEN2-8A-M1 is *aar1-1*. The *aar1* mutant alleles of PS-8A-M2 and PS-8A-M3 are *aar1-9* and *aar1-10*, respectively.

To see whether the *aar1* mutations are allelic with *HML*, KEN2-8A-M5 and KEN2-8A-M6 (having the *aar1-5* and *aar1-6* mutant alleles, respectively) were transformed with plasmid pKT105 (pADA3::*MAT*α; Fig. 1) to the methotrexate-sulfonilamide-resistant phenotype. The resultant transformants, however, showed the α mating type. When an *hml*α*2-102* mutant, SAT140-10B, a tetrad segregant from the PS3-8A-M1 × ATG-3H fusion hybrid, was transformed with the same plasmid, pKT105, the resultant transformants, of which six independent clones have been tested, had the nonmater phenotype but transformants with pADA3 (Fig. 1) showed the α mating type. These results clearly indicate that the *aar1* mutant is not an *hml*α mutant while the *hml*α*2-102* mutant is.

For genetic characterization of two mutants (M30-5-9 and M30-8-2) isolated from M30, the mutants were crossed with an **a** strain, K163 (*ho MAT***a** *HML*α *HMR***a** *SIR*$^+$). The resultant diploids did not sporulate immediately, suggesting a mutation at *MAT*α, but eventually high levels of sporulation were obtained. This must have been caused by the high frequency of occurrence of *sir3*/*sir3* homozygosity in the *sir3*/*SIR*$^+$ heterozygous diploid cells, most probably mediated by the high frequency of mitotic recombination at the tandem multiplicated ribosomal RNA genes, *RDN1*, on chromosome XII (unpublished observation). The asci showed a 2**a**:2α segregation at 25°C in 11 tetrads for M30-5-9 and 17 for M30-8-2, and two of the spore clones in each ascus showed nonmater phenotypes at 34°C, irrespective of their mating type at 25°C. These segregations strongly suggest the homozygosity of the *sir3*(Ts) allele in these diploids, as mentioned above.

When the spore clones from the above-mentioned M30-5-9 × K163 and M30-8-2 × K163 crosses were mated with the wild-type **a** or α strains DC16 or DC17, the resultant diploids from the α spore clones showed a Spo$^-$ phenotype and mated as α. This implies that these mutants have a defect in **a**1-α2 repression. It also suggests that hybrids between M30-5-9 or M30-8-2 and K163 can only have sporulated because of the homozygosity of the *sir3*(Ts) allele, which allowed expression of the α information at the *HML*α allele derived from K163. To confirm this, the mutants M30-5-9 and M30-8-2 were crossed with K123 [*ho MAT***a** *HML*α *HMR***a** *sir3*(Ts)]. The resultant diploids sporulated well at 34°C and showed the 2**a**:2α segregation at 25°C, while two spore clones in each ascus showed a nonmater phenotype at 34°C, just as the original M30-5-9 × K163 and M30-8-2 × K163 crosses eventually did. This implies that M30-5-9 and M30-8-2 both carry a *mat*α mutation that is defective for **a**1-α2 repression but still capable of α2 repression. The result also suggests that the defect is suppressible by the expression of *HML*α$^+$. Thus, the mutant alleles of M30-5-9 and M30-8-2 were designated *mat*α*2-201* and *mat*α*2-202*, respectively.

Nucleotide sequences of the mutant α2 cistrons. Since the *hml*α*2-102*, *mat*α*2-201*, and *mat*α*2-202* mutations appeared to occur in the α2 cistron of their respective loci, their nucleotide sequences were examined. To clone the *hml*α*2-102* mutant gene the genomic DNA extracted from PS3-

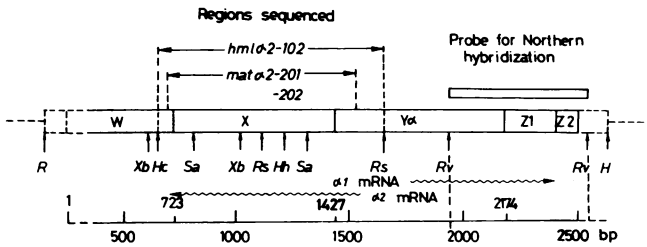

FIG. 2. Restriction map and sequenced regions of the *hmlα2-102* and *matα2* mutant alleles. The wavy arrows represent the region and direction of the α1 and α2 transcripts. Symbols Rv, Hh, Hc, Rs, Sa, and Xb indicate the restriction sites for *Eco*RV, *Hha*I, *Hin*cII, *Rsa*I, *Sau*96I, and *Xba*I, respectively. The W, X, and Yα regions have, respectively, 723, 704, and 747 bp (2).

8A-M1 was double digested with *Eco*RI and *Hin*dIII. The digest was electrophoresed on a polyacrylamide slab gel, blotted onto a nylon membrane (Biodyne; Pall Biosupport, East Hills, N.Y.), and hybridized with a ^{32}P-labeled 3.3-kilobase-pair (kb) *Eco*RI-*Hin*dIII fragment bearing the *MATα* gene prepared from plasmid 2.5 (Fig. 1) as probe. We found a 3.0-kb band hybridized with the probe DNA as described by Abraham et al. (1) (data not shown). The 3.0-kb fragments eluted from the gel were ligated with the larger *Eco*RI-*Hin*dIII fragment of pUC9 (48). The *E. coli* strain JM83 was then transformed with the DNA to the ampicillin-resistant phenotype. About 3,000 transformant colonies were selected by colony hybridization (14) with the same ^{32}P-labeled 3.3-kb *MATα* DNA as probe, and one hybridization-positive clone was obtained. It was confirmed that the cloned DNA fragment has the same restriction map as the *HMLα* locus (data not shown). The plasmid thus obtained was designated pKK4 (Fig. 1).

To confirm that the DNA fragment cloned on pKK4 carries the *hmlα2-102* mutant allele, a chimeric plasmid, pKK22, carrying the cloned DNA fragment from pKK4 and the *LEU2* DNA fragment of *S. cerevisiae* was constructed as shown in Fig. 1. Plasmid pKK22 was used to transform the yeast strain KEN8-5B (*ho MAT***a** *HMLα HMR***a** *PHO83 leu2-3,112*) to the Leu$^+$ phenotype. As a control, the same yeast strain was transformed with plasmid pKK24 (Fig. 1) bearing the wild-type *MATα* DNA. The Leu$^+$ transformants with pKK22 showed a bimating phenotype upon cross-streaking with the **a** (KYC53) and α (KYC54) testers, but the transformants with pKK24 showed the **a** mating type. The bimating phenotype of the transformants with pKK22 can be explained by the appearance of two types of cells, those harboring pKK22 and those that had lost the plasmid in the cell population of a single colony. These observations indicate that the 3.0-kb *Eco*RI-*Hin*dIII fragment cloned on pKK4 contains the *hmlα2-102* allele. The nucleotide sequence of the 1.1-kb *Hin*cII-*Rsa*I region (2) bearing the whole α2 coding frame (Fig. 2) of the *hmlα2* fragment was determined by the method of Maxam and Gilbert (24).

In the case of the two *matα2* mutants isolated from strain M30, 4.2-kb *Hin*dIII fragments prepared from these two mutants and from the wild-type strain M30 were cloned into M13mp10 (25) at the *Hin*dIII site, because the *MATα* region is known to be carried on such a fragment (31). The phage clones were selected by plaque hybridization (3) with the ^{32}P-labeled 4.2-kb *MATα* *Hin*dIII fragment as probe. That the *matα2* DNA was inserted into the M13 vector in the correct orientation was confirmed by digestion of replicative forms of the recombinant phage DNAs with appropriate restriction enzymes. Recombinant M13 phages were then

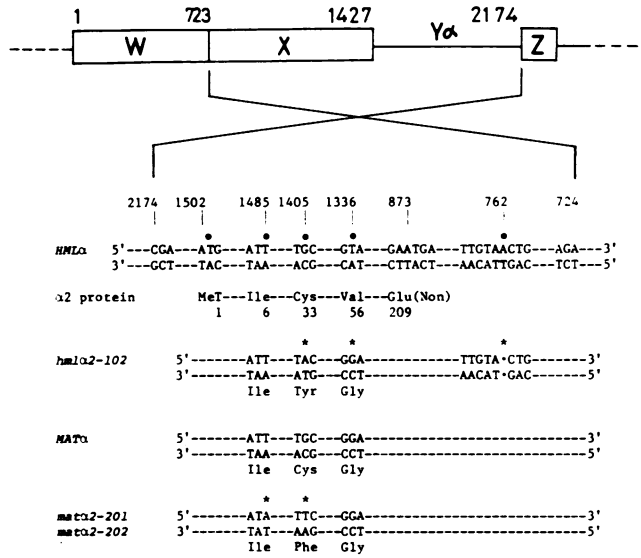

FIG. 3. Nucleotide sequences of the region coding for the α2 cistron of the *HMLα*, *hmlα2-102*, *MATα*, *matα2-201*, and *matα2-202* alleles. The *HMLα* sequence was adopted from Astell et al. (2). The nucleotide pairs that deviate from the corresponding wild-type allele are indicated by asterisks on the mutant sequences. Amino acid sequences of the wild-type and mutant α2 cistrons are shown on the line below the coding sequences. The α2 open reading frame starts at the nucleotide position 1502 and ends at 874 in this figure. The sequences are shown only where they differ. The W, X, and Yα regions have 723, 704, and 747 bp, respectively (2).

sequenced (34) directly for a 663-bp region from nucleotide positions 1530 to 870 which bears the α2 cistron (Fig. 2). This was done by using synthetic oligonucleotides (5'-ATTA TCAACTTACACAG-3' corresponding to nucleotide positions 1201 to 1185 and 5'-CAGCTTAGAAGTGGGCA-3' corresponding to positions 1547 to 1531) as primers.

Mutant nucleotide sequences were compared with the published data for the α2 cistron (2). We found three apparent deviations in the *hmlα2-102* mutant sequence from that of the wild-type DNA (Fig. 3). One is a single A:T pair deletion in the mutant DNA at nucleotide position 762 of the wild-type sequence, downstream of the α2 open reading frame. The second is the substitution of a G:C pair for T:A at nucleotide position 1336, which substitutes a glycine codon for valine at the 56th codon from the translation initiation codon, ATG. The third is an A:T-for-G:C substitution at nucleotide position 1405, which gives rise to a tyrosine-for-cysteine substitution at the 33rd codon from ATG. Since the first deviation is at a site distal from the stop codon of the α2 cistron and the second, at position 1336, has been observed in a *MATα*$^+$ DNA (2) and in a *HMLα2*$^+$ DNA (42), these deviations cannot be responsible for the Aar$^-$ phenotype. Therefore, the *hmlα2-102* mutation is presumably caused by the base substitution at nucleotide position 1405, which causes a codon change from cysteine to tryosine at the 33rd codon of the α2 cistron.

The nucleotide sequences at the α2 cistron of the *matα2-201* and *matα2-202* mutant DNAs were identical and deviated at three nucleotide positions, 1336, 1405, and 1485 (Fig. 3), from the published sequence of the α2 cistron in *HMLα* (2). However, the G:C-for-T:A substitution at position 1336 cannot be responsible for the mutation since the same deviation was found in the *MATα* DNA of M30 and should be regarded as a polymorphism in the α2 cistron, as de-

scribed above. The second deviation, the A:T-for-T:A substitution at position 1485, is silent in its amino acid coding. Therefore, the T:A-for-G:C substitution at nucleotide position 1405 seems responsible for the *matα2* mutations. The nucleotide substitution at this site results in an amino acid substitution of phenylalanine for cysteine in the α2 protein at exactly the same position as the tyrosine-for-cysteine substitution in the *hmlα2-102* mutation. In conclusion, the *hmlα2-102*, *matα2-201*, and *matα2-202* mutations are caused by missense mutations at the 33rd codon and result in the amino acid substitution of tyrosine or phenylalanine for cysteine at the corresponding site of α2 protein.

Characteristics of the *aar1* mutation. We have noted that diploids homozygous for *hmlα2-102* and for the *MAT***a**, *HMR***a**, and *sir3* mutant alleles had the α mating type and were Spo$^-$. In contrast, diploids homozygous for *MAT***a**, *HMLα*, and *HMR***a**, and the *sir3* mutation and carrying any two *aar1* mutant alleles showed the α mating type but have the Spo$^+$ phenotype, and all asci showed a 4α:0**a** segregation. To see the phenotype of the *aar1* mutation without the α information, diploids were constructed by crossing KEN2-8A-M1 (*ho MAT***a** *HMLα HMR***a** *sir3 aar1-1*), KEN2-8A-M5 (*ho MAT***a** *HMLα HMR***a** *sir3 aar1-5*), or KEN2-8A-M6 (*ho MAT***a** *HMLα HMR***a** *sir3 aar1-6*) with strain KO7-2A (*ho MAT***a** *HML***a** *HMR***a** *sir3 AAR*$^+$) and subjected to tetrad analysis. All the diploids gave rise to asci showing the 2**a**:2α, 2**a**:1non:1α or 2**a**:2non segregations in 5 to 10 asci tested for each cross. Since all the asci contain two **a** spores, a cell having the *ho MAT***a** *HML***a** *HMR***a** *sir3 aar1* genotype should have the **a** mating type as do cells having the *ho MAT***a** *HML***a** *HMR***a** *sir3 AAR1*$^+$ genotype. That the segregant spore clones showing the **a** mating type are composed of two different classes, one having the *aar1* mutation and the other *AAR1*$^+$, was confirmed by examination of diploids constructed by crossing these spore clones with one of the original mutants, PS3-8A-M2 (*ho MAT***a** *HMLα HMR***a** *sir3 aar1*). The diploids homozygous for the *aar1* mutation (but heterozygous for *HMLα*/*HML***a**) still showed the α mating type, while the diploids heterozygous for *aar1*/*AAR*$^+$ were nonmaters. All these diploids, irrespective of the homozygosity or heterozygosity of the *aar1* mutation, showed a high degree of sporulation (data not shown).

To examine whether sporulation occurs in a diploid homozygous for *aar1* without α information, a diploid was constructed by cell fusion of strains SH1240 (*ho MAT***a** *HML***a** *HMR***a** *sir3-4 aar1*) and SH1242 (*ho MAT***a** *HML***a** *HMR***a** *sir3-4 aar1*), both of which have the **a** mating type. (That strains SH1240 and SH1242 both have the *aar1* mutation was verified by mating them with KEN2-8A-M6, the original *aar1-6* mutant.) The diploid has the **a** mating type and Spo$^-$ phenotype. These results indicate that the *aar1* mutants without α information cannot sporulate and that the recessive *aar1* mutation causes a defect of the **a**1-α2 repression of the α-specific and haploid-specific genes but does not affect the **a**/α-specific genes. Thus, the *AAR1* gene differs from *RME1* or other *RME*-like genes in its function, because the *rme1*/*rme1* diploid can sporulate irrespective of the **a** and α information (21, 27, 33).

It was confirmed that the *aar1* mutation is nonallelic with the *rme1* mutation by examination of diploids constructed by crosses of strain SH1240 (*ho MAT***a** *HML***a** *HMR***a** *sir3-4 aar1-6*) with strains 714 (*ho mat***a**1 *HMLα HMRα sir1 rme1*), 540 (*ho mat***a**1 *HMLα HMRα sir2 rme1*), 529 (*ho mat***a**1 *HMLα HMRα sir3 rme1*), and 528 (*ho mat***a**1 *HMLα HMRα sir4 rme1*). All the diploids had the **a** mating type and Spo$^-$ phenotype, except for the SH1240 × 529 cross, which was a nonmater diploid and had a Spo$^+$ phenotype caused by the homozygosity of *sir3* mutant alleles. These observations also indicate that *AAR1* is not allelic with the *SIR* genes. It is obvious that *AAR1* differs from *SCA* (12) and *CSP1* (17), because the *sca* gene mutation was identified as an allele of the *sir2* mutation (23) and the *csp1* mutation was suggested to be allelic with the *rme1* mutation (33).

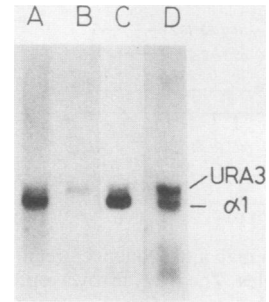

FIG. 4. Detection of the α1 transcript in the *hmlα2-102* and *aar1* mutants by Northern blot hybridization. Total RNA was prepared from strain PS3-8C (*MATα HMLα HMR***a** *SIR*$^+$) (lane A), PS3-8A (*MAT***a** *HMLα HMR***a** *sir3*) (lane B), PS3-8A-M1 (*MAT***a** *hmlα2-102 HMR***a** *sir3-4*) (lane C), and KEN2-8A-M1 (*MAT***a** *HMLα HMR***a** *sir3-4 aar1*) (lane D). RNA (5 to 10 μg) was added to each slot and electrophoresed on an agarose gel (0.8%). The gels were subjected to Northern blot hybridization with a mixture of the ^{32}P-labeled (2.1 × 10^7 Cerenkov cpm/μg of DNA) 1.1-kb *Eco*RV fragment bearing the α1 cistron (Fig. 2) prepared from plasmid 2.5 and ^{32}P-labeled YIp5 DNA (41) (4.7 × 10^7 Cerenkov cpm/μg of DNA) having the *URA3* gene as probes.

α1 transcription in the *hmlα2-102* and *aar1* mutants. To investigate the effects of *hmlα2-102* and *aar1* mutations on the transcription of the α1 cistron, total RNA was prepared from cells of PS3-8A-M1 (*hmlα2-102*) and KEN2-8A-M1 (*aar1*) cultivated in nutrient medium and from cells of their original strain, PS3-8A, and the wild-type strain PS3-8C. The amount of the α1 mRNA in the total RNA samples was estimated by Northern (RNA) blot hybridization with a ^{32}P-labeled 1.1-kb *Eco*RV fragment bearing the α1 cistron (Fig. 2) prepared from plasmid 2.5 (Fig. 1) as probe. It was found that the α1 transcription occurred in the *MAT***a** *hmlα2-102 HMR***a** *sir3* (PS3-8A-M1; Fig. 4, lane C) and *MAT***a** *HMLα HMR***a** *sir3 aar1* (KEN2-8A-M1; Fig. 4, lane D) mutant cells, while that of the *MAT***a** *HMLα HMR***a** *sir3* cells (PS3-8A; Fig. 4, lane B) was severely repressed. These results indicate that both the *hmlα2-102* and *arr1* mutations confer a defect in the **a**1-α2 repression for the α1 cistron.

DISCUSSION

Three kinds of mutations, *hmlα2-102*, *matα2*, and *aar1*, were isolated. It is clear that the *hmlα2-102* and the *matα2* were caused by the substitution of tyrosine or phenylalanine for cysteine at the 33rd amino acid codon in the α2 cistron. These observations strongly suggest that the *hmlα2-102* and *matα2* mutants have a defect in the α2 protein that prevents them from interacting productively with the **a**1 protein, while it retaining the ability to repress the **a**-specific genes. It was predicted by Hall and Johnson (15) that a domain for interaction or multimerization with an **a**1 polypeptide in an α2 polypeptide is in the region from amino acid position 20 to position 141. The mutation sites in the *hmlα2-102* and *matα2* mutant genes are in this region. Therefore, the cysteine residue at the 33rd codon might be essential for the **a**1-α2 complex formation, while it is dispensable for repression of

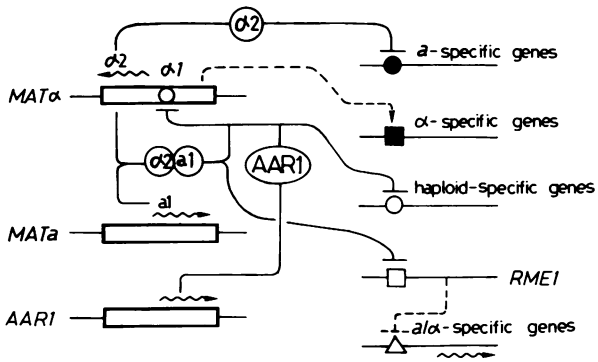

FIG. 5. The possible function of the *AAR1* gene in the regulatory hierarchy of *MAT* genes. Positive control functions are denoted by straight arrows, and T bars represent negative controls. Wavy arrows indicate expression. The broken lines indicate the functions pausing in the a/α diploid cell. Symbols represent target sites for regulatory proteins.

the a-specific genes. It is possible that the cysteine residue forms the a1-α2 heterodimer by a disulfide bridge or serves as a metal ligand to form metal-linked dimers as described for the tat protein of human immunodeficiency virus (11).

Recently, Strathern et al. (40) have also isolated a *matα2* mutation, *matα2d-807*, showing the Aar$^-$ phenotype and found that this mutation has a base change which confers a serine for leucine substitution at the 196th codon of α2 protein. The *matα2d-807* mutation is, however, in the region suggested to encode a domain for sequence-specific DNA binding of the α2-α2 homodimer or tetramer (13, 15).

The *AAR1* gene, a new regulatory gene for cell-type control, might contribute in another way. It has been shown that the a1-α2 repression pathway involves several genes or factors (5, 10). Our observations indicate that the haploid *aar1* mutants having both the a and α information (because of the expression of the *MAT*a, *HML*α, and *HMR*a genes by the *sir3* mutation) express both the α1 cistron and the Ty-controlled *PHO5* gene. The homozygous diploids with the same genotype could sporulate, while they still retained an α mating phenotype. We have also noted that these haploid *aar1* mutants occasionally showed the bimating phenotype. However, the *aar1* mutants without α information but with a information showed an a mating phenotype and could not sporulate, even if they were diploids. These phenotypes suggest that the *AAR1* gene acts cooperatively with or after the *MAT* gene products in the regulation hierarchy of cell-type-specific genes.

The simplest hypothesis which interprets the most frequently seen phenotype of mating as α and sporulation proficient is that the *AAR1* product is responsible for repression of the α1 cistron and haploid-specific genes but not for repression of the a/α-specific genes. In other words, expression of the α1 cistron, whose product is a positive regulator of the α-specific genes, and the haploid-specific genes are repressed by the a1-α2 complex with cooperative functioning of the *AAR1* gene product (Fig. 5). In contrast, expression of the a/α-specific genes is repressed by the *RME1* gene product (27) whose expression is in turn repressed by the a1-α2 complex, and the *AAR1* product is not concerned in these mechanisms. Thus, the *AAR1* and *RME1* genes have divergent functions in transmission of the a1-α2 signals to different categories of cell-type genes. The occasional bimating phenotype can be explained by a possible disturbance in the molecular equilibrium of the α2-α2, a1-α2, and a1-α2-AAR1 complexes by the *aar1* mutation or by existence of a proportion of the cells in a colony that does not express the *HML*α and *HMR*a genes adequately, probably because of the *sir3* mutation, and thus retain their ability to mate as a cells.

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